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THE UNIVERSITY OF OKLAHOMA
GRADUATE COLLEGE

BIOENERGETIC PERSPECTIVES OF SYNTROPHIC SUBSTRATE DEGRADATION

A dissertation

submitted to the graduate faculty

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

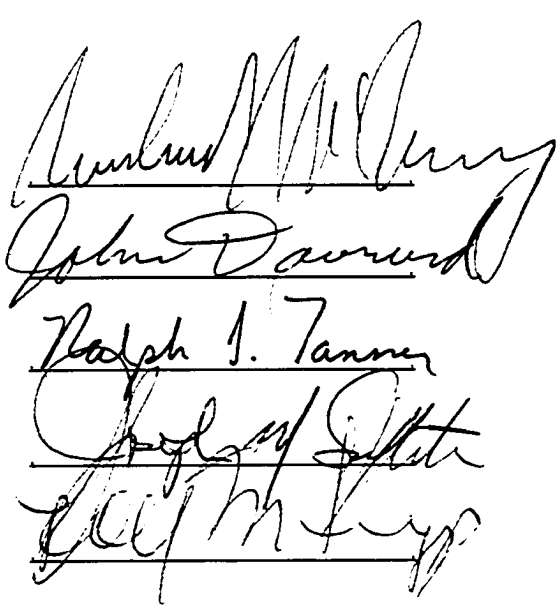
by

Bradley Eugene Jackson
Norman, Oklahoma
1999

BIOENERGETIC PERSPECTIVES OF SYNTROPHIC SUBSTRATE DEGRADATION

A Dissertation Approved for the Department of Botany and Microbiology

By



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Preface

This research investigates the ubiquity of substrate threshold formation and whether a critical amount of free energy is needed to sustain bacterial metabolism. Researchers from our laboratory first observed the phenomenon that cell suspensions of an *Syntrophus aciditrophicus* coculture under sulfate-reducing conditions degraded benzoate to a threshold concentration that persisted indefinitely. They concluded that the buildup of products thermodynamically controls the extent of substrate degradation. My research is founded upon this initial observation and extends these concepts to a variety of syntrophic bacteria, both aromatic and aliphatic substrates, and multiple terminal electron-accepting regimes. Specifically, Chapter I is a study on benzoate metabolism for all members of the genus *Syntrophus*, and Chapter II investigates butyrate metabolism in *Syntrophus aciditrophicus* and *Syntrophomonas wolfei*. Chapter III characterizes syntrophic benzoate and butyrate metabolism with respect to the Gibb's free energy remaining once a substrate threshold is formed and suggests a new hypothesis for defining the energetic limits of bacterial metabolism. All chapters and the appendix are written according to the guidelines for submission to the journal *Applied and Environmental Microbiology*, except Chapter III, which is written according to the guidelines for submission to the journal *Science*.

Chapter I

Thermodynamic Aspects of Syntrophic Benzoate Degradation.

ABSTRACT

The anaerobic bacterium *Syntrophus aciditrophicus* strain SB in coculture with a hydrogen-consuming partner, syntrophically degraded benzoate to a minimum threshold concentration under methanogenic, sulfate-reducing, and nitrate-reducing conditions. The benzoate threshold remained even after extended incubation and was directly influenced by the concentration of acetate in the medium. In cocultures amended with 60 mM acetate, benzoate was degraded to a threshold concentration of 1.5 ± 0.06 mM for methanogenic, 0.96 ± 0.58 mM for sulfate-reducing, and 2.8 ± 1.3 μM for nitrate-reducing conditions, respectively. The benzoate threshold was not due to a loss of metabolic activity, a kinetic inhibition due to low

substrate concentration, or a species specific phenomenon. In syntrophic cocultures containing *Syntrophus gentianae* or *Syntrophus buswellii* and a hydrogen-consuming partner, benzoate thresholds also occurred and were influenced by an accumulation of acetate. Once benzoate metabolism ceased, the Gibb's free energy ($\Delta G'$) for benzoate degradation ultimately depended on the terminal electron-accepting condition, and was independent of the final acetate concentration or syntrophic organism tested. These data show that thresholds for syntrophic benzoate degradation occur under multiple electron-accepting scenarios, that thresholds are thermodynamically controlled, and that once the benzoate threshold is reached, the amount of free energy available corresponds to a given terminal electron-accepting regime.

INTRODUCTION

In natural environments, the anaerobic degradation of aromatic compounds often involves a complex network of microbial species (2, 6, 21). The initial transformation of aromatic compounds to hydroxylated or carboxylated homologues often yields phenol or benzoic acid as products. Phenol and other substituted derivatives are commonly converted to benzoate prior to aromatic ring cleavage (3, 7-9). Thus, benzoate serves as the central intermediate for the anaerobic mineralization of numerous

aromatic compounds (3). It is likely that the factors controlling the rate and extent of benzoate degradation will also influence the effective conversion of aromatic compounds that are known to be degraded via the benzoyl-CoA pathway.

Syntrophic associations are characterized by having at least two microbial groups that are obligately interdependent for growth and substrate metabolism. In anaerobic ecosystems, benzoate can be degraded to acetate, bicarbonate, and hydrogen (or formate) by syntrophically fermenting bacteria (Equation 1) (2, 12). However, this primary reaction is highly endergonic under standard conditions and must be coupled to product removal

$$Benzoate^- + 7\ H_2O \rightarrow 3\ Acetate^- + 3\ H_2 + HCO_3^- + 3\ H^+ \quad (1)$$

$$\Delta G^{\circ\prime} = +70.6\ kJ \bullet reaction^{-1} \quad (6)$$

$$4\ H_2 + HCO_3^- \rightarrow CH_4 + 3\ H_2O \quad (2)$$

$$\Delta G^{\circ\prime} = -135.6\ kJ \bullet reaction^{-1} \quad (18)$$

$$4\ Benzoate^- + 19\ H_2O \rightarrow 12\ Acetate^- + 3\ CH_4 + HCO_3^- + 9\ H^+ \quad (3)$$

$$\Delta G^{\circ\prime} = -124.6\ kJ \bullet reaction^{-1}$$

by a secondary microbial group such as methanogens or sulfate-reducers (Equations 2). By keeping the hydrogen concentration at low levels via

interspecies hydrogen transfer, the oxidation of benzoate becomes thermodynamically favorable (Equation 2)(11). Thus, benzoate is effectively degraded only through the cooperation of metabolically different microbial groups.

Our laboratory has recently isolated a bacterium, *Syntrophus aciditrophicus* strain SB (5), that is capable of metabolizing benzoate and certain fatty acids, in syntrophic association with a hydrogen/formate-using bacterium. Concentrated cell suspensions of *Syntrophus aciditrophicus* strain SB and a sulfate-reducing bacterium were recently shown to degrade benzoate to a threshold concentration, at which no further benzoate degradation occurred (20). Using *S. aciditrophicus* , we wanted to determine whether the benzoate threshold concentration was associated with a specific $\Delta G'$. A minimum quantum of energy has been proposed as the limiting factor for bacterial metabolism (13-15, 19). If such a concept is true, then this $\Delta G'_{crit}$ value should represent the point at which metabolism ceases for all organisms, regardless of their environmental conditions. To test this hypothesis, we monitored syntrophic benzoate degradation and determined the $\Delta G'$ for all species of the genus *Syntrophus* under several different terminal electron-accepting conditions. In contrast to current hypothesis, a universal $\Delta G'_{crit}$ was not observed for all terminal electron-accepting processes, but rather a clustering of similar $\Delta G'$ values was found that corresponded to a given terminal electron-accepting condition. Thus,

the energetic minimum at which substrate degradation ceases depends on the redox process that is operative.

MATERIALS AND METHODS

Organisms and growth conditions. *Syntrophus aciditrophicus* strain SB^T (ATCC 700169^T) was grown in coculture with *Methanospirillum hungateii* strain JF1 under methanogenic conditions. *S. aciditrophicus* was also grown in coculture with *Desulfovibrio* sp. strain G11 under sulfate- or nitrate-reducing conditions depending whether sulfate or nitrate was added. *Methanospirillum hungateii* strain JF1 and *Desulfovibrio* sp. strain G11 were obtained from the culture collection of M.P. Bryant (Urbana, IL). A pure culture of *Syntrophus buswellii* strain DM-2^T (DSM 2612M^T) was obtained from the DSMZ culture collection (Braunschweig, Germany) and grown in coculture with *Desulfovibrio* sp. strain G11 under sulfate-reducing conditions. A coculture of *S. gentianae* strain HQGö1^T (DSM 8423^T) with *M. hungateii* was provided by Dr. Bernhard Schink (University of Konstanz, Germany) and was grown under methanogenic conditions.

Methods for preparation and use of anoxic medium were essentially those of Balch and Wolfe (1). A basal medium (10) modified with 5 mM sodium benzoate and 0.05% clarified rumen fluid, was used for the routine growth of all cocultures. Sodium sulfate and nitrate were added at a final

concentration of 10 mM. Acetate, sulfate, and nitrate were added by syringe transfer from anoxic, sterile stock solutions. Medium was dispensed into 18 x 150 mm serum culture tubes. Tubes were fitted with rubber stoppers, sealed with aluminum crimp seals, and sterilized by autoclaving. Cocultures containing *S. gentianae* were incubated at 30°C and cocultures containing *S. aciditrophicus* or *S. buswellii* were incubated at 37°C.

Benzoate degradation experiments. Benzoate degradation was monitored to ascertain whether thresholds were formed in growing syntrophic cocultures with different terminal electron-acceptors. Cultures were incubated for at least 30 days, and up to 360 days in some cases, to determine whether a true benzoate threshold was formed. Experiments contained initially 2.5 to 8 mM benzoate, and were sampled with time to monitor changes in benzoate, acetate, H_2, formate, and bicarbonate concentrations. All experiments were performed in duplicate or triplicate.

Cell suspensions. Concentrated cell suspensions of benzoate-degrading coculture were added to some experiments when the benzoate threshold was reached to determine whether the benzoate threshold was due to a kinetic inhibition. Approximately 3 liters of each benzoate degrading coculture was grown to late-exponential phase and anoxically harvested by centrifugation (12,000 x g, 30 min., 4°C). The resulting cell pellet was resuspended with a 0.05% dithiothreitol, 50 mM sodium phosphate buffer (pH 7.5), then the protocol was repeated. The final cell

pellets were resuspended in serum tubes with 5 ml of reduced, basal medium without benzoate under a 80% N_2 : 20% CO_2 (35 kPa overpressure).

Analytical procedures. Benzoate was measured by using a high pressure liquid chromatograph (HPLC) equipped with a reverse phase C - 18 column and a UV detector set at an absorbance of 254 nm. The HPLC was operated at a flow rate of 1.0 ml/min, using a mobile phase of 80% (vol./vol.) sodium acetate (50 mM, pH 4.5) and 20% (vol/vol) acetonitrile. The benzoate detection limit was approximately 250 nM. Formate was measured using a Dionex DX-500 ion chromatograph, equipped with an AS-11 anion exchange column and a CD 20 conductivity detector. The ion chromatograph was operated at a flow rate of 1.5 ml/min using a mobile phase of 0.5 mM NaOH. Formate was also measured spectrophotometrically by monitoring the formation of NADH at 320 nm from the enzymatic reduction of NAD by formate dehydrogenase (20). The detection limit for both methods was 1 µM. Acetate was measured by using a gas chromatograph equipped with a flame ionization detector (FID) and a 6' glass column packed with Carbopack B DA 80/20 4% Carbowax 20M resin. The GC was set to a flow rate of 24 ml/min helium and operated with an injector temperature of 180°C, an oven temperature of 165°C, and a detector temperature of 200°C. Carbon dioxide was quantified using a GC equipped with a thermal conductivity detector and a 6' stainless steel

column packed with Porapak Q resin. The GC was set to a flow rate of 20 ml/min helium and operated with an injector temperature of 100°C, an oven temperature of 50°C, and at a filament temperature of 180°C. Methane was quantified with a gas chromatograph (FID), equipped with a 6' stainless steel column packed with Porapak Q resin. The GC was set to a flow rate of 30 ml/min helium and operated with an injector temperature of 100°C, an oven temperature of 60°C, and a detector temperature of 125°C. Hydrogen was quantified with a mercury vapor reduction gas analyzer (17). The cell density of cultures was measured spectrophotometrically at 600 nm.

RESULTS

Syntrophic growth and metabolism with benzoate. Using benzoate as the energy source, cocultures of *Syntrophus aciditrophicus* and *Methanospirillum hungateii* grew and produced methane during the course of a 32 day incubation (Figure 1A). The relationship of the two microorganisms was obligately syntrophic, since growth and products of benzoate metabolism were not observed in benzoate-containing axenic cultures of *S. aciditrophicus* or *M. hungateii* . After 14 days of incubation, the coculture degraded approximately 5.5 mM benzoate and produced 17 mM acetate (Figure 1 B). For each mol of benzoate degraded, 3.04 mol of acetate and 0.61 mol of methane were produced, which is in good

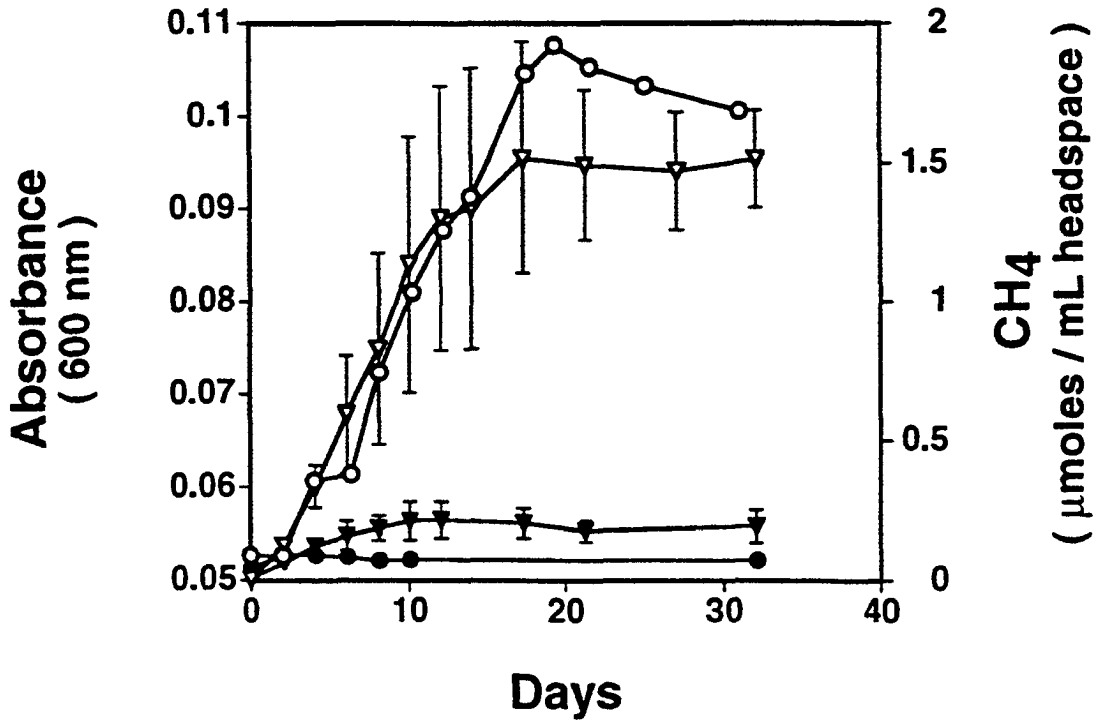

Figure 1 A. Growth and metabolism of 5 mM benzoate by a *Syntrophus aciditrophicus* coculture under methanogenic conditions. Open symbols represent cocultures incubated with benzoate and closed symbols represent cocultures incubated without benzoate. Methane production is represented by triangles (—▼—, —▽—) and biomass production is represented by circles (—●—, —○—).

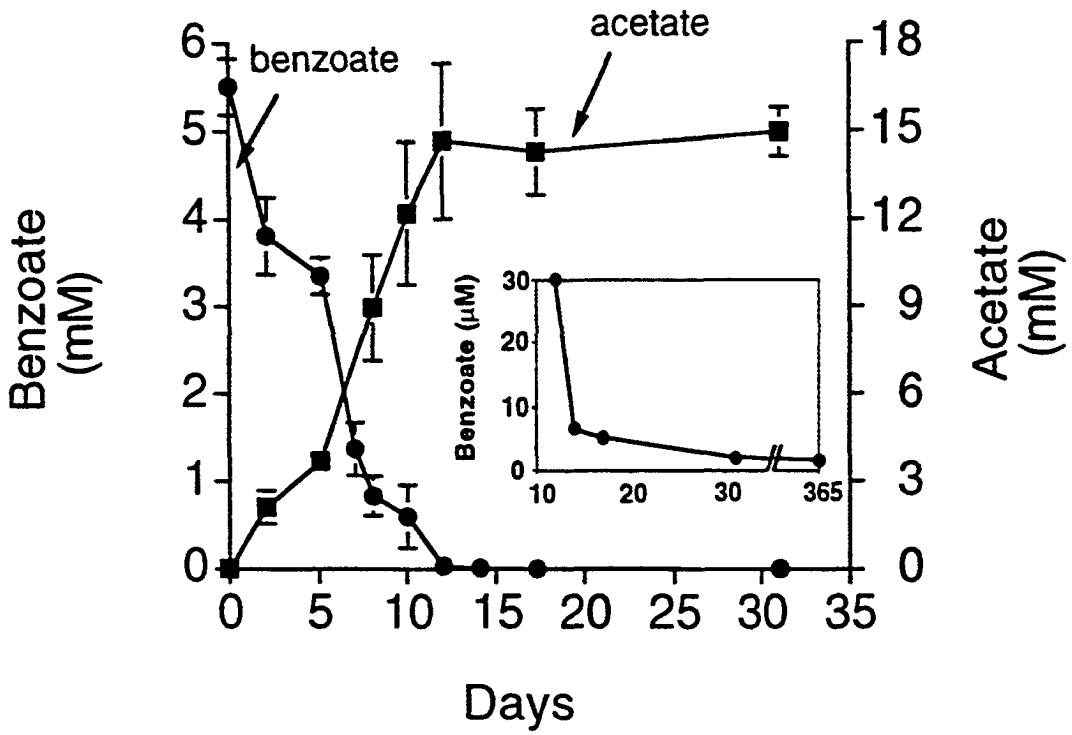

Figure 1 B. Benzoate metabolism and threshold formation by a *Syntrophus aciditrophicus* coculture grown under methanogenic conditions. Data are averages and standard devaitions of triplicate determinations.

agreement with the theoretical stoichiometry for syntrophic benzoate degradation coupled to hydrogenotrophic methanogenesis (Equation 3). A carbon recovery of 96% and a hydrogen recovery of 97% was obtained for this coculture.

The *S. aciditrophicus* and *M. hungateii* coculture degraded benzoate to a minimum threshold concentration of 1.9 µM after 32 days of incubation (Figure 1 B inset). Once a threshold was established, further benzoate degradation did not occur, even after 360 days of incubation. In cultures that had reached a benzoate threshold, degradation could be reinitiated with reamendment of the substrate, indicating that cells were still metabolically active.

At low substrate concentration, however, it is possible that microbial degradation of benzoate may be governed by kinetic constraints. If this hypothesis is indeed true, this phenomenon should be overcome by the addition of a large population of actively metabolizing cells. This hypothesis was tested by using an *S. aciditrophicus* coculture that had degraded benzoate to a threshold of 66 µM, and then reamending it with either 2 mM benzoate or with a concentrated suspension (approx. 10^9 cells/ml) of a benzoate-metabolizing coculture (data not shown). After 2 weeks of incubation, the cultures reamended with 2 mM benzoate degraded benzoate to a threshold concentration. However, in the tubes amended with an active cell suspension, benzoate was not further degraded. A similar experiment

was conducted for all cocultures under each electron-accepting condition. These results along with the work of Warikoo et. al. (20) suggest that the formation of a benzoate threshold is not due to kinetic constraints.

Effect of acetate on benzoate grown *S. aciditrophicus* cocultures with different terminal electron-acceptors. In cocultures containing *S. aciditrophicus* and *M. hungateii*, the effect of acetate on the extent of benzoate metabolism was investigated (Table 1). The coculture was grown with approximately 2.5 mM benzoate and different initial concentrations of acetate ranging from 0 mM to 60 mM. For all of the experimental condition tested benzoate was incompletely degraded to a threshold concentration. Increased acetate concentrations generally resulted in increased benzoate threshold concentrations. In tubes with less than 20 mM acetate (final concentration), the benzoate threshold was near 0.4 mM. However, in tubes containing a final acetate concentration near 70 mM, the benzoate thresholdconcentration was significantly higher, approximately 1.5 mM. In control tubes, the addition of sodium chloride instead of sodium acetate did not influence the benzoate threshold. Hydrogen was detected at low levels, 1.5 to 3.0 Pa, and formate was not detected. These hydrogen concentrations are typical for methanogenic conditions and suggest that inefficient interspecies hydrogen transfer did not limit benzoate degradation. Once the threshold concentration was reached,

Table 1. The effect of acetate on the thermodynamics and threshold value of benzoate degradadtion by growing cocultures of *S. aciditrophicus* under methanogenic conditions[a].

Addition[b]	acetate (mM)	benzoate (μM)	H_2[c] (Pa)	ΔG' (kJ/mol)
none	8.5 ± 0.6	0.4 ± 0 .1	1.9 ± 0.5	-22.4 ± 2.1
10 mM acetate	18.2 ± 1.0	0.4 ± 0.2	1.7 ± 0.5	-17.0 ± 1.2
30 mM acetate	38.0 ± 0.5	4.4 ± 0.7	1.6 ± 0.2	-17.5 ± 1.4
60 mM acetate	67.0 ± 0.8	1460 ± 60	3.0 ± 0.3	-24.0 ± 0.9
60 mM NaCl	7.1 ± 0.2	0.3 ± 0.1	1.5 ± 0.6	-24.3 ± 2.9

[a] Values are means with standard deviation of triplicate determination. Values were determined after 32 days of incubation.

[b] All tubes contained approximately 2.5 mM benzoate

[c] Formate was not detected (1 μM)

metabolism could be reinitiated upon reamendment with benzoate, indicating that cells were still viable even in the presence of high (60 - 80 mM) acetate concentrations (data not shown). Once a benzoate threshold was established, the change in free energy ($\Delta G'$) for benzoate oxidation was calculated based on the direct measurement of each substrate and product. Interestingly, the $\Delta G'$ was nearly identical at all concentrations of acetate tested, regardless of the benzoate threshold concentration, averaging –21.0 ± 3.5 kJ/mol, ranging from –17 to –24 kJ/mol.

Sulfate-reducing cocultures containing *S. aciditrophicus* and *Desulfovibrio* sp. strain G11 were grown with approximately 8 mM benzoate in the presence of different acetate concentrations (Table 2). When the final acetate concentration was increased from 24 mM to 80 mM, the benzoate threshold concentration increased from 1.3 μM to 960 μM. In all conditions, the extent of benzoate metabolism was directly influenced by the amount of acetate present; as the acetate levels increased, the benzoate threshold concentration increased correspondingly. Since hydrogen was detected in the coculture at levels characteristic of sulfate-reduction, 0.2 to 0.6 Pa, and formate was not detected, the hypothesis that the benzoate threshold was due to inhibitory levels of hydrogen or formate was not supported. The effect of sodium acetate on benzoate degradation was a direct result of the acetate anion and not a

Table 2. The effect of acetate on the thermodynamics and threshold value of benzoate degradadtion by growing cocultures of *S. aciditrophicus* under sulfate-reducing conditions[a].

Addition[b]	acetate (mM)	benzoate (μM)	H_2[c] (Pa)	ΔG' (kJ/mol)
none	24.1 ± 3.2	1.3 ± 0.5	0.2 ± 0.1	-32.7 ± 3.3
10 mM acetate	34.8 ± 1.5	2.3 ± 1.8	0.4 ± 0.3	-28.4 ± 5.6
30 mM acetate	52.4 ± 3.6	33.6 ± 15.8	0.4 ± 0.0	-30.5 ± 2.6
60 mM acetate	83.6 ± 6.6	959.9 ± 576.6	0.6 ± 0.2	-33.8 ± 2.1
60 mM NaCl	24.1 ± 1.2	4.3 ± 1.7	0.6 ± 0.3	-30.5 ± 4.5

[a] Values are means and standard deviation of triplicate determination.

[b] All tubes contained approximately 8.0 mM benzoate

[c] Formate was not detected (1 μM)

consequence sodium cation, since the benzoate threshold concentration in the control tubes containing 60 mM sodium chloride was nearly identical to that observed in tubes without sodium acetate. As observed in the methanogenic cocultures, a grouping of similar $\Delta G'$ values was observed at all concentrations of acetate tested, averaging -31.2 ± 2.1 kJ/mol, ranging from -28 to -34 kJ/mol.

S. aciditrophicus and *Desulfovibrio* strain G11 cocultures were adapted to use nitrate as the terminal electron acceptor and were grown with 2.5 mM benzoate (Table 3). A benzoate threshold was observed only when the final acetate concentration was greater than approximately 39 mM. Relative to methanogenic and sulfate-reducing conditions, the observed benzoate threshold for nitrate-reducing conditions was low, 0.4 µM when the acetate concentration was 39 mM and 2.8 µM when the acetate concentration was 68 mM, respectively. Formate was not detected and hydrogen was < 0.1 Pa, indicating that the benzoate threshold was not due to an inhibitory buildup of intermediates. The free energy change for benzoate degradation at threshold was higher than observed for methanogenic and sulfate-reducing conditions, averaging -42.5 ± 3.1 kJ/mol, ranging from -38 to -45 kJ/mol.

Effect of acetate on benzoate grown *S. gentianae* and *S. buswellii* cocultures. Experiments were conducted to determine if a

Table 3. The effect of acetate on the thermodynamics and threshold value of benzoate degradadtion by growing cocultures of *S. aciditrophicus* under nitrate-reducing conditions[a].

Addition[b]	acetate (mM)	benzoate (μM)	H_2[c] (Pa)	ΔG' (kJ/mol)
none	8.0 ± 0.2	BDL[d]	0.07 ± 0.02	- 45.9 ± 3.2
10 mM acetate	17.7 ± 1.2	BDL[d]	0.08 ± 0.02	- 38.3 ± 1.7
30 mM acetate	39.3 ± 5.2	0.38 ± 0.25	0.04 ± 0.03	- 40.3 ± 7.4
60 mM acetate	68.3 ± 3.2	2.77 ± 1.26	0.02 ± 0.01	- 44.7 ± 1.5
60 mM NaCl	7.3 ± 0.2	BDL[d]	0.10 ± 0.01	- 43.2 ± 0.5

[a] Values are means of triplicate determination. Values were determined after 50 days of incubation.

[b] All tubes initially contained approximately 2.5 mM benzoate.

[c] Formate was not detected (1 μM).

[d] A benzoate concentration of 0.1 nM was assumed below the detection limit.

benzoate threshold occurred with other species of the genus *Syntrophus* and whether the ΔG' for benzoate degradation at threshold depended on the terminal-electron-accepting process as well. Similar to *S. aciditrophicus* cocultures, a benzoate threshold was observed for all experimental conditions for *S. gentianae* and *S. buswellii* cocultures, respectively (Tables 4 and 5). Cocultures were grown with approximately 2.5 mM benzoate and final concentration of acetate varied from 10 mM and 75 mM. As observed with *S. aciditrophicus* , the resulting benzoate threshold concentration depended on the amount of sodium acetate but not sodium chloride present. The benzoate threshold was between 2 μM to 943 μM for *S. gentianae* methanogenic cocultures and between 2 μM to 370 μM for the *S. buswellii* sulfate-reducing cocultures, respectively. Hydrogen and formate levels were low and in the range expected for methanogenic and sulfate-reducing conditions. Though the benzoate threshold concentration dramatically increased with increasing acetate concentration, the ΔG' values were remarkably consistent for all conditions for a given terminal electron-accepting process. For the *S. gentianae* methanogenic cocultures, the ΔG' at threshold ranged from –20 to –24 kJ/mol, which was not statistically different than the overall average of –22.8 ± 1.8 kJ/mol. For the *S. buswellii* sulfate-reducing cocultures, the ΔG' at threshold ranged from –28 to –31 kJ/mol, which is not statistically than the overall average –29.7 ± 1.3 kJ/mol.

Table 4. The effect of acetate on the thermodynamics and threshold value of benzoate degradadtion by growing cocultures of *S. gentianae* under methanogenic conditions[a].

Addition[b]	acetate (mM)	benzoate (μM)	H_2[c] (Pa)	ΔG' (kJ/mol)
none	12.6 ± 2.3	1.5 ± 0.7	1.5 ± 0.2	-20.9 ± 2.9
10 mM acetate	19.0 ± 0.1	12.1 ± 0.0	1.1 ± 0.2	-25.1 ± 1.0
30 mM acetate	42.8 ± 0.8	519.9 ± 52.5	1.9 ± 0.3	-23.9 ± 1.2
60 mM acetate	59.6 ± 2.0	942.9 ± 164.2	2.4 ± 0.2	-21.1 ± 0.0
60 mM NaCl	14.7 ± 1.0	39.4 ± 18.4	3.0 ± 0.1	-22.9 ± 0.6

[a] Values are means and average deviation of duplicate determination. Values were determined after 60 days of incubation.

[b] All tubes contained approximately 3.0 mM benzoate

[c] Formate was not detected (1 μM)

Table 5. The effect of acetate on the thermodynamics and threshold value of benzoate degradadtion by growing cocultures of *S. buswellii* under sulfate-reducing conditions[a].

Addition[b]	acetate (mM)	benzoate (μM)	H_2[c] (Pa)	ΔG' (kJ/mol)
none	11.7 ± 0.6	1.6 ± 0.5	0.8 ± 0.2	-29.9 ± 1.6
10 mM acetate	19.4 ± 0.1	2.2 ± 0.1	0.7 ± 0.4	-28.0 ± 3.9
30 mM acetate	43.7 ± 6.2	17.9 ± 0.0	0.6 ± 0.2	-29.0 ± 0.4
60 mM acetate	73.9 ± 0.1	370.4 ± 5.2	0.7 ± 0.4	-31.3 ± 3.1
60 mM NaCl	12.3 ± 1.0	4.3 ± 1.0	1.0 ± 0.1	-30.3 ± 0.1

[a] Values are means and average deviation of duplicate determination. Values were determined after 50 days of incubation.

[b] All tubes contained initially 3.0 mM benzoate

[c] Formate was not detected (1 μM)

In addition, the average $\Delta G'$ values for *S. gentianae* and *S. buswellii* cocultures were not statistically different than the average $\Delta G'$ values obtained for *S. aciditrophicus* cocultures when grown under the same respective terminal electron-accepting condition.

For each terminal electron-accepting condition, an analysis of variance (ANOVA) was performed within and among different treatment groups. This was done to answer the question whether the means for $\Delta G'$ were statistically different. An F-test at the 1% level of significance (α = 0.001) showed that the means within groups i.e. different acetate concentrations were the same, and that the means between terminal electron-accepting conditions were different. Therefore, the averages for $\Delta G'$ values are statistically the same for a given terminal electron-accepting condition but are statistically different between terminal electron-accepting conditions.

DISCUSSION

The thermodynamics and influence of acetate on syntrophic benzoate degradation. Our experiments show that benzoate thresholds are a phenomenon for all known species of the genus *Syntrophus* growing syntrophically regardless of the acetate concentration or the terminal electron-accepting condition. In the presence of acetate

concentrations > 60 mM, the benzoate threshold could be quite large, e.g. 1.5 mM for methanogenic *S. aciditrophicus* cocultures and 960 μM for *S. aciditrophicus* sulfate-reducing cocultures. This is the first body of research that shows that benzoate thresholds are formed by different syntrophic species growing under a variety of terminal electron accepting processes. This work here also highlights the fact that an accumulation of acetate results in an increase in the threshold concentration, and that a specific $\Delta G'$ value corresponds to each terminal electron-accepting scenario once a threshold is reached.

It is likely that thermodynamics ultimately governs the extent of syntrophic benzoate degradation. Previous work from our laboratory has shown that benzoate thresholds are not due to a nutritional limitation, a kinetic inhibition, or a toxicity by the undissociated form of acetate (20). Additionally, this work shows that formation of a benzoate threshold is not a result of a kinetic inhibition of syntrophic metabolism at low benzoate concentrations, since after the addition of dense, actively degrading cells, the benzoate threshold was unaffected. Consistent with a thermodynamic explanation of a threshold, only 5 % of benzoate was degraded by *S. aciditrophicus* cocultures when hydrogen accumulated to levels more than 80 Pa compared to benzoate degradation greater than 99 % when hydrogen was kept below 1 Pa (5). In methanogenic cocultures with *S. gentianae*, benzoate degradation ceased with a buildup of hydrogen but

was restored by replacing the atmosphere with a N_2/CO_2 mix or by adding a hydrogen-using sulfate-reducing bacterium (16). Since both hydrogen and acetate are produced in stoichiometric ratios of 3 mol per mol benzoate degraded, the accumulation of acetate should also influence the extent of benzoate metabolism. In cocultures of *Syntrophus aciditrophicus*, *S. gentianae*, or *S. buswellii* for all terminal electron-accepting conditions, the benzoate threshold increased as the acetate concentration was increased. Once a threshold was established, the addition of an acetate-using sulfate-reducer or an acetate-using methanogen allowed further benzoate degradation in *S. aciditrophicus* or *S. gentianae* cocultures (16, 20). Thus, interspecies acetate transfer in addition to interspecies hydrogen transfer is important for the complete, syntrophic degradation of benzoate.

Interestingly, independent of the actual benzoate threshold or final acetate concentration, the free energy for benzoate degradation corresponded to the terminal electron-accepting process (Tables 1-5). Under methanogenic conditions for cocultures of both *S. aciditrophicus* and *S. gentianae*, the $\Delta G'$ at the benzoate threshold was near –22 kJ/mol. Under sulfate-reducing conditions with *S. aciditrophicus* and *S. buswellii* cocultures, the $\Delta G'$ was near –30 kJ/mol, and with *S. aciditrophicus* cocultures under nitrate-reducing conditions, the $\Delta G'$ was approximately –42 kJ/mol. It appears that the syntrophic organism's energy metabolism may be controlled by the redox process operative in the environment. Although we

have no direct explanation at this time, this phenomenon may actually be a function of the partner bacterium's capacity to consume hydrogen to progressively lower concentrations as the redox reaction becomes more thermodynamically favorable. In theory, the syntrophic bacterium can most effectively degrade benzoate when the hydrogen concentration is a its lowest i.e. nitrate-reducing conditions. Since more substrate is metabolized, one would assume that the bacterium can generate more ATP. We observed that for a given amount of substrate, nitrate-reducing cocultures of *S. aciditrophicus* grew to a greater absorbance than did methanogenic and sulfate-reducing cocultures (data not shown). Possibly, the distinct difference in remaining free energy ($CH_4 < SO_4 < NO_3$) observed at threshold between electron acceptors may be a result of additional ATP consumption that is needed to fuel the increased biomass production associated with a more thermodynamically favorable redox process.

This research attempts to elucidate whether a universal $\Delta G'$ can accurately describe the point at which benzoate metabolism stops. A universal $\Delta G'$ value of approximately –23 kJ/mol corresponding to 1/3 of an ATP equivalent has been hypothesized to be the minimum quantum of free energy for bacterial metabolism (13-15). If this value does indeed describe an absolute minimum for metabolism, then we would predict that substrate metabolism will cease near this value (e.g. not significantly different from this value), regardless of the substrate, organism, or physiological condition

tested. We observed significantly higher ΔG' values for benzoate degradation at threshold for different *Syntrophus* species when sulfate and nitrate were used as electron-acceptors. Schöcke and Schink (16) also reported ΔG' values higher than the postulated minimum quantum, from –31.8 to –45.8 kJ/mol for *S. gentianae* cocultures. For syntrophic butyrate degradation, however, the ΔG' values at threshold are consistently lower (< –15 kJ/mol) than observed for benzoate degradation and predicted for a minimum quantum (4). Taken collectively, these data for syntrophic benzoate and butyrate degradation argue strongly against the hypothesis that a minimum amount of free energy exists for bacterial metabolism.

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Chapter II

The energetics of syntrophic butyrate degradation and its implications for anaerobic digestion processes.

ABSTRACT

The anaerobic bacteria *Syntrophomonas wolfei* and *Syntrophus aciditrophicus* grown in coculture with a hydrogen-consuming partner degraded butyrate to a threshold concentration, which depended on the concentration of acetate in the medium. Butyrate remained even after extended incubation under methanogenic, sulfate-reducing, and nitrate-reducing conditions. The formation of a threshold appeared to be controlled thermodynamically and was not due to a loss of metabolic activity, an inhibitory accumulation of hydrogen or formate, toxicity of acetate, or a species specific phenomenon. For both *S. wolfei* and *S. aciditrophicus*

cocultures, the butyrate threshold concentration increased with increasing acetate concentrations. For *S. wolfei* cocultures, the butyrate threshold concentration decreased as the terminal electron-accepting regime became more favorable, with the highest threshold observed under methanogenic conditions and the lowest threshold observed under nitrate-reducing conditions. In the presence of > 60 mM acetate, *S. wolfei* degraded butyrate to a threshold of 91 μM under methanogenic conditions, 51 μM under sulfate-reducing conditions, and 45 μM under nitrate-reducing conditions, respectively. Independent of the final acetate concentration or the organism tested, the ΔG' at the butyrate threshold was specific for the terminal electron-accepting process and was – 4.1 ± 3.0 for methanogenesis, – 10.1 ± 2.3 for sulfate-reduction, and – 15.3 ± 1.6 kJ/mol for nitrate-reduction. This research shows that thresholds exist for syntrophic butyrate degradation, that the threshold is thermodynamically influenced by the accumulation of acetate, that the final ΔG' corresponds to the terminal electron-accepting scenario, and that substrate metabolism ceases when thermodynamic equilibrium is approached rather than when a critical ΔG' value is reached.

INTRODUCTION

The anaerobic degradation of organic matter via syntrophic interactions is a widespread process, occurring in both natural and man-made environments

such as waterlogged soils, freshwater sediments, municipal landfills, hydrocarbon-contaminated soils, and wastewater treatment facilities (9, 10, 14, 29, 30). For a given anaerobic environment, the mineralization of organic matter is not typically dominated by a single microbial group but is usually governed by a network of diverse, microbial activities (26). Syntrophism within a bacterial community is characterized by an interdependence between microbial groups for the effective degradation of substrate. Butyrate degradation is a classic example of a syntrophic interaction, especially under methanogenic conditions (24). The oxidation of butyrate to acetate and hydrogen by a single fermentative bacterium is endergonic under standard conditions (Equation 1). When the butyrate degradation reaction is coupled to the consumption of products by

$$\text{butyrate}^- + 2\,H_2O \rightarrow 2\,\text{acetate}^- + 2\,H_2 + H^+ \quad (1)$$

$\Delta G^{\circ\prime} = +\,48.3$ kJ/reaction (35)

$$4\,H_2 + HCO_3^- \rightarrow CH_4 + 3\,H_2O \quad (2)$$

$\Delta G^{\circ\prime} = -\,135.6$ kJ/reaction (35)

$$2\,\text{butyrate}^- + HCO_3^- + H_2O \rightarrow 4\,\text{Acetate}^- + CH_4 + H^+ \quad (3)$$

$\Delta G^{\circ\prime} = -\,39.4$ kJ/reaction

another microbial group (equation 2), the overall reaction becomes thermodynamically favorable (equation 3). Common partners for syntrophic bacteria are hydrogenotrophic methanogens and sulfate-reducing bacteria, which effectively consume hydrogen to levels below 3 Pa, thus allowing butyrate oxidation to occur via interspecies hydrogen transfer. This stepwise manner of metabolism appears to be a microbial motif for anaerobic environments, with metabolite production from one bacterial group serving as the substrate source for the next group. To that end, the complete degradation of organic compounds in anaerobic ecosystems is most effectively accomplished by an intimate cooperation between several microbial trophic groups.

The degradation and/or accumulation of fatty acids such as butyrate, propionate, and acetate is an important consideration in assessing anaerobic decomposition of complex organic matter within wastewater treatment digestors. Recent studies by Ahring et. al. (3) suggest that monitoring volatile fatty acid accumulation, in particular butyrate and isobutyrate, is a good indicator of impending anaerobic digestor instability. Because of the application to wastewater treatment systems, the factors that contribute to butyrate accumulation and, consequently, potential digestor failure is of great socioeconomic importance. Our laboratory has investigated previously the factors that govern substrate degradation in syntrophic systems, and using benzoate as a model aromatic compound, we

have observed the formation of persistent, benzoate threshold concentrations (18, 19, 40). These results with benzoate have prompted us to question whether this phenomenon, e.g. persistent substrate thresholds, are also observed with other syntrophic systems.

Using the syntrophically fermenting bacterium, *Syntrophomonas wolfei*, which metabolizes aliphatic fatty acids, or *Syntrophus aciditrophicus*, which metabolizes both aromatic and aliphatic acids, in coculture with hydrogen-using microorganisms, we investigated the factors that control the extent of butyrate degradation. Butyrate-degrading cocultures were challenged with varying acetate concentrations, and once metabolism had ceased, the $\Delta G'$ was determined based upon direct measurements of all reactants and products. We report on the physiological and thermodynamic limitations for syntrophic butyrate decay under methanogenic, sulfate-reducing, and nitrate-reducing conditions. Ultimately, these data yield information on whether a universal minimum quantum of energy exists for substrate degradation.

MATERIALS AND METHODS

Organisms and growth conditions. *Syntrophomonas wolfei* was grown in coculture with *Methanospirillum hungateii* strain JF1 under methanogenic conditions. *S. wolfei* was grown in coculture with *Desulfovibrio* sp. strain G11 under sulfate- or nitrate-reducing conditions depending whether sulfate or nitrate was added. *Syntrophus aciditrophicus* strain SB^T (ATCC 700169^T) was grown with *Desulfovibrio* sp. strain G11 under sulfate-reducing conditions. *Syntrophomonas wolfei*, *Methanospirillum hungateii* strain JF1, and *Desulfovibrio* sp. strain G11 were obtained from the culture collection of M.P. Bryant (Urbana, IL).

Methods for preparation and use of anoxic medium were essentially those of Balch and Wolfe (6). A basal medium (24) modified with 10 mM sodium benzoate and 0.05% clarified rumen fluid, was used for the routine growth of all cocultures. Sodium sulfate and nitrate were added at a final concentration of 10 mM. Acetate, sulfate, and nitrate were added by syringe transfer from anoxic, sterile stock solutions. Medium was dispensed into 18 x 150 mm serum culture tubes. Tubes were fitted with rubber stoppers, sealed with aluminum crimp seals, and sterilized by autoclaving. Cocultures were incubated at 35°C.

Butyrate degradation experiments. Butyrate metabolism was monitored to determine whether thresholds were formed in growing

syntrophic cocultures with different terminal electron-acceptors. Cultures were incubated up to 120 days, to determine whether a stable butyrate threshold was formed. Once a threshold was established, the addition of concentrated cell-suspensions did not alter the threshold concentration, however, a reamendment with additional butyrate stimulated substrate degradation. Experiments contained initially 2.5 to 20 mM butyrate, and were sampled with time to monitor changes in butyrate, acetate, H_2, and formate concentrations. All experiments were performed in duplicate or triplicate.

Analytical procedures. Butyrate and acetate were measured using a gas chromatograph equipped with a flame ionization detector (FID) and a 6' glass column packed with Carbopack B DA 80/20 4% Carbowax 20M resin. The GC column temperature was increased from 165°C to 185°C over 3.5 min, with an injector temperature of 180°C and a detector temperature of 200°C. The GC was set to a flow rate of 24 ml/min helium. Formate was measured using a Dionex DX-500 ion chromatograph, equipped with an AS-11 anion exchange column and a CD 20 conductivity detector. The ion chromatograph was operated at a flow rate of 1.5 ml/min using a mobile phase of 0.5 mM NaOH. Formate was also measured spectrophotometrically by monitoring the formation of NADH at 320 nm from the enzymatic reduction of NAD by formate dehydrogenase (40). The detection limit for both methods was 1 µM. Hydrogen was quantitated with a

mercury vapor reduction gas analyzer (33). Methane was quantified with a gas chromatograph (FID), equipped with a 6' stainless steel column packed with Porapak Q resin. The GC was set to a flow rate of 30 ml/min helium and operated with an injector temperature of 100°C, an oven temperature of 60°C, and a detector temperature of 125°C.

RESULTS

The effect of acetate on butyrate metabolism by *Syntrophomonas wolfei* cocultures with different terminal electron-acceptors. Growing cocultures of *Syntrophomonas wolfei* and *Methanospirillum hungateii* were used to ascertain whether butyrate was degraded to a threshold concentration and to determine the thermodynamics of butyrate degradation in the presence of varying acetate concentrations. Table 1 summarizes the effect of 5 to 60 mM acetate on the extent of degradation with 2.5 mM butyrate, the hydrogen partial pressure, and the $\Delta G'$ for butyrate metabolism once degradation ceased under methanogenic conditions. Cocultures were allowed to degrade butyrate until an apparent equilibrium was established and substrate degradation had ceased, typically 30 to 45 days. Additional butyrate decay was not observed beyond this period of time. Over 95 % of the butyrate degraded was recovered as acetate in a ratio of 1:2, in accordance with the expected

Table 1. The effect of acetate on the thermodynamics and threshold value of butyrate degradation by cocultures of *S. wolfei* under methanogenic conditions. [a]

Addition[b]	acetate (mM)	butyrate (μM)	H_2[c] (Pa)	ΔG' (kJ/mol)
none	5.1 ± 0.7	3.6 ± 0.2	1.3 ± 0.0	- 4.9 ± 0.6
10 mM acetate	15.0 ± 1.0	14.8 ± 3.8	1.8 ± 0.1	- 1.2 ± 0.8
30 mM acetate	34.5 ± 0.5	38.4 ± 5.6	1.4 ± 0.0	- 0.9 ± 0.2
60 mM acetate	68.2 ± 4.9	106.0 ± 14.2	1.2 ± 0.1	- 1.2 ± 0.4
60 mM NaCl	6.8 ± 0.5	8.5 ± 2.3	1.9 ± 0.3	- 3.7 ± 1.8

[a] Values are means and average deviations of duplicate determination.

[b] All tubes initially contained approximately 2.5 mM butyrate

[c] Formate was not detected (1 μM)

TAB 1

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stoichiometry outlined in Equation 1. In the methanogenic cocultures, methane production was confirmed by gas chromatography and was produced in the predicted stoichiometric quantities (data not shown) (equation 3). For every acetate concentration tested, a butyrate threshold was detected, ranging from 5 μM to 91 μM. Butyrate was not degraded when incubated with axenic cultures of *S. wolfei* or *M. hungateii* , and the substrate threshold was dramatically effected only by the presence of sodium acetate and not sodium chloride. A trend was observed that as the acetate concentration was increased, a corresponding increase occurred in the butyrate threshold concentration. Effective hydrogen (or formate) transfer did not limit butyrate degradation based upon the fact that hydrogen partial pressure was less that 2.0 Pa and formate was not detected. This result of low hydrogen partial pressures and of undetectable formate levels was observed for all cocultures tested. Interestingly, the $\Delta G'$ values were similar regardless of the acetate concentration tested, ranging from – 0.9 kJ/mol to – 4.9 kJ/mol (Table 1).

Similar trends were also observed for butyrate-degrading *S. wolfei* cocultures when grown under sulfate-reducing and nitrate-reducing conditions. A butyrate threshold was observed in all sulfate-reducing cocultures when grown with 2.5 mM butyrate and challenged with sodium acetate concentrations from 5 mM to 65 mM (final concentration) (Table 2). A butyrate threshold was detected for butyrate degradation by *S. wolfei*

Table 2. The effect of acetate on the thermodynamics and threshold value of butyrate degradation by cocultures of *S. wolfei* under sulfate-reducing conditions. [a]

Addition[b]	acetate (mM)	butyrate (μM)	H_2[c] (Pa)	ΔG' (kJ/mol)
none	5.5 ± 0.2	3.1 ± 0.5	0.24 ± 0.00	-12.9 ± 0.2
10 mM acetate	14.6 ± 0.4	11.3 ± 0.7	0.32 ± 0.01	-9.7 ± 0.1
30 mM acetate	34.1 ± 1.6	35.4 ± 0.3	0.27 ± 0.02	-9.1 ± 0.2
60 mM acetate	64.2 ± 1.4	51.1 ± 0.3	0.21 ± 0.02	-8.1 ± 0.3
60 mM NaCl	4.9 ± 0.5	3.2 ± 0.4	0.32 ± 0.06	-12.2 ± 0.9

[a] Values are means and average deviations of duplicate determination.

[b] All tubes initially contained approximately 2.5 mM butyrate

[c] Formate was not detected (1 μM)

under nitrate-reducing conditions, albeit only at high acetate concentrations, 50 mM to 80 mM (Table 3). For both sulfate- and nitrate-reducing conditions, the butyrate threshold appeared to be directly related to the acetate concentration, with the lowest threshold observed in cocultures with the least amount of acetate, and the highest threshold observed in cocultures with the most amount of acetate. For sulfate-reducing conditions, the butyrate threshold was between 3 μM and 51 μM, and for nitrate-reducing conditions, the threshold concentration ranged from < 2 μM up to 45 μM, respectively. Formate was not detected, and hydrogen partial pressures were low, in the expected range for each respective terminal electron-accepting process . Once a butyrate threshold had been established, the ΔG' value for a given terminal electron accepting process tightly clustered regardless of acetate concentration used. For sulfate-reduction, the ΔG' ranged from – 8.1 to – 12.9 kJ/mol butyrate, and for nitrate-reduction, the ΔG' ranged from – 15.1 to – 15.3 kJ/mol butyrate.

Figure 1 summarizes the relationship between the terminal electron acceptor and the butyrate threshold concentration when 60 mM acetate was added to the medium. When the electron acceptor in *S. wolfei* cocultures was changed from bicarbonate, to sulfate or nitrate, butyrate was degraded to a threshold concentration which decreased from101 μM, to 52 μM, to 38 μM, respectively. Thus, the terminal electron accepting process as well as acetate concentrations affected the butyrate threshold concentration.

Table 3. The effect of acetate on the thermodynamics and threshold value of butyrate degradation by cocultures of *S. wolfei* under nitrate-reducing conditions.[a]

Addition[b]	acetate (mM)	butyrate (μM)	H_2[c] (Pa)	ΔG' (kJ/mol)
none	19.0 ± 0.4	< 2	0.07 ± 0.01	NA
10 mM acetate	28.4 ± 1.2	< 2	0.05 ± 0.01	NA
30 mM acetate	48.8 ± 0.1	16.9 ± 2.4	0.04 ± 0.01	-15.1 ± 0.6
60 mM acetate	77.5 ± 3.4	45.2 ± 7.4	0.04 ± 0.01	-15.3 ± 0.3
60 mM NaCl	17.9 ± 1.0	< 2	0.06 ± 0.01	NA

[a] Values are means and average deviations of duplicate determination.

[b] All tubes initially contained approximately 10 mM butyrate

[c] Formate was not detected (1 μM)

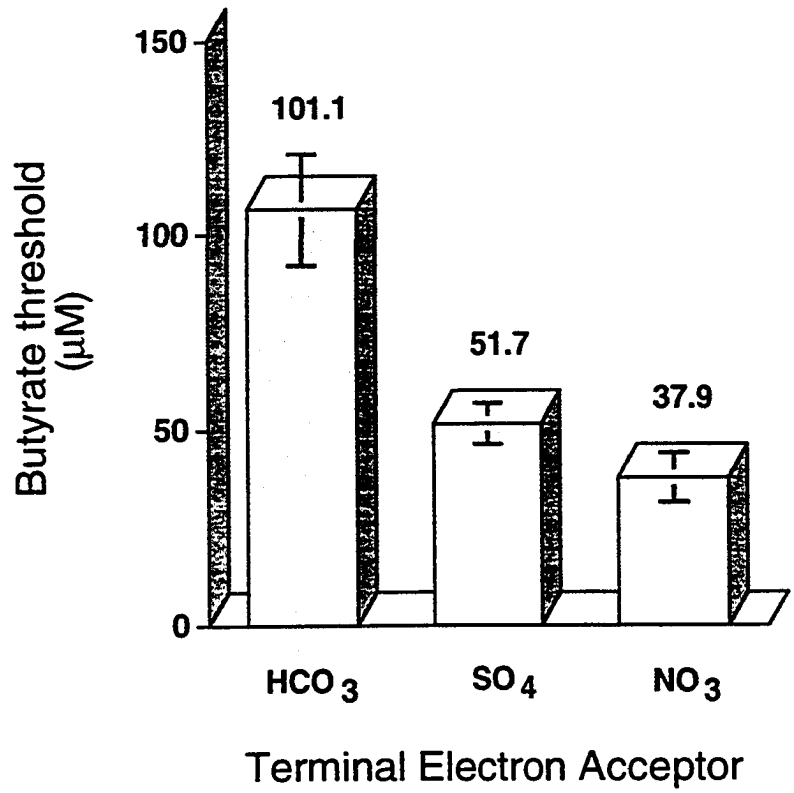

Figure 1. The butyrate threshold concentration for *S. wolfei* cocultures amended with 60 mM acetate under methanogenic (HCO_3^-),sulfate-reducing (SO_4^{-2}),and nitrate-reducing (NO_3^-) conditions. The final acetate concentration was normalized to 65 mM since culturescontained different final acetate concentrations after substrate metabolism. Data are the average and standard deviation of triplicate deterrminations.

The effect of acetate on the thermodynamics of sulfate-reducing *Syntrophus aciditrophicus* cocultures. Cocultures of *S. acidtrophicus* and *Desulfovibrio* strain G11 were grown on butyrate to determine if thresholds were formed by other syntrophic butyrate-using species, and whether the extent of butyrate metabolism with different acetate concentrations was similar to that observed for *S. wolfei.* Sulfate-reducing, *S. aciditrophicus* cocultures degraded butyrate to a threshold concentration under all conditions tested, and the addition (or accumulation) of acetate greatly influenced the extent of butyrate degradation (Figure 2). The highest final acetate concentration resulted in the highest butyrate threshold, and an exponential curve fit of the data shows a high degree of correlation (97 %) between final acetate concentration and butyrate threshold concentration. Once a butyrate threshold was established, tne $\Delta G'$ for all conditions tested averaged – 11.0 ± 1.4 kJ/mol, a value very similar to the average observed for sulfate-reducing, *S. wolfei* cocultures (– 10.4 ± 2.1).

For each terminal electron-accepting condition, an analysis of variance (ANOVA) was performed within (i.e. different acetate concnetrations) and among (i.e. different terminal electron-acceptors) statistical groups. This was done to answer the question whether the means for $\Delta G'$ were statistically different. An F-test at the 1% level of significance (α = 0.001) showed that the means obtained for each acetate concentration for a given terminal electron-accepting process were the same, and that the

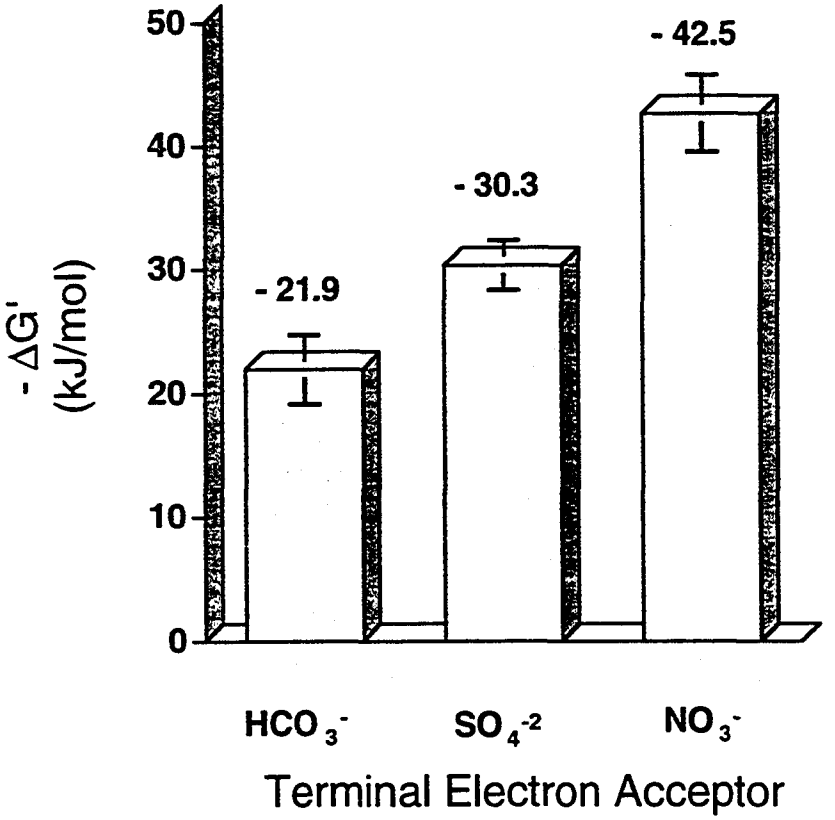

Figure 2. Thel free energy (ΔG') value at the substrate threshold concentration for benzoate-grown cocultures under methanogenic(HCO_3^-), sulfate-reducing (SO_4^{-2}), and nitrate-reducing (NO_3^-) conditions.

means obtained for each terminal electron-acceptor were different from each other. Therefore, the averages for $\Delta G'$ values are statistically the same for a given terminal electron-accepting condition but are statistically different between terminal electron-accepting conditions, regardless of the species or acetate concentration used.

DISCUSSION

In this study, butyrate thresholds for growing, syntrophic cultures are reported for the first time. Thus, the phenomenon of persistent, syntrophic substrate thresholds is not an isolated occurrence, being observed in phylogenetically distant genera, *Syntrophomonas* and *Syntrophus*, with different terminal electron-accepting scenarios and with different substrates, butyrate and benzoate (20, 32, 40). The actual butyrate threshold concentration depended on the amount of final acetate concentration as well as the specific terminal electron-accepting process, and was detected at concentrations as high as 250 μM. Thus, it is likely that the butyrate threshold concentration is ultimately governed by thermodynamic limitations, and has been argued for syntrophic benzoate degradation as well (32, 40).

The persistence of remnant concentrations of butyrate could not be attributed to numerous additional physiological factors. Threshold formation resulting from a of loss of metabolic activity due to an acetate toxicity was not

a feasible explanation, since thresholds were observed even when acetate concentrations were low (< 5 mM), and since butyrate metabolism could be reinitiated in cocultures that had reached a threshold, even in the presence of high acetate concentrations (60 to 80 mM). An accumulation of hydrogen or formate is well documented (1, 11, 30, 39) to inhibit syntrophic substrate degradation and was thus monitored in our experiments. All active cocultures efficiently consumed hydrogen to levels expected for the respective terminal electron-accepting condition, never exceeding a partial pressure of 2 Pa; formate was not detected. In contrast, butyrate degradation was nonexistent or severely limited when hydrogen accumulated in controls tubes containing cocultures deficient of an electron-acceptor or in cultures of the syntrophic bacterium alone (data not shown). Butyrate threshold formation even with low hydrogen partial pressure is consistent with a thermodynamic explanation, as also observed for syntrophic benzoate degradation (32, 40).

In accordance with equation 1, the buildup of acetate should exhibit as much thermodynamic backpressure as the buildup of hydrogen, since both compounds are produced in stoichiometric ratios of 2 mol per mol of butyrate oxidized. This hypothesis indeed appears to be true, since a consistent trend that was observed for all cocultures was that an increase in acetate concentration resulted in an increase in the butyrate threshold concentration. This argument is also strengthened by previous observations

in syntrophic benzoate- and butyrate-degrading cocultures that substrate degradation resumes once the accumulated acetate is removed by addition of an aceticlastic methanogen or sulfate-reducing bacterium (2, 7, 32, 40). These results indicate that bacterial substrate degradation proceeds to a thermodynamically controlled threshold concentration, with the extent of metabolism ultimately determined by an accumulation of endproducts until an equilibrium is reached which is defined by the mass-action ratio.

Operational improvements in the methanogenic degradation of sewage and other industrial wastes has been based on controlling the inhibitory effects of increased hydrogen (15, 27, 34) and/or formate concentrations in reactors (37, 38) and on maintaining low concentrations of the undissociated form of volatile fatty acids (4, 12, 13). High rate anaerobic digestion systems have been developed using biofilms or naturally immobilized microbial populations (granules) to avoid the washout of the slow growing populations and to bring syntrophic bacteria in close physical proximity to methanogenic bacteria (22). Thiele et. al. (39), noted that the selection of different syntrophic bacteria and acetate-using methanogens may have been the reason for improved rates of volatile fatty acid degradation in their granules. Models based on the inhibitory effects of high hydrogen concentration (23, 34) or on the toxicity of the undissociated form of volatile fatty acids (4, 12, 13) have been used to predict volatile fatty acid degradation. The influence of acetate on the threshold for butyrate and

benzoate degradation (20, 32, 40) suggests that the concentration of acetate also influences the energetics of these reactions.

At low hydrogen partial pressures (about 3 Pa) the degradation of propionate is favorable at acetate concentrations up to 100 mM, and butyrate degradation is favorable up to acetate concentrations of 60 mM, assuming a substrate concentration of 0.1 mM (25). However, if conditions in the reactor result in a higher hydrogen partial pressure (about 30 Pa), then acetate concentrations must be much lower for the degradation of propionate and butyrate to be favorable, about 0.1 and 5 mM, respectively. Thus, if either hydrogen or acetate become elevated, syntrophic metabolism becomes more sensitive to perturbation by an increase in the concentration of the other product. It may be that acetate controls anaerobic digestion by affecting the energetics of syntrophic metabolism rather than by a toxicity of its undissociated form. Acetate was shown to inhibit the extent of benzoate degradation by syntrophic cocultures (30, 32) and butyrate degradation by fluidized bed reactors (21) even though the H_2 pressure was low.

The impetus driving this research was to answer the question: If thermodynamics ultimately govern the extent of substrate metabolism, then does a critical $\Delta G'$ exist that can universally describe the point at which microbial metabolism ceases? The concept of a minimum or quantum of energy value was proposed by Thauer and Morris (36) and expanded by Schink and Thauer (31) in order to explain energy conservation in

organisms that grow with reactions where the $\Delta G'$ is less than that required to make 1 mol of ATP. These theories are still used today to explain bacterial metabolism (30). Based upon intracellular concentrations of ATP, ADP, and P_i in actively growing *Echerichia coli* cells, a free energy of about + 50 kJ/mol is needed to synthesize ATP and an additional + 20 kJ/mol is believed to be lost as heat. Thus, about +70 kJ is needed to irreversibly conserve 1 mol of ATP (28). If one assumes that a molecule of ATP is formed through vectoral translocation of 3 ions across an energized membrane, then the smallest unit of potential energy gained is the transport of one ion or $^1/_3$ of an ATP unit (28). Combined with the above calculations, the passage of one ion across the bacterial membrane corresponds to approximately 23 kJ/mol as the minimum quantum of free energy necessary to sustain microbial metabolism (28, 30). However, systematic testing of this hypothesis has been lacking.

Our research has focused on the $\Delta G'$ once a threshold equilibrium is established, in order to surmise the energetic situation of the bacterium when substrate metabolism ceases. The $\Delta G'$ at threshold for syntrophic butyrate degradation under all terminal electron-accepting conditions tested, was significantly lower, e.g. less than –15 kJ/mol, than that postulated for the minimum energy concept. This apparent discrepancy was consistently observed for two phylogenetically distinct syntrophic genera, *Syntrophomonas* and *Syntrophus*, for many different acetate concentrations

from 5 to 75 mM, and for different terminal electron-accepting processes. Although previous research has not centered exclusively on substrate thresholds, other authors have noted ΔG' values for butyrate degradation similar to ours, ranging from –3.2 kJ/mol in digesting sludge (17), –3.8 kJ/mol for methanogenic cocultures (11), and –17.0 kJ/mol for sulfate-reducing cocultures (11). For syntrophic benzoate degradation, significantly higher average ΔG' values than predicted by the minimum quantum energy concept were found, e.g. near –30 kJ/mol, in washed cell suspensions of sulfate-reducing cocultures when a benzoate threshold was reached in the presence of > 30 mM acetate (40). We have also shown that growing sulfate- and nitrate-reducing syntrophic cocultures stop metabolizing benzoate at ΔG' values near –30 kJ/mol and –42 kJ/mol, respectively (20). Schöcke and Schink (32) observed ΔG' values from approximately –30 kJ/mol to –45 kJ/mol for apparent benzoate thresholds with *Syntrophus gentianae* and *Methanospirillum hungateii* cocultures. Thus, there does not appear to be a single ΔG' value that accurately predicts where substrate metabolism ceases.

These results obviously suggest a reconsideration of our current energetic hypotheses and raises the question of why such variance is observed. The calculated ΔG' with regard to the metabolic state of the system is crucial in understanding the bacterial energetic circumstance, and our research highlights threshold formation (where substrate metabolism

ceases) as the reference point for $\Delta G'$ determination. Two apparent trends stand out from investigating syntrophic substrate degradation and threshold formation: a) the remaining free energy is a function of the terminal electron-accepting regime, and b) less free energy remains for the syntrophic degradation of butyrate than for the degradation of benzoate. Since methanogenesis even under optimal conditions is the most energetically challenged relative to sulfate- and nitrate-reduction, it is possible that methanogenic processes have evolved to become the most efficient in the conservation of what little energy is available and, by necessity, lends itself to the tightest metabolic coupling with syntrophic metabolism.

The disparity observed between butyrate and benzoate degradation with respect to residual free energy may be explained as the difference in energy required for the syntrophic bacterium to activate the respective substrate. Activation of benzoate by all known *Syntrophus* species, *S. aciditrophicus*, *S. gentianae*, and *S. buswellii*, have been shown to occur via a benzoyl-CoA ligase reaction, which potentially consumes 2 mol of ATP per mol of benzoate activated (5, 8, 16). Additional ATP may also be needed to dearomatize the highly stable benzene ring, as is the case for *Thauera aromatica* and *Rhodopseudomonas palustris* (16). For *Syntrophomonas wolfei*, however, Wofford et. al. (41) demonstrated that the activation of butyrate was neutral with regard to energy consumption and is

accomplished via a butyryl:acetyl-CoA transferase. The biochemistry of activation may account for why less free energy remains for syntrophic butyrate degradation than for benzoate degradation. These data suggest that the respective physiologic status of the cell and the biochemical reaction involved may ultimately determine the extent of substrate metabolism. These results and queries indicate that much research is still needed to understand of how syntrophic interactions are governed at the energetic limits of metabolism.

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Chapter III

Bacterial metabolism: Does a biological energy quantum exist?

Abstract. In order to answer the question whether an energetic minimum exists that limits bacterial substrate metabolism, we investigated the anaerobic degradation of benzoate and butyrate by syntrophic associations. We observed that substrate degradation ceases at a threshold concentration, which was thermodynamically controlled. Once a substrate threshold concentration was reached, a unique $\Delta G'$ value was observed that depended on the bacterial species, terminal electron-accepting condition, or substrate tested. In contrast to the current bioenergetic hypothesis, we found that substrate metabolism could proceed at values close to thermodynamic equilibrium ($\Delta G' \sim 0$ kJ/mol) rather than ceasing at the value postulated to be the smallest quantum of metabolically convertible energy ($\Delta G' \sim -20$ kJ/mol).

The anaerobic degradation of organic compounds through syntrophic cooperations is a ubiquitous process, occurring in both man-made and natural environments such as sewage digesters, municipal landfills, hydrocarbon-contaminated soils, freshwater sediments, and waterlogged soils (1 - 5). The complete anaerobic degradation of organic matter is not typically dominated by a single microbial group, but requires the intimate interaction among numerous microbial species (6). Syntrophic interactions are highlighted by a metabolic interdependence between species, and this scheme of metabolism allows the degradation of substrate to occur in environments where a single species is unable to degrade a given compound due to thermodynamic or biochemical constraints (6). Table 1 shows examples of some common reactions involved in syntrophic metabolism and the $\Delta G^{o'}$ for the oxidation of a given substrate. For example, the degradation of benzoate or butyrate to hydrogen and acetate as endproducts by a single organism is endergonic under standard conditions (Table 1). However, when butyrate or benzoate fermentation is coupled to hydrogen-consumption by bacteria such as methanogens, the combined process becomes exergonic. Thus, a syntrophic cooperation via interspecies hydrogen (or formate) transfer permits the degradation of energetically unfavorable substrates to occur. However, even under conditions where hydrogen levels are low, the free energy available (–20 to

Table 1. Reactions involved in syntrophic metabolism[a]

Reaction	$\Delta G^{o'}$ (kJ/reaction)
Methanogenic H_2 consumption	
$4\ H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3\ H_2O$	- 135.6
Syntrophic degradation without H_2 consumption	
$Butyrate^- + 2\ H_2O \rightarrow 2\ Acetate^- + H^+ + 2\ H_2$	+ 48.3
$Benzoate^- + 7\ H_2O \rightarrow 3\ Acetate^- + HCO_3^- + 3\ H^+ + 3\ H_2$	+ 70.6[b]
Syntrophic degradation with H_2 consumption	
$2\ Butyrate^- + HCO_3^- + H_2O \rightarrow 4\ Acetate^- + H^+ + CH_4$	- 39.4
$4\ Benzoate^- + 19\ H_2O \rightarrow 12\ Acetate^- + HCO_3^- + 9\ H^+ + 3\ CH_4$	- 124.4

[a] Calculated from the data given in Thauer et al (8).
[b] Calculated using the free energy of formation for benzoate given in Kaiser and Hanselmann (9).

–40 kJ/mol) for metabolism is much less than that required to make ATP (Table 1)(2, 7).

Syntrophism and interspecies hydrogen transfer are also important from an evolutionary standpoint. Martin and Müller (8) recently proposed a novel hypothesis for the origin of eukaryotic cells based upon a syntrophic relationship between an anaerobic bacterium such as a methanogen and a heterotrophic eubacterium. In this model, the symbiont (the eubacterium) metabolizes organics to hydrogen and acetate, while the host (the methanogen) consumes the waste products, namely hydrogen, of the symbiont. This hypothetical syntrophic scenario for the evolution of the first eukaryote is highly plausible because interspecies hydrogen transfer provides a strong selective pressure for a strict dependence between host and symbiont. Therefore syntrophism has far reaching implications for the evolution of multicellular life and for the cycling of organic matter in natural and man-made environments.

The concept that free energy transduction in bacteria, particularily fermentative anaerobes, is coupled to the stoichiometric formation of ATP (11) has given rise to the view that free energy is conserved in quantal packets and that there is a biological energy quantum necessary to synthesize ATP (12). It is believed that approximately + 70 kJ of free energy is required to irreversibly synthesize 1 mol of ATP in a living bacterial cell (2). Based upon the Mitchell hypothesis of respiration, the formation of ATP

is coupled to the vectoral translocation of ions, protons or sodium, across an energized biological membrane. If the ratio of ions transported to ATP formed is 3:1, then the smallest quantum of energy utilizable by a bacterium is that generated through the translocation of one ion across the bacterial membrane or the equivalent of $^1/_3$ of an ATP. These concepts have led to the conclusion that a free energy change of approximately – 23 kJ/mol corresponds to a minimum quantum of energy, which can support microbial metabolism (2, 7, 12).

In order to understand the lower limits of microbial bioenergetics, we investigated anaerobic syntrophic associations as the representative bacterial system since they proliferate and thrive on minimal amounts of free energy (2). To explain how organisms involved in syntrophic associations conserve energy from reactions that yield significantly less energy than that needed to make an ATP, it has been proposed that the change in free energy of the overall reaction is shared equally by all partners and that this amount is in the range of the minimum energy quantum of about –20 kJ/mol which can be exploited for ATP synthesis (7, 12). We have previously observed the phenomenon that benzoate and butyrate grown syntrophic cocultures incompletely degraded their substrate to a threshold concentration, which could be quite large, up to 1.5 mM (13, 14). The substrate threshold was persistent for up to 360 days of incubation, and its formation was not due to a nutritional limitation, a loss of metabolic activity,

an inhibition by hydrogen or formate, a kinetic inhibition, or a toxicity from the buildup of undissociated acetate concentrations (13, 14, 15). The substrate threshold concentration was influenced by endproduct accumulation (specifically acetate) and we concluded that the extent of substrate degradation was controlled thermodynamically by a mass-action effect and that substrate metabolism ceased when a critical free energy value was reached (13, 14). By manipulating substrate degradation and threshold formation, we wanted to experimentally answer the question: is there a minimum quantum of free energy that is needed to sustain microbial activity? Based upon the tenets of the universal biological energy quantum theory (7, 11), we posit the following predictions: 1) substrate degradation will cease at a threshold concentration that corresponds to a critical free energy value ($\Delta G'_{crit}$) regardless of the species involved; 2) the $\Delta G'_{crit}$ value at threshold will be constant (not appreciably lower or higher than – 23 kJ/mol); and 3) the $\Delta G'_{crit}$ will not be affected by different substrates or terminal electron-accepting scenarios.

Experiments were designed to determine the critical $\Delta G'$ for syntrophic substrate degradation using defined cocultures containing a syntrophically oxidizing bacterium and a hydrogen consuming partner (16). Benzoate and butyrate served as model substrates because each are key intermediates in the degradation of diverse organic compounds (2, 17). The energetic status of the system was assessed by calculating the change in

free energy for substrate degradation at the point where a stable threshold concentration was established (18).

Table 2 shows the results of butyrate degradation by a typical *Syntrophus aciditrophicus* coculture grown under sulfate-reducing conditions when stressed with different concentrations of acetate. Approximately 2.5 mM butyrate was incompletely degraded to a threshold concentration that ranged from 5 μM to 250 μM. The extent of butyrate degradation depended on the amount of the acetate in the medium, i.e. increased acetate concentrations resulted in increased butyrate threshold concentrations, indicating that an accumulation of endproducts controlled the degree of substrate degradation thermodynamically. Interestingly, even though the final acetate concentrations and butyrate threshold concentrations differed between treatments by nearly two orders of magnitude, the $\Delta G'$ value was remarkably constant for all conditions (Table 1). The average $\Delta G'$ for butyrate degradation was -11.5 ± 1.2 kJ/mol; however, this value is significantly different than that expected according to the biological energy quantum theory. These results prompted us to further test the validity of this theory by investigating the change in free energy of substrate thresholds for other substrates, organisms, and electron-accepting regimes.

Figures 1 and 2 show the average $\Delta G'$ values for substrate thresholds for all of the syntrophic species tested grown under multiple

TABLE 2. The effect of acetate on the thermodynamics and threshold value of butyrate degradation by growing cocultures *of S. aciditrophicus* under sulfate-reducing conditions.[a]

Addition[b]	acetate (mM)	butyrate (μM)	ΔG' (kJ/mol)
none	5.1 ± 0.4	4.7 ± 1.6	-12.4 ± 0.5
10 mM acetate	14.0 ± 0.3	13.9 ± 1.0	-11.6 ± 0.2
30 mM acetate	34.6 ± 1.4	50.9 ± 6.8	-10.1 ± 0.5
60 mM acetate	64.0 ± 1.6	251.1 ± 87.0	-10.4 ± 1.2
60 mM NaCl	5.2 ± 0.6	5.0 ± 1.9	-12.9 ± 0.7

[a] Values are means and standard deviation of triplicate determination.

[b] All tubes contained approximately 2.5 mM butyrate.

[c] Formate was not detected (1 μM)

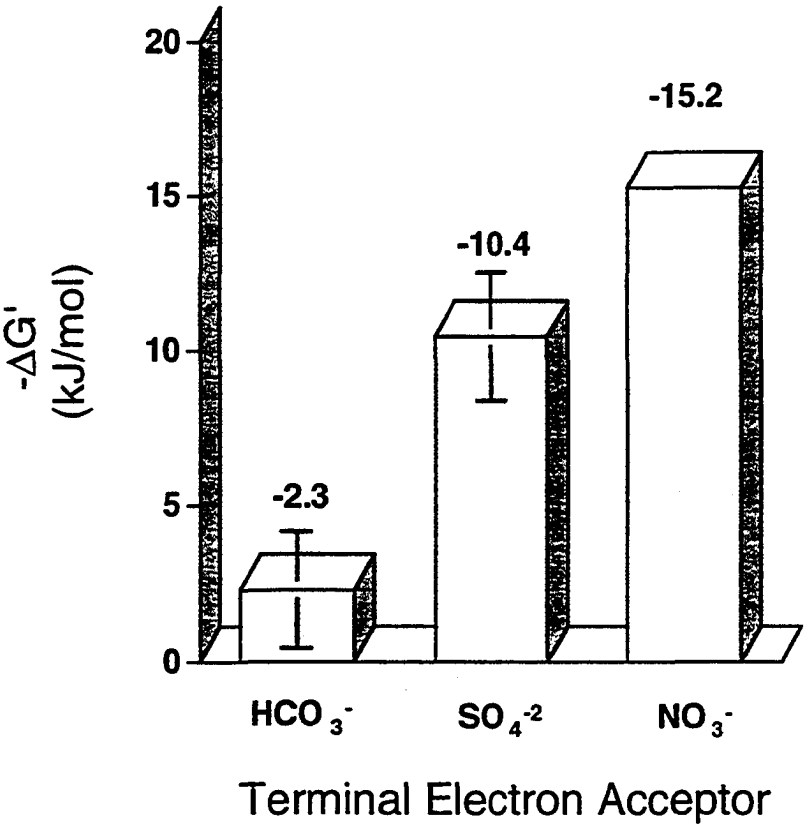

Figure 1. The averagel free energy (ΔG')value at the substrate threshold concentration for butyrate-grown cocultures under methanogenic (HCO_3^-), sulfate-reducing (SO_4^{-2}), and nitrate-reducing (NO_3^-) conditions.

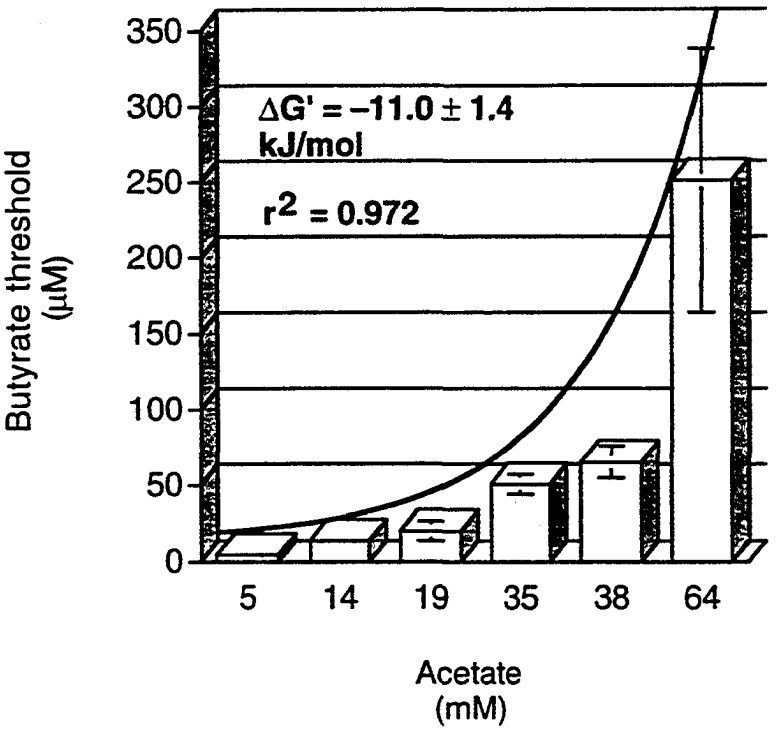

Figure 2. Relationship of butyrate threshold to the final acetate concentration in *S. aciditrophicus* cocultures under sulfate-reducing conditions. Data are average and standard deviations of triplicate determinations. The ΔG' value is the average for all acetate concentrations tested.

electron-accepting conditions. For syntrophic butyrate degradation, the average $\Delta G'$ was -2.3 ± 1.8 under methanogenic conditions, -10.4 ± 2.1 under sulfate-reducing conditions, and -15.2 ± 0.1 under nitrate reducing conditions, respectively (Figure 1). For syntrophic benzoate degradation, the average $\Delta G'$ ranged from -21.9 ± 2.8 under methanogenic conditions, -30.3 ± 2.0 under sulfate-reducing conditions, and -42.5 ± 3.1 under nitrate reducing conditions, respectively (Figure 2). These $\Delta G'$ values for syntrophic substrate degradation at threshold were consistently different than the postulated $\Delta G'_{crit}$ value of approximately –23 kJ/mol, with $\Delta G'$ values for butyrate degradation significantly lower and $\Delta G'$ values for benzoate degradation significantly higher than the postulated minimum quantum of energy value. The variability of $\Delta G'$ values might be explained by the difference observed in the amount of energy required for butyrate and benzoate activation, respectively. All menbers of the genus *Syntrophus* have been shown to utilize the enzyme benzoyl-CoA ligase, which requires the stoichiometric consumption of ATP for the activation of benzoate (19, 20, 21). This is in contrast to the activation stategy employed by *Syntrophomonas wolfei*, which uses an energy-neutral butyryl-acetyl CoA transferase to enzymatically activate butyrate (22). These data indicate that the $\Delta G'$ value when substrate metabolism stops is not constant and depends upon both the activation steps used for substrate metabolism and the terminal electron-accepting process tested. The observation that the

remaining free energy is highly variable when metabolism stops is in direct contrast to current bioenergetic hypotheses and shows that for certain substrates, bacterial metabolism can proceed near thermodynamic equillibrium, a condition that is often thought to be a biological impossibility.

A number of arguable points can be raised regarding the assumptions used in formulating the minimum quantum theory. One major assumption is that bacteria require on the order of 70 kJ/mol of free energy to conserve 1 mol of ATP under the physiological conditions that are presumed to prevail in a cell. This assumption is based on actively growing *E. coli* cells ([ATP] = 10 mM; [ADP] = 1 mM; [Pi] = 10 mM), which, in all probability, may not accurately represent the phosphorylation potential of a bacterium nearing its thermodynamic limits for substrate metabolism. Tran and Unden (23) recently showed that the ADP/ATP ratio changes depending on the metabolic state of the cell. Another major assumption is that the stoichiometry for ATP synthesis via ion translocation is a fixed ratio (24). If the stoichiometry of ion transported to ATP formed is not a fixed ratio of 3 to 1 or if the ratio is variable depending on the energetic state of the cell, then a constant energy quantum may not be required as the energetic limits of substrate metabolism are reached. Variable stoichiometries of 2 to 4 mol H^+ / mol ATP have been reported for ATP-driven proton pumps in *E. coli* (25). The variability observed for the Gibb's free energy values when substrate thresholds were reached implies that the above assumptions are not

universally appropriate, especially for anaerobic bacteria, and must be reassessed for proper description of energetic processes within the bacterial cell.

This is the first body of research to systematically test the biological energy quantum hypothesis by investigating whether a universal critical $\Delta G'$ exists for different bacterial species, substrates, and electron accepting scenarios once substrate metabolism halts. Contrary to the conventional thought for microbial bioenergetics, substrate metabolism can proceed beyond a $\Delta G'_{crit}$ value of approximately – 23 kJ/mol postulated to be the minimum free energy needed to support microbial activity. Our work suggests that the change in free energy at substrate threshold for syntrophic metabolism is quite variable. It appears that as the redox reaction becomes more thermodynamically favorable, substrate metabolism ceases at a more negative $\Delta G'$. Syntrophic bacteria may attempt to maximize energy conservation when thermodynamic constraints begin to limit substrate degradation by altering their phosphorylation potential or electromotive membrane potential such that ATP synthesis becomes more favorable, possibly in response to signals from their hydrogen-using partner. Finally, organisms that have evolved pathways for substrate activation that do not require a large input of energy continue to metabolize at free energy changes approaching thermodynamic equilibrium.

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or in coculture with *Desulfovibrio* sp. strain G11 under sulfate-reducing or nitrate-reducing conditions. S. acidtrophicus was also grown on butyrate in coculture with *Desulfovibrio* sp. strain G11 under sulfate-reducing conditions. *Syntrophus gentianae* was grown on benzoate in coculture with *Methanospirillum hungateii* under methanogenic conditions. *Syntrophus buswellii* was grown on benzoate in coculture with *Desulfovibrio* sp. strain G11 under sulfate-reducing conditions [B.E. Jackson et al, Arch. Microbiol. 171, 107 (1999); Jackson, B.E., Ph.D. Thesis, University of Oklahoma (1999)].

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18. Metabolism was monitored until a stable, substrate threshold was observed, typically between 45 to 90 days. Once a threshold was established, the $\Delta G'$ for butyrate and benzoate degradation was calculated [R.K. Thauer et al, Microbiol. Rev. 41, 100 (1977)] based upon the measured substrates and products concentrations. Values are reported as the mean $\pm$ standard deviation (n = 3 to 10). An analysis of variance (ANOVA) was performed within and among data sets to determine statistical significance (a = 0.001). Benzoate was measured using an high pressure liquid chromatograph (HPLC)

equipped with a reverse phase C - 18 column and a UV detector set at an absorbance of 254 nm. The HPLC was operated at a flow rate of 1.0 ml/min, using a mobile phase of 80% (vol./vol.) sodium acetate (50 mM, pH 4.5) and 20% (vol/vol) acetonitrile. The benzoate detection limit was approximately 250 nM. Formate was measured using a Dionex DX-500 ion chromatograph, equipped with an AS-11 anion exchange column and a CD 20 conductivity detector. The ion chromatograph was operated at a flow rate of 1.5 ml/min using a mobile phase of 0.5 mM NaOH. Formate was also measured spectrophotometrically by monitoring the formation of NADH at 320 nm from the enzymatic reduction of NAD by formate dehydrogenase [V. Warikoo et al, Appl. Environ. Microbiol. 62, 26 (1996)]. The detection limit for both methods was 1 μM. Butyrate and acetate were measured using a gas chromatograph equipped with a flame ionization detector (FID) and a 6' glass column packed with Carbopack B DA 80/20 4% Carbowax 20M resin. The GC column temperature was increased from 165°C to 185°C over 3.5 min, with an injector temperature of 180°C and a detector temperature of 200°C. The GC was set to a flow rate of 24 ml/min helium. Carbon dioxide was quantified using a GC equipped with a thermal conductivity detector and a 6' stainless

steel column packed with Porapak Q resin. The GC was set to a flow rate of 20 ml/min helium and operated with an injector temperature of 100°C, an oven temperature of 50°C, and at a filament temperature of 180°C. Methane was quantified with a gas chromatograph (FID), equipped with a 6' stainless steel column packed with Porapak Q resin. The GC was set to a flow rate of 30 ml/min helium and operated with an injector temperature of 100°C, an oven temperature of 60°C, and a detector temperature of 125°C. Hydrogen was quantitated with a mercury vapor reduction gas analyzer [W. Seiler et al, Atmos. Technol. 12, 40 (1980). Absorbence of cultures was measured spectrophotometrically at 600 nm.

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Appendix

Thiosulfate metabolism in *Desulfotomaculum thermobenzoicum*: Growth by disproportionation.

ABSTRACT

Desulfotomaculum thermobenzoicum, *D. nigrificans*, *D. ruminis*, and *D. orientis* were tested for the ability to grow and disproportionate thiosulfate or sulfite. *D. thermobenzoicum* grew by disproportionation of thiosulfate, forming stoichiometric amounts of sulfate and sulfide; sulfite was not disproportionated. The disproportionation of thiosulfate or sulfite was not observed by any other of the species tested. The addition of acetate enhanced growth and thiosulfate disproportionation by *D. thermobenzoicum* compared to that observed with thiosulfate alone. Interestingly, *D.*

thermobenzoicum initially oxidized thiosulfate to sulfate when hydrogen was added in levels in excess of that needed to stoichimetrically reduce thiosulfate to sulfide. However, cultures with limiting amounts of hydrogen reduced thiosulfate to hydrogen sulfide, with no apparent production of sulfate as an intermediate. These data indicate that thiosulfate transformation by disproportionating bacteria may be regulated by the relative abundance electron donor. This is the first report of a gram positive, thermophilic bacterium capable of obstining energy for growth by thiosulfate disproportionation.

INTRODUCTION

Since Bak (1, 2) and coworkers first reported on the disproportionation of inorganic sulfur oxyanions in *Desulfovibrio sulfodismutans*, research has shown that thiosulfate disproportionation is an important process throughout aquatic environments (6-9). Bacterial thiosulfate disproportionation can be described as an inorganic fermentation, occurring via an intramolecular redox change at each of the sulfur atoms (19). The stoichiometry of thiosulfate disproportionation of thiosulfate is as follows (equation 1):

$$S_2O_3^{-2} + H_2O \rightarrow SO_4^{-2} + HS^- + H^+ \qquad (1)$$

$\Delta G^{o'} = -21.9$ kJ/mol (17)

The disproportionation of thiosulfate produces one mol of sulfate and one mol of sulfide, with a Gibb's free energy change of approximately - 22 kJ/mol (equation 1). Multiple genera of sulfate-reducers have been reported to disproportionate thiosulfate, sulfur, or sulfite, however few are able to conserve energy for growth by these reactions, likely due to the small change in free energy of each respective reaction (11). Although only a few bacteria have been identified to grow by this reaction, the microorganisms capable of this metabolism are numerically abundant in both freshwater and marine environments (1,6).

The disproportionation of thiosulfate is an important process in nature. Jorgensen and coworkers (6-9) have studied the fate of thiosulfate in marine sediments, freshwater sediments, and bethnic cyanobacterial mats and observed that in addition to oxidation and reduction transformations of thiosulfate, a significant proportion of thiosulfate was disproportionated. Research revealed that thiosulfate disproportionation was a common link between aerobic and anaerobic metabolism of sulfur (9), and that sulfoxyanions could be simultaneously oxidized and reduced by a single

group of organisms. Thus, a dynamic cycling of sulfur occurs via bacterial disproportionation in many environments.

To date, the only microorganisms capable of an inorganic sulfur compound disproportionation metabolism are members of the diverse microbial group the sulfate-reducing bacteria (11). Disproportionating sulfate-reducing bacteria are typically mesophilic, gram-negative bacteria phylogenetically grouped within the delta subclass of the proteobacteria. *Desulfovibrio sulfodismutans, Desulfocapsa thiozymogenes, Desulfocapsa sulfoexigens*, and strain NTA 3 are the only known microorganisms capable of growth by thiosulfate disproportionation (1, 2, 4, 5). In this communication, we report on growth and metabolism of *Desulfotomaculum* sp. in the presence of sulfoxyanions. This is the first report of growth by thiosulfate disproportionation by a gram positive, thermophilic sulfate-reducing bacterium.

MATERIALS AND METHODS

Organisms and methods of cultivation. The type strains of *Desulfotomaculum thermobenzoicum* (49756) , *Desulfotomaculum nigrificans* (19998), *Desulfotomaculum ruminis* (23193), and *Desulfotomaculum orientis* (19365) were obtained from the American Type Culture Collection (Rockville, MD). Microorganisms were cultivated in a

basal medium containing (per liter): 0.4 g $MgCl_2 \cdot 6\ H_20$, 0.1 g $CaCl_2 \cdot 2\ H_20$, 1.0 g NaCl, 0.25 g NH_4Cl, 0.1 g KH_2PO_4, 0.1 g yeast extract (Difco), 2.0 g $NaHCO_3$, 2.0 g TES (N-tris (Hydroxymethy)methyl-2-aminoethanesulfonic acid), 0.001 g resazurin, and 0.5 g dithiothreitol. A trace metal solution and vitamin solution (12) were added to the medium in amounts of 5.0 and 10.0 ml per liter, respectively. The trace metal solution contained the following (per l): 2.0 g nitrilotriacetic acid (adjusted to pH 6.0 with 1.0 M KOH), 1.0 g $MnCl_2 \cdot 4\ H_20$, 1.0 g $FeCl_2 \cdot 4\ H_20$, 0.2 g $CoCl_2 \cdot 6\ H_20$, 0.2 g $ZnCl_2$, 0.5 g H_3BO_3, 0.02 g $CuCl_2 \cdot 2\ H_20$, 0.02 g $NiCl_2 \cdot 6\ H_20$, 0.02 g $Na_2MoO_4 \cdot 2\ H_20$, 0.02 g Na_2SeO_4, and 0.02 g Na_2WO_4. The pH of the medium was adjusted to a pH of 7.4 and boiled under a gas stream of 80:20 N_2:CO_2.

Basal medium was dispensed in 10 ml aliquots into 18 x 150 mm serum tubes with a headspace of 80:20 N_2:CO_2 at atmospheric pressure, sealed with blue rubber stoppers, crimped with aluminum seals, and autoclaved. All procedures for the anaerobic preparation and use of media and solutions were essentially those of Balch and Wolfe (3). In experiments where hydrogen was used as the electron donor, tubes were aseptically amended with an 80:20 H_2:CO_2 gas mixture and shaken at 200 rpm. Additions of thiosulfate, sulfate, sulfite, and acetate were made from filter sterilized, anoxic stock solutions by using syringes and needles. The initial concentration of thiosulfate ranged from 5 to 15 mM. For some experiments, acetate was used as a carbon source at an initial concentration of 3 mM. For

all experiments, cultures of *Desulfotomaculum* species were grown on hydrogen and thiosulfate to late exponential phase of growth, anoxically and aseptically harvested by centrifugation (11,500 X g; 15 min; 4°C), and resuspended to 20 times the original cell concentration (vol./vol.) in anoxic, sterile basal medium. Two hundred µl of the cell suspension was used to inoculate each tube. Incubation times ranged from 5 to 12 days. *D. ruminis* and *D. orientis* were incubated at 37°C and *D. thermobenzoicum* and *D. nigrificans* were incubated at 62°C without shaking.

Analytical techniques. Growth of cultures was monitored spectrophotometrically by measuring the increase in absorbance at 600 nm. Direct cell counts were performed at 400-fold magnification with a phase-contrast microscope and a Petroff-Hausser counting chamber (10). For measurement of thiosulfate transformation, 500 µl of each sample was immediately analyzed for volatile sulfides and total reduced inorganic sulfur. Volatile sulfides were quantified spectrophotometrically by the methylene blue assay as described by Tanner (14). Total reduced inorganic sulfur was determined by using a modified single-extraction chromium reduction assay as previously reported (19). Elemental sulfur was determined as previously reported (18). Sulfoxyanions (thiosulfate, sulfate, and sulfite) were quantified by suppressed ion chromatography using a Dionex Ion Cromatograph DX-500 equpped with a CD-20 conductivity detector and an AS-11 column. Thiosulfate and sulfite were also quantified with the ion

chromatography system by measuring the absorbance at 230 nm with an AD-20 absorbance detector. The chromatograph was run isocratically with a mobile phase of 7 mM NaOH at a flow rate of 2.0 ml/min for 5 min., then the mobile phase concentration was linearily increased to 35 mM NaOH for an additional 7 min..

RESULTS

Growth of *Desulfotomculum* sp. by disproportionation of sulfoxyanions. A small amount of growth (a change of 0.06 absorbance units) was observed when *D. thermobenzoicum* but not *D. nigrificans*, *D. ruminis*, and *D. orientis* were incubated in medium with thiosulfate (Figure 1). *D. thermobenzoicum* transformed approximately 4.5 mM thiosulfate to aproximately 4.0 mM sulfate. The ratio of sulfate produced to thiosulfate consumed was about 0.9, which is close to that theoretically predicted for thiosulfate disproportionation (equation 1). In medium without thiosulfate, no increase in absorbance was observed and sulfate was not produced. Abiotic transformation of thiosulfate to sulfate or sulfide was not observed. Additionally, *D. thermobenzoicum* cultures did not increase in absorbance in medium with 5 mM sulfate, indicating that the small amount of yeast extract in the medium was not used as an energy source. Figure 2 is a typical

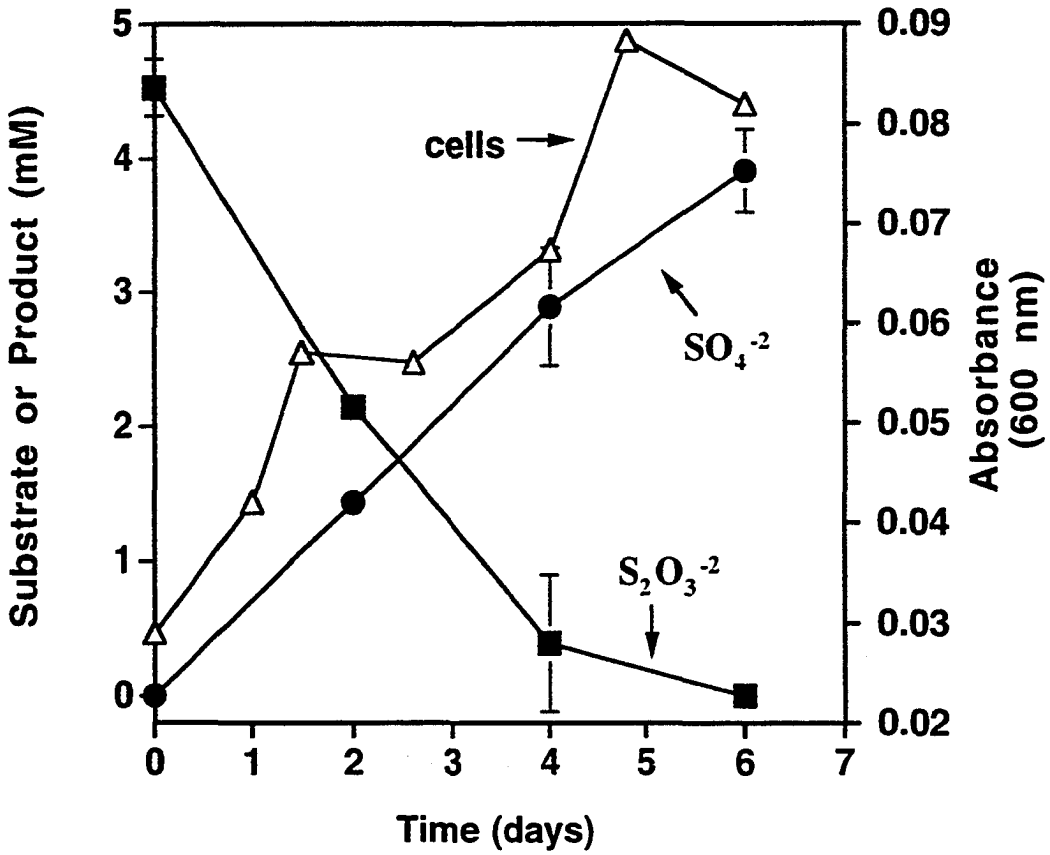

Figure 1. Growth and thiosulfate disproportionation by D. thermobenzoicum. Results represent the mean and standard deviation of triplicate determinations.

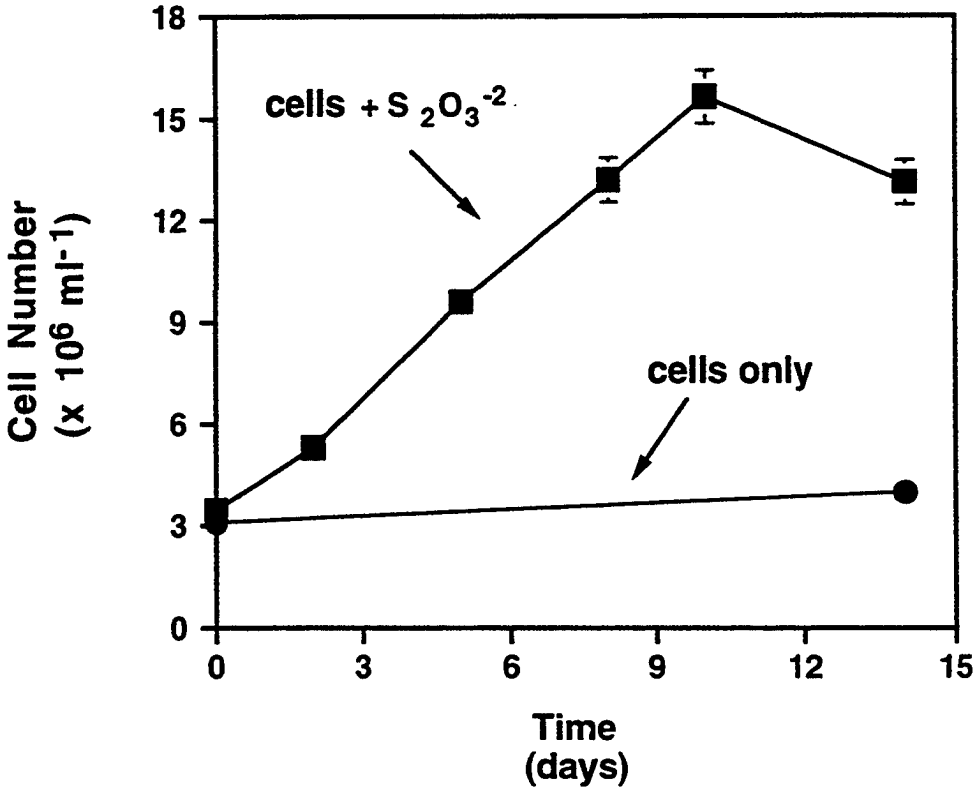

Figure 2. Growth of D. thermobenzoicum by utilization of thiosulfate as the sole energy source. Cell counts are means and average deviations of duplicate incubations with and without thiosulfate.

growth profile of *D. thermobenzoicum* with thiosulfate as the sole source of energy. Cell numbers increased linearly from approximately 3.5 X 10^6 to 1.6 X 10^7 cells/ml over a period of 10 days in sulfate-free medium with 8 mM thiosulfate, however in medium without thiosulfate, cell number did not increase. Growth was sustained in basal medium with thiosulfate through at least 4 successive transfers.

Thiosulfate transformation was not observed with *D. orientis*, *D. ruminis*, or *D. nigrificans* in basal medium amended with or without 3 mM acetate as a carbon source. Sulfite (5 mM) was not transformed by any of the four *Desulfotomaculum* species tested. However, all of the *Desulfotomaculum* species tested grew with hydrogen (70 kPa of 80:20 H_2:CO_2 gas phase) and 10 mM thiosulfate or 5 mM sulfite as the electron-acceptor, indicating that this medium does support growth of these organisms if a suitable electron donor is supplied (data not shown).

Stoichiometry and effect of acetate on thiosulfate disproportionation by *D. thermobenzoicum*. *D. thermobenzoicum* disproportionated approximately 26 µmol of thiosulfate to 24 µmol of acid volatile sulfide and 25 µmol of sulfate when grown only with thiosulfate (Table 1). The ratio of sulfate to sulfide was 1.0, a value that is nearly identical for the theoretical stoichiometry of thiosulfate disproportionation (equation 1). The sulfur recovery was 101 % with a small amount of sulfur (approximately 4 µmol or 7% of the total sulfur) recovered in the total reduced inorganic sulfur pool.

Table 1. Disproportionation of thiosulfate by *D. thermobenzoicum* in the presence and absence of acetate. [a]

Addition	Thiosulfate used (µmol)	Sulfite formed (µmol)	Sulfate formed (µmol)	Sulfide formed (µmol)	Growth (Δ O.D. 600nm)	Ratio sulfate:sulfide
S_2O_3	26.4 ± 1.7[b]	0.5 ± 1.0	24.9 ± 3.1	23.8 ± 2.5	0.05	1
Acetate + S_2O_3	145.5 ± 10.6[c]	9.8 ± 9.8	135.4 ± 21.3	168.7 ± 5.3	0.11	0.8
Acetate	BDL[d]	BDL[d]	BDL[d]	0.4 ± 0.4	0.0	NA[e]
S_2O_3 (no cells)	2.7	BDL[d]	BDL[d]	BDL[d]	0.0	NA[e]

[a] Incubated at 62°C for 7 days

[b] 101% sulfur recovery with remaining sulfur recovered as iron sulfides; elemental sulfur was not detected.

[c] 107% sulfur recovery

[d] Below detection limit

[e] Not applicable

This recovered sulfur may be in the form of iron sulfides since elemental sulfur was not detected; however this observation was not investigated further.

When 3 mM acetate was added to the medium, the extent of both growth and thiosulfate disproportionation significantly increased (Table 1). *D. thermobenzoicum* is not known to utilize oxidize acetate as an electron donor for sulfate or thiosulfate reduction and presumably uses acetate only as a carbon source for biosynthesis (15). Consistent with this, we did not observe any growth of *D. thermobenzoicum* in the presence of acetate and sulfate. *D. thermobenzoicum* converted approximately 146 µmoles of thiosulfate to 135 µmoles of sulfate, 169 µmoles of sulfide, and 10 µmoles of sulfite. The sulfur recovery was 107 % and the ratio of sulfate to sulfide was 0.8, again in agreement with stoichiometry of thiosulfate disproportionation (equation 1). Growth was not observed in tubes that contained acetate alone. Thiosulfate has been reported to chemically decompose at elevated temperatures (13). However, less than 2 % of the initial thiosulfate was abiotically transformed in control tubes amended only with thiosulfate (minus cells) under experimental conditions used here, e.g. 62°C and 35 kPa overpressure. In addition, thiosulfate was not transformed in the presence of heat-killed cells.

Transformations of thiosulfate by *D. thermobenzoicum* in the presence of hydrogen. A novel observation was made when *D. thermobenzoicum* was grown with different amounts of hydrogen as the

electron donor and 8 mM (100 µmol) thiosulfate as the electron acceptor. When *D. thermobenzoicum* was grown with approximately 75 µmol hydrogen, a stoichimetric amount of thiosulfate was reduced and hydrogen sulfide was formed according to equation 2 (data not shown). However,

$$4\ H_2 + S_2O_3^{-2} \rightarrow 2\ HS^- + 3\ H_2O \qquad (2)$$

when the amount of hydrogen was doubled to 150 µmol of hydrogen, the thiosulfate was initially transformed to predominantly sulfate (Figure 3). Approximately 5 mM thiosulfate was consumed with the production of 6 mM sulfate and 1 mM sulfide during the first 2 days of incubation. After 7 days, sulfate and thiosulfate were completely reduced to hydrogen sulfide. Under this condition, the amount of electrons derived from hydrogen oxidation exceeds the amount needed for the reduction of thiosulfate to hydrogen sulfide. Sulfate was detected only in tubes containing stoichiometrically excess amounts amounts of hydrogen and was not observed in control tubes that contained a headspace replaced with a gas phase of N_2:CO_2.

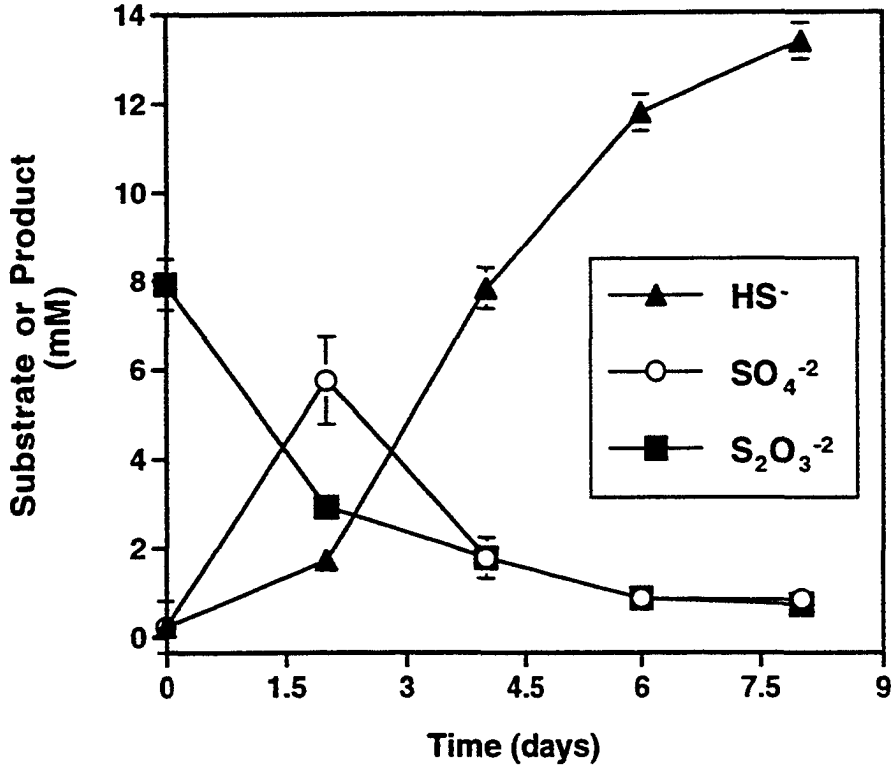

Figure 3. Metabolism of *D. thermobenzoicum* grown with hydrogen as the electron donor and thiosulfate as the electronacceptor. The hydrogen amount (150 µmol) was in stoichiometric excess than that needed to completely reduce thiosulfate to sulfide (100 µmol). Results represent the standard deviation of triplicate determinations.

DISCUSSION

D. thermobenzoicum is the first obligate thermophile reported to grow by disproportionation of thiosulfate and is phylogenetically distinct from previously described disproportionating bacteria, clustering with low % G + C Gram-positive bacteria (clostridia) within a separate division of the domain Eubacteria. To date, disproportionation metabolism has only been conclusively documented within the Gram-negative, mesophilic sulfate-reducing bacterial group, which is phylogenetically clustered within the delta subclass of the Proteobacteia. Physiologically, our results are consistent with the fact that the ability to disproportionate sulfoxyanions is limited to sulfate-reducing bacteria only (11). However, a disproportionation metabolism is apparently not unique to gram-negative sulfate-reducing bacteria but may have diverged along with gram-positive Eubacteria as well.

D. thermobenzoicum disproportionated thiosulfate to stoichiometric quantities of sulfate and sulfide, in accordance with equation 1. The disproportionation of thiosulfate supported growth, in both the presence and absence of acetate, however, the addition of acetate significantly stimulated both growth as well as thiosulfate disproportionation. *D. thermobenzoicum* achieved twice the cell density and disproportionated six-fold more thiosulfate relative to when acetate was not present. The degree of growth observed for *D. thermobenzoicum* was very similar to that observed by Bak

and Pfennig (2) for *Desulfovibrio sulfodismutans*. *D. thermobenzoicum* grew repeatedly by thiosulfate disproportionation in medium without acetate as a carbon source; although yeast extract was present. However it is unlikely that this small amount of yeast extract (0.05 g/L) could support the degree of growth observed. This observation is consistant with the fact that *D. thermobenzoicum* posesses the enzymatic capability to assimilate carbon via the carbon monoxide dehydrogenase pathway (16). To date, only one bacterium has been observed to grow autochemolithotrophically by thiosulfate disproportionation, strain NTA 3 (1).

Interestingly, sulfate and not sulfide was initially produced from thiosulfate metabolism when *D. thermobenzoicum* was grown in the presence of stoichiometrically excess amounts of hydrogen. These results suggests that the concentration of electron donor may regulate whether thiosulfate is directly reduced to sulfide or whether thiosulfate is disproportionated to sulfate. When hydrogen is in excess, the disproportionation of thiosulfate to sulfate may provide an energetic advantage over sulfate-reducers that cannot disproportionate. A pathway that combines both thiosulfate disproportionation and dissimilatory sulfate reduction allows for ATP production by both substrate level and electron transport phosphorylation. Additionally, an excess supply of electron donor may provide a continued source for the electrons needed to catalyze thiosulfate reductase, thus allowing the electrons generated from a reverse

ADP sulfurylase (11) to be used for conservation of energy above that observed for dissimilatory thiosulfate reduction alone.

Disproportionating bacteria are numerically significant and have been detected in high numbers in freshwater mud and marine sediments (2, 9). The genus *Desulfotomaculum* has the unique ability among sulfate-reducing bacteria to form endospores, indicating that bacteria possessing a disproportionation metabolism can persist throughout extreme, unfavorable environmental conditions. Ecologically, *Desulfotomaculum* sp. are commonly associated with petroleum reservoirs. The formation of sulfide by sulfate-reducing bacteria within petroleum reservoirs is responsible for many deleterious effects on commerial oil and gas production, therefore sulfur compound transformation by these bacteria is of great concern to the petroleum industry. The finding that *D. thermobenzoicum* effectively disproportionates thiosulfate indicates that complex sulfur transformations may also exist in hydrocarbon-rich environments, such as commercial petroleum reservoirs, in addition to freshwater and marine environments.

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