Biochemistry and bioenergetics of syntrophic propionate-oxidizing bacteria

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STELLINGEN

1. De bewering dat er bewijs gevonden is voor het optreden van omgekeerd elektronentransport tijdens syntrofe benzoaat-oxidatie, moet worden afgezwakt.
   Wallrabenstein, C., and B. Schink. 1994. Evidence of reversed electron transport in syntrophic butyrate or benzoate oxidation by Syntrophomonas wolfei and Syntrophus buswellii. Arch. Microbiol. 162: 136-142.

2. Na de recente ontdekking van een pathogene *Desulfovibrio*-soort moet bij ophopingen van sulfaat-reducerende bacteriën enige voorzichtigheid in acht worden genomen.
   McDougall, R., J. Robson, D. Paterson, and W. Tee. 1997. Bacteremia caused by a recently described novel Desulfovibrio species. J. Clinic. Microbiol. 35: 1805-1808.

3. De ene pot ijzer(II)chloride is de andere niet.
   Dit proefschrift, hoofdstuk 7.

4. Dat samenwerken uiteindelijk voor beide partners een beter resultaat kan opleveren, is door de microbiële wereld allang begrepen.

5. Om mogelijk biotechnologisch interessante stoffen uit de oceaan te leren kennen, moeten meer farmaceutische bedrijven de sprong in het diepe wagen.
   Fenical, W. 1997. New pharmaceuticals from marine organisms. Tibtech 15: 339-341.

6. Bij de aanstellingsduur van een AIO/OIO binnen de microbiologie zou rekening gehouden moeten kunnen worden met de verdubbelingssnelheid van het micro-organisme waarmee wordt gewerkt.

7. In een biofilm bepalen de hoofdrolspelers grotendeels zelf hun scenario.

8. De term wachtgeld is goed gekozen aangezien veel wachtgelders op hun geld moeten wachten als gevolg van de administratieve wanorde bij de USZO.

9. Door het verplicht stellen van cursussen voor AIO's/OIO's binnen een onderzoekschool komt de nadruk te liggen op school in plaats van onderzoek.

10. Telemarketing is vaak antimarketing.

11. De nieuwste kreet, "een slimme meid krijgt haar kind op tijd" lijkt vooral bedoeld als excuus voor iedere "slimme meid die op haar toekomst is voorbereid", maar die erachter komt dat het ook na het "kiezen van exact" moeilijk is om een goede plaats op de arbeidsmarkt te veroveren.

12. Het plezierjacht wordt minder bekritiseerd dan de plezierjacht.

13. Het aantal files op ongebruikelijke plaatsen neemt af.

Stellingen behorende bij het proefschrift *Biochemistry and bioenergetics of syntrophic propionate-oxidizing bacteria.*

Ine van Kuijk

Wageningen, 18 februari 1998

CONTENTS

1

General Introduction

Degradation of organic material under methanogenic conditions

In methanogenic environments, organic matter is degraded to methane and carbon dioxide by microorganisms via fermentation processes, and respiration processes with protons and bicarbonate as electron acceptors. Examples of methanogenic environments are freshwater sediments (e.g. wetlands, paddy fields and swamps), anaerobic bioreactors (e.g. upflow anaerobic sludge bed reactors and fluidized bed reactors), and the intestinal tracts of insects and higher animals (Bryant 1977; Oremland 1988; Boone 1991; Iza 1991; Lettinga and Hulshoff Pol 1991).

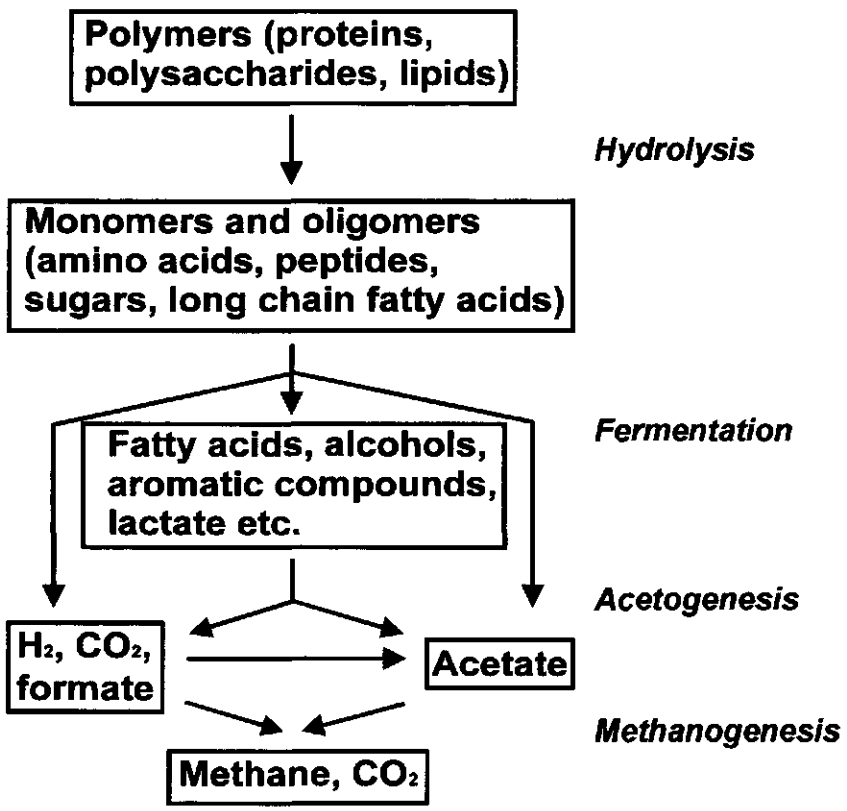

Fig.1. Anaerobic degradation of organic matter under methanogenic conditions.

At least three different trophic groups of microorganisms are involved in the complete anaerobic conversion of organic matter (Fig. 1). The first one consists of fermenting bacteria which hydrolyze organic polymers into monomers and oligomers like sugars, amino acids, and long chain fatty acids; subsequently these compounds are fermented to acetate, hydrogen, formate, and reduced compounds such as alcohols, short and branched chain fatty acids, and lactate. The reduced compounds are oxidized to acetate, carbon dioxide, hydrogen and formate by the second trophic group, consisting of hydrogen-producing acetogenic bacteria. Finally, methane and carbon dioxide are formed from acetate, hydrogen, and formate by the methanogenic archaea, which form the third trophic level (Boone 1982; Gujer and Zehnder 1983).

Syntrophic conversions

Almost all conversions that are carried out by hydrogen-producing acetogenic bacteria are endergonic reactions under thermodynamic standard conditions, i.e. 1 M for solutes or 1 atm for gases, pH 7, and a temperature of 298 °K (Table 1). Therefore, in methanogenic environments, these conversions can only proceed if the products, hydrogen, formate and (to some extent) acetate, are efficiently removed by methanogenic archaea. Consequently, the capacity of acetogenic bacteria to degrade substrates depends on the action of methanogens, while the methanogenic archaea depend (partially) on the action of acetogenic bacteria for substrate supply. Such a co-operation in which two organisms feed together is called syntrophism (Schink 1992, 1997; Stams 1994).

The process of transfer of reducing equivalents from the acetogens to the methanogens is called interspecies electron transfer. Both hydrogen and formate have been proposed to be used as intermediate in the electron transfer (Bryant et al. 1967; McInerney et al. 1979). Hydrogen transfer has been considered as the most important mechanism for electron exchange for a long time. However, experiments by Thiele and Zeikus (1988) and Boone et al. (1989) showed the importance of formate transfer in syntrophic growth as well. The role of formate and hydrogen in syntrophic degradation has been studied more directly by constructing defined cocultures and tricultures (Dong et al. 1994). The syntrophic propionate-oxidizing bacterium strain MPOB

degraded propionate in coculture with the methanogens *Methanospirillum hungatei* or *Methanobacterium formicicum*, which are able to use both hydrogen and formate. However, in cocultures with *Methanobrevibacter arboriphilus* strains or *Methanosaeta soehngenii*, which are only able to use hydrogen or acetate, respectively, propionate was not degraded unless bacteria were present which were able to convert formate into hydrogen plus carbon dioxide.

Table 1. Some reactions involved in syntrophic conversions under methanogenic conditions

Acetogenic reactions	**$\Delta G^{\circ'}$ (kJ/mol)**
Fatty acids	
$Acetate^- + 4\ H_2O \rightarrow 2\ HCO_3^- + H^+ + 4\ H_2$	+104.6
$Propionate^- + 3\ H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 3\ H_2$	+ 76.1
$Butyrate^- + 2\ H_2O \rightarrow 2\ Acetate^- + H^+ + 2\ H_2$	+ 48.1
Alcohols	
$Methanol + 2\ H_2O \rightarrow HCO_3^- + H^+ + 3\ H_2$	+ 23.5
$Ethanol + H_2O \rightarrow Acetate^- + H^+ + 2\ H_2$	+ 9.6
Aromatic compounds	
$Benzoate^- + 7\ H_2O \rightarrow 3\ Acetate^- + HCO_3^- + 3\ H^+ + 3\ H_2$	+ 58.9
$Phenol + 5\ H_2O \rightarrow 3\ Acetate^- + 3\ H^+ + 2\ H_2$	+ 5.5
$Hydroxybenzoate^- + 6\ H_2O \rightarrow 3\ Acetate^- + HCO_3^- + 3\ H^+ + 2H_2$	+ 5.2
Organic acids	
$Glycolate^- + 3\ H_2O \rightarrow 2\ HCO_3^- + H^+ + 3\ H_2$	+ 19.3
$Lactate^- + 2\ H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 2\ H_2$	- 4.2
Amino acids	
$Glutamate^- + 7\ H_2O \rightarrow Acetate^- + 3\ HCO_3^- + NH_4^+ + 2\ H^+ + 5\ H_2$	+ 70.3
$Alanine + 3\ H_2O \rightarrow Acetate^- + HCO_3^- + NH_4^+ + H^+ + 2\ H_2$	+ 7.5
$Aspartate^- + 4\ H_2O \rightarrow Acetate^- + 2\ HCO_3^- + NH_4^+ + H^+ + 2\ H_2$	- 14.0
Methanogenic reactions	
$4\ H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	- 33.9
$4\ Formate^- + H_2O + H^+ \rightarrow CH_4 + 3\ HCO_3^-$	- 32.6
$Acetate^- + H_2O \rightarrow CH_4 + HCO_3^-$	- 31.0

Interspecies electron transfer can also influence the metabolism of fermenting bacteria. A shift in product formation can occur if fermenting bacteria are cocultivated with hydrogen-consuming microorganisms. This effect was first shown for *Ruminococcus albus* growing in the presence and absence of *Wolinella* (*Vibrio*) *succinogenes*. During growth with glucose in pure culture, *R. albus* produces hydrogen, carbon dioxide, ethanol and acetate as end-products, while in coculture with *W. succinogenes* no ethanol is formed (Ianotti et al. 1973). During the fermentation of glucose NADH is formed which has to be reoxidized to NAD and hydrogen. However, for thermodynamic reasons the formation of hydrogen from NADH becomes impossible at high hydrogen concentrations. Therefore, *R. albus* has to couple the oxidation of NADH to the reduction of acetyl-CoA to ethanol. In coculture, hydrogen is removed by *W. succinogenes*; in this case, it is no longer necessary to produce ethanol. Similar effects of interspecies hydrogen transfer have been described for many other microorganisms; for an overview see Stams (1994).

A remarkable characteristic of syntrophic conversions is that the Gibbs free energy change is relatively low, resulting in only small amounts of energy available for growth. In addition, this small amount of energy has to be shared by the partner organisms. This may explain the low growth rates and yields of syntrophic cultures. As a consequence of the slow growth and low growth yields syntrophic cultures are difficult to isolate. Moreover, the acetogenic bacteria of syntrophic cultures are mostly not able to grow on plates or in agar shake cultures and therefore have to be purified in dilution series in liquid media (Stams et al. 1993; Wallrabenstein et al. 1994, 1995a and b). Nevertheless, a variety of syntrophic bacteria that oxidize fatty acids, alcohols or aromatic acids have been isolated in the last decade (for an overview, see Schink 1992 and 1997 or Stams 1994). Syntrophic propionate-oxidizing bacteria were studied in this thesis research, and will therefore be discussed in more detail below.

Syntrophic propionate-oxidation

The volatile fatty acid propionate is one of the reduced compounds that are formed as intermediates during the anaerobic breakdown of organic material. Under methanogenic conditions, propionate can only be degraded syntrophically.

Table 2. Characteristics of syntrophic propionate-oxidizing bacteria.

	Syntrophobacter wolinii	*Syntrophobacter pfennigii*	Strain MPOB
Morphology	rod	egg-shaped rod	lemon-shaped rod
Size in syntrophic culture	0.6-1.0 x 1.0-4.5 μm	1.0-1.2 x 2.2-3.0 μm	1.1-1.6 x 1.8-2.5 μm
Motility	no	yes	no
Gram-stain	negative	negative	negative
Mol % G + C of DNA	not determined	57.3 ± 0.2 mol %	60.6 ± 0.2 mol %
Optimum growth temp.	not determined	37 °C	37 °C
pH optimum	not determined	7.0-7.3	7.2
Substrates in pure culture	propionate + sulfate, pyruvate	propionate or lactate + sulfate, sulfite or thiosulfate	propionate, formate or succinate + sulfate; propionate, hydrogen , or formate + fumarate; fumarate; pyruvate; malate; aspartate
Cytochromes	cyt. *b* (428, 530, 560 nm) cyt. *c* (419, 523, 552 nm)	cyt. *b* (429, 529, 561 nm) cyt. *c* (419, 522, 553 nm)	cyt. *b* (429, 527, 557 nm) cyt. *c* (420, 523, 553 nm)
Quinones	menaquinone-7	menaquinone-7	menaquinone-6 and -7
Habitat	anaerobic digestor sludge	anaerobic sewage sludge	anaerobic granular sludge
References	Boone and Bryant 1980; Wallrabenstein et al. 1994	Wallrabenstein et al. 1995b	Stams et al. 1993; Harmsen 1996; Chapter 7 of this thesis.

So far, three syntrophic propionate-oxidizing bacteria have been described, namely *Syntrophobacter wolinii* (Boone and Bryant 1980; Wallrabenstein et al. 1994), *Syntrophobacter pfennigii* (Wallrabenstein et al. 1995b), and strain MPOB (Stams et al. 1993; Harmsen 1996). Characteristics of these bacteria are presented in Table 2. In addition, some highly purified enrichment cultures of syntrophic propionate-oxidizing bacteria have been described, including culture T13 (Mucha et al. 1988), strain LX-2 (Mah et al. 1990), a mixed culture which contains sporeforming thermophilic propionate-oxidizing bacteria in coculture with defined methanogenic archaea (Stams et al. 1992), an enrichment of two mesophilic sporeforming syntrophic propionate-oxidizing bacteria (Harmsen 1996), strain SYN7 (Harmsen 1996), and strain HP1.1 (Zellner et al. 1996).

Comparison of the 16S rRNA gene sequences revealed that *S. wolinii*, *S. pfennigii*, strain MPOB, and strain HP1.1 are closely related and form one group within the delta subdivision of proteobacteria (Harmsen et al. 1993, 1995; Zellner et al. 1996) (Fig. 2). The closest related bacterium is the sulfate-reducer *Desulforhabdus amnigenus*, which has a 16S rRNA gene sequence identity with e.g. *S. wolinii* of 93.1 %, but is not able to grow syntrophically with propionate (Oude Elferink et al. 1995). The syntrophic propionate-oxidizing bacteria are not restricted to this group within the delta subdivision of proteobacteria; two enriched sporeforming syntrophic propionate-oxidizing bacteria are related to the Gram-positive sulfate-reducing bacteria of the genus *Desulfotomaculum*, and culture SYN7 is related to the syntrophic benzoate-oxidizing bacteria of the genus *Syntrophus* (Harmsen 1996).

Methylmalonyl-CoA pathway

Studies with ^{14}C- and ^{13}C-labelled propionate have indicated that syntrophic propionate-oxidizing bacteria degrade propionate via the methylmalonyl-CoA pathway (Fig. 3) (Koch et al. 1983; Houwen et al. 1987; Robbins 1987, 1988; Mucha et al. 1988; Mah et al. 1990). The same pathway is involved in the formation and degradation of propionate by the sulfate-reducing bacterium *Desulfobulbus propionicus* (Stams et al. 1984; Kremer and Hansen 1988). The involvement of the methylmalonyl-CoA pathway in syntrophic propionate-oxidation was later confirmed

by enzyme activity measurements in *Syntrophobacter wolinii* (Houwen et al. 1990), strain MPOB (Plugge et al. 1993), and in *S. pfennigii* (Wallrabenstein et al. 1995b). There is a small difference in the process of propionate activation between strain MPOB, and *S. wolinii* and *S. pfennigii*. In *S. wolinii* and *S. pfennigii*, a propionate kinase is involved in the activation of propionate, while in strain MPOB propionate is activated via a propionate:acetyl-CoA HS-CoA transferase (Houwen et al. 1990; Plugge et al. 1993; Wallrabenstein et al. 1995b).

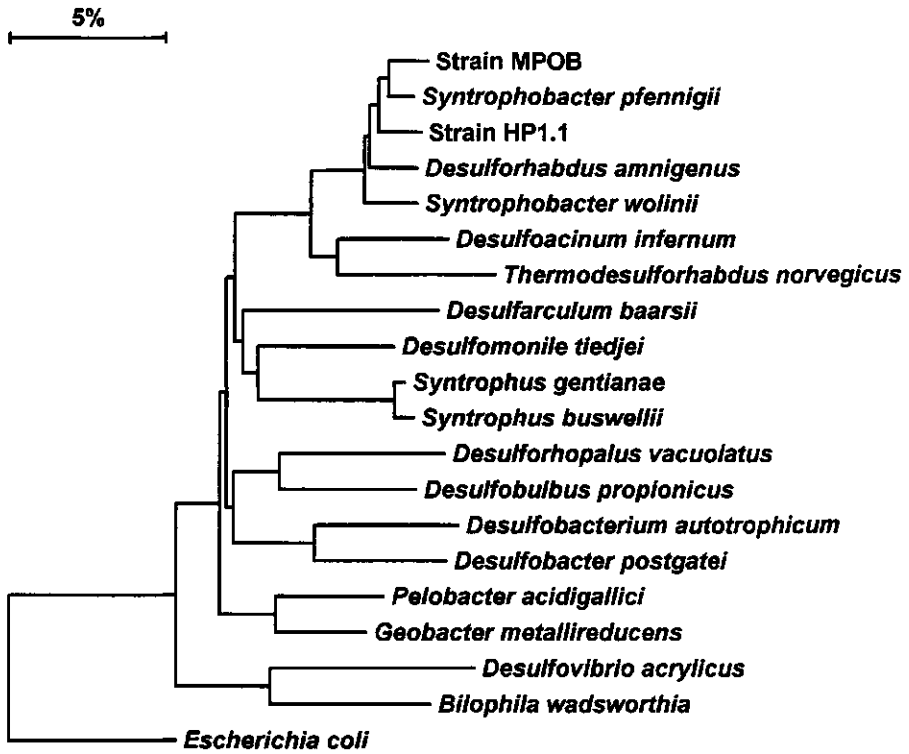

Fig. 2. Phylogenetic dendrogram based on 16 S rRNA gene sequence comparison indicating that syntrophic propionate-oxidizing bacteria form a group within the delta subclass of proteobacteria. The tree is based on a distance analysis using only those nucleotides that could unambiguously be aligned and was constructed via the neighbor-joining method as implemented in TREECON for Windows. The scale bar represents 5 % estimated sequence divergence.

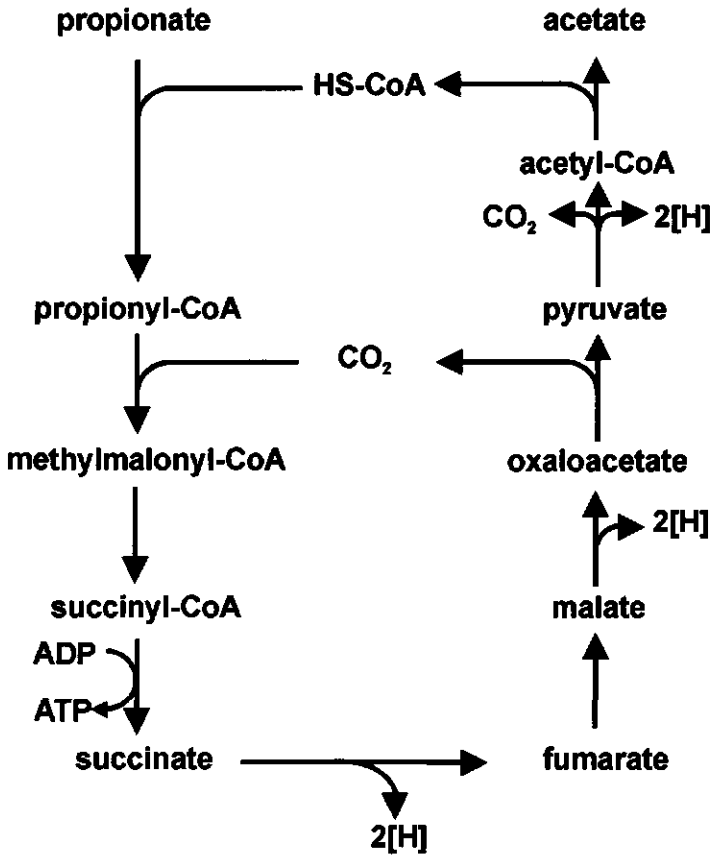

Fig. 3. Methylmalonyl-CoA pathway involved in syntrophic propionate-oxidation in strain MPOB (Houwen et al. 1987; Plugge et al. 1993).

Propionate oxidation through the methylmalonyl-CoA pathway releases electrons in three reactions, namely the oxidation of succinate to fumarate ($E^{o'}$= +30 mV), the oxidation of malate to oxaloacetate ($E^{o'}$= -176 mV), and the oxidation of pyruvate to acetyl-CoA ($E^{o'}$= -470 mV). These oxidation reactions are in equilibrium at hydrogen partial pressures of 10^{-15}, 10^{-8} and >1 bar, respectively (Schink 1997; Schink and

Friedrich 1994). At a hydrogen partial pressure of 10^{-4} to 10^{-5} bar, which is common in methanogenic environments (Zehnder et al. 1982), the electrons of pyruvate and (presumably) malate oxidation can be released as molecular hydrogen. However, the hydrogen partial pressure cannot be maintained low enough by the methanogenic partner to allow the propionate-oxidizing bacterium direct proton reduction with the electrons released during the oxidation of succinate to fumarate. Therefore, it has been hypothesized that the electrons which are released at the oxidation of succinate have to be shifted to a lower redox potential via reversed electron transport, which would be driven by the hydrolysis of $^2/_3$ ATP (Thauer and Morris 1984; Schink 1992). Because the methylmalonyl-CoA pathway yields one ATP via substrate-level phosphorylation, only $^1/_3$ ATP is left for growth if such a reversed electron transport is occurring.

Reversed electron transport in syntrophic conversions

A general characteristic of syntrophic conversions is the presence of at least one energetically very unfavourable oxidation step in the metabolism, which is thought to be driven by an energy-consuming reversed electron transport process. For syntrophic butyrate-, benzoate- and glycolate-oxidation, the involvement of such an ATP-consuming reversed electron transport has been studied by localization of the involved enzymes, by investigation of the effect of ionophores and ATPase-inhibitors on the oxidation reaction, and by determination of the presence of electron carriers. In syntrophic butyrate-oxidation, the oxidation of butyryl-CoA to crotonyl-CoA coupled to proton reduction requires a hydrogen partial pressure of approximately 10^{-10} bar, which is too low to be established by methanogens (Schink 1997). The enzymes involved in the oxidation of butyryl-CoA, namely butyryl-CoA dehydrogenase and hydrogenase, were found to be partly membrane-associated in *Syntrophomonas wolfei*. Furthermore, hydrogen production from butyrate by intact *S. wolfei* cells was inhibited in the presence of the protonophore carbonylcyanide-m-chlorophenyl-hydrazone (CCCP) or the ATPase inhibitor N, N' dicyclohexylcarbodiimide (DCCD), indicating that an ATP-dependent step was involved in this process. In *S. wolfei* only menaquinone-7 was detected, but it could not be determined whether this electron carrier is involved in a reversed electron transport mechanism (Wallrabenstein and

Schink 1994). More direct evidence for the involvement of reversed electron transport can be obtained via studies with membrane vesicles. However, the preparation of active inside-out vesicles of *S. wolfei* cells appeared to be not possible (Wallrabenstein and Schink 1994).

In syntrophic benzoate-oxidation, the oxidation of glutaryl-CoA to glutaconyl-CoA is the energetically most difficult reaction. Wallrabenstein and Schink (1994) investigated the involvement of an ATP-driven reversed electron transport in this conversion by *Syntrophus buswellii*. Exact localization of glutaryl-CoA dehydrogenase, which is involved in the proposed reversed electron transport, was not possible in *S. buswellii* because of its low activity. The formation of hydrogen from benzoate by cell suspensions of *S. buswellii* was inhibited by CCCP and DCCD. This suggested that the conversion of benzoate requires an intact proton potential that is maintained by the hydrolysis of ATP. However, positive control experiments could not be performed. Therefore, it cannot be excluded that the protonophore or ATPase inhibitor had an effect on substrate uptake instead of hydrogen formation from benzoate. *S. buswellii* contains a menaquinone as electron carrier, which possibly is involved in reversed electron transport. No cytochromes were detected in the bacterium (Wallrabenstein and Schink 1994).

The best-studied case of the involvement of reversed electron transport in syntrophic conversions is for syntrophic glycolate-oxidation, in which proton reduction coupled to the oxidation of glycolate to glyoxylate is energetically unfavourable. The enzymes glycolate dehydrogenase and hydrogenase, which may be involved in reversed electron transport, were found to be membrane-bound in the syntrophic glycolate-oxidizing bacterium *Syntrophobotulus glycolicus* strain FlGlyR (Friedrich and Schink 1993; Friedrich et al. 1996). Hydrogen formation from glycolate in membrane vesicles of strain FlGlyR occurred only in the presence of ATP. Addition of CCCP or other protonophores and the ATPase inhibitors DCCD or diethylstilbestrol abolished hydrogen formation entirely. This indicated that a membrane potential is required for hydrogen formation from glycolate, and that the process is driven by ATP-hydrolysis. The electron carriers menaquinone 7, 8, 9, and 10 were found in the membranes of strain FlGlyR, with MK-9 as the most dominant one. However, the

involvement of these menaquinones in the reversed electron transport could not be demonstrated unambiguously (Friedrich and Schink 1993). In addition to syntrophic glycolate-oxidation, strain FlGlyR is able to grow in pure culture by glyoxylate fermentation. In this case, the bacterium is able to reduce glyoxylate with hydrogen as the electron donor, which is the reversed conversion of the energetically unfavourable oxidation of glycolate to glyoxylate. Studies with membrane vesicles of strain FlGlyR demonstrated that ATP-formation was uncoupled from glyoxylate reduction in the presence of protonophores or ATPase inhibitors. This indicated that ATP is formed via electron transport phosphorylation at the reduction of glyoxylate with hydrogen, and that this process is the reversal of the ATP-driven reversed electron transport mechanism which is involved in the oxidation of glycolate (Friedrich and Schink 1995).

Growth of syntrophic acetogenic bacteria in pure culture

Bacteria formerly regarded as obligate syntrophs have also been shown to grow in pure culture with substrates that are more oxidized than the substrates used in the case of syntrophic growth. The syntrophic butyrate-oxidizing bacteria *Syntrophomonas wolfei* and *Syntrophospora bryantii* were found to grow in pure culture by the dismutation of crotonate to acetate and butyrate (Beaty and McInerney 1987; Zhao et al. 1990). *Syntrophus buswellii* and *S. gentianae*, which are syntrophic benzoate-oxidizing bacteria, appeared to grow in pure culture with crotonate, and hydroquinone or gentisate, respectively (Wallrabenstein et al. 1995a). The syntrophic glycolate-oxidizing bacterium *Syntrophobotulus glycolicus* strain FlGlyR could be grown in pure culture by glyoxylate fermentation (Friedrich and Schink 1995). In the absence of methanogenic archaea, the syntrophic propionate-oxidizing bacteria *Syntrophobacter wolinii*, *S. pfennigii*, strain HP1.1, and strain MPOB were able to couple propionate oxidation to sulfate reduction (Wallrabenstein et al. 1994, 1995b; Zellner et al. 1996; Chapter 2 of this thesis). This coincides very well with their phylogenetic relationship with sulfate-reducing bacteria. In addition, *S. wolinii* is able to grow in pure culture with pyruvate (Wallrabenstein et al. 1994), *S. pfennigii* with lactate and sulfate (Wallrabenstein et al. 1995b), and strain MPOB by fumarate disproportionation and by

fumarate reduction with propionate, hydrogen or formate as the electron donor (Stams et al. 1993).

Growth of syntrophs in pure culture allows detailed biochemical investigation of their metabolism. So far, the enzyme acetoacetyl-CoA reductase of *S. wolfei* was partly purified and characterized (Amos and McInerney 1993), the pathway of crotonate metabolism in *S. wolfei* was investigated via enzyme activity measurements (McInerney and Wofford 1992), and the pathway of fumarate disproportionation in strain MPOB was studied (Plugge et al. 1993). By using NMR techniques with labelled substrates, and by enzyme measurements it was shown that the acetyl-CoA cleavage pathway is involved in fumarate disproportionation in strain MPOB (Fig. 4). This pathway is also present in some sulfate-reducing bacteria such as *Desulfobacterium autotrophicum* and *Desulfotomaculum acetoxidans* (Schauder et al. 1986; Spormann and Thauer 1988), and is the reversal of the pathway of acetate formation in homoacetogenic bacteria (Diekert and Wohlfarth 1993).

In this thesis research the metabolism of the syntrophic propionate-oxidizing bacterium strain MPOB was studied in detail. To obtain more insight into the oxidation of propionate by strain MPOB, and the energetically unfavourable oxidation of succinate to fumarate, the enzymes fumarate reductase, fumarase, and malate dehydrogenase were isolated and characterized. Below, a short overview is given about what has been reported in literature on these enzymes.

Fumarate reductase/ succinate dehydrogenase

Fumarate reductase catalyzes the reduction of fumarate to succinate, while the reversed reaction is catalyzed by succinate dehydrogenase (Fig. 5A). Both enzymes are structurally related. Most fumarate reductases and succinate dehydrogenases are membrane-bound enzymes, which consist of three or four non-identical subunits. The largest subunit (60-75 kDa) contains covalently bound flavin adenine dinucleotide (FAD) and the substrate-binding site. The second subunit (24-33 kDa) contains three iron-sulfur clusters of the [2Fe-2S], [4Fe-4S] and [3Fe-4S] type. These two subunits form the membrane-extrinsic catalytic domain of the enzyme and are anchored in the

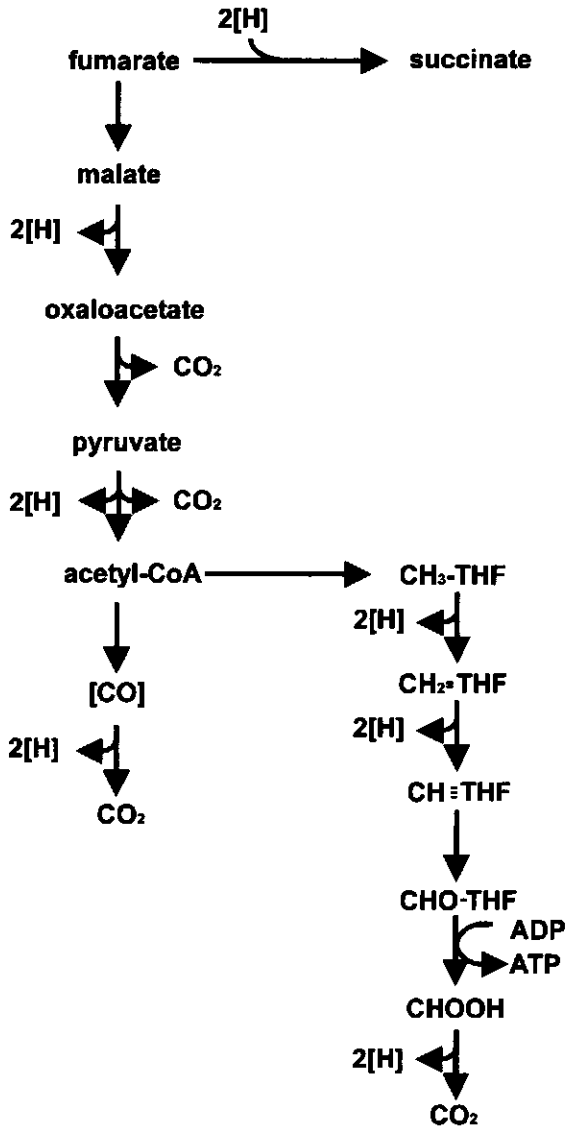

Fig.4. Pathway of fumarate disproportionation in strain MPOB. THF, tetrahydrofolate. According to Plugge et al. (1993).

membrane by one or two smaller hydrophobic subunits. In addition, these membrane anchor subunits, which are usually associated with a cytochrome-*b*, are involved in quinone reduction and oxidation (reviewed by Kröger 1978; Cole et al. 1985; Ackrell et al. 1992; van Hellemond and Tielens 1994). Ubiquinone is usually the electron acceptor of succinate oxidation, while reduced menaquinone serves as the electron donor of fumarate reductase. However, succinate dehydrogenase of *Bacillus subtilis* uses menaquinone as the electron acceptor (Lemma et al. 1991).

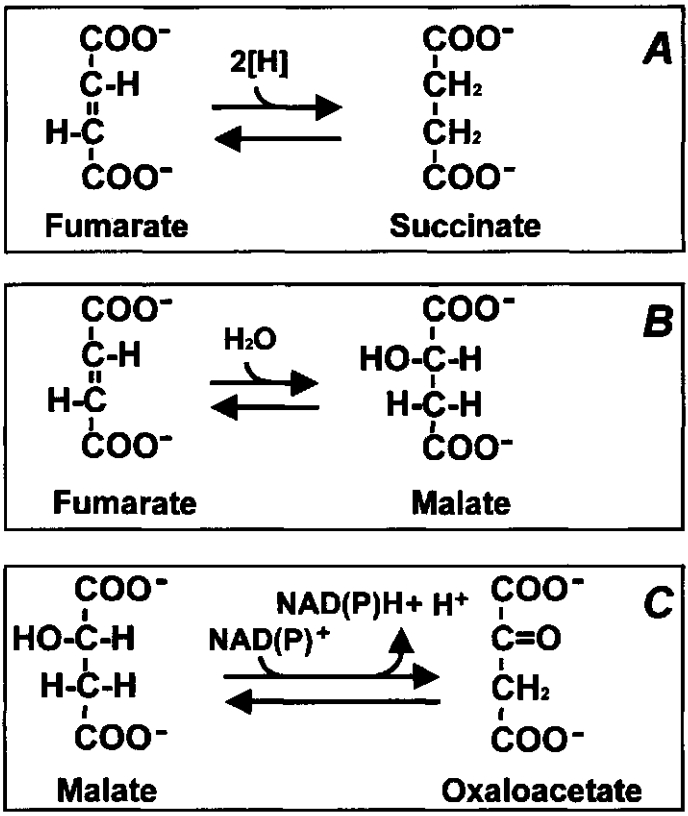

Fig. 5. Reactions catalyzed by **A:** fumarate reductase and succinate dehydrogenase, **B:** fumarase, and **C:** malate dehydrogenase.

Not all fumarate reductases have the structural composition as described above. The sulfate-reducing bacterium *Desulfovibrio multispirans* contains a fumarate reductase which consists of four subunits with molecular masses of 46, 32, 30, and 27 kDa. The FAD component is non-covalently bound to the largest subunit of the enzyme (He et al. 1986). Another fumarate reductase with uncommon properties is that of *Methanobacterium thermoautotrophicum*; the enzyme uses 2-mercaptoethanesulfonic acid (HS-CoM) and *N*-(7-mercaptoheptanoyl)threonine-O-phosphate (HS-HTP) as electron donors, and consists of only two subunits with molecular masses of 50 and 58 kDa (Bobik and Wolfe 1989). The most unique fumarate reductase is that of *Shewanella putrefaciens*. This bacterium contains a soluble fumarate reductase (Myers and Myers 1992), which is in fact a flavocytochrome *c* with fumarate reductase activity (Morris et al. 1994). Flavocytochromes *c* of other organisms do not contain fumarate reductase activity (Pealing et al. 1992). The enzyme is composed of a single subunit (63 kDa) with non-covalently bound FAD, contains no iron-sulfur clusters, and is related to the flavin-containing subunits of the commonly occurring fumarate reductases and succinate dehydrogenases (Pealing et al. 1992; Morris et al. 1994).

Fumarate reductase and succinate dehydrogenase are not only structurally, but also genetically related. Complete DNA sequences have been obtained for the fumarate reductase genes from e.g. *Escherichia coli* (Cole 1982; Cole et al. 1982), *Wolinella succinogenes* (Körtner et al. 1990; Lauterbach et al. 1990), and *Proteus vulgaris* (Cole 1987), and for the succinate dehydrogenase genes from e.g. *E. coli* (Darlison and Guest 1984; Wood et al. 1984) and *B. subtilis* (Magnusson et al. 1986; Phillips et al. 1987). All these genes are arranged as operons. The genes that encode the largest subunit of fumarate reductase and succinate dehydrogenase show striking sequence homology, which is concentrated in eleven domains (Fig. 6). Most of these domains are involved in substrate binding and the binding of FAD (Lauterbach et al. 1990). In the genes that encode the iron-sulfur subunit, eleven cysteine residues are highly conserved. These cysteines provide the ligands to the three iron-sulfur clusters (Lauterbach et al. 1990). On the basis of their high sequence similarity, it seems likely that the genes that code for the catalytic domain of fumarate reductase and succinate dehydrogenase arose from a common ancestor or resulted from a gene duplication

(Cole et al. 1982; Wood et al. 1984). In contrast to the catalytic subunits, less similarity is found among the membrane anchor subunits of the different fumarate reductases and succinate dehydrogenases. Even the number of membrane anchor subunits varies from one to two.

Although fumarate reductase and succinate dehydrogenase are closely related enzymes which are usually able to catalyze both fumarate reduction and succinate oxidation, some facultatively anaerobic bacteria such as *E. coli* and *Bacillus macerans* contain two distinct enzymes (Cole 1982; Darlison and Guest 1984; Schirawski and Unden 1995). Succinate dehydrogenase is synthesized during aerobic growth where it functions in the citric acid cycle. In contrast, fumarate reductase is synthesized under anaerobic growth conditions where it participates in anaerobic respiration with fumarate as the electron acceptor. The expression of the fumarate reductase and succinate dehydrogenase genes in *E. coli* is regulated by various transcriptional regulator systems: the cAMP receptor protein system (CRP), the aerobic respiration control system (ARC), the fumarate and nitrate reduction system (FNR) and the nitrate reduction system (NAR). At low oxygen concentrations, the expression of the succinate dehydrogenase genes is repressed by the ARC system and that of the fumarate reductase genes is induced by the transcriptional regulator FNR (reviewed by Unden et al. 1994). In *E. coli*, nitrate respiration is preferred to fumarate respiration. Therefore, the expression of the fumarate reductase genes is repressed by the NAR system if nitrate is present under anaerobic growth conditions (Jones and Gunsalus 1985, 1987). Since no biochemical data have been reported, it is not known whether strictly anaerobic microorganisms contain distinct fumarate reductases and succinate dehydrogenases, or only a single enzyme that is involved in both fumarate reduction and succinate oxidation.

Fig. 6. Alignment of the amino acid sequences of the flavin subunits of the fumarate reductases of *Wolinella succinogenes* (Lauterbach et al. 1990), *Escherichia coli* (Cole 1982), and *Proteus vulgaris* (Cole 1987), and the succinate dehydrogenases of *Escherichia coli* (Wood et al. 1984), and *Bacillus subtilis* (Phillips et al. 1987). Conserved regions are marked with boxes.
Frd, fumarate reductase; Sdh, succinate dehydrogenase. Adapted from Lauterbach et al. 1990.

Fumarase

Fumarase, which catalyzes the interconversion of fumarate and L-malate (Fig. 5B), is widely distributed among microorganisms, animals, and plants because of its role in the citric acid cycle. Two classes of fumarases are known. The class I fumarases are homodimers with a molecular mass of 120 kDa (2 x 60 kDa), which are oxygen-sensitive and contain a [4Fe-4S] iron-sulfur cluster. Class I fumarases are members of the family of iron-dependent hydrolyases, which includes aconitase (Kennedy et al. 1983), serine dehydratase (Grabowski and Buckel 1991), and maleate hydratase (Dreyer 1985). Examples of class I fumarases are fumarase A and B of *Escherichia coli* (Miles and Guest 1984; Bell et al. 1989; Ueda et al. 1991; Flint et al. 1992), fumarase of the alga *Euglena gracilis* (Shibata et al. 1985) and $fumA_{Bst}$ of *Bacillus stearothermophilus* (Reany et al. 1993). Class II fumarases are oxygen-stable homotetramers of 200 kDa (4 x 50 kDa) and do not contain an iron-sulfur cluster. Examples of class II fumarases are fumarase C of *E. coli* (Woods et al. 1986) and the fumarases of *Bacillus subtilis* (Miles and Guest 1985), *Saccharomyces cerevisiae* (Wu and Tzagoloff 1987), *Sulfolobus solfataricus* (Puchegger et al. 1990; Colombo et al. 1994), and mammals.

For a long time, it was assumed that iron-sulfur clusters in proteins only had a role in redox reactions. However, the discovery that aconitase and other hydrolyases contain a [4Fe-4S] cluster which has a role in the catalytic mechanism, demonstrated that iron-sulfur clusters could function in non-redox reactions as well (Emptage et al. 1983). Like other hydrolyases, class I fumarases contain a [4Fe-4S] cluster which participates in catalysis by activating the hydroxyl group of the substrate to facilitate the elimination or attachment, depending on the direction of the conversion (Flint et al. 1992). Upon inactivation of the enzyme in the presence of oxygen, the [4Fe-4S] cluster is converted into a [3Fe-4S] cluster (Flint et al. 1993). The class II fumarases do not contain an iron-sulfur cluster. Therefore, their catalytic mechanism is probably different from that of the class I fumarases. Currently, it is not clear why fumarases with as well as without iron-sulfur clusters exist. Some years ago, it was discovered that the iron-sulfur cluster containing aconitase functions as an iron regulatory element-binding protein in the regulation of iron uptake. When the enzyme is devoid

of its iron-sulfur cluster it binds to iron regulatory elements in the cell; this was not observed when the cluster is present. In this way, the state of the iron-sulfur cluster of the aconitase regulates the uptake of iron (Haile et al 1992; Kennedy et al 1992). It may be speculated that the iron-sulfur cluster containing fumarases also have such an additional regulatory role in iron uptake, but so far there is no experimental evidence for this possibility (Flint 1994).

Most organisms contain only one fumarase gene, which usually encodes a class II type fumarase. *E. coli*, however, possesses three fumarase genes, *fum A*, *fum B* and *fum C* (Guest and Roberts 1983; Guest et al. 1985), with differentially regulated promoters. *Fum A* is activated under aerobic conditions by the cyclic AMP-CRP complex and repressed anaerobically by the ArcA regulator (Park and Gunsalus 1995), whereas *fum B* is controlled by the anaerobic transcriptional activator FNR (Woods and Guest 1987). The gene products of *fum A* and *fum B*, fumarase A and fumarase B, are closely related; the amino acid sequences are 90 % identical (Bell et al. 1989). The *fum C* gene product is expressed under both aerobic and anaerobic growth conditions and its expression is controlled by the *soxRS* regulon, which acts as a defensive system at oxidative stress. Possibly, fumarase C functions as an alternative fumarase that can substitute for fumarase A and B, which are susceptible to oxidative stress conditions (Liochev and Fridovich 1992; Park and Gunsalus 1995).

Malate dehydrogenase

Malate dehydrogenase, a key metabolic enzyme in most organisms, catalyzes the reversible conversion of L-malate to oxaloacetate, using NAD or NADP as cofactor (Fig. 5C). An exception is the malate dehydrogenase of *Desulfobacter postgatei*, which does not couple with NAD or NADP but with a quinone. Moreover, this enzyme is, in contrast to other known malate dehydrogenases, membrane-bound (Brandis-Heep et al. 1983). Malate dehydrogenase has been studied extensively in many organisms. The enzymes that have been isolated are either homodimers or homotetramers with a subunit molecular mass of 30-38 kDa (Murphy et al. 1967; Banaszak and Bradshaw 1975; Sundaram et al. 1980). Amino acid sequence comparison revealed the presence of residues which are conserved in most malate dehydrogenases. The N-terminal part

of the sequence contains the cofactor-binding site with the highly conserved sequence motif GAXGXXG/A (Charnock et al. 1992). The active site is located near the C-terminal end of the subunit sequence. This domain is less conserved among the malate dehydrogenases than the cofactor-binding site, except for six amino acid residues that are directly involved in substrate binding and catalysis (McAlister-Henn 1988).

Because amino acid sequence data of malate dehydrogenase are available from many bacteria, archaea and eukarya, the enzyme is often chosen as model enzyme to study molecular adaptation to extreme environmental conditions like high temperature (Nishiyama et al. 1986), low temperature (Ohkuma et al. 1996), or high salt concentrations (Dym et al. 1995). The availability of amino acid sequences of many malate dehydrogenases also allowed the investigation of the evolutionary history of this enzyme (Joh et al. 1987; McAlister-Henn 1988; Honka et al. 1990). It is thought that three different lineages of malate dehydrogenase separated early in the evolution. The first lineage is represented by cytosolic malate dehydrogenases of eukaryotes and malate dehydrogenases of some bacteria. Mitochondrial malate dehydrogenases of eukaryotes and the malate dehydrogenases of other bacteria represent the second lineage. The third lineage is represented by the archaeal malate dehydrogenases (Joh et al. 1987; McAlister-Henn 1988; Honka et al. 1990; Cendrin et al. 1993).

Outline of this thesis

The aim of the research presented in this thesis was to study the biochemistry and bioenergetics of syntrophic propionate-oxidation. Special attention was given to the energetically most difficult conversion in syntrophic propionate-oxidation, namely the oxidation of succinate to fumarate coupled to hydrogen or formate formation. The syntrophic propionate-oxidizing bacterium strain MPOB, which can grow in the absence of a methanogenic partner, was used as the model-organism in these studies. Strain MPOB was isolated from granular sludge of an upflow anaerobic sludge bed reactor treating wastewater from a sugar refinery. By using 16S rRNA-based oligonucleotide probes, it has been shown that strain MPOB is the most abundant propionate-oxidizing bacterium in this granular sludge, and that the bacterium is juxtapositioned with methanogens in syntrophic microcolonies (Harmsen et al. 1996).

Syntrophobacter wolinii and *S. pfennigii*, which are related to strain MPOB, had been shown to grow in pure culture by coupling propionate-oxidation to sulfate-reduction. In chapter 2 is presented that strain MPOB is also able to grow by sulfate-reduction. In chapter 3 and 4 the purification and characterization of fumarase and malate dehydrogenase from strain MPOB are described, respectively. Chapter 5 describes the isolation of fumarate reductase of strain MPOB; the iron-sulfur cluster composition, N-terminal amino acid sequence, and some other properties of this enzyme are presented. Energetics of the fumarate metabolism of strain MPOB, and the investigation of the electron transport chain that is involved in fumarate reduction in strain MPOB are reported in chapter 6. Chapter 7 describes several physiological properties of strain MPOB, and its classification as a new member of the genus *Syntrophobacter*, *S. fumaroxidans*. Finally, the results of this thesis are discussed and summarized in Chapter 8.

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2

Sulfate reduction by a syntrophic propionate-oxidizing bacterium

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ABSTRACT

The syntrophic propionate-oxidizing bacterium strain MPOB was able to grow in the absence of methanogens by coupling the oxidation of propionate to the reduction of sulfate. Growth on propionate plus sulfate was very slow (μ = 0.024 day^{-1}). An average growth yield was found of 1.5 g (dry weight) per mol of propionate. Strain MPOB grew even slower than other sulfate-reducing syntrophic propionate-oxidizing bacteria. The growth rates and growth yields of strict sulfate-reducing bacteria (*Desulfobulbus* sp.) grown on propionate plus sulfate are considerably higher.

INTRODUCTION

In methanogenic environments reduced organic compounds like fatty acids, primary alcohols and some aromatic acids are degraded by consortia of acetogenic bacteria and methanogenic archaea. The conversion of most of these compounds to acetate, CO_2, H_2 and formate is energetically unfavourable unless the product concentrations are kept low. This results in obligate syntrophic growth of the acetogenic bacteria with methanogens (Schink 1992; Stams 1994).

The amount of energy which can be obtained from syntrophic oxidation reactions is relatively low (Schink 1992). It has been proposed that part of the ATP generated by substrate-level phosphorylation has to be invested in a reversed electron transport to drive the energetically unfavourable redox reactions with protons as the electron acceptor (Thauer and Morris 1984). As a result only a small amount of ATP is available for growth. Most acetogenic bacteria are not obligate syntrophs, but are also able to grow on some other substrates in the absence of a partner organism. The syntrophic butyrate-oxidizing bacteria *Syntrophomonas wolfei* and *Syntrophospora bryantii*, and the syntrophic benzoate-oxidizer *Syntrophus buswellii* grow in pure culture with crotonate and some unsaturated fatty acids (Beaty and McInerney 1987; Amos and McInerney 1990; Zhao et al. 1990; McInerney et al. 1992). In addition *Syntrophospora bryantii* is able to grow with pentenoate (Dong et al. 1994). A

characteristic of almost all syntrophic propionate-oxidizing bacteria that have been isolated up to now is their ability to couple the oxidation of propionate to the reduction of sulfate (Dörner 1992; Wallrabenstein et al. 1994). Phylogenetic analysis of *Syntrophobacter wolinii* has revealed that this bacterium is indeed related with sulfate-reducing bacteria (Harmsen et al. 1993).

Here we present the growth of the mesophilic propionate-oxidizing bacterium MPOB on propionate and sulfate. This bacterium is also able to couple the oxidation of propionate to the reduction of fumarate. In addition, strain MPOB ferments fumarate to succinate and CO_2 or reduces fumarate to succinate with hydrogen or formate as electron donor (Stams et al. 1993). Recently a phylogenetic analysis of this bacterium also showed a relatedness with *Syntrophobacter wolinii* and sulfate-reducing bacteria (Harmsen et al. 1995).

MATERIALS AND METHODS

Organism and cultivation. Strain MPOB was grown at 37 °C in 1-L serum bottles containing 500 ml of a bicarbonate-buffered medium as described before (Stams et al. 1993). Propionate and sulfate were added from stock solutions (1 M) to a final concentration of 20 mM. Media were inoculated with 10 % (v/v) of substrate-adapted cultures.

Analytical methods. During growth samples were taken for analysis of substrate and product concentrations. Propionate and acetate were measured by gas chromatography using a CP9000 gas chromatograph (Chrompack, Middelburg, The Netherlands) equipped with a flame ionization detector and molecular sieve as described previously (Stams et al. 1993). Sulfide was determined colorimetrically (Trüper and Schlegel 1964). Bacterial growth was followed by protein determination. Cell pellets of 5 ml culture samples were resuspended in 1 ml 1-M NaOH. After heating at 100 °C for 15 min the samples were treated further according to the method of Bradford (1976). Bovine serum albumin was used as a standard.

RESULTS

The syntrophic propionate-oxidizing bacterium strain MPOB is able to couple the oxidation of propionate to acetate to the reduction of sulfate to sulfide (Fig. 1). The stoichiometry of the conversion is shown in Table 1. Strain MPOB grew with a very low growth rate of μ=0.024 day^{-1}. This value was determined from the logarithmic phase in the acetate accumulation from four separate cultures. The growth yield was determined from the total protein content of the culture. It was assumed that 1 g of dry cells corresponds to 0.5 g of protein. An average yield value was found of 1.5 g dry weight per mol propionate.

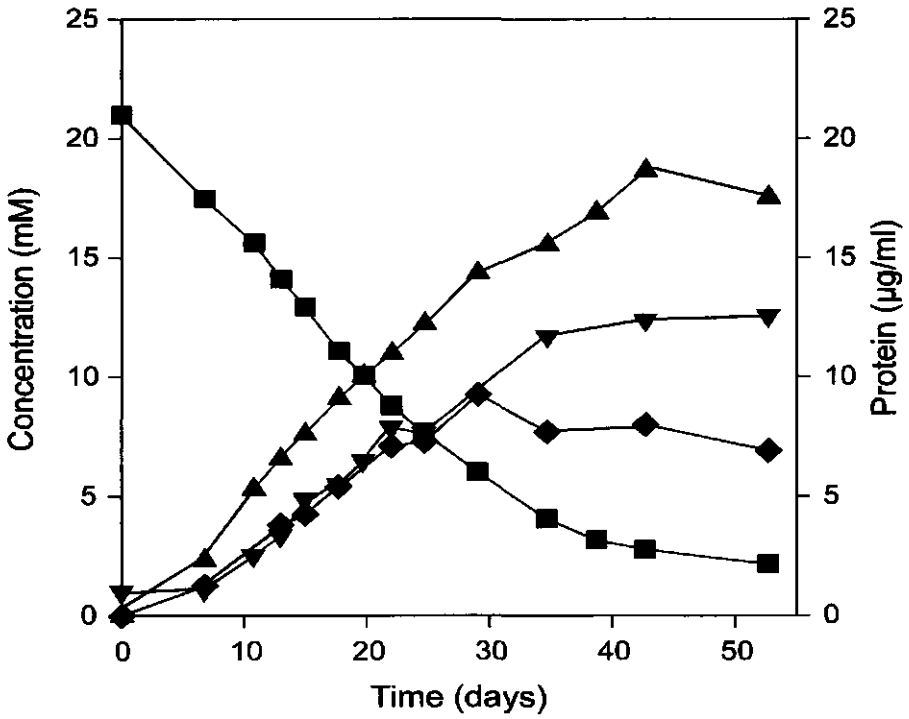

Fig. 1. Growth of the syntrophic propionate-oxidizing bacterium strain MPOB with propionate plus sulfate: (■) propionate; (▲) acetate; (▼) sulfide; (◆) protein.

Table 1. Stoichiometry of the conversion of propionate + sulfate by strain MPOB.

Substrates converted (mmol)		Products formed (mmol)		Carbon recovery (%)	Electron[a] recovery (%)
Propionate	Sulfate	Acetate	Sulfide		
9.2	n.d.[b]	8.6	5.3	95	88

[a]For the calculation of carbon and electron recovery the assumption was used that 1 g of dry cells corresponds to 0.5 g of protein. <$C_5H_7O_2N$> was used as the structural formula for biomass.
[b]n.d.=not determined.

DISCUSSION

Results presented here show that strain MPOB can couple the oxidation of propionate to the reduction of sulfate. These findings confirm the phylogenetic relatedness of strain MPOB with sulfate-reducing bacteria (Harmsen et al. 1995). Previously it was demonstrated that strain MPOB is also able to oxidize propionate to acetate with fumarate as electron acceptor (Stams et al. 1993). Fumarate reduction is a common property among sulfate-reducing bacteria (Widdel and Hansen 1992).

In Table 2 the growth rate and growth yield of strain MPOB on propionate plus sulfate was compared with those of other sulfate-reducing bacteria. Strain MPOB grew even much slower than other syntrophic propionate-oxidizing bacteria. The growth rates of the sulfate-reducing bacteria *Desulfobulbus propionicus* and *Desulfobulbus elongatus* on propionate plus sulfate are considerably higher.

Although both syntrophic propionate-oxidizers and propionate-degrading sulfate-reducers can couple the oxidation of propionate to acetate to the reduction of sulfate, they clearly represent different groups of bacteria. Propionate-degrading *Desulfobulbus* sp. cannot grow syntrophically (Laanbroek et al. 1982) and the

acetogenic bacteria prefer to oxidize propionate syntrophically when they are cocultured with *Desulfovibrio* sp. in the presence of sulfate (Boone and Bryant 1980;

Table 2. Growth rates and growth yields of syntrophic propionate-oxidizing bacteria and sulfate-reducing bacteria grown on propionate + sulfate.

Bacterium	Growth rate (day^{-1})	Growth yield (g dry weight/ mol substrate)	Reference
Syntrophic propionate-oxidizing bacteria			
Strain MPOB	0.024	1.5	This study
Syntrophobacter wolinii	0.062	n.d.[a]	Wallrabenstein et al. 1994
Strain KoProp1	0.063	3.0	Dörner 1992
Strain PW	0.23	n.d.	Wu et al. 1992
Propionate-oxidizing sulfate-reducing bacteria			
Desulfobulbus propionicus	0.89	3.8	Stams et al. 1984
	1.66	4.4	Widdel and Pfennig 1982
Desulfobulbus elongatus	1.39	n.d.	Samain et al. 1984

[a]n.d.=not determined.

Dörner 1992). However, strain PW, described by Wu et al. (1992), which is morphologically similar to *Desulfobulbus propionicus* is able to grow syntrophically on propionate. Strain PW grows much faster than the other syntrophic propionate-oxidizing bacteria.

It is not clear what the ecological importance is of the two different groups of bacteria in the degradation of propionate to acetate in anaerobic environments. The sulfate-reducing bacteria compete with the syntrophic acetogenic bacteria for propionate in anaerobic environments when sulfate is present. It is assumed that the sulfate-reducing bacteria out-compete the acetogenic bacteria in the presence of high sulfate concentrations, because of their better growth kinetic properties (Visser et al.

1993; Oude Elferink et al. 1994). At low sulfate concentrations, the affinity for propionate will determine the outcome of the competition between the two groups of bacteria. Differences in affinity for sulfate between different types of sulfate-reducing bacteria will also influence this competition. Studies in energy-limited chemostats have shown that *Desulfovibrio* sp. have a higher affinity for sulfate than *Desulfobulbus* sp. (Laanbroek et al. 1984). Therefore, it is likely that at low sulfate concentration propionate is oxidized syntrophically by consortia of acetogenic bacteria and sulfate-reducers rather than being oxidized directly by sulfate-reducing bacteria.

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3

Purification and characterization of fumarase from the syntrophic propionate-oxidizing bacterium strain MPOB

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ABSTRACT

Fumarase from the syntrophic propionate-oxidizing bacterium strain MPOB was purified 130-fold under anoxic conditions. The native enzyme had an apparent molecular mass of 114 kDa and was composed of two subunits of 60 kDa. The enzyme exhibited maximum activity at pH 8.5 and approximately 54 °C. The K_m values for fumarate and L-malate were 0.25 mM and 2.38 mM, respectively. Fumarase was inactivated by oxygen, but the activity could be restored by addition of Fe^{2+} and β-mercaptoethanol under anoxic conditions. EPR spectroscopy of the purified enzyme revealed the presence of a [3Fe-4S] cluster. Under reducing conditions, only a trace amount of a [4Fe-4S] cluster was detected. Addition of fumarate resulted in a significant increase of this [4Fe-4S] signal. The N-terminal amino acid sequence showed similarity to the sequences of fumarase A and B of *Escherichia coli* (56%) and to the sequence of fumarase A of *Salmonella typhimurium* (63%).

INTRODUCTION

The anaerobic oxidation of propionate to acetate, carbon dioxide, hydrogen, and/or formate is an important process in methanogenic environments (Gujer and Zehnder 1983). Under thermodynamical standard conditions, propionate oxidation is endergonic ($\Delta G^{o'}$ = +76 kJ/mol propionate; Thauer et al. 1977). Bacteria are only able to grow by this conversion when the concentration of the products is kept low. This results in obligate syntrophic growth of propionate-oxidizing bacteria with hydrogen-, formate-, and acetate-consuming methanogens (Schink 1992; Stams 1994). Because of their syntrophic growth, little is known about the biochemistry of propionate-oxidizing bacteria. However, the propionate-oxidizing strain MPOB is able to grow in the absence of a syntrophic partner organism by the fermentation of fumarate (Plugge et al. 1993; Stams et al. 1993).

Enzyme measurements and ^{13}C-NMR experiments have indicated that the methylmalonyl-CoA pathway is involved in propionate oxidation by syntrophic cultures (Koch 1983; Houwen et al. 1987, 1990, 1991). Characteristic of this pathway is the coupling of the carboxylation of propionyl-CoA to methylmalonyl-CoA to the decarboxylation of

oxaloacetate to pyruvate via a transcarboxylase. During the oxidation of propionate, reducing equivalents are released in the conversions of succinate to fumarate, malate to oxaloacetate, and pyruvate to acetyl-CoA. These reducing equivalents are used to produce molecular hydrogen or formate, which have to be consumed by methanogens. The energetically most unfavourable reaction in this pathway is the oxidation of succinate to fumarate coupled to the reduction of H^+ to hydrogen or to the reduction of bicarbonate to formate. It can be calculated that this conversion becomes just feasible at a hydrogen partial pressure of about 10 Pa and/or a formate concentration of 10 μM, a ratio between succinate and fumarate in the cell of 10^4, and a membrane potential of 180 mV. Therefore, it has been proposed that probably two-thirds of the ATP generated by substrate-level phosphorylation during propionate oxidation has to be used to drive this energetically unfavourable redox reaction, via a reversed electron transport (Thauer and Morris 1984). To pull the reaction of succinate oxidation in the direction of the products, the hydrogen and the fumarate concentration has to be kept low. To remove fumarate effectively, the bacterium needs an active fumarase (EC 4.2.1.2), which catalyzes the reversible hydration of fumarate to L-malate, with a high affinity for fumarate. In this paper, we report on the purification and characterization of fumarase of strain MPOB. The properties of the enzyme from strain MPOB are compared with those of fumarases from other species.

MATERIALS AND METHODS

Organism and cultivation. Strain MPOB (DSM 10017) was cultured on 20 mM fumarate in 25-l carboys containing 20 l of a bicarbonate-buffered mineral medium as described by Stams et al. (1993). Cells were harvested anoxically by continuous-flow centrifugation (Biofuge 28RS, Heraeus Sepatech, Osterode, Germany) and stored under N_2 at –20 °C until used.

Purification of fumarase. All purification steps were performed at room temperature under strict anoxic conditions in an anaerobic chamber with N_2/H_2 (96:4;v/v) as gas phase. Traces of oxygen were removed by a platinum catalyst (gift of BASF, Arnhem, The Netherlands). Frozen cell paste (60 g wet weight) was thawed and washed twice in 100

mM Tris-HCl (pH 7.5). The cells were resuspended in 100 mM Tris-HCl (pH 7.5) containing 250 mM sucrose, 20 mM KCl, 5 mM $MgCl_2$ and 5 mM dithiothreitol and disrupted by passing twice through a French pressure cell at 110 MPa. Cell debris was removed by centrifugation for 20 min at 16,000 x *g*. The supernatant which formed the crude extract was centrifuged for 1.5 h at 140,000 x *g* and 4 °C to separate the membrane fraction from the cytoplasmic fraction.

The cytoplasmic fraction (90 ml) was applied in two steps to a column of Q-Sepharose (3.2 x 9.3 cm) equilibrated with 50 mM Tris-HCl (pH 7.6) containing 2.5 mM dithiothreitol (buffer A). Fumarase did not adsorb to the column material and was eluted with equilibration buffer (1 ml/min). After addition of $(NH_4)_2SO_4$ to a final concentration of 1 M, the enzyme solution was applied to a column of Phenyl-Sepharose (5.0 x 10.8 cm) equilibrated with 1 M $(NH_4)_2SO_4$ in buffer A. Protein was eluted in a 1,090-ml discontinuous linear gradient of 1-0 M $(NH_4)_2SO_4$ in buffer A at a flow rate of 2.0 ml/min. (1-0.35 M in 400 ml; 290 ml 0.35 M; 0.35-0 M in 400 ml). Fumarase eluted at 0.35 M $(NH_4)_2SO_4$. The fractions with the highest fumarase activity were pooled and concentrated in an Amicon ultrafiltration cell (Rotterdam, The Netherlands) with a PM10 filter. The concentrated enzyme solution (47.5 ml) was applied to a column (2.2 x 10 cm) of Matrex Gel Red A equilibrated with 20 mM Tris-HCl (pH 7.5) containing 2.5 mM dithiothreitol (buffer B). After the column was washed with buffer B to remove unbound material, a linear gradient of 0-1.5 M KCl in buffer B was applied at a flow rate of 1 ml/min. Fumarase eluted at 0.55 M KCl. The fractions containing fumarase activity were pooled and applied to a column of Superdex 200 (1.6 x 70.5 cm) equilibrated with buffer A containing 250 mM NaCl. Fumarase was eluted with a flow rate of 0.5 ml/min. The active fractions were stored under N_2 at 4 °C until used.

Determination of fumarase activity and protein concentration. Fumarase activity was determined spectrophotometrically by measuring the conversion of L-malate to fumarate or fumarate to L-malate at 250 nm and 37 °C as described by Stams et al. (1984). One unit of enzyme activity is defined as the amount of the enzyme that converts 1 μmol substrate per min under standard assay conditions. Protein was determined by the method of Bradford (1976) with bovine serum albumin as the standard.

Determination of molecular mass. The molecular mass of the native enzyme was estimated by gel filtration using a Superdex 200 column (1.6 x 70.5 cm) equilibrated with 50 mM Tris-HCl (pH 7.6) containing 250 mM NaCl and 2.5 mM dithiothreitol. The column was calibrated with the protein standards catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa). The void volume was determined with blue dextran.

The molecular mass of the subunits was determined by SDS-PAGE according to the method of Laemmli (1970). SDS gels were calibrated using a Pharmacia low-molecular-mass calibration kit containing phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Gels were stained with Coomassie brilliant blue G250.

pH and temperature optimum. The fumarase activity over the pH range from 3.0 - 8.0 was determined in a 0.1 M citric acid/ 0.2 M Na_2HPO_4 buffer. In the pH range from 8.0 - 13.0, a 0.1 M glycine/ NaCl buffer mixed with 0.1 M NaOH was used. The temperature optimum of fumarase was determined with a temperature-controlled Beckman DU-7500 diode-array spectrophotometer.

Electron paramagnetic resonance. EPR spectroscopy was carried out with a Bruker EPR 200 D spectrometer using peripheral equipment and data handling as described previously (Pierik and Hagen 1991). The modulation frequency was 100 kHz.

N-Terminal amino acid sequence determination. The N-terminal amino acid sequence was determined by Eurosequence (Groningen, The Netherlands) by automated Edman degradation with a protein sequencer (Applied Biosystems 477A, Warrington, U.K.). The amino acid derivatives were identified by HPLC (Applied Biosystems 120A).

Materials. Q-Sepharose, Phenyl-Sepharose, Superdex 200, and molecular mass standards for SDS-PAGE and gel filtration were purchased from Pharmacia Biotech (Roosendaal, The Netherlands). Matrex Gel Red A was obtained from Amicon (Rotterdam, The Netherlands).

RESULTS

Initial experiments showed that fumarase of strain MPOB was inactivated by molecular oxygen (data not shown). Therefore, the purification required strict anoxic conditions. In four steps, a 130-fold purified enzyme was obtained, with a recovery of 25% (Table 1). The enzyme was not purified to complete homogeneity; trace amounts of a few contaminating proteins remained visible on SDS polyacrylamide gels (Fig. 1). In the first two chromatographic steps, malate dehydrogenase copurified with fumarase. Fumarase was separated from malate dehydrogenase by chromatography on Matrex Red A. Fumarase was purified further by size-exclusion chromatography on Superdex 200. The apparent molecular mass of the native protein was 114 kDa, as estimated by gel filtration; SDS-PAGE (Fig. 1) revealed one type of subunit with a molecular mass of 60 kDa, suggesting that the native enzyme is a homodimer.

Table 1. Purification of fumarase from strain MPOB

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Cytoplasm	1,350	10,019	7.4	100	1
Q-Sepharose	488	7,991	16.4	80	2.2
Phenyl-sepharose	32.3	6,492	201	65	27.2
Matrex Gel Red A	3.4	2,784	819	28	111
Superdex 200	2.6	2,525	971	25	131

Fumarase showed a sharp pH optimum at pH 8.5. At pH values below 5.5 and above 11.5, no fumarase activity could be measured. The activity of the enzyme was maximal between 53 and 55 °C.

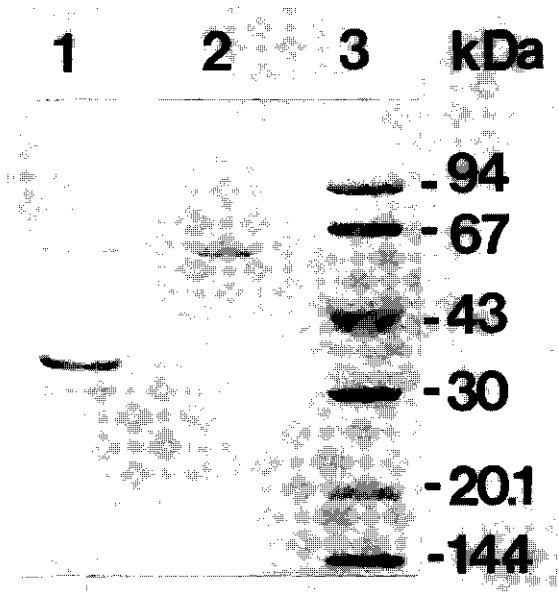

Fig. 1. SDS-PAGE (10%) of fumarase from strain MPOB. Lane 1, pooled fractions from Phenyl-Sepharose; lane 2, purified fumarase (4 μg); lane 3, low molecular mass markers. The gel was stained with Coomassie blue G250.

The kinetic properties of fumarase were determined at pH 7.5 and 37 °C. The reaction rate at various fumarate and L-malate concentrations followed Michaelis-Menten kinetics. The K_m of fumarase for L-malate was 2.38 mM. The K_m for fumarate was 0.25 mM. The k_{cat} for fumarate hydration and malate dehydration was 690 s^{-1} and 540 s^{-1}, respectively. Fumarase of strain MPOB showed no activity with D-malate, maleate, mesaconate, *cis*-aconitate and L-tartrate.

Fumarase activity was stable for at least one month when stored under N_2 at 4 °C. The enzyme lost 50% of its activity within 20 min when exposed to air at room temperature. Air-inactivated fumarase could be reactivated with ferrous ammonium sulfate and β-mercaptoethanol under anoxic conditions (Table 2). Zn^{2+} and Mn^{2+} could not replace Fe^{2+}. Reactivation was also observed with Fe^{2+} in the absence of β-mercaptoethanol. However, this may have been caused by the presence of dithiothreitol in the buffer.

Table 2. Reactivation of fumarase from strain MPOB. Air-inactivated fumarase was incubated with ferrous ammonium sulfate, $MnCl_2$, $ZnCl_2$, and β-mercaptoethanol at room temperature. After 1 h, the fumarase activity was measured in the different samples. The percentage reactivation was related to the original activity of fumarase before air-inactivation.

Effectors	Reactivation (%)
No addition	5
0.5 mM Fe^{2+} + 50 mM β-mercaptoethanol	74
5 mM Fe^{2+} + 50 mM β-mercaptoethanol	104
5 mM Fe^{2+}	100
50 mM β-mercaptoethanol	28
5 mM Mn^{2+} + 50 mM β-mercaptoethanol	13
5 mM Zn^{2+} + 50 mM β-mercaptoethanol	2

The EPR spectrum of the isolated protein in the presence of 2.5 mM dithiothreitol exhibited a weak, near-isotropic signal around $g = 2.02$, characteristic for a $[3Fe\text{-}4S]^{+}$ cluster (not shown). Upon reduction with 2 mM sodium dithionite, this signal disappeared and a very weak rhombic signal was observed (Fig. 2A), which was too weak to be quantified. Attempts were made to convert the [3Fe-4S] cluster to a [4Fe-4S] cluster by addition of Fe^{2+} ions. However, both the shape and the amplitude of the EPR signal did not change after addition of 5 mM ferrous ammonium sulfate under reducing conditions. Addition of methyl viologen to the enzyme under reducing conditions to enhance the reduction of the [4Fe-4S] cluster had no effect on the signal. However, the addition of 20 mM fumarate resulted in the appearance of a signal with effective g values of 2.03, 1.90 and 1.83 (Fig. 2B). No signals were found at lower field. The shape and temperature dependence of this signal point to the presence of a $[4Fe\text{-}4S]^{1+}$ cluster with S=1/2 ground state. The intensity of this signal corresponded to 12% of the total enzyme concentration.

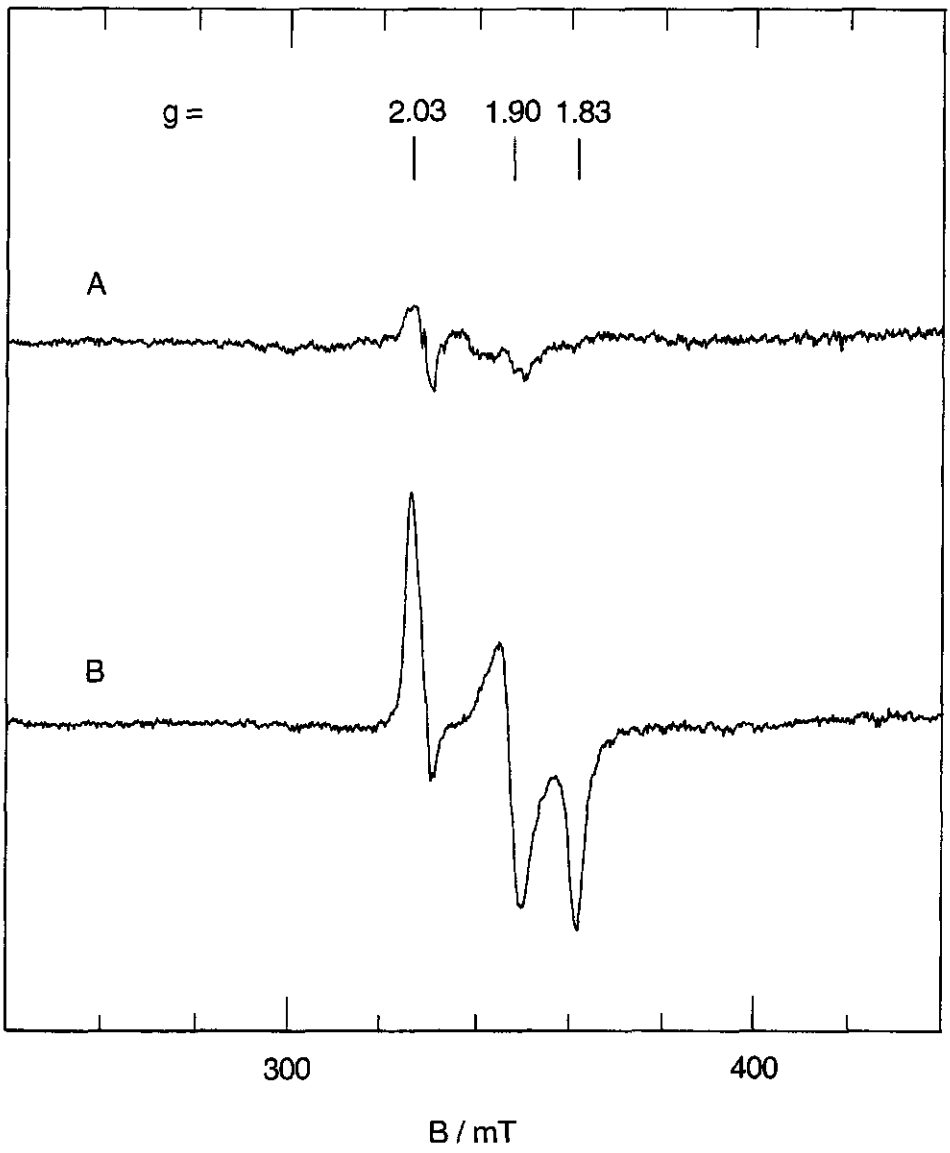

Fig. 2. EPR spectra of purified fumarase from strain MPOB. The protein concentration was 3.7 mg/ml in 50 mM Tris-HCl (pH 7.5). Trace A, dithionite-reduced fumarase, trace B, same as A except after addition of 20 mM fumarate. EPR conditions: microwave frequency, 9.181 GHz; microwave power, 12.5 mW; modulation amplitude, 1.6 mT; temperature, 13 K.

The first 16 amino acids of fumarase from strain MPOB showed similarity with the class I fumarase A and B from *Escherichia coli* (56%) (Miles and Guest 1984; Bell et al. 1989) and fumarase A from *Salmonella typhimurium* (63%) (Collins and Hackett 1991) (Fig. 3). No similarity was found with sequences of class II fumarases.

```
                                  *              **  *
Strain MPOB                       AEFVYQDPFPAWKDET
                                    :  ::  :::   ::  :
E. coli          fumarase A       SNKPFHYQAPFPLKKDDT
                                    :  ::  :::   ::  :
E. coli          fumarase B       MSNKPFIYQAPFPMGKDNT
                                    :  ::  :::   ::  :
S. typh          fumarase A       SNKPFFYQDPFPLKKDDT
```

Fig. 3. Comparison of the N-terminal amino acid sequence of fumarase from strain MPOB with those of fumarase A and B from *Escherichia coli* and fumarase A from *Salmonella typhimurium*. Amino acids marked with an *asterisk* could not be identified unambiguously. A *colon* (:) indicates identical residues.

DISCUSSION

Fumarase is widely distributed in plants, animals, and microorganisms because of its central role in the citric acid cycle. Two different classes of fumarase have been described (Woods et al. 1988; Yumoto and Tokushige 1988). Class I fumarases are oxygen-sensitive homodimers with a molecular mass of 120 kDa and contain an Fe-S cluster. They belong to the family of Fe-dependent hydrolyases, which includes aconitase (Kennedy et al. 1983; Beinert and Kennedy 1989), serine dehydratase (Grabowski and Buckel 1991), and dihydroxy-acid dehydratase (Flint et al. 1993). Examples are fumarase A and B of *Escherichia coli* (Ueda et al. 1991; Flint et al. 1992), fumarase of *Euglena gracilis* (Shibata et al. 1985) and $fumA_{Bst}$ of *Bacillus stearothermophilus* (Reany et al. 1993). Class II fumarases are oxygen-stable homotetramers with a molecular mass of 200 kDa and do not contain an Fe-S cluster. Class II fumarases include fumarase C of *E. coli* (Yumoto and Tokushige 1988), fumarase of *Sulfolobus solfataricus* (Puchegger et al. 1990; Colombo et

al. 1994), fumarase of *Saccharomyces cerevisiae* (Wu and Tzagoloff 1987), and mammalian fumarases. Based on its oxygen sensitivity, subunit molecular mass, dimeric structure, and N-terminal amino acid sequence, fumarase of strain MPOB clearly belongs to the class I fumarases.

The EPR spectrum of the isolated enzyme exhibited a signal typical for a [3Fe-4S] cluster. The EPR spectrum of the enzyme in the reduced state showed only a trace of a [4Fe-4S] cluster. It is known for many Fe-S enzymes that oxidative damage can lead to the formation of [3Fe-4S] clusters (Beinert and Thomson 1983). However, our experiments were carried out with highly active enzyme and the intensity of the [4Fe-4S] signal remained the same after addition of iron. Comparable results have been found for other Fe-dependent hydrolyases, for instance, L-serine dehydratase from *Peptostreptococcus asaccharolyticus* (Hofmeister et al. 1994). EPR spectroscopy has shown that this enzyme contains a $[3Fe\text{-}4S]^{+}$ cluster in its oxidized, inactive state. Incubation with Fe^{2+} under reducing conditions restores the activity, but a $[4Fe\text{-}4S]^{+}$ signal has never been observed. The authors have proposed that the catalytically active L-serine dehydratase contains a $[4Fe\text{-}4S]^{2+}$ cluster, but that either the reduction of the cluster is too slow, or the reduction potential is too low to be able to detect this cluster (Hofmeister et al. 1994). Flint et al. (1992) have been able to detect a [4Fe-4S] cluster in the reduced state of fumarase A of *E. coli*, although the spectrum integrated to only 0.05 spin/mol. The $[4Fe\text{-}4S]^{+}$ cluster exists as a mixture of S=1/2 and S=3/2 ground states. Addition of fumarate to fumarase A causes a drastic increase of the $[4Fe\text{-}4S]^{+}$ S=1/2 EPR signal and a concomitant disappearance of the S=3/2 signal of the same cluster. Similarly, addition of substrate to strain MPOB fumarase caused a change in the EPR signal. The $[4Fe\text{-}4S]^{+}$ signal increased drastically; however, unlike *E. coli* fumarase A, no shift in *g* values was observed.

The effect of substrate on the EPR signal of the Fe-S cluster and the inactivation of the enzyme by oxygen and reactivation by Fe^{2+} strongly suggests that the Fe-S cluster of strain MPOB fumarase has the same function in substrate binding and catalysis as described for fumarase A of *E. coli* (Flint et al. 1992) and other Fe-dependent hydrolyases such as aconitase (Kennedy et al. 1983; Beinert and Kennedy 1989). In these enzymes, one atom of the [4Fe-4S] cluster acts as a Lewis acid to activate the hydroxyl group of the substrate for addition or elimination. This Fe atom is lost at oxidation and a [3Fe-4S] cluster is

formed. Addition of Fe^{2+} under reducing conditions leads to reincorporation of the Fe atom and, as a result, to restoration of the enzyme activity.

There is no experimental evidence for the presence of a class II fumarase in strain MPOB. So far, strain MPOB is the only strictly anaerobic bacterium from which fumarase has been studied. Fumarase has been studied in several aerobic or facultatively anaerobic bacteria. These bacteria contain, in contrast to strain MPOB, either only a class II fumarase (Puchegger et al. 1990; Colombo et al. 1994) or both class I and class II fumarases (Acuna et al. 1991; Ueda et al. 1991; Flint et al. 1992; Reany et al. 1993). The green alga *Euglena gracilis* is the only other organism known that has only a class I fumarase (Shibata et al. 1985).

The two class I fumarases that are present in *E. coli*, fumarase A and fumarase B, are closely related. The amino acid sequences are 90% identical to each other (Miles and Guest 1984; Bell et al. 1989), and the kinetic constants of the enzymes are similar in both directions (Flint 1994). The difference between these class I fumarases is that fumarase A accounts for 80% of the fumarase activity in *E. coli* during aerobic growth, when fumarate is converted to L-malate. Fumarase B is the most dominant fumarase under anoxic conditions, when L-malate is converted to fumarate. Based on the analytical function in vivo, fumarase of strain MPOB is probably more related to fumarase A than to fumarase B.

Fumarase of strain MPOB is a more efficient fumarate hydratase than L-malate dehydratase. The K_m for fumarate is approximately tenfold less than that for L-malate. Secondly the k_{cat} for fumarate hydration is higher than that for malate dehydration. These kinetic properties of the purified fumarase of strain MPOB support our hypothesis that the enzyme has to remove fumarate efficiently during the syntrophic oxidation of propionate, in order to facilitate the energetically unfavourable succinate oxidation.

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4

Purification and characterization of malate dehydrogenase from the syntrophic propionate-oxidizing bacterium strain MPOB

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ABSTRACT

Malate dehydrogenase from the syntrophic propionate-oxidizing bacterium strain MPOB was purified 42-fold. The native enzyme had an apparent molecular mass of 68 kDa and consisted of two subunits of 35 kDa. The enzyme exhibited maximum activity with oxaloacetate at pH 8.5 and 60 °C. The K_m for oxaloacetate was 50 μM and for NADH 30 μM. The K_m values for L-malate and NAD were 4 and 1.1 mM, respectively. Substrate inhibition was found at oxaloacetate concentrations higher than 250 μM. The N-terminal amino acid sequence of the enzyme was similar to the sequences of a variety of other malate dehydrogenases from plants, animals and microorganisms.

INTRODUCTION

The oxidation of propionate to hydrogen or formate, carbon dioxide and acetate is an endergonic process, which occurs under methanogenic conditions. The $\Delta G^{o'}$ of this reaction is +76 kJ/mol propionate. Acetogenic bacteria are only able to oxidize propionate at low product concentrations. Therefore, they grow syntrophically with hydrogen-, formate- or acetate-consuming methanogens (Schink 1992). The syntrophic propionate-oxidizing bacteria which have been investigated up to now are also able to grow in the absence of a partner organism by coupling the oxidation of propionate to the reduction of sulfate (Wallrabenstein et al. 1994; 1995; van Kuijk and Stams 1995). In addition, strain MPOB grows in pure culture by the fermentation of fumarate or by the reduction of fumarate with hydrogen, formate or propionate as the electron donor (Stams et al. 1993). Strain MPOB was isolated from anoxic granular sludge (Stams et al. 1993). Analysis of the 16S rRNA sequence revealed that strain MPOB forms one cluster with the other syntrophic propionate-oxidizing bacteria *Syntrophobacter wolinii* and *S. pfennigii*, within the sulfate-reducing bacteria (Harmsen et al. 1995).

In syntrophic propionate-oxidizing bacteria propionate is oxidized via the methylmalonyl-CoA pathway (Schink 1992). The energetically most difficult reactions in this pathway are the oxidation of succinate to fumarate and the oxidation of malate to

oxaloacetate coupled to hydrogen formation; hydrogen partial pressures of 10^{-15} and 10^{-8} bar, respectively, are required (Schink 1992). In this paper, we report on the purification and characterization of malate dehydrogenase (EC 1.1.1.37) from strain MPOB. This enzyme catalyzes the NAD(H)-dependent interconversion of malate and oxaloacetate.

MATERIALS AND METHODS

Organism and cultivation. Strain MPOB (DSM 10017) was grown anaerobically at 37°C on 20 mM fumarate in 25-l carboys containing 20 l mineral medium, as described previously (Stams et al. 1993). Cells were harvested anoxically by continuous-flow centrifugation and stored at –20 °C until used.

Purification of malate dehydrogenase. Washed cells (60 g wet weight) were suspended in 100 mM Tris-HCl (pH 7.5) containing 250 mM sucrose, 20 mM KCl, 5 mM $MgCl_2$ and 5 mM dithiothreitol, and disrupted by passing twice through a French pressure cell at 110 MPa. Cell debris was removed by centrifugation at 16 000 x *g* for 20 min. The resulting supernatant was ultracentrifuged at 140 000 x *g* for 90 min at 4 °C. The soluble fraction was applied in two successive portions to a column (3.2 x 9.3 cm) of Q-Sepharose equilibrated with 50 mM Tris-HCl (pH 7.6) containing 2.5 mM dithiothreitol (buffer A). Malate dehydrogenase did not bind to the column and was eluted with 45 ml of equilibration buffer. After addition of $(NH_4)_2SO_4$ to a concentration of 1 M, the enzyme solution was loaded onto a column (5.0 x 10.8 cm) of Phenyl-Sepharose equilibrated with 1 M $(NH_4)_2SO_4$ in buffer A. Protein was eluted with a gradient of 1-0 M $(NH_4)_2SO_4$ in buffer A. Malate dehydrogenase eluted at 0.25 M $(NH_4)_2SO_4$. The fractions containing the highest enzyme activity were pooled, concentrated (Amicon ultrafiltration with a PM10 filter) and applied to a column (2.2 x 10 cm) of Matrex Gel Red A equilibrated with 20 mM Tris-HCl (pH 7.5) containing 2.5 mM dithiothreitol (buffer B). Bound proteins were eluted with a gradient of 0-1.5 M KCl in buffer B. Malate dehydrogenase eluted at 0.95 M KCl. The fractions

containing malate dehydrogenase were pooled and separated on a column of Superdex 200 (1.6 x 70.5 cm) with buffer A containing 250 mM NaCl as the elution buffer. After addition of $(NH_4)_2SO_4$ to a concentration of 1 M, the active fractions were applied on a Phenyl-Superose column that was equilibrated with 1 M $(NH_4)_2SO_4$ in buffer A. Proteins were eluted with a gradient of 1-0 M $(NH_4)_2SO_4$ in buffer A. Malate dehydrogenase eluted at 0.7 M $(NH_4)_2SO_4$. The purified malate dehydrogenase was stored at 4 °C.

Enzyme and protein assays. Malate dehydrogenase was assayed spectrophotometrically at 37 °C by following the oxidation of NADH or the reduction of NAD at 340 nm. The formation of malate was assayed in 1 ml 50 mM Tris-HCl (pH 7.2) containing 0.25 mM oxaloacetate, 0.15 mM NADH and enzyme. The oxidation of malate was measured at pH 9.0 in a mixture (1 ml) of 0.4 M glycine, 0.32 M hydrazine, 4 mM NAD, 10 mM L-malate and enzyme. One unit of malate dehydrogenase activity was defined as the amount of enzyme that catalyzes the oxidation or reduction of 1 μmol of NADH or NAD per min. Protein concentrations were determined by the method of Bradford (1976) with serum albumin as the standard.

The purity of the enzyme was determined by SDS- and native-PAGE according to Laemmli (1970). Gels were stained with Coomassie Blue R-250 or incubated at 37 °C in an activity-staining solution composed of 20 mM L-malate, 2 mM NAD, 0.1 mM phenazine methosulfate and 0.12 mM *p*-nitroblue tetrazolium in 50 mM Tris-HCl (pH 7.5).

Determination of molecular mass and N-terminal amino acid sequence. The molecular masses of the native enzyme and the subunits were estimated by gel filtration and SDS-PAGE, respectively (van Kuijk et al. 1996). The N-terminal amino acid sequence was determined by automated Edman degradation (van Kuijk et al. 1996).

RESULTS AND DISUSSION

Purification and molecular mass of malate dehydrogenase. Malate dehydrogenase from strain MPOB was purified 42-fold in five steps to a specific activity of 1728 U/mg protein (Table 1). The enzyme was not sensitive to oxygen. The identity of malate dehydrogenase was confirmed by activity staining on a native polyacrylamide gel. The purified enzyme showed a single band on SDS-PAGE at a migration distance corresponding to a molecular mass of approximately 35 kDa (Fig. 1). The molecular mass of the native enzyme was estimated by gel filtration to be 68 kDa, indicating that the native enzyme is a homodimer. Malate dehydrogenase has been purified from a variety of sources, including bacteria, archaea and eukaryotes (Murphey et al. 1967; Banaszak and Bradshaw 1975; Sundaram et al. 1980). In all cases the enzyme was a homodimer or a homotetramer with a subunit molecular mass of 30-38 kDa. The malate dehydrogenase of strain MPOB belongs to the dimeric malate dehydrogenases.

Table 1. Purification of malate dehydrogenase from strain MPOB

	Protein (mg)	Activity (U)	Spec. act. (U/mg)	Yield (%)	Purif. factor (- fold)
Soluble cell-fraction	1,350	54,645	41	100	1
Q Sepharose	488	53,358	109	98	2.7
Phenyl Sepharose	32	24,629	770	45	18.8
Matrex Gel Red A	5.1	5,939	1,165	11	28.4
Superdex-200	3.3	5,160	1,564	9	38.1
Phenyl Superose	0.8	1,382	1,728	3	42.1

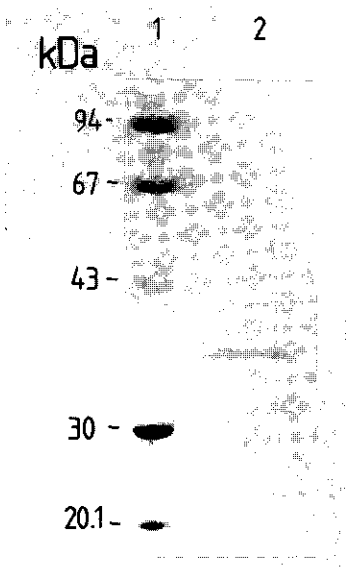

Fig. 1. SDS-PAGE (12.5%) of purified malate dehydrogenase from strain MPOB. In lane 1 are the low molecular mass markers and in lane 2 is the purified malate dehydrogenase. The gel was stained with Coomassie Blue R-250.

Catalytic properties. The malate dehydrogenase showed selectivity for the cofactor NAD(H); only a low activity was found with NADP and NADPH, which was 5% and 3%, respectively, of the activity using NAD and NADH. The optimum pH and temperature of the purified enzyme were 8.5 and 60 °C with oxaloacetate as the substrate. The kinetic properties were determined at 37 °C and a pH of 7.5 for the reduction of oxaloacetate and a pH of 9.0 for the oxidation of L-malate. All saturation curves followed Michaelis Menten kinetics, with the exception of the oxaloacetate saturation curve, which showed substrate inhibition at concentrations higher than 250 μM. This substrate inhibition at high concentrations of oxaloacetate is a common feature of malate dehydrogenases (Honka et al. 1990). For oxaloacetate a K_m value of 50 μM was found, the K_m for NADH being 30 μM. The K_m for L-malate and NAD were 4 and 1.1 mM, respectively. The purified enzyme showed a k_{cat}[oxaloacetate] of 1360 s^{-1} and a k_{cat}[L-malate] of 140 s^{-1}. From these kinetic parameters it is clear that malate dehydrogenase of strain MPOB catalyzes the reduction of oxaloacetate more efficiently than the oxidation of malate. This is contradictory to the function of the enzyme in vivo where it catalyzes the oxidation of malate (Schink 1992). The $\Delta G^{o'}$ value of malate oxidation with NAD is +30 kJ. However, it can be calculated that the

oxidation of malate at 37 °C and pH 8.5 becomes thermodynamically just possible if the concentrations of malate, NAD, oxaloacetate and NADH are equal to the K_m values as determined for these substrates.

N-terminal amino acid sequence. The amino acid sequence of the first 21 residues of the N-terminus of the enzyme was determined and compared with 10 sequences from the more than 30 malate dehydrogenase sequences that are available in literature and databases (Fig. 2). The sequence of malate dehydrogenase from strain MPOB was similar to sequences from microorganisms, animals and plants. A very high degree of sequence homology was observed with the enzyme from Actinomycetales strains (Rommel et al. 1989) and that of the archaeon *Thermus flavus* (Nishiyama et al. 1986). The domain GAXGXXG/A (amino acids 11-17 in Fig. 2) is highly conserved in the N-terminal sequence of malate dehydrogenases. This domain is thought to be involved in cofactor binding (McAlister-Henn 1988; Charnock et al. 1992).

Strain MPOB	AKKPVRVTVTGAAGQIGYALL
Streptosporangium roseum	AQTPVKVTVTGAAGQIGYALL
Planomonospora venezuelensis	AQTPVKVTVTGAAGQIGYALL
Streptomyces atratus	TRTPVNVTVTGAAGQIGYALL
Thermus flavus	MKAPVRVAVTGAAGQIGYSLL
Escherichia coli	----MKVAVLGAAGGIGQALA
Mouse (mitochondrial)	AQNNAKVAVLGASGGIGQPLS
Mouse (cytoplasmic)	MSCPIRVLVTGAAGQIAYSLL
Saccharomyces cerevisiae (mit.)	----YKVTVLGAGGGIGQPLS
Saccharomyces cerevisiae (cyt.)	EQDSLKIAILGAAGGIGQPLS
Watermelon (mitochondrial)	SVPERKVAVLGAAGGIGQPLA

Fig. 2. Comparison of the N-terminal amino acid sequence of malate dehydrogenase from strain MPOB with a selection of malate dehydrogenase-sequences that were derived from the SWISSPROT and NCBI GenBank databases. The sequence of strain MPOB has been submitted to the SWISSPROT database with accession number P80648.

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5

Isolation and properties of the oxygen-sensitive fumarate reductase of the syntrophic propionate-oxidizing bacterium strain MPOB

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ABSTRACT

The membrane-bound fumarate reductase of the syntrophic propionate-oxidizing bacterium strain MPOB was purified after solubilization with the detergent Triton-X100. The enzyme consisted of three subunits with a molecular mass of 70.5, 33.5, and 23.5 kDa. The N-terminal amino acid sequences of the two largest subunits showed similarity with sequences of fumarate reductases and succinate dehydrogenases of other organisms. By using electron paramagnetic resonance (EPR) spectroscopy, a [4Fe-4S] cluster (although very weak) and a [2Fe-2S] cluster were detected in the purified enzyme. However, EPR data of membranes of strain MPOB, suggest that an additional [3Fe-4S] cluster is present in the fumarate reductase of strain MPOB. Formate, hydrogen, reduced benzyl viologen, and reduced 2,3-dimethyl-1,4-naphtoquinone ($DMNH_2$) served as electron donors for fumarate reduction in membranes of strain MPOB, whereas NADH and $FMNH_2$ did not. The K_m for fumarate, measured with benzyl viologen, was 0.025 mM. The ratio of fumarate reductase activity over succinate dehydrogenase activity in membranes of strain MPOB was comparable at different growth conditions, suggesting that fumarate reductase catalyzes the oxidation of succinate to fumarate during propionate-oxidation as well.

INTRODUCTION

The oxidation of propionate to acetate, hydrogen or formate, and carbon dioxide is an endergonic reaction at thermodynamic standard conditions ($\Delta G^{o'}$= + 76 kJ/mol). Therefore, in methanogenic environments, acetogenic bacteria are only able to carry out this conversion if the products are efficiently removed. As a result propionate is oxidized by syntrophic cooperations of acetogenic bacteria and methanogenic archaea (Schink 1997).

Labelling experiments with ^{13}C-NMR, and enzyme measurements revealed that propionate is oxidized via the methylmalonyl-CoA pathway (Houwen et al. 1987,

1990). The energetically most difficult reaction in this pathway is the oxidation of succinate to fumarate ($\Delta G^{o'}$= +86.2 kJ). Even at the lowest hydrogen or formate concentrations that can be reached by methanogenic archaea, this reaction is endergonic. Therefore, it has been hypothesized that the oxidation of succinate requires a proton motive force-dependent reversed electron transport which utilizes $^2/_3$ of an ATP (Thauer and Morris 1984; Schink 1997). Involvement of such an energy-driven reversed electron transport has been demonstrated in syntrophic glycolate-oxidation (Friedrich and Schink 1993). Also for syntrophic butyrate- and benzoate-oxidation some indications have been obtained for the involvement of a reversed electron transport (Wallrabenstein and Schink 1994).

So far, a few acetogenic syntrophic propionate-oxidizing bacteria have been isolated, namely *Syntrophobacter wolinii* (Boone and Bryant 1980; Wallrabenstein et al. 1994), *S. pfennigii* (Wallrabenstein et al. 1995), and strain MPOB (Stams et al. 1993; Chapter 7). Based on 16S rRNA gene sequence analysis, these syntrophic propionate-oxidizing bacteria are closely related and form one group within the delta subclass of proteobacteria (Harmsen et al. 1993, 1995). The biochemical and bioenergetical characterization of the syntrophic propionate-oxidizing bacteria was initially hampered by the presence of the partner organism. Some years ago, however, these studies became possible after the observation that syntrophic propionate-oxidizing bacteria are also able to grow in pure culture (Stams et al. 1993; Wallrabenstein et al. 1994, 1995). The syntrophic propionate-oxidizing bacterium strain MPOB, which was used as the model-organism in our studies, grows in pure culture by fumarate reduction to succinate with hydrogen, formate or propionate as electron donor, or by fumarate disproportionation into succinate and carbon dioxide (Stams et al. 1993). In addition, strain MPOB can couple the oxidation of propionate to sulfate-reduction in pure culture (van Kuijk and Stams 1995). The reduction of fumarate to succinate with hydrogen or formate as the electron donor is the reversed conversion of the energetically unfavourable oxidation of succinate during syntrophic propionate-oxidation. In the present study fumarate reductase, which catalyzes the reduction of fumarate to succinate, was purified from strain MPOB. Some properties of the isolated enzyme were determined, and it was investigated whether fumarate

reduction and succinate oxidation in strain MPOB are reversible processes, which are catalyzed by the same enzyme.

MATERIALS AND METHODS

Organism and cultivation. Strain MPOB (DSM 10017) was cultured anaerobically at 37°C in a bicarbonate-buffered mineral medium as described by Stams et al. (1993). Mass cultivation for enzyme purification was done in 25-l carboys containing 20 l of medium with 20 mM fumarate as the substrate. For the preparation of smaller volumes of cell extracts, the bacterium was grown in 3-l serum bottles containing 1.5 l medium, and 20 mM fumarate, or 20 mM propionate plus 20 mM sulfate as the substrate.

Preparation of cell fractions. Cell fractions were prepared under anoxic conditions. Cultures of 1.5 l were centrifuged at 16,000 x *g* for 10 min. Large culture volumes (20 l) were harvested at a similar speed by continuous-flow centrifugation (Biofuge 28RS, Heraeus Sepatech, Osterode, Germany). Cell pellets were washed with 100 mM Tris-HCl, pH 7.5, suspended in 100 mM Tris-HCl, pH 7.5, containing 250 mM sucrose, 5 mM dithiothreitol (DTT), 20 mM KCl, and 5 mM $MgCl_2$ (buffer A), and disrupted by passing twice through a French pressure cell at 110 MPa. After a DNase incubation of 15 min at room temperature, cell debris was removed by centrifugation for 20 min at 16,000 x *g*. The supernatant was centrifuged for 1.5 h at 140,000 x *g* and 4 °C to separate the membrane fraction from the soluble fraction. The membrane fraction was suspended in buffer A.

Enzyme and protein assays. Enzyme activities were measured spectrophotometrically at 37 °C in N_2-flushed cuvettes, which were closed with rubber stoppers. One unit of enzyme activity corresponds to the amount of enzyme that catalyzes the conversion of 1 µmol substrate per min. Fumarate reductase was assayed with reduced benzyl viologen (Boonstra et al. 1975), reduced 2,3-dimethyl-1,4-naphthoquinone ($DMNH_2$) (Möller-Zinkhan and Thauer 1988), reduced flavin mononucleotide ($FMNH_2$) (Hiraishi

1988) or NADH (Meinhardt and Glass 1994) as electron donor. The activity of fumarate reductase with hydrogen or formate as electron donor was measured according to the method of Macy et al. (1976). The assay mixture contained 50 mM Tris-HCl, pH 7.6, (saturated with hydrogen), 50 mM $MgCl_2$, 1.8 mM GTP, 1.8 mM phosphoenolpyruvate, 0.075 mM NADH, 1.85 mM acetyl-CoA, 5 U succinyl-CoA synthetase, 10 U pyruvate kinase, 25 U lactate dehydrogenase, and membrane fraction. The reaction was started by addition of fumarate (saturated with hydrogen) to a concentration of 2 mM. The oxidation of NADH was followed at 340 nm and 37 °C. The reduction of fumarate by formate was measured by a small-modified assay as described for fumarate reduction with hydrogen; the Tris-buffer and the fumarate solution were saturated with nitrogen, and formate was added to the assay mixture to a concentration of 10 mM.

Succinate dehydrogenase was assayed with 2,6-dichlorophenolindophenol (DCPIP) and phenazine methosulfate (PMS) according to Schirawski and Unden (1995).

Protein was determined by the method of Bradford (1976), with bovine serum albumin as the standard.

Purification of fumarate reductase. All purification steps were performed at room temperature under strictly anoxic conditions in an anaerobic chamber with N_2/H_2 (96:4; v/v) as gas phase. Membrane fraction was isolated from 24 g (wet weight) MPOB cells as described above. The membranes were solubilized by the addition of Triton-X100 to a final concentration of 3% (vol/vol). After stirring at 4 °C for 15 h the membrane fraction was centrifuged at 140,000 x *g* and 4 °C for 90 min. The solubilized enzyme preparation (supernatant) was applied to a Q-Sepharose column (3.2 x 9.3 cm) equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.1% Triton-X100 and 2.5 mM DTT (buffer B). A linear gradient of 0-2 M KCl in buffer B was applied at a flow rate of 1.5 ml/min. Fumarate reductase eluted partially in the unbound protein fraction and partially at 120 mM KCl. The active fractions were pooled, concentrated in an Amicon ultrafiltration cell with a PM30 filter and applied to a hydroxyapatite column (1.2 x 8.8 cm) which was equilibrated with buffer B. Fumarate reductase was eluted with a linear gradient from 0-250 mM potassium

phosphate in buffer B at a potassium phosphate concentration of approximately 125 mM. The fractions with fumarate reductase activity were concentrated in an Amicon ultrafiltration cell with a PM30 filter and applied to a column of Superdex 200 (1.6 x 70.5 cm) equilibrated with buffer B, containing 250 mM NaCl. Protein was eluted at a flow rate of 0.5 ml/min. The purified fumarate reductase was stored under N_2 at 4 °C until used.

Molecular mass determination. The molecular mass of the subunits was determined by SDS-PAGE according to Laemmli (1970). The gels were calibrated using the low-molecular-mass calibration kit of Pharmacia (Roosendaal, The Netherlands) containing phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Gels were stained with Coomassie Brilliant Blue-R250.

N-Terminal amino acid sequence determination. Protein samples for N-terminal amino acid sequence determination were obtained by electroblotting onto a polyvinylidine difluoride (PVDF) membrane (Immobilan Millipore, Etten-Leur, The Netherlands) after separation by SDS-PAGE. Sequence determination was performed by Eurosequence (Groningen, The Netherlands) by automated Edman degradation with an Applied Biosystems 477A protein sequencer (Warrington, UK). The amino acid derivatives were identified by HPLC (Applied Biosystems 120A, Warrington, UK).

Electron paramagnetic resonance (EPR). EPR spectroscopy was carried out on a Bruker 200 D spectrometer with cryogenics, peripheral equipment, and data acquisition/ manipulation facilities as described previously (Pierik and Hagen 1991).

Materials. Q-Sepharose, Superdex 200 and molecular mass standards for SDS-PAGE were obtained from Pharmacia Biotech (Roosendaal, The Netherlands). Hydroxyapatite was obtained from BioRad (Utrecht, The Netherlands). DMN was a gift of R. Schauder (University of Frankfurt, Germany). All other chemicals were at least of analytical grade.

RESULTS

Purification and molecular composition of fumarate reductase. Fumarate reductase of strain MPOB was inactivated by oxygen. In membrane fractions, the enzyme lost 50 % of its activity within four hours in the presence of air. Therefore, all purification steps were carried out under strictly anoxic conditions in an anaerobic chamber and all the N_2-flushed buffers contained DTT. A typical purification of fumarate reductase from the membrane fraction of strain MPOB is outlined in Table 1. The enzyme was solubilized with Triton-X100 and purified by ion exchange, hydroxyapatite, and gel filtration in the presence of the detergent. The specific activity of the purified fumarate reductase was 28.3 U/mg protein with benzyl viologen as electron donor. This value, (and therefore also the purification factor), can be an underestimation because of the instability of the enzyme. Although strict anoxic conditions were applied, the enzyme lost part of its activity during the purification procedure. At the end of the purification procedure, only 1 % of the original enzyme activity was left.

Table 1. Purification of fumarate reductase from strain MPOB.

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Membrane	274	1754	6.4	100	1
Triton-extract	232	1160	5.0	66	0.8
Q-Sepharose	34.3	172	5.0	9.8	0.8
Hydroxyapatite	7.0	62	8.8	3.5	1.4
Gel filtration	0.6	17	28.3	1	4.4

SDS-polyacrylamide gel electrophoresis of the purified enzyme revealed three protein bands, indicating that the fumarate reductase consists of three subunits (Fig. 1).

The apparent molecular mass of these subunits was calculated to be 70.5 kDa, 33.5 kDa and 23.5 kDa. The smallest subunit was not always recovered; in some initial purifications the fumarate reductase was isolated as a catalytic dimer. With the assumption that the stoichiometry of the subunits is 1:1:1, the apparent molecular mass of the native enzyme is ± 130 kDa.

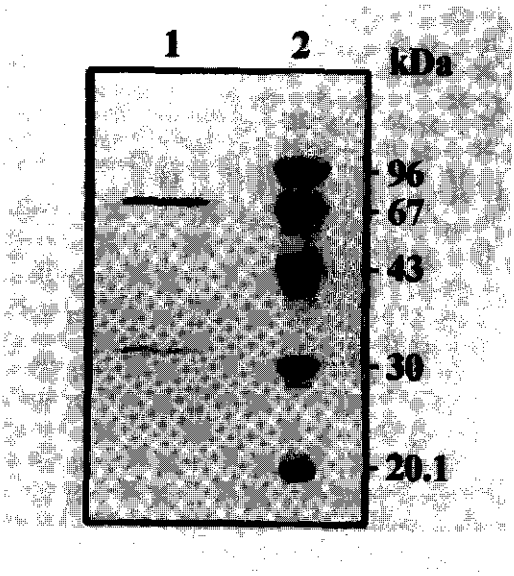

Fig. 1. SDS-PAGE of purified fumarate reductase of strain MPOB. Lane 1, purified fumarate reductase; lane 2, molecular mass markers. The gel was stained with Coomassie Brilliant Blue- R250.

The N-terminal amino acid sequences of the two largest subunits showed similarity to N-terminal sequences of the flavin and iron-sulfur subunits of fumarate reductases and succinate dehydrogenases of other bacteria (Fig. 2).

70.5 kDa subunit	**A**
	********
FRD Strain MPOB	MDTFYTDLLXVGAGLAGERVA
FRD *Escherichia coli*	MQTFQADLAIVGAGGAGLRAA
FRD *Wolinella succinogenes*	MKVQYCDSLVIGGGLAGLRAA
FRD *Proteus vulgaris*	MQTFNADIAIIGAGGAGLRAA
FRD *Haemophilus influenzae*	MQTVNVDIAIVGAGGGGLRAA
FRD *Mycobacterium tuberculosis*	MTAQHNIVVIGGGGAGLRAA
FRD *Helicobacter pylori*	MKITYCDALIIGGGLAGLRAS
SDH *Coxiella burnetii*	MSSIRVKQYDALIVGAGGAGLRAA
SDH *Rickettsia prowazekii*	MTKAYNIIHHKFDVVVVGAGGAGLRSA
SDH *Escherichia coli*	MKLPVREFDAVVIGAGGAGIARA
SDH *Bacillus subtilis*	MSQSSIIVVGGGLAGLMAT
	********

33.5 kDa subunit	**B**
FRD Strain MPOB	MGRPLKFSVFRFNPLD
FRD *Escherichia coli*	MAEMKNLKIEVVRYNPEVDTAP
FRD *Wolinella succinogenes*	MGRMLTIRVFKYDPQSAVSK
FRD *Proteus vulgaris*	MADDMKHVKMEVMRYNPETDDAP
FRD *Mycobacterium tuberculosis*	MMDRIVMEVSRYRPEIESAP
SDH *Escherichia coli*	MRLEFSIYRYNPDVDDAP

Fig. 2. Comparison of the N-terminal amino acid sequences of the 70.5 kDa **(A)**, and the 33.5 kDa **(B)** subunits of fumarate reductase of strain MPOB with sequences of fumarate reductases **(FRD)** and succinate dehydrogenases (SDH) from other bacteria. The sequences were derived from the NCBI GenBank database. The amino acid in Fig. A marked "X" could not be determined unambiguously. The conserved domain V/I-V/I-G-A/G-G-G/L-A-G in the largest subunit is indicated in Fig. A with *asterisks*. The sequences in Fig. B were aligned on the basis of the conserved cysteine residues in the sequences of the iron-sulfur subunits of the other bacteria.

Catalytic properties of the fumarate reductase. The reduction of fumarate was measured photometrically with different electron donors in membrane fractions of strain MPOB (Table 2). Reduced benzyl viologen, formate, hydrogen, and $DMNH_2$

served as electron donors. NADH- or $FMNH_2$-linked fumarate reductase activity could not be detected. The purified fumarate reductase had an apparent K_m for fumarate of 0.025 mM. This value was determined at a benzyl viologen concentration of 1.6 mM, and a temperature of 37 °C.

Table 2. Fumarate reductase activity in the membrane fraction of strain MPOB with different electron donors. N. a., no activity.

Electron donor	Fumarate reductase activity (U/mg protein)
Benzyl viologen	12.6
H_2	0.05
Formate	0.05
$DMNH_2$	1.5
NADH	n.a.
$FMNH_2$	n.a.

EPR analysis of fumarate reductase. EPR analysis of the fumarate reductase as isolated, in the presence of 2.5 mM DTT, showed a weak, rhombic signal with g-values of 2.037, 1.941, and 1.895. Anaerobic incubation with 5 mM dithionite for 10 min at ambient temperature resulted in the full development of this signal, as shown in Figure 3A. The shape and saturation behaviour of the signal are indicative for a dinuclear $[2Fe\text{-}2S]^{1+}$ cluster. An indication for a broad, underlying signal was obtained when the same spectrum was run under conditions of higher microwave power, higher gain, and lower temperature (Fig. 3A). When the enzyme, as isolated, was anaerobically oxidized with 5 mM potassium ferricyanide, the spectrum of Fig. 3A disappeared, and no other signals were observed (not shown). By analogy with previously characterized mammalian and bacterial fumarate reductases and succinate

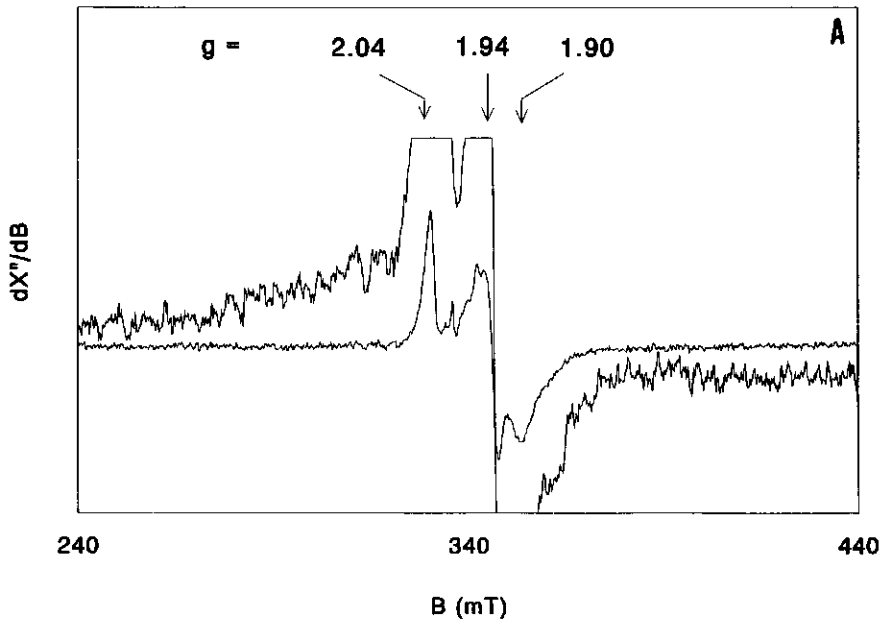

Fig. 3. **A.** EPR spectrum of purified fumarate reductase of strain MPOB. The protein, 3 mg/ml in 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 % Triton-X100 and 2.5 mM DTT, was anaerobically incubated with 5 mM sodium dithionite for 10 min at ambient temperature. The spectrum is characteristic for a $[2Fe\text{-}2S]^{1+}$ cluster. The blow-up shows some indication for a broad signal presumably from a $[4Fe\text{-}4S]^{1+}$ cluster. EPR conditions: microwave frequency, 9397 MHz; microwave power, 0.08 mW (blow-up: 20 mW); modulation frequency, 100 kHz; modulation amplitude, 1.0 mT; temperature, 15 K (blow-up: 8 K).

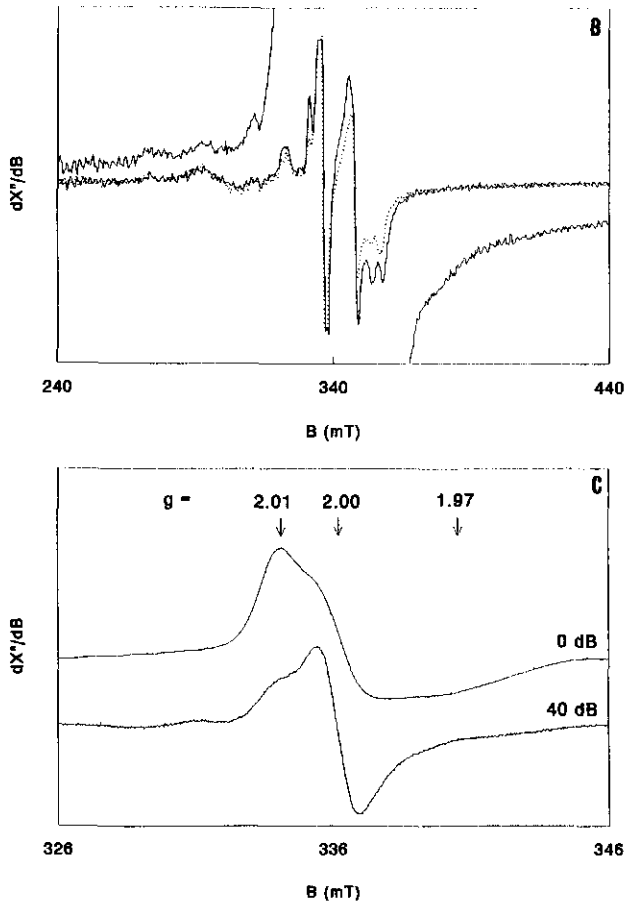

Fig. 3. **B.** EPR spectra of membranes of strain MPOB incubated with fumarate (dotted trace) or succinate (solid trace, and blow-up). The membranes, approx. 22.5 mg/ml protein in 50 mM Tris-HCl buffer, pH 7.5, were anaerobically incubated with 50 mM substrate for 10 min, at room temperature. EPR conditions: microwave frequency, 9395 mHz, microwave power, 0.12 mW (blow-up: 20 mW); modulation frequency, 100 kHz; modulation amplitude, 1.0 mT; temperature, 15 K (blow-up: 11 K).
C. EPR spectra of membranes of strain MPOB oxidized with ferricyanide. The membranes (same preparation as for Fig. B) were anaerobically incubated with 5 mM potassium ferricyanide for 10 min. at room temperature. The figure shows a high-power trace (0 dB) dominated by a $[3Fe\text{-}4S]^{1+}$ signal and a low-power trace (40 dB) dominated by a radical signal. EPR conditions: microwave frequency, 9395 MHz; microwave power, 200 mW (0 dB) or 0.02 mW (40 dB); modulation frequency, 100 kHz; modulation amplitude, 0.63 mT; temperature, 8 K.

dehydrogenases (reviewed by Ackrell et al. 1992), we expected the enzyme to exhibit a sharp $[2Fe\text{-}2S]^{1+}$ signal and a very broad $[4Fe\text{-}4S]^{1+}$ signal in its reduced state, and a sharp $[3Fe\text{-}4S]^{1+}$ signal in its oxidized state. Our data, however, which were obtained with two independent enzyme preparations, are only suggestive of the presence of the [4Fe-4S] cluster in fumarate reductase of strain MPOB, and no [3Fe-4S] signal is observed. This may be a result of (partial) cluster desintegration during solubilization and/or purification of the enzyme. Therefore, we have tried to identify EPR signals from the three putative iron-sulfur clusters of fumarate reductase in membrane preparations of strain MPOB. Figure 3B shows the spectrum of membranes incubated with succinate and fumarate, respectively. These spectra are significantly more complex than those of the purified enzyme, due to the presence of a range of iron-sulfur clusters. However, the additional intensity of the spectrum after succinate reduction clearly corresponds to the sharp $[2Fe\text{-}2S]^{1+}$ signal found for the purified fumarate reductase. Moreover, a blow-up of this spectrum (high power, high-gain, low temperature) more clearly showed the broad signal that is typical for the $[4Fe\text{-}4S]^{1+}$ cluster in fumarate reductases or succinate dehydrogenases. Furthermore, anaerobic incubation of the membranes with potassium ferricyanide resulted in a clear signal characteristic for a $[3Fe\text{-}4S]^{1+}$ cluster in its g-values and temperature dependence. In Fig. 3C it is shown that this signal was slightly disturbed by a (much more readily saturating) radical signal presumably from the semiquinone form of FAD and/or menaquinone.

Ratio of fumarate reductase and succinate dehydrogenase activity. To investigate whether the fumarate reduction and succinate oxidation in strain MPOB are catalyzed by the same enzyme, fumarate reductase and succinate dehydrogenase activities were determined in membrane fractions of strain MPOB grown with fumarate, and with propionate plus sulfate. During growth with fumarate, strain MPOB needs an active fumarate reductase, while during growth with propionate and sulfate succinate dehydrogenase is required. The ratio of fumarate reductase over succinate dehydrogenase activity was 23 ± 0.4 in cells grown with fumarate, and 16 ± 0.7 in cells grown with propionate plus sulfate.

DISCUSSION

The structurally similar enzymes fumarate reductase and succinate dehydrogenase catalyze the interconversion of fumarate to succinate. Most fumarate reductases and succinate dehydrogenases are membrane-bound enzymes consisting of three or four subunits. The largest subunit (60-75 kDa) is a flavoprotein, containing covalently bound FAD and the substrate-binding site. The second subunit is an iron-sulfur protein containing three iron-sulfur clusters of the [2Fe-2S], [4Fe-4S] and [3Fe-4S] type. These two subunits form the membrane-extrinsic catalytic part of the enzyme. The smallest subunits anchor the catalytic subunits to the membrane and are responsible for electron transfer to quinones (reviewed by Cole et al. 1985; Ackrell et al. 1992; van Hellemond and Tielens 1994). The fumarate reductase of strain MPOB resembles other fumarate reductases and succinate dehydrogenases with respect to the location in the membrane, the subunit composition, and the N-terminal amino acid sequences of the catalytic subunits. The membrane fraction of strain MPOB showed the EPR signals typical for fumarate reductases and succinate dehydrogenases, namely a $[2Fe\text{-}2S]^{1+}$, a $[3Fe\text{-}4S]^{1+}$, and a very broad $[4Fe\text{-}4S]^{1+}$ signal (reviewed by Ackrell et al. 1992). In the purified enzyme, however, the 3Fe-4S signal was not observed, and the signal from the [4Fe-4S] cluster was significantly less pronounced than in the membranes. It is possible that the [4Fe-4S] cluster was partially destroyed in the purified fumarate reductase; alternatively a structural rearrangement near the cluster had occurred. Because the fumarate reductase of strain MPOB resembles other fumarate reductases with respect to subunit-composition, N-terminal amino acid sequences of the catalytic subunits, and location in the membrane, we expect that the iron-sulfur cluster composition of the enzyme is similar to that of other fumarate reductases. We assume that the integrity of the [3Fe-4S] cluster of fumarate reductase of strain MPOB was lost upon solubilization and/or purification of the enzyme. Difficulties in detecting the [3Fe-4S] cluster have been reported for other purified fumarate reductases and succinate dehydrogenases (Ackrell et al. 1992; Singer and Johnson 1985).

In initial purifications, fumarate reductase of strain MPOB was isolated as a catalytic dimer. The fumarate reductase of *Escherichia coli* has also been purified in

two forms: a tetrameric holoenzyme (Lemire et al. 1982), and a dimeric catalytic unit (Dickie and Weiner 1979). When the enzyme of *E. coli* was purified via hydrophobic exchange chromatography, the hydrophobic membrane anchor subunits were tightly bound to the column material. In that case, only the catalytic part of the enzyme was isolated (Dickie and Weiner 1979). It is not clear why the smallest subunit of the fumarate reductase of strain MPOB was lost during initial purifications. It is possible that the subunit remained in the membrane because of the use of an insufficient detergent concentration.

The ratio of fumarate reductase activity over succinate dehydrogenase activity in strain MPOB was similar in cells grown with fumarate and with propionate plus sulfate. At growth with fumarate as the sole substrate strain MPOB needs fumarate reductase (Plugge et al. 1993), whereas at growth by the oxidation of propionate coupled to sulfate reduction, succinate dehydrogenase is active (Houwen et al. 1987; 1990). This similar ratio of fumarate reductase activity over succinate dehydrogenase activity at different growth conditions, suggests that in strain MPOB a single enzyme catalyzes both fumarate reduction and succinate oxidation. Some facultatively anaerobic bacteria like *E. coli* and *Bacillus macerans* contain two different enzymes for the interconversion of fumarate and succinate; succinate dehydrogenase is synthesized during aerobic growth whereas fumarate reductase is active under anaerobic growth conditions (Cole et al. 1985; Schirawski and Unden 1995). In *B. macerans*, the ratio of fumarate reductase activity over succinate dehydrogenase activity is almost one thousand times higher at anaerobic growth compared to aerobic growth conditions (Schirawski and Unden 1995). Because strain MPOB is strictly anaerobic, the bacterium is not expected to have an oxygen-regulated expression of fumarate reductase and succinate dehydrogenase as has been found to occur in facultatively anaerobic bacteria.

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6

Investigation of the fumarate metabolism of the syntrophic propionate-oxidizing bacterium strain MPOB

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Arch. Microbiol. (1998) in press

ABSTRACT

The growth of the syntrophic propionate-oxidizing bacterium strain MPOB in pure culture by fumarate disproportionation into carbon dioxide and succinate, and by fumarate reduction with propionate, formate or hydrogen as electron donor was studied. The highest growth yield, 12.2 g dry cells/ mol fumarate, was observed for growth by fumarate disproportionation. In the presence of hydrogen, formate or propionate the growth yield was more than twice as low; 4.8, 4.6, and 5.2 g dry cells/ mol fumarate, respectively. The location of enzymes that are involved in the electron transport chain during fumarate reduction in strain MPOB was analyzed. Fumarate reductase, succinate dehydrogenase, and ATPase were membrane-bound, while formate dehydrogenase and hydrogenase were loosely attached to the periplasmic side of the membrane. The cells contained cytochrome *c*, cytochrome *b*, menaquinone-6 and menaquinone-7 as possible electron carriers. Fumarate reduction with hydrogen in membranes of strain MPOB was inhibited by 2-(heptyl)-4-hydroxyquinoline-N-oxide (HOQNO). This inhibition, together with the activity of fumarate reductase with reduced 2,3-dimethyl-1,4-naphtoquinone ($DMNH_2$), and the observation that cytochrome *b* of strain MPOB was oxidized by fumarate, suggested that menaquinone and cytochrome *b* are involved in the electron transport during fumarate reduction in strain MPOB. The growth yields of fumarate reduction with hydrogen or formate as electron donor were similar to that of *Wolinella succinogenes*. Therefore, it can be assumed that strain MPOB gains the same amount of ATP from fumarate reduction as *W. succinogenes*, i.e. 0.7 mol ATP/ mol fumarate. This value supports the hypothesis that syntrophic propionate-oxidizing bacteria have to invest $^2/_3$ of an ATP via reversed electron transport in the succinate oxidation step during the oxidation of propionate. The same electron transport chain that is involved in fumarate reduction may operate in the reversed direction to drive the energetically unfavourable oxidation of succinate during syntrophic propionate-oxidation, because cytochrome *b* was reduced by succinate, and succinate oxidation was similarly inhibited by HOQNO as fumarate reduction.

INTRODUCTION

In methanogenic environments, organic matter is completely degraded to methane and carbon dioxide via the concerted action of different groups of microorganisms. The oxidation of some reduced intermediates that are formed during the degradation process, such as fatty acids and alcohols, is thermodynamically unfavourable unless the concentrations of the products are kept low. This results in syntrophic growth of acetogenic bacteria with hydrogen- or formate-consuming methanogenic archaea (Stams 1994; Schink 1997).

A characteristic of the degradation pathways that are involved in the syntrophic oxidation of these compounds is the presence of at least one energetically unfavourable oxidation step. The electrons that are released at these oxidations have to be coupled to proton or bicarbonate reduction. However, at the lowest hydrogen partial pressures and formate concentrations that can be reached by methanogenic archaea, some of these reactions are still endergonic (Stams 1994; Schink 1997). Therefore, it has been hypothesized that the electrons can only be coupled to proton or bicarbonate reduction if they are shifted to a lower redox potential via reversed electron transport which is driven by the hydrolysis of ATP (Thauer and Morris 1984; Schink 1997). Involvement of such reversed electron transport has been demonstrated in the oxidation of glycolate to glyoxylate during syntrophic glycolate-oxidation (Friedrich and Schink 1993). Also for syntrophic butyrate- and benzoate-oxidation some evidence has been obtained for the presence of an energy-consuming reversed electron transport system, in the oxidation of butyryl-CoA to crotonyl-CoA and the oxidation of glutaryl-CoA to glutaconyl-CoA, respectively (Wallrabenstein and Schink 1994).

In syntrophic propionate-oxidation, the energetically most unfavourable reaction is the oxidation of succinate to fumarate coupled to hydrogen or formate production. It has been proposed that the bacteria have to invest $^2/_3$ ATP in this reaction (Thauer and Morris 1984). To obtain more insight in a possible reversed electron transport mechanism during syntrophic propionate-oxidation, we studied the growth of strain MPOB which is able to grow in pure culture by the reduction of fumarate with hydrogen, formate or propionate as electron donor, and by fumarate disproportionation

(Stams et al. 1993). Fumarate reduction with hydrogen as electron donor is the reversed conversion of the energetically unfavourable succinate oxidation step in syntrophic propionate-oxidation. Growth rates and growth yields of strain MPOB during growth via the reduction or disproportionation of fumarate were determined, and compared to those of other microorganisms which are able to grow by the same conversions. Several enzymes that could be involved in electron transport processes in strain MPOB were localized and the presence and function of cytochromes and menaquinones in the cell was investigated.

MATERIALS AND METHODS

Organism and cultivation. Strain MPOB (DSM 10017) was grown at 37 °C in mineral medium as described before (Stams et al. 1993), but no yeast extract was added. Bacteria were cultivated in 1-l serum bottles containing 500 ml medium (for growth studies) or in 3-l serum bottles containing 1.5 l medium (for the preparation of cell extracts). Bottles were closed with butyl-rubber stoppers and screw-caps, and provided with a gas phase of N_2/CO_2 or H_2/CO_2 (80:20 v/v; 170 kPa). Fumarate, sodium propionate, sodium formate and sodium sulfate were added from sterile stock solutions (1M) to a final concentration of 20 mM. Media were inoculated with 5% of substrate-adapted cultures.

Growth determination. Growth was followed by the determination of substrate, product and protein concentrations. Organic acids were measured by HPLC as described previously (Stams et al. 1993). For protein determination, cell pellets of 5-ml culture samples were resuspended in 1 ml 1 M NaOH. After 15 min incubation at 100°C, the samples were treated further according to the protein determination method of Lowry et al.(1951). Bovine serum albumin was used as the standard. The dry weight was calculated from the protein concentration assuming that 50 % of the dry weight consists of protein (Macy et al. 1986). With the assumption that the organic fraction comprises for 90 % of the cell dry weight and that the structural formula of this

organic fraction is $C_5H_7O_2N$ (M=113), the dry weight data were converted to molar units for the calculation of the carbon and electron recovery (McCarty 1975).

Preparation of cell fractions. Cell fractions were prepared under anoxic conditions in an anaerobic chamber with N_2/H_2 (96:4; v/v) as gas phase. Bacteria from 1.5-l cultures were harvested by centrifugation at 16,000 x *g* and washed with 100 mM Tris-HCl pH 7.5. The cells were resuspended in 100 mM Tris-HCl pH 7.5 containing 250 mM sucrose, 20 mM KCl, 5 mM $MgCl_2$ and 5 mM dithiothreitol (buffer A), and disrupted by passing twice through a French pressure cell at 110 MPa. After an incubation with DNase for 15 min at room temperature, cell debris was sedimented by centrifugation at 16,000 x *g*. The crude extract was centrifuged for 90 min at 140,000 x *g* and 4 °C to separate the membrane fraction (pellet) from the soluble fraction (supernatant). The membrane fraction was suspended in buffer A.

Enzyme activities. Enzyme activities were measured spectrophotometrically at 37 °C in 1-ml cuvettes, which were closed with rubber stoppers and flushed with N_2. One unit of enzyme activity is the amount of enzyme that catalyzes the conversion of 1 µmol substrate per min. Fumarate reductase was assayed with reduced benzyl viologen as described by Boonstra et al. (1975). The activity of fumarate reductase with hydrogen as electron donor was measured according to the method of Macy et al. (1976). The assay mixture contained 50 mM Tris-HCl, pH 7.6, (saturated with hydrogen), 50 mM $MgCl_2$, 1.8 mM GTP, 1.8 mM phosphoenolpyruvate, 0.075 mM NADH, 1.85 mM acetyl-CoA, 5 U succinyl-CoA synthetase, 10 U pyruvate kinase, 25 U lactate dehydrogenase and membrane fraction. The reaction was started by addition of fumarate (saturated with hydrogen) to a concentration of 2 mM. The oxidation of NADH was followed at 340 nm and 37 °C. Succinate dehydrogenase activity was measured with 2,6-dichlorophenol-indophenol (DCPIP) as described by Schirawski and Unden (1995). Hydrogenase and formate dehydrogenase were assayed with methyl viologen as electron acceptor according to Odom and Peck (1981). Fumarase was analyzed according to Stams et al. (1984). ATPase was assayed as described by Vogel and Steinhart (1976). Protein concentrations of the cell fractions were determined

according to the method of Bradford (1976), with bovine serum albumin as the standard.

Detection of cytochromes and quinones. Cytochromes were determined in cell fractions by difference spectroscopy. Redox difference spectra (dithionite-reduced *minus* air-oxidized) were recorded on a Beckman DU7500 spectrophotometer. To investigate whether oxidation or reduction of the cytochromes occurred, 15 mM fumarate or succinate was added to an anoxic cuvette which contained 1 ml 200 mM potassium phosphate buffer, pH 7.5, and membrane fraction.

Quinones were extracted from freeze-dried cells and identified by HPLC by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH, Braunschweig, Germany.

RESULTS

Determination of growth parameters. Growth curves of strain MPOB in batch cultures with fumarate and fumarate plus hydrogen are presented in Fig. 1. During fumarate degradation malate was excreted transiently. The concentration of fumarate which is given in Fig. 1 includes both measured fumarate and malate. In Table 1 the stoichiometry of the fumarate disproportionation and fumarate reduction is presented for one representative culture; the growth rate and yield data are means of at least duplicate cultures. The growth rates of strain MPOB, which were calculated from the exponential growth phase, were low; the doubling time ranged between 7.5 and 17 days. Cell yields were calculated from the amount of protein formed and the amount of substrate used. The highest growth yield, 12.2 g dry cells/ mol fumarate, was observed for growth by fumarate disproportionation. In the presence of hydrogen, formate or propionate the growth yield was more than twice as low; 4.8, 4.6, and 5.2 g dry cells/ mol fumarate, respectively.

Table 1. Stoichiometry of fumarate disproportionation and reduction by strain MPOB

Substrate	Substrate converted (mmol)	Products formed (mmol)		Biomass formed (mg)	Carbon[a] recovery (%)	Electron[a] recovery (%)	Yield[b] (g/mol)	Growth rate (day^{-1})
		succinate	acetate					
Fumarate	9.2	6.5	-[c]	112	95	101	12.2	0.09
Fumarate + Hydrogen	8.9	8.2	-	43	97	99	4.8	0.06
Fumarate + Formate	8.9 8.7	8.0	-	40	94	96	4.6	0.04
Propionate + Fumarate	3.5 9.5	8.7	3.3	49	90	96	5.2	0.05

[a]For the calculation of the carbon and/or electron recovery, the amount of CO_2 formed and H_2 used were estimated from the amount of succinate formed by using the known stoichiometric equations of the different conversions (Stams and Plugge 1995).

[b]Yield in g dry cells/mol fumarate. With the assumption that 1 g dry cells corresponds to 0.5 g protein (Macy et al. 1986).

[c]- = measured but not detected

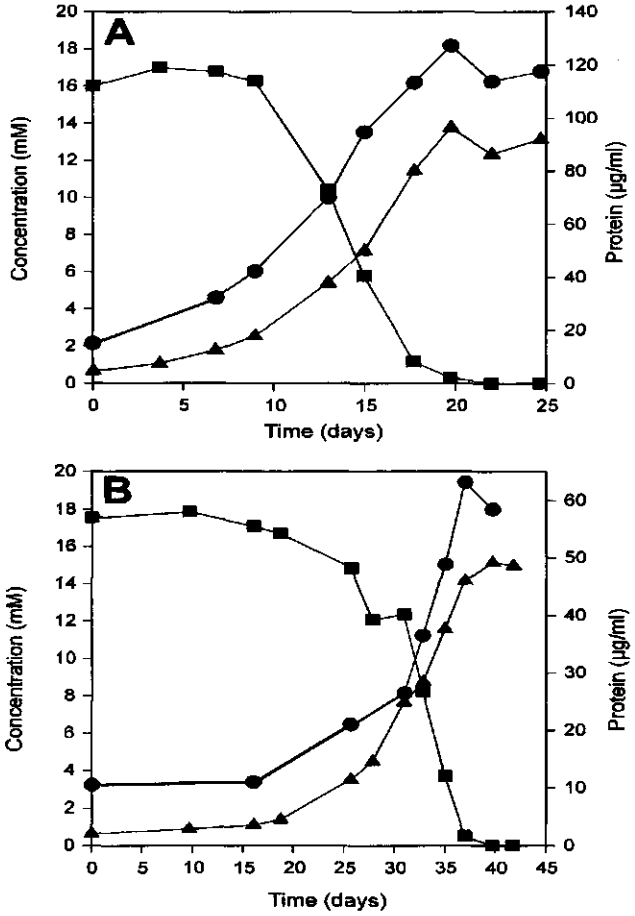

Fig. 1. Growth of strain MPOB with fumarate (A) and hydrogen + fumarate (B). Symbols: (■) fumarate + malate; (▲) succinate; (●) protein.

Enzyme localization. In Table 2 the distribution of enzymes in cell fractions of strain MPOB is given. Fumarase, measured as a marker enzyme for the soluble cell fraction, was almost completely recovered in the soluble fraction, indicating a succesful subcellular fractionation. Fumarate reductase, succinate dehydrogenase and ATPase were found to be membrane-bound. Hydrogenase and formate dehydrogenase were partially detected in the membrane fraction and partially in the soluble fraction.

Table 2. Distribution of enzyme activities in cell fractions of strain MPOB grown with 20 mM fumarate. One unit of activity is the amount of enzyme that catalyzes the conversion of 1 μmol substrate per min. The activity of the enzymes in the cell extract, which is a combination of the soluble fraction and the membrane fraction, was fixed at 100%.

Enzyme activity	Cell fraction	Activity (U)	Protein (mg)	Specific Activity (U/mg)	Percentage of total activity
Fumarate reductase	Extract	101.9	29.0	3.5	100
	Soluble	3.1	19.2	0.2	3
	Membrane	91.6	8.8	10.4	90
Succinate dehydrogenase	Extract	30.2	29.0	1.0	100
	Soluble	0.2	19.2	0.01	0.6
	Membrane	20.9	8.8	2.4	69
Hydrogenase	Extract	29.3	29.0	1.0	100
	Soluble	20.7	19.2	1.1	70.7
	Membrane	7.0	8.8	0.8	24
Formate dehydrogenase	Extract	85.1	29.0	2.9	100
	Soluble	59.9	19.2	3.1	70.4
	Membrane	24.2	8.8	2.8	28.4
Fumarase	Extract	280.2	29.0	9.7	100
	Soluble	272.6	19.2	14.2	97.3
	Membrane	13.6	8.8	1.6	4.9
ATPase	Extract	7.8	29.0	0.3	100
	Soluble	0.3	19.2	0.01	3.4
	Membrane	3.2	8.8	0.4	40.4

In order to gain further insight into the location of hydrogenase, formate dehydrogenase and fumarate reductase, the enzyme activities were measured with methyl viologen with intact cells, and with the same cells after permeabilization by addition of 1 % Triton-X100. The activities of hydrogenase and formate dehydrogenase in intact and permeabilized cells were comparable, 0.16 and 0.12

U/mg, respectively, for hydrogenase and 0.11 and 0.07 U/mg, respectively, for formate dehydrogenase. With intact cells only a low fumarate reductase activity was found (0.02 U/mg protein). After permeabilization of the cells, an up to 10-fold increased fumarate reductase activity was measured.

Cytochromes and quinones. Redox difference spectra of the soluble fraction of strain MPOB cells showed absorption maxima at 420, 523, and 553 nm, indicating the presence of *c*-type cytochromes. The redox difference spectrum of the membrane fraction of the cells showed absorption maxima at 429, 527, and 557 nm, which is indicative for the presence of *b*-type cytochromes. Both the *b*- and *c*-type cytochromes were detected in fumarate-grown cells as well as in cells grown with propionate plus sulfate. Cytochrome *b* in the membrane fraction was oxidized in the presence of fumarate, and reduced when succinate was added (Fig. 2).

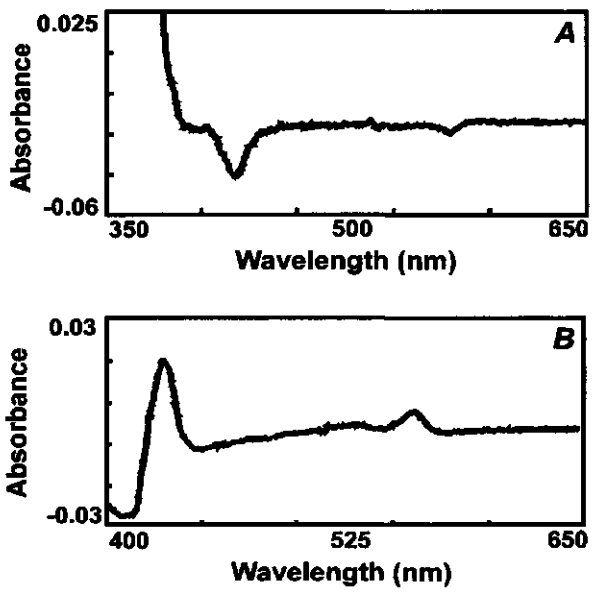

Fig. 2 **A**. Effect of the addition of fumarate to the redox difference spectrum (blank) of the membrane fraction of strain MPOB. **B**. Effect of the addition of succinate to the spectrum of oxidized membrane fraction (blank) of strain MPOB.

Menaquinone-6 and menaquinone-7 were detected in strain MPOB. The amount of menaquinone-7 in the cells was approximately 10-times higher than the amount of menaquinone-6.

HOQNO inhibition. Incubation of the membrane fraction of strain MPOB with 2-(heptyl)-4-hydroxyquinoline-*N*-oxide (HOQNO) inhibited the fumarate reductase activity measured with hydrogen as electron donor (Fig. 3), and also the succinate dehydrogenase activity. Fumarate reduction was inhibited completely at an HOQNO-concentration higher than 0.6 μM, and the succinate oxidation at an HOQNO-concentration higher than 2.3 μM. When fumarate reductase was assayed with the artificial electron donor benzyl viologen, which circumvents the electron transport chain, the activity was hardly affected by HOQNO.

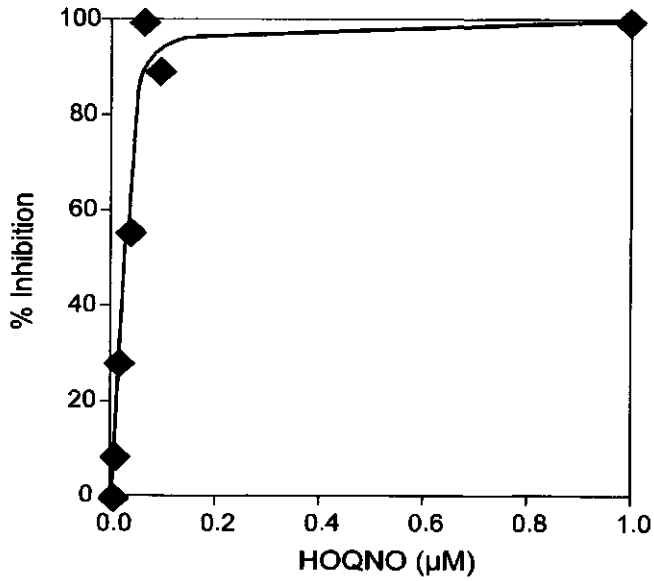

Fig. 3. Effect of HOQNO on the reduction of fumarate with hydrogen as electron donor.

DISCUSSION

In this paper, the growth of strain MPOB by fumarate disproportionation and fumarate reduction with propionate, formate or hydrogen as electron donor was studied. When the growth rates and molar growth yields of strain MPOB are compared with those of other microorganisms (Table 3), it is clear that strain MPOB grows much slower than these microorganisms, but that the growth yields are similar (Kröger 1974; Bernhard and Gottschalk 1978; Dorn et al. 1978; Bronder et al. 1982; Macy et al. 1986; Dehning and Schink 1989).

Table 3. Comparison of growth rates and growth yields of strain MPOB with those of other microorganisms, grown on the same substrates. If necessary, growth yield data were converted to g dry cells/mol substrate using the assumption that 1 g dry cells corresponds to 0.5 g protein (Macy et al. 1986).

Microorganism	Growth rate (day^{-1})	Growth yield (g dry cells/ mol substrate)	Reference
Cells grown with 7 fumarate → 6 succinate + 4 CO_2			
Strain MPOB	0.09	12.2	This study
Proteus rettgeri	1.3	4.8	Kröger 1974
Malonomas rubra (GraMal1)	4.6	11.0	Dehning and Schink 1989
Cells grown with hydrogen + fumarate → succinate			
Strain MPOB	0.06	4.8	This study
Wolinella succinogenes	4.8	3.2	Bronder et al. 1982
Escherichia coli	4.2	4.8	Bernhard et al. 1978
Cells grown with formate + fumarate → succinate + CO_2			
Strain MPOB	0.04	4.6	This study
Wolinella succinogenes	5.2	4.8	Bronder et al. 1982
	9.2	7.0	Macy et al. 1986
Clostridium formicoaceticum	-	5.0	Dorn et al. 1978

The molar growth yield of strain MPOB was highest when grown by fumarate disproportionation. However, when hydrogen or formate was present in addition to fumarate, the molar growth yield was considerably lower. This indicates the presence of an energy yielding step in the oxidation of fumarate to carbon dioxide, which is part of the pathway of fumarate disproportionation in strain MPOB. Fumarate is oxidized to carbon dioxide via the acetyl-CoA cleavage pathway (Plugge et al. 1993), which is also present in some sulfate-reducing and homoacetogenic bacteria (Schauder et al. 1986; Spormann and Thauer 1988; Diekert and Wohlfarth 1995). In this pathway, one ATP is generated during the formation of formate from formyl-tetrahydrofolate (Diekert and Wohlfarth 1995). Another possible energy-yielding step is the conversion of CO to CO_2, which is sufficiently exergonic to be coupled to ATP-synthesis via electron transport phosphorylation (Diekert et al. 1986).

The ability to gain energy from anaerobic respiration with fumarate as terminal electron acceptor is a common property among facultative and strictly anaerobic bacteria (see e.g. Kröger et al. 1992). The ATP must be formed by electron transport phosphorylation, because ATP formation by substrate level phosphorylation can be excluded for growth with fumarate as terminal electron acceptor and hydrogen or formate as electron donor. The electron transport chain catalyzing fumarate reduction with formate as electron donor has been investigated extensively for *Wolinella succinogenes*. In this bacterium, formate dehydrogenase is located at the periplasmic side of the membrane, whereas fumarate reductase is bound to the cytoplasmic side of the membrane (Kröger et al. 1980). Electrons are transferred from formate dehydrogenase to fumarate reductase via *b*-type cytochromes and menaquinone (Kröger and Innerhofer 1976a and b). In strain MPOB, the electron transport chain involved in fumarate reduction is likely to be arranged in the same way as in *W. succinogenes*. Fractionation studies with strain MPOB revealed that fumarate reductase and ATPase are membrane bound. Hydrogenase and formate dehydrogenase were found in the membrane fraction as well as in the soluble fraction, indicating that both enzymes are probably loosely attached to the membrane. Methyl viologen-dependent hydrogenase and formate dehydrogenase could be measured with intact cells, whereas only 10 % of the fumarate reductase activity was detected with whole

cells. Because methyl viologen does not pass the cell membrane (Jones and Garland 1977), this result indicates that hydrogenase and formate dehydrogenase are associated with the periplasmic side of the membrane and that fumarate reductase is located at the inner aspect of the cytoplasmic membrane. Other results obtained in this research suggest that both menaquinone and cytochrome *b* are intermediary electron carriers in the electron flow from formate or hydrogen to fumarate in strain MPOB. Incubation of the membrane fraction of strain MPOB with the menaquinone antagonist HOQNO, which is thought to block the electron flow from menaquinone to cytochrome *b* by binding at the menaquinone binding site on cytochrome *b* (Smirnova et al. 1995), inhibited the fumarate reduction with hydrogen as electron donor. In contrast, the activity of fumarate reductase was hardly influenced by HOQNO if measured with benzyl viologen, which donates the electrons directly to the fumarate reductase. Moreover, the menaquinone analogue $DMNH_2$ acted as an electron donor for fumarate reduction in membranes of strain MPOB (Chapter 5), and cytochrome *b* was oxidized by fumarate.

The molar growth yields of strain MPOB grown with fumarate and hydrogen or formate were similar to molar growth yields reported for *W. succinogenes*, *Escherichia coli* or *Clostridium formicoaceticum* (Bernhard and Gottschalk 1978; Dorn et al. 1978; Bronder et al. 1982; Macy et al. 1986). Therefore, it is likely to assume that the ATP yield in the case of fumarate reduction is similar to the ATP yield determined for these bacteria, i.e. $^2/_3$ mol ATP/mol fumarate. Provided that the mechanism of fumarate reduction and succinate oxidation in strain MPOB is the same, this would support the hypothesis that during syntrophic propionate-oxidation the bacterium has to invest $^2/_3$ mol ATP to couple the oxidation of succinate to proton or bicarbonate reduction via a reversed electron transport (Thauer and Morris 1984; Schink 1997). To find direct evidence for energy-dependent succinate-oxidation in strain MPOB, studies with membrane vesicles in the presence of ionophores or ATPase inhibitors are essential. Unfortunately, we could not prepare active membrane vesicles of strain MPOB which produced significant amounts of hydrogen during succinate oxidation.

It may be envisaged that in strain MPOB the same electron transport chain which is involved in fumarate reduction is also involved in succinate oxidation. Reversibility of

an electron transport system has been shown to occur in the syntrophic glycolate-oxidizing bacterium strain FlGlyR during glycolate oxidation/glyoxylate reduction (Friedrich and Schink 1993), and in *Desulfuromonas acetoxidans* for succinate oxidation/fumarate reduction (Paulsen et al. 1986). In the case of strain MPOB the possibility of reversibility of the electron transport chain involved in fumarate reduction/succinate oxidation is supported by the observations that cytochrome *b* in membranes of strain MPOB was reduced by succinate, and succinate oxidation was similarly inhibited by HOQNO as fumarate reduction.

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7

Description of *Syntrophobacter fumaroxidans* sp. nov., a syntrophic propionate-degrading sulfate-reducing bacterium

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ABSTRACT

A syntrophic propionate-oxidizing bacterium, strain MPOB, was isolated from a culture enriched from anaerobic granular sludge. It oxidizes propionate syntrophically in coculture with the hydrogen- and formate-utilizing *Methanospirillum hungatei*, and it can oxidize propionate and other organic compounds in pure culture with sulfate or fumarate as electron acceptor. Additionally, it is able to ferment fumarate. 16S rRNA gene sequence analysis revealed a relationship with *Syntrophobacter wolinii* and *S. pfennigii*. The G+C content of its DNA is 60.6 mol percent, which is in the same range as that of other *Syntrophobacter* species. DNA-DNA hybridization studies showed less than 26 % hybridization among the different genomes of the *Syntrophobacter* species and strain MPOB. This justifies the assignment of strain MPOB to the genus *Syntrophobacter* as a new species. We propose the name *S. fumaroxidans*.

INTRODUCTION

For a long time *Syntrophobacter wolinii* was the only described bacterium which could oxidize propionate syntrophically in coculture with the hydrogen-consuming *Desulfovibrio* G11 (Boone and Bryant 1980). Several methanogenic syntrophic cocultures were enriched, but obtaining defined cocultures remained difficult. Only recently *S. wolinii* was obtained in pure culture and found to be able to grow on pyruvate or on propionate and sulfate (Wallrabenstein et al. 1994). Two related syntrophic propionate-oxidizing bacteria, *Syntrophobacter pfennigii*, previously indicated as KoProp1, and strain HP1.1 were isolated with propionate and sulfate (Wallrabenstein et al. 1995; Zellner et al. 1996).

A mesophilic bacterium (MPOB) enriched by us on propionate was able to ferment fumarate to succinate and carbon dioxide without syntrophic partner (Stams et al. 1993). This strain could oxidize propionate by using fumarate or sulfate as electron acceptors (Stams et al. 1993; van Kuijk and Stams 1995). Similar to *S. wolinii*, strain MPOB oxidized propionate via the methylmalonyl-CoA pathway, but in contrast to *S. wolinii*, strain MPOB activates propionate by the use of HS-CoA transferase (Plugge et al. 1993).

16S rRNA gene sequence analysis of *S. wolinii*, *S. pfennigii*, strain HP1.1, and strain MPOB revealed that these syntrophic bacteria are closely related and belong to the delta subclass of proteobacteria (Harmsen et al. 1993; Harmsen et al. 1995; Zellner et al. 1996). Remarkably, it was observed that another bacterium was related to this group, *Desulforhabdus amnigenus*, a sulfate-reducing bacterium which is not able to grow syntrophically on propionate (Oude Elferink et al. 1995).

Recently, we obtained a pure culture of strain MPOB. Its morphological and physiological characterization are presented here, and its taxonomic position within the genus *Syntrophobacter* is discussed.

MATERIALS AND METHODS

Bacterial strains, cultivation and isolation procedures. Strain MPOB originated from a propionate-degrading culture enriched from granular sludge of an upflow anaerobic sludge bed reactor treating wastewater from a sugar refinery (Stams et al. 1993). *Syntrophobacter wolinii* (DSM 2805) and *Desulfobulbus propionicus* (DSM 2505) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). *Syntrophobacter pfennigii* was kindly provided by B. Schink (University of Konstanz, Germany). *Desulforhabdus amnigenus* was provided by S. Oude Elferink (Wageningen Agricultural University, The Netherlands).

A bicarbonate-buffered medium as described previously was used for isolation and cultivation (Stams et al. 1993). For isolation of strain MPOB the roll-tube-dilution method (Hungate 1969) and direct dilution series in liquid media with fumarate as carbon and energy source were used. Purity was checked by growth in Wilkins-Chalgren anaerobe broth (Oxoid, Basingstoke, UK) and in media with 1 % yeast extract and 20 mM glucose, and by microscopy.

Phylogeny and DNA analysis. Phylogenetic analysis of the strain was described previously (Harmsen et al. 1995; Oude Elferink et al. 1995). The nucleotide sequence of strain MPOB was aligned using the Dedicated Comparative Sequence Editor (DSCE) v2.60 alignment program with other 16S rRNA gene sequences derived from the NCBI

collection and the Antwerp Ribosomal RNA database. The sequence of strain HP1.1 was kindly provided by G. Zellner (Wageningen Agricultural University, The Netherlands). Only nucleotides that could unambiguously be aligned were taken into account. A phylogenetic tree based on the neighbor-joining method was constructed from a distance matrix according to the two parameter model of Kimura, as implemented in the TREECON for Windows software package (van de Peer and de Wachter 1994).

DNA hybridizations were performed by filter hybridization according to Johnson et al. (1981). Modifications of this method on DNA labeling and hybridization conditions were described previously (Klijn et al. 1994). The final stringent washing was done at 65 °C in 0.03 M NaCl and 0.003 M sodium citrate with 1 % SDS. The percentage of binding was determined by measuring the radioactive hybridization signals relative to that found in the homologous hybridizations.

The percentage G+C of the DNA was analyzed at the DSMZ (Braunschweig, Germany) using standard methods (Tamaoka and Komagata 1984; Mesbah et al. 1989; Visuvanathan et al. 1989).

Chemical analyses. All compounds except amino acids, were measured by gas chromatography and HPLC as described previously (Stams et al. 1993). Amino acids were measured as described by Kengen and Stams (1994). The presence of cytochromes was analyzed by recording air-oxidized versus dithionite-reduced spectra of cell-free extracts and membrane fractions. The types of quinones were analyzed at the DSMZ by HPLC (Braunschweig, Germany).

RESULTS AND DISCUSSION

Isolation and morphological characterization. The origin and enrichment of the syntrophic propionate-oxidizing coculture from methanogenic granular sludge was described previously (Stams et al. 1993). The *Methanospirillum* sp. was removed from the coculture by continuous subculturing on malate and fumarate. However, this enrichment culture still contained a contaminating small coccoid bacterium, which could be enriched

and isolated on media containing yeast extract. The roll-tube technique was applied to try to eliminate the contaminating bacteria. A culture, containing about 10^8 cells/ml was diluted in anaerobic roll-tubes with fumarate as carbon source, and this resulted after two months in the formation of yellowish colonies of about 2 mm in diameter in the 10^4 and 10^5 dilution. However, when these colonies were cultured in liquid medium with fumarate as substrate and 0.05 % yeast extract, still contaminating bacteria were found. Therefore, dilution series of this liquid enrichment were made, with fumarate as substrate and 0.05 % yeast extract, to simultaneously purify the bacterium and check for the growth of the contaminant. Growth was observed up to a dilution of 10^8. This dilution appeared to be free of the contaminating coccoid. This culture (strain MPOB) consisted only of the gram-negative, non motile, rod-shaped bacterium (1.1 - 1.6 μm wide and 1.8 - 2.5 μm long) described previously (Stams et al. 1993).

Physiological characterization. Strain MPOB grew on fumarate between 20 °C and 40 °C with an optimum at 37 °C. The pH range was from 6.0 to 8.0 with an optimum growth around pH 7. Addition of 0.05 % yeast extract was not necessary, but it stimulated growth. In coculture with *M. hungatei* strain MPOB had a growth rate on propionate of approximately 0.17 day^{-1}. In pure culture, lower growth rates were observed; 0.09 day^{-1} during fermentative growth on fumarate and 0.024 day^{-1} during growth on propionate and sulfate (van Kuijk and Stams 1995). Both *c*-and *b*-type cytochromes as well as the menaquinones MK-6 and MK-7 were present in fumarate-grown cells.

Strain MPOB utilizes propionate syntrophically in coculture with *M. hungatei*, and in pure culture using sulfate or fumarate as electron acceptors (Table 1). In these cases it oxidizes propionate stoichiometrically to acetate and CO_2, with a concomitant production of methane, sulfide or succinate, respectively. Thiosulfate could also serve as an electron acceptor, but nitrate was not utilized. Strain MPOB ferments fumarate to succinate and CO_2, and reduces fumarate to succinate with hydrogen or formate as electron donor (Stams et al. 1993). In addition, strain MPOB grew chemotrophically on some organic compounds (Table 1).

Table 1. Organic compounds tested as electron donors and carbon sources. The substrate concentrations are given in mM in parentheses.

Utilized :	**Products:**
In coculture with *Methanospirillum hungatei*;	
propionate (20)	acetate, CO_2, and methane
In pure culture;	
propionate (20) + fumarate (20)	acetate, CO_2, and succinate
fumarate (20)	succinate, and CO_2
malate (20)	succinate, and CO_2
aspartate (20)	succinate, NH_4^+, and CO_2
H_2 (80 %) + fumarate (20)	succinate
formate (20) + fumarate (20)	succinate, and CO_2
pyruvate (20)	acetate, succinate, and CO_2
In pure culture in presence of 20 mM sulfate;	
propionate (20)	acetate, and CO_2
formate (20)	CO_2
succinate (20)	CO_2
hydrogen (80 %) [poor growth]	

Not utilized:

Tested in the presence of 20 mM sulfate;
Butyrate (10), isobutyrate (10), acetate (20), citrate (10), lactate (20), butanol (10), propanol (20), ethanol (20), methanol (10), glucose (20), fructose (10), xylose (10), glutamate (20).

During the course of the experiments it was observed that strain MPOB shows better growth when $FeCl_3$ was used in the trace element solution instead of $FeCl_2$. After storage under air, commercial $FeCl_2$ is partially oxidized. Trace elements solution from such partially oxidized $FeCl_2$ also gave good growth of strain MPOB.

Taxonomy. The 16S rRNA gene sequence of strain MPOB was analyzed, and this showed that it was closely related to *S. wolinii* and *S. pfennigii* (Harmsen et al. 1995). Using the 16S rRNA gene sequence data obtained previously, a new phylogenetic tree was contructed that includes the syntrophic propionate-oxidizing bacteria *S. wolinii, S. pfennigii*, strain HP1.1, strain MPOB and the sulfate-reducing bacterium *Desulforhabdus amnigenus*, and members of the delta subclass of proteobacteria (Harmsen et al. 1993, 1995; Oude Elferink et al. 1995; Zellner et al. 1996) (Fig. 1). This tree clearly shows that the syntrophic propionate-oxidizing bacteria form one cluster with *D. amnigenus* and belong, according to the grouping of Devereux et al. (1989), to group 7 of the delta subclass of proteobacteria. This is a phylogenetically distinct group of the line of complete-oxidizing sulfate-reducing bacteria.

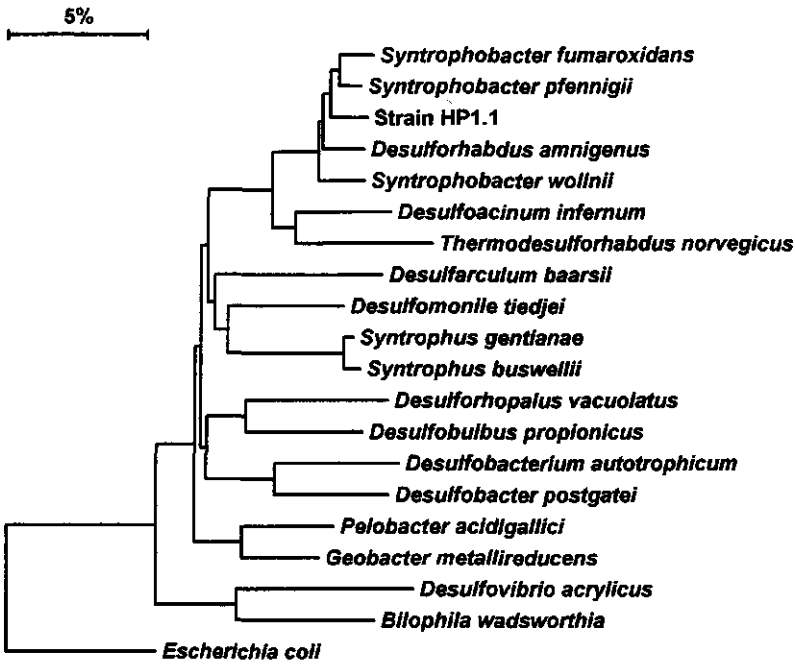

Fig. 1. Phylogenetic relationship of strain MPOB with members of the delta-subclass of proteobacteria. The dendrogram is based on a distance analysis using only those nucleotides that could unambiguously be aligned and was constructed via the neighbor-joining method as implemented in TREECON for Windows (van de Peer and de Wachter 1994). The scale bar represents 5% estimated sequence divergence.

To investigate this relationship on a species level, DNA-DNA hybridizations were performed. Labeled total chromosomal DNA of each of the four strains and *Desulfobulbus propionicus* as an unrelated strain, was hybridized with membrane-fixed total chromosomal DNA of the same strains (Table 2). The homology observed between the different strains was always below 26 %, indicating that all strains belong to different species.

Table 2. Percentage of DNA-DNA hybridization of strain MPOB, *Syntrophobacter wolinii*, *S. pfennigii*, *Desulforhabdus amnigenus* and *Desulfobulbus propionicus*. DNA samples from five strains mentioned in the column were bound to the filter and hybridized with DNA samples from strains given in the row.

	Strain MPOB	*S. wolinii*	*S. pfennigii*	*D. amnigenus*	*D. propionicus*
Strain MPOB	100	9.4	16.4	24.7	11.4
S. wolinii	23.2	100	16.3	26.0	8.8
S. pfennigii	19.8	5.0	100	13.0	6.1
D. amnigenus	14.4	2.3	8.2	100	4.2
D. propionicus	22.0	6.5	15.4	8.1	100

Strain MPOB resembles the syntrophic propionate-oxidizing species *S. wolinii* and *S. pfennigii* by its growth on propionate in coculture with *M. hungatei* and in pure culture by sulfate-reduction. Furthermore, *S. wolinii* can also utilize fumarate and pyruvate (Wallrabenstein et al. 1994; Stams, unpublished results). In addition, *S. pfennigii* can utilize in coculture propanol and lactate (Wallrabenstein et al. 1995). However, the utilization of aspartate, succinate, and formate as substrates for sulfate-reduction was not observed in the two other bacteria. The sulfate-reducing species *D. amnigenus* can not oxidize propionate syntrophically and does not utilize fumarate (Oude Elferink et al. 1995). The G+C content of DNA of strain MPOB is 60.6 mol percent, which is slightly higher than that of other *Syntrophobacter* species (Wallrabenstein et al. 1995; Zellner et al. 1996).

The morphological and physiological differences mentioned above and the results obtained from the DNA-DNA hybridization, showed that the syntrophic strains do not

belong to the same species. Therefore, it appears justified to describe strain MPOB as a new species within the genus *Syntrophobacter* and we propose the name *S. fumaroxidans* sp. nov..

Syntrophobacter fumaroxidans. fu.mar.ox'i.dans. The one that oxidizes fumarate, as the ability to oxidize fumarate played an important role in the isolation and physiological characterization of this bacterium. Non motile, rod- to eye-shaped cells, 1.1 to 1.6 by 1.8 to 2.5 μm in size, with round ends, single or in pairs. Gram negative. Endospores are not formed. The mol percentage G+C is 60.6 ± 0.2 % (n=4). Contains *c*- and *b*-type cytochromes, and quinones MK-6 and MK-7. The strictly anaerobic bacterium grows syntrophically on propionate in the presence of hydrogen- and formate-utilizing bacteria or methanogens, e.g. *Methanospirillum hungatei*. The bacterium ferments fumarate, malate, aspartate, and pyruvate. Propionate, succinate, and formate are oxidized coupled to sulfate-reduction. Besides sulfate, thiosulfate and fumarate can serve as electron acceptors, but nitrate is not reduced. The growth is optimal in freshwater medium with a pH of 7.0-7.6, and at 37 °C. Habitat is granular sludge from an upflow anaerobic sludge bed (UASB) reactor treating sugar-beet processing wastewater. Type strain: *Syntrophobacter fumaroxidans* strain MPOB (DSM 10017).

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8

Summary and Concluding remarks

Propionate is one of the important intermediates that are formed during the anaerobic breakdown of organic matter. In methanogenic environments, like freshwater sediments or anaerobic bioreactors, the oxidation of propionate to acetate, hydrogen or formate, and carbon dioxide is thermodynamically unfavourable. This conversion can only occur if the concentrations of the products are kept low. As a result, propionate is converted in methanogenic environments by syntrophic consortia of propionate-oxidizing acetogenic bacteria and methanogenic archaea (Schink 1997). For a long time, detailed studies on the biochemistry of syntrophic propionate-oxidation were hampered by the presence of the partner organism. Some years ago, however, the research described in this thesis became possible after the discovery that propionate-oxidizing acetogenic bacteria are also able to grow in the absence of methanogenic archaea (Stams et al. 1993; Wallrabenstein et al. 1994, 1995). Strain MPOB, which was isolated from granular sludge of an UASB-reactor treating sugar beet-processing wastewater, was used as model-organism. Energetics of growth of the bacterium was studied, some key-enzymes that are involved in propionate-oxidation were purified and characterized, and the electron transport chain of strain MPOB was investigated. In **Chapter 1** an overview is given of syntrophic degradation, with emphasis on the syntrophic oxidation of propionate. In addition, characteristics of the enzymes fumarate reductase/succinate dehydrogenase, fumarase, and malate dehydrogenase are reviewed in this chapter.

Strain MPOB could be obtained in pure culture using fumarate as a substrate (Stams et al. 1993). Two other syntrophic propionate-oxidizing bacteria, *Syntrophobacter wolinii* and *S. pfennigii*, had been shown to grow in the absence of a methanogenic partner by coupling the oxidation of propionate to sulfate-reduction (Wallrabenstein et al. 1994, 1995). In **Chapter 2** it is presented that strain MPOB is also able to reduce sulfate. The growth rate with propionate and sulfate of strain MPOB and the two *Syntrophobacter* species, however, was very low compared to that of propionate-oxidizing sulfate-reducing *Desulfobulbus* species. Recently, the highly enriched syntrophic propionate-oxidizing bacterium strain HP1.1 was also shown to couple the oxidation of propionate to the reduction of sulfate (Zellner et al. 1996). Based on the 16S rRNA gene sequences, the two *Syntrophobacter* species, strain MPOB, and strain

HP1.1 are closely related. They form one group within the delta subclass of proteobacteria, and are related to Gram-negative sulfate-reducing bacteria (Harmsen et al. 1993, 1995; Zellner et al. 1996). Also three other enriched syntrophic propionate-oxidizing bacteria, which do not group within the genus *Syntrophobacter*, are related to sulfate-reducing bacteria (Harmsen 1996). Therefore, it may be speculated that syntrophic propionate-oxidizing bacteria are a special group of sulfate-reducing bacteria, which exploits the ability to grow syntrophically in order to survive in environments with very low sulfate concentrations.

During the syntrophic oxidation of propionate, reducing equivalents are released at three intermediate reactions, namely the oxidation of succinate to fumarate, malate to oxaloacetate, and pyruvate to acetyl-CoA. The reducing equivalents are used to reduce protons or bicarbonate to hydrogen or formate, respectively. To let the oxidation of propionate proceed, it is important that the formed hydrogen or formate is taken away by the methanogenic archaea. To facilitate the intermediate succinate oxidation, also the fumarate concentration has to be kept low. Therefore, the bacteria need an active fumarase, which catalyzes the interconversion of fumarate and malate. Fumarase of strain MPOB (**Chapter 3**) is an oxygen-sensitive homodimeric enzyme with a molecular mass of 120 kDa. The N-terminal amino acid sequence of the enzyme showed similarity to that of two fumarases of *Escherichia coli*. Electron paramagnetic resonance (EPR) spectroscopy revealed the presence of a [4Fe-4S] cluster in the enzyme. This cluster reacted with fumarate. The characteristics of the fumarase described above, indicated that strain MPOB contains a class 1 fumarase. Based on the kinetic properties of the purified enzyme, fumarase of strain MPOB is a more efficient fumarate hydratase than malate dehydratase. This supports the hypothesis that the enzyme has to remove fumarate efficiently during the syntrophic oxidation of propionate, in order to facilitate the energetically unfavourable succinate oxidation.

Chapter 4 describes the purification and characterization of malate dehydrogenase of strain MPOB. This enzyme catalyzes the NAD-dependent conversion of malate to oxaloacetate, which is one of the energetically unfavourable oxidation reactions during syntrophic propionate-oxidation. Malate dehydrogenase of strain MPOB consists of two subunits with molecular masses of 35 kDa. The N-terminal amino acid sequence

of the subunits contains the conserved sequence motif (GAXGXXG/A) that has been found in most malate dehydrogenases. MPOB's malate dehydrogenase had a K_m of 4 mM for malate and 1.1 mM for NAD. The K_m values for NADH and oxaloacetate were 30 μM and 50 μM, respectively. The kinetic properties of the purified malate dehydrogenase indicate that in vitro the enzyme prefers the conversion of oxaloacetate to malate. This is contradictory to the function of the enzyme in vivo, where it has to catalyze the reversed conversion during the syntrophic oxidation of propionate or the disproportionation of fumarate. However, if the Gibbs free energy change for the conversion of malate to oxaloacetate at 37°C and pH 8.5 is calculated using concentrations of malate, oxaloacetate, NADH, and NAD equal to the measured K_m-values, it can be concluded that this conversion is energetically possible. Of course, also the kinetic properties of the propionyl-CoA:oxaloacetate transcarboxylase determine the rate at which malate is oxidized to oxaloacetate in vivo. The low affinity of the malate dehydrogenase for malate may also explain the transient accumulation of malate which was observed during growth of strain MPOB with fumarate (**Chapter 6**), and the high specific activity of the purified enzyme (1728 U/mg protein).

The isolation and characterization of fumarate reductase of strain MPOB is described in **Chapter 5**. The membrane-bound enzyme was purified anoxically after solubilization with the detergent Triton-X100. The fumarate reductase consisted of three subunits with molecular masses of 70.5, 33.5, and 23.5 kDa, respectively. The N-terminal amino acid sequences of the two largest subunits showed similarity with sequences of the flavin and iron-sulfur subunits of fumarate reductases and succinate dehydrogenases of other organisms. In the purified enzyme a [2Fe-2S] cluster was detected and weak indications were obtained for the presence of a [4Fe-4S] cluster. A [3Fe-4S] cluster, which is usually present as the third iron-sulfur cluster in fumarate reductases and succinate dehydrogenases, could not be detected in the purified enzyme. EPR-analysis of the membrane fraction of strain MPOB incubated with succinate and fumarate, however, showed the signals that are typical for a $[2Fe\text{-}2S]^{1+}$, $[3Fe\text{-}4S]^{1+}$, and $[4Fe\text{-}4S]^{1+}$ cluster. Therefore, it is likely that the [3Fe-4S] cluster, which may be present in the fumarate reductase of strain MPOB in vivo, was degraded during solubilization and/or purification of the enzyme. Possibly, the fumarate

reductase of strain MPOB catalyzes the oxidation of succinate as well, because the ratio of the fumarate reductase and succinate dehydrogenase activity in cells grown with fumarate, in which fumarate reductase is active, was similar to that in cells grown with propionate and sulfate, in which succinate dehydrogenase is active.

The energetically most unfavourable reaction during the syntrophic oxidation of propionate is the oxidation of succinate to fumarate coupled to hydrogen or formate formation. Even at the lowest hydrogen or formate concentrations that can be reached by methanogenic archaea, this reaction is endergonic (Schink 1997). Therefore, it has been hypothesized that the electrons that are released at the oxidation of succinate can only be coupled to proton or bicarbonate reduction if they are shifted to a lower redox potential via reversed electron transport. It is thought that this reversed electron transport is driven by the hydrolysis of $^2/_3$ ATP (Thauer and Morris 1984; Schink 1997). The involvement of such a reversed electron transport mechanism in the oxidation of succinate was supported by the observation that strain MPOB can grow in pure culture by the reversed conversion, namely the reduction of fumarate with hydrogen or formate as electron donor. Growth studies (**Chapter 6**) indicated that strain MPOB yields approximately $^2/_3$ ATP per mol fumarate from the reduction of fumarate. Because substrate-level phosphorylation can be excluded at growth with hydrogen or formate and fumarate, ATP has to be formed via electron transport phosphorylation. The electron transport chain that is involved in fumarate reduction in strain MPOB was investigated (**Chapter 6**). Fumarate reductase, succinate dehydrogenase, and ATPase were shown to be membrane-bound enzymes. Hydrogenase and formate dehydrogenase were found partially in the membrane fraction and partially in the soluble fraction of the cell. Possibly, both enzymes are only loosely bound to the membrane. However, the existence of multiple hydrogenases and formate dehydrogenases in strain MPOB can not be excluded. Fumarate reductase was located at the cytoplasmic side, and hydrogenase and formate dehydrogenase at the periplasmic side of the membrane. Strain MPOB contained menaquinone-6 and –7, and *b*-type and *c*-type cytochromes as potential electron carriers. The inhibition of hydrogen-oxidation coupled to fumarate reduction by HOQNO, the activity of fumarate reductase with the reduced menaquinone analogue 2,3-dimethyl-1,4-

naphtoquinone, and the oxidation of cytochrome *b* upon addition of fumarate indicated the involvement of menaquinone and cytochrome *b* in the oxidation of hydrogen coupled to fumarate reduction in strain MPOB. The electron transport chain that is involved in fumarate reduction in strain MPOB likely is organized in the same way as in *Wolinella succinogenes* (Fig. 1) (Kröger et al. 1992).

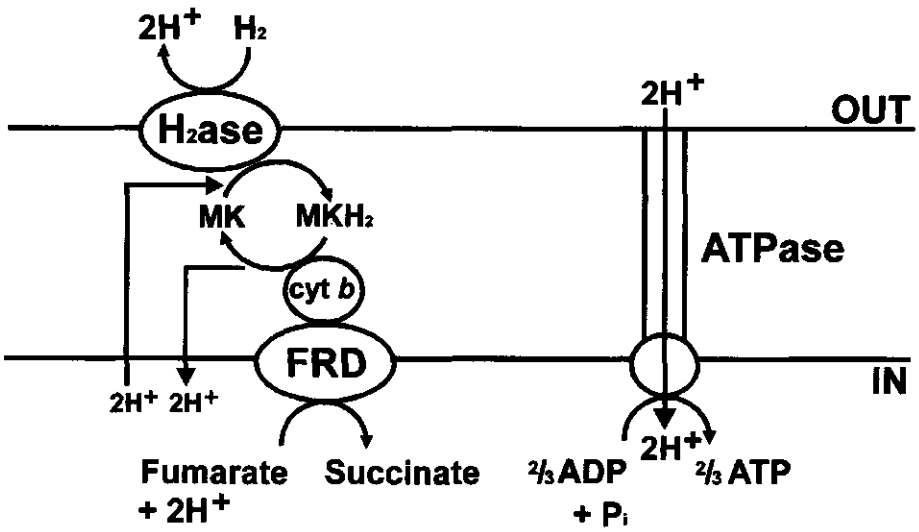

Fig. 1. Model of the electron transport chain from hydrogen to fumarate in the cytoplasmic membrane of strain MPOB. H_2ase, hydrogenase; FRD, fumarate reductase; MK, menaquinone; cyt b, cytochrome b. The model was adapted from Kröger et al. (1992).

If the oxidation of succinate in strain MPOB is coupled to a reversed electron transport mechanism, it is likely that the electron transport chain involved in fumarate reduction operates in the reversed direction. Direct evidence for the proposed energy-dependent succinate oxidation during the syntrophic oxidation of propionate can only be obtained via similar experiments with membrane vesicles as were used to demonstrate the

involvement of reversed electron transport in succinate oxidation and glycolate oxidation in *Desulfuromonas acetoxidans* and *Syntrophobotulus glycolicus*, respectively (Paulsen et al. 1986; Friedrich and Schink 1993). The effect of ionophores and ATPase inhibitors on the production of hydrogen during the oxidation of succinate to fumarate in membrane vesicles of strain MPOB could answer the question whether the oxidation of succinate is coupled to an ATP-consuming reversed electron transport mechanism. Unfortunately, however, we could not perform such experiments so far, because we were not able to prepare active membrane vesicles of strain MPOB that produced significant amounts of hydrogen during the oxidation of succinate to fumarate.

Finally, **Chapter 7** describes the physiological characterization of strain MPOB. On the basis of its characteristics the bacterium was identified as a new member of the genus *Syntrophobacter*. The name *Syntrophobacter fumaroxidans* was proposed for strain MPOB.

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Samenvatting

"Biochemie en bio-energetica van syntrofe propionaat-oxiderende bacteriën"

Propionaat is één van de belangrijke tussenproducten die worden gevormd tijdens de anaërobe afbraak van complex organisch materiaal. In methanogene milieus, zoals zoetwatersedimenten en anaërobe bioreactoren, is de afbraak van propionaat tot acetaat, kooldioxide en waterstof of formiaat een endergone omzetting onder thermodynamische standaardcondities. Dit betekent dat deze omzetting niet plaats kan vinden, tenzij de gevormde producten worden weggenomen. Het gevolg is dat propionaat wordt omgezet door zogenaamde syntrofe consortia, bestaande uit propionaat-oxiderende acetogene bacteriën en waterstof- of formiaat-consumerende methanogene archaea. Uit het Grieks vertaald betekent syntroof letterlijk "samen (syn) voedend (trophos)".

Gedetailleerde bestudering van het metabolisme van de propionaat-oxiderende acetogene bacteriën werd tot nu toe gehinderd door de noodzakelijke aanwezigheid van een waterstof- of formiaat-consumerende partner. Enkele jaren geleden echter werd duidelijk dat syntrofe propionaat-oxiderende bacteriën ook zonder een methanogene partner kunnen groeien. Dankzij de groei van deze bacteriën in reinculturen werd het in dit proefschrift beschreven onderzoek, waarbij biochemische en energetische aspecten van syntrofe propionaat-oxiderende bacteriën werden bestudeerd, mogelijk. In **hoofdstuk 1** wordt een inleiding gegeven over syntrofie met speciale aandacht voor de syntrofe omzetting van propionaat. Tevens worden in **hoofdstuk 1** eigenschappen gepresenteerd van de enzymen fumaraat reductase/ succinaat dehydrogenase, fumarase en malaat dehydrogenase, omdat deze enzymen werden gezuiverd uit de syntrofe propionaat-oxiderende bacterie stam MPOB, die als modelorganisme werd gebruikt tijdens dit onderzoek. Stam MPOB werd enkele jaren geleden geïsoleerd uit korrelslib van een anaërobe reactor waarmee afvalwater van een suikerfabriek werd gezuiverd. De bacterie is strikt anaëroob; dit betekent dat stam MPOB in aanwezigheid van zuurstof niet kan overleven. Stam MPOB werd geïsoleerd als een propionaat-omzettende syntrofe culture met methanogene archaea, maar kon in reinculture worden verkregen met behulp van het substraat fumaraat.

Tijdens de uitvoering van dit onderzoek werd bekend dat andere syntrofe propionaat-oxiderende bacteriën, namelijk *Syntrophobacter wolinii* en *S. pfennigii*, de oxidatie van propionaat kunnen koppelen aan sulfaatreductie in reinculturen. In

hoofdstuk 2 wordt beschreven dat stam MPOB ook in staat is om sulfaat te reduceren. De groeisnelheid van stam MPOB met propionaat en sulfaat was, net als die van de twee *Syntrophobacter* soorten, erg laag vergeleken met de groeisnelheden van propionaat-oxiderende sulfaat-reducerende *Desulfobulbus* soorten.

Tijdens de oxidatie van propionaat komen bij drie tussenreacties reductie-equivalenten vrij, namelijk bij de oxidatie van succinaat tot fumaraat, malaat tot oxaalacetaat, en pyruvaat tot acetyl-CoA. Deze reductie-equivalenten komen vrij in de vorm van waterstof of formiaat, welke vervolgens verwijderd moeten worden door methanogene archaea om de propionaat-oxidatie te kunnen laten voortgaan. Om de oxidatie van succinaat tot fumaraat te vergemakkelijken is het niet alleen van belang dat de waterstof of formiaat concentratie laag gehouden wordt, maar ook de fumaraat concentratie. Hiervoor heeft de bacterie een fumarase nodig dat de omzetting van fumaraat tot malaat efficiënt katalyseert. De zuivering van dit enzym uit stam MPOB wordt beschreven in **hoofdstuk 3**. Het fumarase van stam MPOB bleek instabiel te zijn in aanwezigheid van zuurstof en werd daarom gezuiverd onder anaërobe condities in een anaërobe tent. Het enzym bestaat uit twee subunits die elk een moleculaire massa van 60 kDa hebben. De N-terminale aminozuurvolgorde van het fumarase van stam MPOB vertoonde duidelijke overeenkomsten met onder andere die van twee fumarases uit *Escherichia coli*. Met behulp van elektron paramagnetische resonantie (EPR) spectroscopie werd de aanwezigheid van een [4Fe-4S] cluster in het fumarase van stam MPOB aangetoond. Dit cluster reageert met het substraat fumaraat. De katalytische eigenschappen van het fumarase lieten zien dat het enzym efficiënter fumaraat omzet tot malaat dan andersom. Dit is belangrijk voor de vergemakkelijking van de energetisch ongunstige succinaat-oxidatie tijdens de omzetting van propionaat.

In **hoofdstuk 4** wordt de zuivering en karakterisering beschreven van het malaat dehydrogenase van stam MPOB. Dit enzym katalyseert één van de tussenreacties tijdens de syntrofe propionaat-oxidatie waarbij reductie-equivalenten vrijkomen, namelijk de NAD-afhankelijke omzetting van malaat naar oxaalacetaat. Het malaat dehydrogenase van stam MPOB bestaat uit twee subunits met een moleculaire massa van elk 35 kDa. De N-terminale aminozuurvolgorde van het enzym bevatte het geconserveerde gebied dat in de meeste malaat dehydrogenases, van zowel planten,

dieren als micro-organismen, voorkomt. De affiniteit van het enzym voor oxaalacetaat en NADH was hoger dan de affiniteit voor malaat en NAD, terwijl het enzym in de cel de omzetting van malaat tot oxaalacetaat katalyseert. Dit is mogelijk de reden voor de bijzonder hoge specifieke activiteit van het gezuiverde malaat dehydrogenase van stam MPOB (1728 U/mg eiwit).

De zuivering van het fumaraat reductase uit stam MPOB, beschreven in **hoofdstuk 5**, verliep erg moeizaam, hetgeen voornamelijk werd veroorzaakt door de zuurstofgevoeligheid van het enzym en het feit dat het een membraan-gebonden enzym betrof. Het gezuiverde enzym bevatte drie subunits die qua moleculaire massa, namelijk 70,5, 33,5 en 23,5 kDa, overeenkwamen met reeds bekende fumaraat reductases en succinaat dehydrogenases. Ook op grond van de N-terminale aminozuurvolgorde van de twee grootste subunits kon gezegd worden dat het fumaraat reductase van stam MPOB verwant is aan fumaraat reductases en succinaat dehydrogenases van andere micro-organismen. De meeste andere bekende fumaraat reductases en succinaat dehydrogenases bevatten drie verschillende ijzer-zwavel clusters, namelijk een [2Fe-2S], [4Fe-4S] en [3Fe-4S] cluster. Met behulp van EPR-spectroscopie konden deze drie clusters worden aangetoond in membranen van stam MPOB. In het gezuiverde fumaraat reductase konden echter alleen het [2Fe-2S] cluster en het [4Fe-4S] cluster worden aangetoond. Het signaal van het [4Fe-4S] cluster was bovendien erg zwak. Mogelijkerwijs zijn de [3Fe-4S] en [4Fe-4S] clusters gedeeltelijk verloren gegaan tijdens de zuiveringsprocedure, of na blootstelling aan zuurstof gedeeltelijk afgebroken.

De oxidatie van succinaat tot fumaraat en waterstof of formiaat is verreweg de energetisch ongunstigste tussenreactie tijdens syntrofe propionaat-oxidatie. Omdat deze reactie zelfs bij de voor methanogene archaea laagst haalbare waterstof- of formiaat-concentraties nog steeds endergoon is, wordt verondersteld dat de bacterie energie moet investeren in de oxidatie van succinaat. Men denkt dat de elektronen die bij de oxidatie van succinaat vrijkomen, via een omgekeerd elektronentransport naar een lagere redox potentiaal moeten worden geleid waarbij de elektronen wel gekoppeld kunnen worden aan de reductie van protonen of bicarbonaat. Het omgekeerd elektronentransport zou worden gedreven door de hydrolyse van $^2/_3$ ATP.

Uit groeistudies met stam MPOB, gekweekt op fumaraat met waterstof of formiaat als elektronendonor (**hoofdstuk 6**), is gebleken dat de bacterie ongeveer $^2/_3$ ATP verkrijgt uit de reductie van 1 mol fumaraat. Deze waarde ondersteunt de hypothese dat de bacterie in de omgekeerde reactie $^2/_3$ ATP moet investeren. In **hoofdstuk 6** wordt tevens de lokalisering beschreven van enkele enzymen die betrokken zijn bij de reductie van fumaraat. De enzymen fumaraat reductase, succinaat dehydrogenase en ATPase zijn gebonden aan de cytoplasmatische membraan. Hydrogenase en formiaat dehydrogenase zijn slechts zwak geassocieerd met deze membraan, waarschijnlijk aan de periplasmatische zijde. Daarnaast wordt in **hoofdstuk 6** beschreven dat stam MPOB twee typen menaquinonen bevat, menaquinon-6 en –7, en twee typen cytochromen, namelijk *b*-type cytochromen in de membraanfractie en *c*-type cytochromen in de oplosbare celfractie. Er zijn aanwijzingen gevonden dat cytochroom *b* en menaquinon onderdeel zijn van de elektronentransportketen tijdens de reductie van fumaraat. Indien de oxidatie van succinaat in stam MPOB gekoppeld is aan een omgekeerd elektronentransportmechanisme is het waarschijnlijk dat hierbij de elektronen-transportketen die betrokken is bij de reductie van fumaraat, in omgekeerde richting wordt gebruikt. Helaas konden hier tot dusver alleen enkele indirecte aanwijzingen voor worden verkregen. Omdat het niet is gelukt om van stam MPOB actieve membraanvesikels te maken, was het niet mogelijk om het effect van ionoforen en ATPase-remmers te bestuderen op de waterstofproductie uit de omzetting van succinaat tot fumaraat. Alleen met dergelijke experimenten is het mogelijk om te bewijzen dat de succinaat-oxidatie tijdens syntrofe propionaat-oxidatie wordt gedreven door een energieverbruikend omgekeerd elektronentransport.

Hoofdstuk 7 beschrijft de fysiologische karakterisering van stam MPOB. Stam MPOB wordt gezien als een nieuwe soort van het geslacht *Syntrophobacter*. Voor stam MPOB wordt de naam *Syntrophobacter fumaroxidans* voorgesteld, omdat de bacterie in reinculture kon worden verkregen dankzij zijn groei met fumaraat.

Tenslotte worden in **hoofdstuk 8** de voorafgaande hoofdstukken samengevat en bediscussieerd.

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Ine

CURRICULUM VITAE

Bernardina Louisa Maria (Ine) van Kuijk werd geboren op 29 augustus 1968 in het Brabantse Lieshout en groeide op in het nabij gelegen Sint-Oedenrode. In 1986, nadat ze geslaagd was voor het eindexamen gymnasium β aan het Mgr. Zwijsen College te Veghel, ging zij biologie studeren aan de Katholieke Universiteit Nijmegen. Na het behalen van het propedeutisch examen, in 1987, koos ze voor de afstudeerrichting Toegepaste Biologie. In het kader van deze richting deed ze een bijvakstage op het gebied van de moleculaire plantkunde bij het voormalig onderzoeksinstituut ITAL te Wageningen. Haar eerste hoofdafstudeervak verrichtte ze bij de afdeling Microbiologie en Evolutiebiologie van de Katholieke Universiteit Nijmegen, waarbij het β-glucosidase uit een anaërobe schimmel werd bestudeerd. Haar tweede hoofdafstudeervak, op het gebied van de moleculaire biologie en immunologie, bestond uit twee delen: het eerste deel was een stage bij Organon Teknika N.V. te Boxtel, waarbij immunoglobuline genen werden gekloneerd en tot expressie gebracht. Het tweede deel werd uigevoerd bij de afdeling Moleculaire biologie van de Katholieke Universiteit Nijmegen; hier werkte ze aan de expressie van een gen uit de malariaparasiet, in prokaryote en eukaryote expressiesystemen. Enkele weken na het behalen van het doctoraalexamen vertrok ze naar Wageningen om als onderzoeker in opleiding te gaan werken bij de vakgroep Microbiologie van de Landbouwuniversiteit. Ze werkte van oktober 1992 tot oktober 1996 aan de bestudering van de biochemie en bio-energetica van syntrofe propionaat-oxiderende bacteriën. Het resultaat van dit onderzoek, dat werd gefinancierd door de stichting Levenswetenschappen (SLW) die deel uitmaakt van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO), staat beschreven in dit proefschrift.