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Growth of *Syntrophomonas wolfei* in pure culture on crotonate

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Abstract. A *Synthrophomonas wolfei-Methanospirillum hungatei* coculture was adapted to catabolize crotonate. *S. wolfei* was then isolated in axenic culture using agar spread plates and roll tubes with crotonate as the sole energy source. *S. wolfei* catabolized crotonate via a disproportionation mechanism similar to that of some *Clostridium* species. Growth on crotonate was very slow (specific growth rate of 0.029 h^{-1}) but the conversion of energy into cell material was very efficient with cell yields of 14.6 g (dry wt.) per mol of crotonate. *S. wolfei* alone did not catabolize butyrate, but butyrate was stoichiometrically degraded to acetate and presumably methane when *S. wolfei* was reassociated with *M. hungatei*. *S. wolfei-M. hungatei* cocultures accumulated some butyrate during growth on crotonate indicating that protons were not the sole electron acceptors used for crotonate oxidation by the coculture.

Key words: *Syntrophomonas wolfei* – Pure culture – Crotonate – Syntrophic – Fatty acid degradation – Anaerobic – Methanogenesis

Syntrophomonas wolfei was originally described as an obligate proton-reducing, interspecies H_2-transfer dependent, anaerobic bacterium which catabolizes C_4 to C_8 straight-chain saturated fatty acids to acetate, propionate (from valerate or heptanoate) and H_2 (McInerney et al. 1979, 1981). Isoheptanoate is also catabolyzed to isovalerate, acetate and H_2. The anaerobic oxidation of saturated fatty acids requires the maintenance of a low H_2 partial pressure which necessitates the inclusion of a H_2-consuming organism in *S. wolfei* cultures (Bryant 1976; McInerney and Bryant 1980; Mah 1982). *S. wolfei* is routinely grown on butyrate medium in coculture with a H_2-using sulfate-reducing bacterium or a methanogen. *S. wolfei* can not be grown on butyrate in the absence of a H_2-using organism (McInerney et al. 1981). *S. wolfei* is representative of a group of bacteria which have become collectively referred to as obligate proton-reducing (H_2-producing) acetogenic bacteria. This group is responsible for the degradation of reduced organic compounds such as primary alcohols, fatty acids, and some aromatic compounds in anaerobic environments (McInerney and Bryant 1980). Their metabolic activities are essential for the complete degradation of organic matter to methane (Balba and Nedwell 1982; Boone 1982; Kasper and Wuhrmann 1978; McCarty et al. 1963; Schink 1985). Since the isolation of *S. wolfei* in cocultures, a number of similar interspecies H_2-transfer dependent associations have been described which use a variety of substrates in either methanogenic or sulfidogenic environments (Boone and Bryant 1980; Eichler and Schink 1985; Henson and Smith 1985; Mountfort and Bryant 1982; Shelton and Tiedje 1984; Stieb and Schink 1985; Zinder and Koch 1984).

Studies on the metabolism of *S. wolfei* and similar organisms have been hampered by the presence of the H_2-consuming organism, the low growth yields and very slow growth rates. Only 0.1 to 0.25 g (wet wt.) of cells of *S. wolfei-Methanospirillum hungatei* coculture is obtained per liter after 4 to 6 weeks incubation (Wofford et al. 1986). Recently extracts of butyrate-grown *S. wolfei* cells were obtained by selective lysis of a cell suspension of *S. wolfei* and *M. hungatei* with lysozyme (Wofford et al. 1986). The *S. wolfei* extracts contain low levels of methanogenic cofactors and have high activities of the enzymes associated with the β-oxidation pathway. However, breakage of *S. wolfei* cells with lysozyme is inefficient resulting in low protein and β-oxidation enzyme yields. Only limited success has been achieved on physically separating *S. wolfei* from *M. hungatei* using Percoll density gradients or in axenically growing *S. wolfei* on butyrate using a rapidly sparged fermentor to lower the H_2 partial pressure (P. S. Beaty and M. J. McInerney, unpublished data). The presence of β-oxidation enzymes suggested to us that *S. wolfei* could possibly be induced to catabolize crotonate. This would bypass butyrate dehydrogenation and increase the amount of free energy available for growth.

In this paper, we describe the isolation of *S. wolfei* from a butyrate-degrading coculture containing *M. hungatei*. The pure culture of *S. wolfei* catabolizes crotonate by a disproportionation reaction in which crotonate is oxidized to two acetate and reduced to butyrate and some caproate. Butyrate catabolism occurs only when *S. wolfei* is reassociated with a H_2-using organism.

Materials and methods

Organisms and growth conditions. Syntrophomonas wolfei (Göttingen strain) (DSM 2245B) in coculture with *Methanospirillum hungatei* JF1 was grown in the butyrate basal medium of McInerney et al. (1979). Methods for the preparation and use of anaerobically prepared media were essentially those of Bryant (1972) as modified by Balch and Wolfe (1976). Crotonate cultures were prepared in a similar manner substituting equimolar amounts of crotonate for butyrate. Culture purity was checked by regular microscopic

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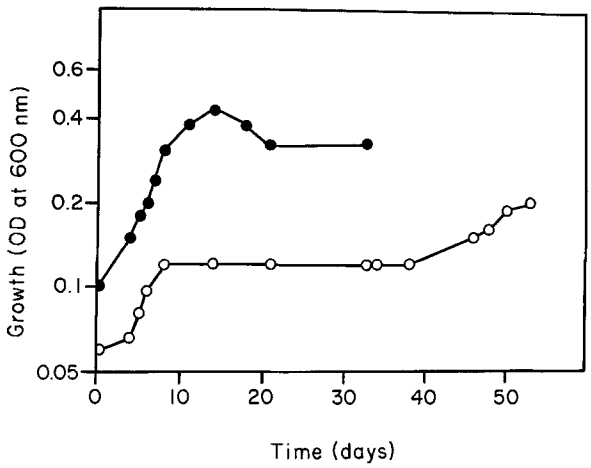

Fig. 1. Growth of acclimated and nonacclimated cocultures of *S. wolfei* with *M. hungatei*. The nonacclimated coculture was grown in butyrate basal medium with 0.1% crotonate (○). The acclimated cocultures were grown in crotonate basal medium (●). Data are means of duplicate cultures inoculated with 1 ml of the respective coculture

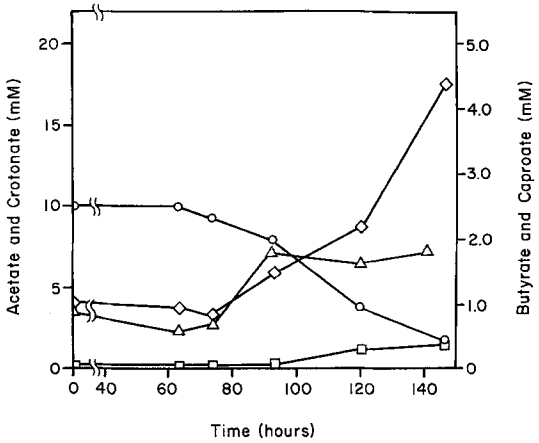

Fig. 2. Fermentation time course of the pure culture of *S. wolfei* in crotonate basal medium. Data are means of triplicate cultures. *Symbols*: crotonate, ○; butyrate, △; acetate, ◇; caproate, □

examination and inoculation of thioglycolate broth (Difco Laboratories, Inc., Detroit, MI, USA), which does not support the growth of either species. Growth was determined spectrophotometrically at 600 nm (McInerney et al. 1979). Generation times were calculated from the linear portion of the growth curve by measuring the time the culture took to double its optical density. Direct microscopic counts were done using a Petroff-Hausser counting chamber as described previously (McInerney et al. 1981).

Analytical methods. Acetate, butyrate, crotonate and caproate were determined as free acids with a gas chromatograph equipped with a flame ionization detector and a glass column (diameter, 6 mm; length, 2 m) packed with 10% SP-1000/1% H_3PO_4 on 100/120 chromosorb W/AW (Supelco, Inc., Bellefonte, PA, USA) (Sharak-Genthner et al. 1981) or a fused silica column (diameter, 530 μm; length, 10 m) packed with Carbowax 20 M (Hewlett-Packard, Palo Alto, CA, USA) (Jenneman et al. 1986). Methane and H_2 were measured with a gas chromatograph equipped with a thermal conductivity detector and a Poropak Q column (Supelco, Inc.) (Baresi et al. 1978). Identification and quantitation of each compound were made by comparing retention times and peak areas with those of known standards.

Results

A butyrate-catabolyzing coculture containing *Syntrophomonas wolfei* and *Methanospirillum hungatei* was inoculated into basal medium containing 0.1% butyrate and 0.1% crotonate. A small amount of growth, was observed during the first 10 days of incubation (Fig. 1), which is typical for this coculture on 0.1% butyrate. However, a second change in absorbance occurred after about 40 days and the coculture reached an absorbance of 0.19 on day 53. This coculture was inoculated into basal medium containing 0.2% crotonate as the sole carbon and energy source. The culture grew with a specific growth rate (μ) of 0.012 h^{-1} and had a final absorbance change of 0.35. Growth did not occur in the basal medium in the absence of crotonate or butyrate, nor in thioglycolate broth. The crotonate grown coculture contained cells morphologically identical to *S. wolfei* and *M. hungatei*. However, the ratio of *S. wolfei* cells to *M. hungatei* cells was much greater in crotonate grown cocultures (47:1) compared to butyrate grown cocultures (3:1).

S. wolfei was isolated on agar spread plates of the basal medium with 0.2% crotonate. The plates were inoculated with a crotonate grown *S. wolfei-M. hungatei* coculture in an anaerobic chamber (Balch and Wolfe 1976). The plates were incubated in a Torbal jar with a 80% N_2/20% CO_2 gas mixture at 37° C for 2 weeks. Opaque, tiny colonies less than 1 mm in diameter, appeared which contained only *S. wolfei* cells. These colonies were inoculated into basal media containing 0.2% crotonate and 0.3% Na_2SO_4. *Desulfovibrio vulgaris* strain G11 (McInerney et al. 1979) was added to several tubes in case a H_2-consuming organism was required. Growth of *S. wolfei* occurred within 2 weeks in all tubes indicating that the H_2 user was not needed. A culture of *S. wolfei* without *D. vulgaris* was then used to inoculate roll tubes containing the basal medium with 0.1% crotonate without sulfate. Colonies appeared between 7 to 14 days at dilutions up to 10^{-6}, only in roll tubes with crotonate. The colonies were greyish-brown, 1 to 2 mm in diameter, smooth with an entire margin and contained only *S. wolfei* cells. No other colony morphology was observed at any dilution. Colonies were picked and inoculated into tubes of crotonate liquid medium without sulfate. When growth occurred, these tubes were used to inoculate a second set of roll tubes. Identical colonies of *S. wolfei* appeared within 2 weeks only in roll tubes with crotonate added.

Crotonate was oxidized to acetate and reduced to butyrate, with the accumulation of a small amount of caproate by pure cultures of *S. wolfei* (Fig. 2). Crotonate catabolism proceeded according to Eq(1),

$$100\ \text{crotonate}^- \leftrightarrow 145\ \text{acetate}^- + 22.5\ \text{butyrate}^- + 3.3\ \text{caproate}^- + 70.9\ H^+ \quad (1)$$

with a 99% carbon recovery and 91% of the reducing equivalents accounted for in butyrate and caproate. Hydrogen was detectable in the headspace at less than 0.5 μmol/ml of gas throughout the growth phase. No methane was detected in any *S. wolfei* pure culture showing that a methanogen

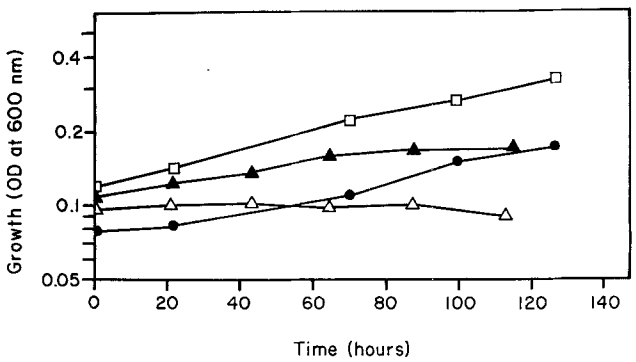

Fig. 3. Growth of the pure culture of *S. wolfei* (△) alone or reassociated with *M. hungatei* (▲) on butyrate basal medium and growth of the acclimated coculture of *S. wolfei* and *M. hungatei* on crotonate (□) or butyrate (●) basal medium. Data are means of triplicate cultures. The reassociated coculture contained 1 ml of a axenically grown *S. wolfei* and 0.4 ml of *M. hungatei*. The acclimated cocultures were inoculated with 1 ml of the coculture

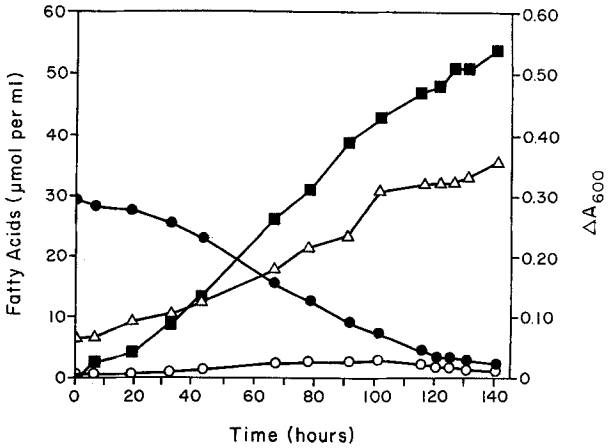

Fig. 4. Fermentation time course of the coculture of *S. wolfei* and *M. hungatei* in crotonate basal medium. The coculture was transferred biweekly for a period of 9 months before being used as the inoculum. Data are means of triplicate cultures. *Symbols*: growth, ■; crotonate, ●; acetate, △; butyrate, ○

Table 1. Use of butyrate by *S. wolfei* alone or in coculture with *M. hungatei*[a]

Culture	Butyrate (mM)	Acetate (mM)	%C recovery
S. wolfei alone	21.1 ± 0.37	<0.5	Not done
S. wolfei + *M. hungatei*	<1.0	38.7 ± 1.83	92.0

[a] Initial butyrate concentration was 21 mM. Data are means of duplicate cultures ± standard deviation

was not present. Growth was completely inhibited by the presence of 20% H_2 in the headspace. The cell yield of the pure culture of *S. wolfei* was 14.6 g (dry wt.) per mol of crotonate.

Growth occurred on butyrate basal medium only when *S. wolfei* was reassociated with either *M. hungatei* (Fig. 3) or *D. vulgaris* (in the presence of sulfate). The reassociated coculture and the original coculture had similar growth rates on butyrate (0.008 h^{-1}). Butyrate was stoichiometrically catabolized to acetate by the reassociated coculture (Table 1). However, *S. wolfei* alone was not capable of any butyrate metabolism. The butyrate grown reassociated coculture grew on crotonate without an extended lag period with a specific growth rate of 0.015 h^{-1}. The reassociated coculture grew very slowly (μ of 0.005 h^{-1}) when transferred to butyrate basal medium after growing on crotonate basal medium.

Initially, the reassociated coculture of *S. wolfei* and *M. hungatei* grew with a specific growth rate of 0.015 h^{-1} with an absorbance change of 0.5 on crotonate basal medium. After 9 months of culturing on crotonate basal medium, the reassociated coculture had a faster specific growth rate of 0.027 h^{-1} although the absorbance change (0.47) was similar. The reassociated coculture now accumulated butyrate during growth on crotonate with about 38% of the reducing equivalents generated during crotonate oxidation being accounted for by butyrate formation (Fig. 4). The butyrate was catabolized after the coculture reached stationary phase. A similar change in growth rate was observed with axenic cultures of *S. wolfei*, with the specific growth rate increasing from 0.011 h^{-1} to 0.021 h^{-1} after about 9 months of culturing on crotonate basal medium. The pure culture of *S. wolfei* now formed 0.22 mol of butyrate and 0.06 mol of caproate per mol of crotonate indicating that more caproate is formed than was originally observed when *S. wolfei* was first isolated on crotonate [Eq. (1)].

The specific growth rate of the pure culture of *S. wolfei* was determined in media with different crotonate concentrations. *S. wolfei* had a specific growth rate of 0.029 h^{-1} when 20, 50 or 70 mM crotonate was used. Slower growth rates were observed at crotonate concentrations of 10 mM (μ of 0.021 h^{-1}) and 90 mM (μ of 0.020 h^{-1}). A similar change in absorbance was observed at crotonate concentrations of 20, 50, 70, or 90 mM.

Discussion

Syntrophomonas wolfei and other proton-reducing acetogenic bacteria were considered to be obligately syntrophic bacteria since these bacteria need a H_2-using bacterium to maintain the low H_2 concentrations required for the metabolism of their energy sources (Bryant et al. 1967; Bryant 1976; McInerney et al. 1979). We have shown that *S. wolfei* can be adapted to grow on crotonate in pure culture. This allows the unique opportunity for the study of synthrophic bacteria since the growth yields and rates of *S. wolfei* are greatly increased with crotonate as the energy source. Also, axenically grown *S. wolfei* will allow the definitive study of the metabolism of this organism without the problems associated with using material which may contain some cellular components from the H_2-using bacterium (Wofford et al. 1986).

Using lysozyme to selectively lyse *S. wolfei* but not *Methanospirillum hungatei*, we have shown that *S. wolfei* contains the enzyme activities associated with β-oxidation, substrate-level phosphorylation and fatty acid activation by an acetyl CoA: butyrate coenzyme A (CoA) transferase reaction in butyrate grown cocultures (Wofford et al. 1986). The proposed butyrate degradation pathway (Wofford et al. 1986; Thauer and Morris 1984) predicts that about 1 mol of ATP is made per mol of butyrate degraded. Thauer and

Morris (1984) hypothesize that about two-thirds of this ATP is used to drive the reverse electron flow associated with H_2 production from electrons generated in the oxidation of butyryl-CoA to crotonyl-CoA since this process is energetically unfavorable even at low H_2 concentrations. The use of crotonate as an energy source bypasses this very unfavorable step and allows *S. wolfei* to grow in pure culture. Previously, it was reported that *S. wolfei* was unable to use crotonate (McInerney et al. 1979). Preliminary studies show that *S. wolfei* grown in pure culture with crotonate contains acetyl CoA:crotonate CoA transferase activity which was not detected in butyrate grown cocultures (N. Q. Wofford and M. J. McInerney, unpublished data). Pure cultures of *S. wolfei* can activate crotonate by CoA transferase reaction while cocultures of *S. wolfei* grown with butyrate cannot activate crotonate. These data suggest that we have selected a mutant of *S. wolfei* possessing an altered CoA transferase activity although it is possible that the induction or derepression of another enzyme specific for crotonate occurred. We obtained crotonate-using pure cultures of *S. wolfei* by growing the *S. wolfei-M. hungatei* cocultures in the presence of both butyrate and crotonate. Thus, a large number of *S. wolfei* cells were present which increased the probability of obtaining a variant/mutant of *S. wolfei* which uses crotonate.

Comparing the growth of *S. wolfei* on crotonate to that of crotonate-using anaerobes shows that *S. wolfei* grows much slower than *Clostridium kluyveri* (μ of 0.058 h^{-1}) (Thauer et al. 1968) or other clostridial species (μ of 0.15 h^{-1}) (Bader et al. 1980). However, the growth yield of *S. wolfei* [14.6 g (dry wt.) per mol of crotonate] is three times higher than that observed for other crotonate users (Bader et al. 1980; Stieb and Schink 1984; Thauer et al. 1968). Cells of *S. wolfei* axenically grown on crotonate contain about 20% poly-β-hydroxybutyrate (PHB) on a dry weight basis (D.A. Amos and M.J. McInerney, unpublished data). Even after correcting for the PHB content, the cells yields of *S. wolfei* on crotonate are much higher than other crotonate-using anaerobes. This suggests that *S. wolfei* is very efficient in conserving energy. This is to be expected since *S. wolfei* probably evolved a very efficient energy conservation mechanism in response to the very small change-in-free energy associated with butyrate degradation (Beaty et al. 1986; Thauer and Morris 1984; Mah 1982).

Studies on the pure culture of *S. wolfei* show that is has the same properties as the organism present in the coculture except for the newly acquired ability to use crotonate. Thus, the studies on pure culture of *S. wolfei* confirm that the properties attributed to this organism based on coculture studies (McInerney et al. 1979, 1981) are properties of *S. wolfei* and not those of the H_2 user. Thus, *S. wolfei* is a valid taxon. This work also shows that well controlled studies of known cocultures are useful in studying the physiology and taxonomy of bacteria.

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