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Evidence for an Anaerobic Syntrophic Benzoate Degradation Threshold and Isolation of the Syntrophic Benzoate Degrader

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An anaerobic, motile, gram-negative, rod-shaped, syntrophic, benzoate-degrading bacterium, strain SB, was isolated in pure culture with crotonate as the energy source. Benzoate was degraded only in association with an H_2-using bacterium. The kinetics of benzoate degradation by cell suspensions of strain SB in coculture with *Desulfovibrio* strain G-11 was studied by using progress curve analysis. The coculture degraded benzoate to a threshold concentration of 214 nM to 6.5 μM, with no further benzoate degradation observed even after extended incubation times. The value of the threshold depended on the amount of benzoate added and, consequently, the amount of acetate produced. The addition of sodium acetate, but not that of sodium chloride, affected the threshold value; higher acetate concentrations resulted in higher threshold values for benzoate. When a cell suspension that had reached a threshold benzoate concentration was reamended with benzoate, benzoate was used without a lag. The hydrogen partial pressure was very low and formate was not detected in cell suspensions that had degraded benzoate to a threshold value. The Gibbs free energy change calculations showed that the degradation of benzoate was favorable when the threshold was reached. These studies showed that the threshold for benzoate degradation was not caused by nutritional limitations, the loss of metabolic activity, or inhibition by hydrogen or formate. The data are consistent with a thermodynamic explanation for the existence of a threshold, but a kinetic explanation based on acetate inhibition may also account for the existence of a threshold.

The degradation of aromatic compounds under methanogenic conditions involves the concerted action of many interacting microbial species (15, 22, 34, 45, 51, 55). The initial transformations of aromatic compounds involve the removal or simplification of substituent groups prior to ring cleavage, resulting in the production of intermediates such as phenol and benzoate (18, 54). Phenol is further transformed to benzoate prior to ring cleavage (23, 44). Thus, benzoate is a central intermediate in the anaerobic mineralization of aromatic compounds, and factors that affect the rate and extent of benzoate degradation will likely affect the anaerobic degradation of aromatic compounds.

In methanogenic environments, benzoate is degraded to the methanogenic substrates acetate, CO_2, H_2, and possibly formate (equation 1) by syntrophic bacteria such as *Syntrophus buswellii* BZ-2 and HQGo1 (33, 34, 45, 47).

$$\text{benzoate}^- + 7H_2O \rightarrow 3\ \text{acetate}^- + 3H^+ + HCO_3^- + 3H_2 \quad (1)$$

$$\Delta G^{o'} = 74\ \text{kJ}\ (37)$$

As shown in equation 1, the oxidation of benzoate with the production of acetate and hydrogen (or formate) is thermodynamically unfavorable, which makes the metabolism of benzoate by syntrophic bacteria dependent on H_2- or formate-using bacteria (30, 34). From a thermodynamic viewpoint, acetate as a product of benzoate metabolism is equivalent to hydrogen, with 3 mol each being formed from benzoate (equation 1). Thus, even if hydrogen levels are kept low, the accumulation of acetate may inhibit benzoate degradation. Dolfing and Tiedje (13) reported that the initial rate of benzoate degradation by a syntrophic coculture of a syntrophic benzoate-degrading bacterium and a methanogen was inhibited by acetate (K_i, 40 mM). However, the rate of benzoate degradation was calculated from the rate of methane production, and it is not known whether benzoate was degraded completely or reached a threshold concentration.

In this paper, we report the isolation of a syntrophic benzoate-degrading bacterium, strain SB, in pure culture on crotonate. We show that a coculture of strain SB with a hydrogen-using sulfate reducer degrades benzoate to a threshold value and that the benzoate threshold concentration depends on the acetate concentration.

MATERIALS AND METHODS

Organisms and growth conditions. Strain SB was isolated from sewage sludge obtained from the secondary anaerobic digestor at the municipal wastewater treatment plant in Norman, Okla. *Desulfovibrio* strain G-11 was obtained from the culture collection of M. P. Bryant, Urbana, Ill. A basal medium (31) with 2% (vol/vol) clarified ruminal fluid and an 80% N_2–20% CO_2 gas phase was used. For the growth of strain SB in coculture with *Desulfovibrio* strain G-11, the basal medium contained 8.2 mM sodium benzoate and 21 mM sodium sulfate. The pure culture of strain SB was grown in the basal medium with 10 mM sodium crotonate. *Desulfovibrio* strain G-11 was grown in pure culture in the basal medium with 21 mM sodium sulfate and an 80% H_2–20% CO_2 gas phase. Solid medium for roll tubes contained 2% agar.

Methods for the preparation and use of anaerobic media and solutions were essentially those of Bryant (10) as modified by Balch and Wolfe (5). The medium was dispensed into aluminum crimp seal culture tubes or 2-liter bottles fitted with black rubber stoppers with the top of a culture tube inserted into the stopper. Cultures were incubated at 37°C. The purity of cultures was checked routinely by microscopic examination, Gram reaction, and inoculation of thioglycolate broth (Difco Laboratories, Inc., Detroit, Mich.), which did not support the growth of the pure culture of strain SB or the coculture of SB and G-11. Growth was monitored by the change in A_{600} (31).

Enrichment and isolation. A methanogenic benzoate-degrading enrichment culture was obtained by inoculating anaerobic digestor sludge (10%, vol/vol) into the basal medium containing 7 mM benzoate. The enrichment was maintained by transferring 50% of the volume into sterile medium every 2 weeks. A benzoate-degrading syntrophic coculture with *Desulfovibrio* strain G-11 was obtained by

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the agar roll tube method, as described previously (31, 34). Strain SB was isolated in pure culture by serial dilution and repeated inoculation of crotonate agar roll tubes, as described previously (7).

Cell suspensions. Cells of the benzoate-degrading coculture were concentrated prior to progress curve analysis. All manipulations of the cells were done inside an anaerobic chamber, which contained 1 to 5% H_2, with the balance being N_2. About 1.5 liters of the coculture was grown to the late exponential phase of growth, and the cells were harvested by centrifugation (1,200 × *g*, 20 min, 4°C). The cell pellet was washed three times by resuspending the pellet in 50 mM sodium phosphate buffer (pH 7.5) and recentrifuging. The final cell pellet was resuspended in 100 ml of basal medium without vitamins and ruminal fluid, with 0.5 to 1 mM sodium benzoate, and with 1.5 to 3.0 mM sodium sulfate. The cell suspension was transferred to a 160-ml serum bottle and closed with a black rubber stopper.

Progress curves. To ensure that the cells were metabolically active, the cell suspension was amended with 1.0 mM benzoate and incubated overnight at 37°C. The consumption of benzoate by the cell suspension indicated that the cells were metabolically active. The cell suspension was then amended with a solution of sodium benzoate, sodium sulfate, or sodium acetate. At different time intervals, 1-ml samples were withdrawn. The rate of benzoate degradation was determined by quantitating the change in benzoate concentration with time. The sampling frequency increased as the rate of benzoate consumption approached the mixed-order region of the progress curve. For experiments with radioactive benzoate, 40 μCi of [U-^{14}C]sodium benzoate (Biomedicals Inc., Costa Mesa, Calif.) was added to suspensions containing 100 μM sodium benzoate to give a final specific activity of 4 μCi of benzoate per μmol.

To determine the effect of acetate on the energetics and threshold value for benzoate degradation, triplicate tubes containing 8 ml of the cell suspension were each amended with 1 mM sodium benzoate and 20 mM sodium acetate. Controls tubes received 20 mM NaCl instead of sodium acetate. Concentrations of benzoate, acetate, and hydrogen were determined at the start of the experiment and when the concentration of benzoate reached the threshold value.

Analytical methods. The concentration of benzoate was determined with high-pressure liquid chromatography (HPLC); the HPLC was equipped with a reverse-phase column (250 by 4.6 mm; 5 μm) and a UV light detector set at 271 nm. HPLC was operated at a flow rate of 1.2 ml/min, using a mobile phase of 80% (vol/vol) sodium acetate (50 mM; pH 4.5)–20% (vol/vol) acetonitrile. To detect [U-^{14}C]sodium benzoate, the column effluent was mixed (1:1) in line with liquid scintillation cocktail (Beckman Ready Flow III), and the mixture was delivered at a flow rate of 2 ml/min to a radioisotope detector (Beckman model 171). [U-^{14}C]sodium benzoate of known specific activity served as the standard. The concentrations of acetate and formate were determined with an HPLC system equipped with an anion-exclusion column (3). Except for values given in Table 1, the concentrations of organic acids are reported as the means of duplicate determinations with the ranges given in parentheses. Hydrogen was quantitated with a mercury vapor reduction analyzer (41). Whole-cell protein was determined colorimetrically, using bovine serum albumin as the standard (28). The samples and the standards were boiled in 0.1 N NaOH for 20 min to digest the cells prior to protein determination.

RESULTS

Isolation of the syntrophic benzoate-degrading bacterium. After 2 to 3 months of incubation at 37°C, colonies of a benzoate-degrading syntrophic bacterium (strain SB) in coculture with *Desulfovibrio* strain G-11 were observed in roll tubes containing benzoate. The colonies were black, entire, and lens shaped, about 1 to 2 mm in diameter. Roll tubes without benzoate had several colony types, but none were black. Several well-isolated colonies were picked and transferred to basal medium with benzoate and sulfate. Growth and degradation of benzoate were observed after 1 month of incubation. These cultures contained small, gram-negative, rod-shaped cells with rounded ends (strain SB) in coculture with strain G-11. A coculture of strain SB with G-11 was obtained by repeated application of the roll tube isolation procedure.

The coculture was inoculated into culture tubes containing the basal medium with crotonate to determine whether SB uses crotonate and thus to provide a means to isolate the syntrophic bacterium. Basal medium without crotonate served as the control. After 60 days of incubation, one tube had a higher turbidity than control tubes. Growth was observed in a second crotonate-containing tube after 90 days of incubation. Growth in the rest of the culture tubes was not observed even after 6 months of incubation. Cultures which grew with crotonate contained large numbers of cells morphologically similar to those of strain SB and a few cells similar to those of strain G-11. Strain SB was isolated in pure culture by using crotonate agar roll tubes (7). The pure culture of strain SB produced white, entire, lens-shaped colonies, about 1 mm in diameter. Cells of strain SB grown in basal medium with crotonate were rod shaped with rounded ends that occurred singly or in pairs and stained gram negative. Some of the cells in the early exponential phase of growth were motile.

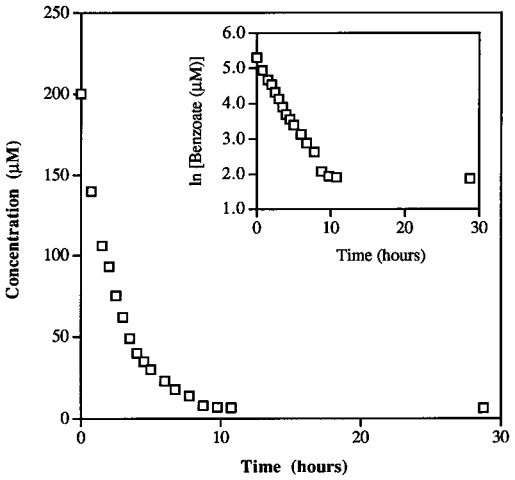

FIG. 1. Progress curve of benzoate utilization by a cell suspension of the syntrophic benzoate-degrading bacterium, strain SB, with *Desulfovibrio* strain G-11. (Inset) Natural logarithm (ln) of the benzoate concentration plotted versus time.

The SB–G-11 coculture grew with benzoate as the energy source with a specific growth rate of 0.005 ± 0.001/h and had an absorbance change of 0.19 after 12 days of incubation. In pure culture with crotonate as the energy source, SB had an absorbance change of 0.4 to 0.6 in 10 to 14 days of incubation. When reassociated with strain G-11, SB degraded 7.6 ± 0.1 mM benzoate and produced 19.5 ± 1.0 mM acetate (73% carbon recovery) after 3 weeks of incubation. Alone, SB was unable to grow with benzoate as the energy source, and only a trace amount of benzoate was metabolized (2.5 μM). Benzoate did not support the growth of strain G-11, and G-11 did not metabolize benzoate. In pure culture, SB produced 18.5 ± 1.0 mM acetate and 1.2 ± 0.6 mM butyrate from 13 ± 0.2 mM crotonate after 7 days of incubation (78% carbon recovery).

Evidence for a threshold for benzoate metabolism. Figure 1 shows a representative progress curve for benzoate degradation by concentrated cell suspensions of the coculture. The protein concentration did not change during the course of the experiment. Initially, the rate of benzoate degradation exhibited zero-order kinetics; it showed mixed-order kinetics when the benzoate concentration was below 40 to 50 μM. Michaelis-Menten kinetics predict (2, 24) that benzoate consumption will eventually exhibit first-order decay. However, a continual decrease in the benzoate degradation was not observed (Fig. 1, inset). The degradation of benzoate in the coculture approached a threshold benzoate concentration, and no further degradation was observed even after extended incubations (7 to 14 days). This pattern of benzoate degradation was consistently observed in repeated experiments with different initial benzoate concentrations and cell densities (data not shown). A typical threshold of 1 to 3 μM benzoate was observed in cell suspensions following an overnight consumption of 1 mM benzoate and the sequential use of 200 μM benzoate. A threshold for benzoate degradation was also observed in growing cul-

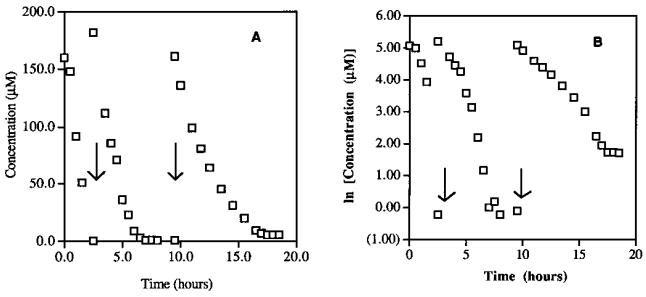

FIG. 2. Effect of acetate on benzoate threshold concentration in cell suspensions of a coculture. Arrows indicate amendments of benzoate and acetate (6 mM). (A) Benzoate concentration versus time; (B) natural logarithm (ln) of the benzoate concentration plotted versus time.

tures. After 19 days of incubation, the SB–G-11 cocultures degraded 9.0 ± 0.1 mM benzoate to a threshold value of 49.5 ± 17 μM benzoate.

Since the detection limit for benzoate with HPLC was 0.2 μM, the experiment was repeated with [U-^{14}C]benzoate to determine whether the threshold observed in cell suspensions was the result of a detection problem. In this experiment, the cell suspension was not incubated overnight with 1.0 mM benzoate. Benzoate degradation was monitored immediately after suspension of the cells. The cell suspension degraded the labeled benzoate to an apparent threshold of about 210 nM. The cell suspension was amended with labeled benzoate and 10 mM sodium acetate, and benzoate was degraded until a new threshold benzoate concentration of 7.5 μM benzoate was reached (data not shown).

Effect of acetate. The threshold value appeared to depend on how much benzoate was added to the cell suspension, since cell suspensions that received additional amendments of benzoate had higher threshold values. This may have been due to the accumulation of acetate. To investigate the possible influence of acetate on the threshold for benzoate degradation, progress curve analysis was conducted with cell suspensions which were serially amended with sodium benzoate and either 6 mM sodium acetate or 6 mM sodium chloride. Resuspension of the cells in the basal medium resulted in the accumulation of acetate with a concentration of 3.1 mM. The cells were incubated overnight with 0.5 mM benzoate and produced 1.5 mM acetate. Thus, a total of 4.6 mM acetate was present in the cell suspension at the start of the progress curve. The cell suspension degraded 200 μM benzoate to below the detection limit of HPLC (0.5 μM) (Fig. 2). The cell suspension was then amended with 200 μM benzoate and 6 mM sodium acetate, and a benzoate threshold concentration of 1.0 μM was observed. The cell suspension again received 200 μM benzoate and 6 mM sodium acetate, and benzoate was degraded to a threshold of 5.5 μM benzoate. In the control cell suspension that received 6 mM sodium chloride instead of sodium acetate, benzoate was degraded to a concentration below the detection limit of the HPLC system after each addition of benzoate, which shows that the sodium ion did not cause the benzoate threshold concentration. The fact that benzoate was degraded without a lag by cell suspensions that had degraded benzoate to a threshold concentration showed that the threshold value was not due to nutritional limitations or the loss of metabolic activity. The initial rate of benzoate degradation decreased after each addition (Fig. 2B), suggesting that inhibitory materials may have accumulated in the suspension. A benzoate threshold could have been caused by the buildup of inhibitory concentrations of H_2 and formate. The H_2 partial pressure was 10 Pa. Formate was not detected by the HPLC method used (<20 μM).

The effect of acetate on the change in free energy for benzoate degradation when the benzoate threshold was reached was determined (Table 1). Cultures that received 20 mM acetate degraded benzoate to a threshold value of 10.6 μM, while cultures that received an equal concentration of NaCl degraded benzoate to a concentration below the detection limit of the HPLC system. In both cases, the final H_2 concentration was very low and much lower than the initial H_2 concentration. The change in Gibbs free energy for benzoate degradation once the threshold value for benzoate degradation was reached was still favorable.

DISCUSSION

Benzoate consumption by a syntrophic coculture did not exhibit a gradual substrate disappearance that would be predicted from first-order or second-order decay models (24, 35) but stopped at a threshold level at which no further degradation of benzoate was observed even after extended incubations. The observed threshold was not the result of an analytical detection limit since a benzoate threshold was observed whether the concentration of benzoate was determined by scintillation counting or spectrophotometrically. The fact that benzoate was consumed each time serial amendments were made indicates that the observed threshold was not due to a nutritional limitation or the loss of metabolic activity. The possibility that the concentration of the undissociated form of acetate accumulated to toxic levels, as has been postulated to explain the toxicity of volatile fatty acids during anaerobic digestion (16, 17), can also be excluded since benzoate degradation would not have been observed after reamendment if toxic levels of acetate were present. Hydrogen levels when the threshold was reached were low, below those found in other syntrophic cultures (12, 14). Thus, inhibitory levels of H_2 cannot account for the existence of a threshold for benzoate degradation. The buildup of formate to inhibitory levels is also unlikely since formate was not detected at the time that the benzoate threshold was reached.

Several lines of evidence suggest a thermodynamic explanation for the existence of a threshold for benzoate degradation.

TABLE 1. Effect of acetate on energetics and threshold value for benzoate degradation by the SB–G-11 coculture

Acetate (mM)		Hydrogen (Pa)		Benzoate (μM)	$\Delta G^{o'}$ (kJ)[a]
Initial	Final	Initial	Final		
3.3 ± 0.72[b]	7.1 ± 0.42	0.20 ± 0.19	0.007 ± 0.007	<0.2	−54.2 ± 3.03
20.3 ± 1.0	26.3 ± 0.82	1.10 ± 0.9	0.011 ± 0.0008	10.6 ± 1.49	−53.5 ± 0.08

[a] The change in free energy for benzoate degradation when the threshold was reached was calculated according to Thauer et al. (48), using the above concentrations and a bicarbonate concentration calculated from the amount of bicarbonate produced (equation 1) plus the initial concentration.

[b] Mean ± standard deviation of three replicates, except for the final hydrogen and $\Delta G^{o'}$ values, which are the means ± standard deviations of two replicates.

The threshold value was influenced by the amount of benzoate that the cell suspension received and, consequently, the resulting acetate concentration. Higher acetate concentrations resulted in higher threshold values for benzoate degradation by the coculture. The data suggest that as the concentration of benzoate becomes small relative to the concentrations or partial pressures of the products (acetate, hydrogen or formate, and bicarbonate), the change in Gibbs free energy approaches a minimum value below which further benzoate degradation is energetically unfavorable. Seitz et al. (42, 43) found that the Gibbs free energy value associated with syntrophic ethanol degradation by *Pelobacter acetylenicus* was independent of the type of hydrogen user and/or the electron acceptor used, indicating that the hydrogen producer was under energy homeostasis, and that a critical Gibbs free energy value for ethanol degradation exists (38). The critical value for ethanol degradation was close to that required to synthesize one-third of an ATP (49). The existence of a critical Gibbs free energy value for benzoate may explain the existence of the threshold. However, there may be other explanations for the existence of a threshold for benzoate degradation. Since the rate of benzoate degradation decreased when the acetate concentration increased (Fig. 2), a kinetic explanation based on acetate inhibition (13) cannot be excluded.

A minimum or threshold value for substrate consumption has been observed for other anaerobic (12, 16, 17, 21, 26, 32, 53) and aerobic (9, 39) bacteria. A threshold for hydrogen use has been proposed to explain why sulfate reducers outcompete methanogens for hydrogen (6, 27). The existence of different threshold values for acetate degradation by different acetoclastic methanogens (16, 32, 53), all of which carry out the same reaction with the same energetics, is obviously due to factors other than a thermodynamic barrier. Jetten et al. (21) proposed that the different threshold values for acetate were due to different uptake systems with differing affinities for acetate. Min and Zinder (32) proposed that the different acetate threshold values may represent different minimum concentrations of acetate below which the microorganism can no longer obtain enough energy to support metabolism. Schmidt et al. (40) proposed that a threshold for growth will be observed when the amount of substrate taken up by a bacterial cell is just adequate to meet its maintenance requirements. Below the threshold value, the cell will die or enter a state of dormancy where no significant metabolism will occur.

Operational improvements in the methanogenic digestion of sewage and other industrial wastes have been based on the control of potentially inhibitory levels of hydrogen (19, 37, 46) and/or formate (50) and prevention of toxic levels of the undissociated forms of volatile fatty acids (4, 16, 17). The influence of acetate on the threshold for benzoate degradation suggests that the concentration of acetate may also influence the energetics of volatile fatty acid degradation. The syntrophic degradation of fatty acids may approach a thermodynamic barrier if the concentration of acetate accumulates. Ahring and Westermann (1) studied the effect of acetate on butyrate degradation by a thermophilic syntrophic culture. Butyrate degradation was severely inhibited by the addition of 75 mM acetate. Our calculations show that the Gibbs free energy would become unfavorable when the butyrate concentration reached 8 mM (initial concentration of 10 mM), assuming a hydrogen partial pressure of 10 Pa and including in the calculation the amount of acetate produced from butyrate. Beaty and McInerney (8) observed that continued butyrate degradation by a syntrophic coculture of *Syntrophomonas wolfei* and *Methanospirillum hungatei* required the presence of an acetate-using methanogen when high levels of acetate were present. If thermodynamic thresholds for the syntrophic degradation of volatile fatty acids exist, then the accumulation of acetate and hydrogen (or formate) may make the degradation of these compounds energetically unfavorable. The in situ concentrations of propionate and butyrate are much lower than that of acetate (11, 29, 52). Thus, the increase in product formation, acetate, hydrogen, or formate, need not be large to make the degradation of these compounds energetically unfavorable.

The existence of threshold concentrations for the biodegradation of synthetic and natural organic compounds (9) may account for the persistence of trace organic compounds in natural waters (20) and may have some interesting implications for modeling substrate decay and predicting the fate of a compound. Many kinetic approaches (2, 24, 25, 35, 36, 39) assume that first-order decay occurs when the substrate concentration is below the apparent K_m. The assumption that continued substrate removal occurs may not always be valid and may lead to an overly optimistic estimate of the persistence of the compound if a threshold for substrate degradation exists. This may be particularly important in methanogenic environments where syntrophic interactions are required for the degradation of many compounds.

Here, we report the isolation of a syntrophic benzoate-degrading bacterium (strain SB) in pure culture, using crotonate as the sole source of energy. Preliminary characterization of strain SB shows that it is morphologically similar to *S. buswellii* (33) but differs from *S. buswellii* and other syntrophic bacteria that degrade aromatic compounds (33, 34, 45, 47) in that fatty acids also support its growth in coculture with a hydrogen-using bacterium (8a). With the exception of strain PA-1 (6), no other syntrophic bacterium that uses both aromatic compounds and fatty acids for growth is known. Further characterization is required to determine the taxonomic status of strain SB.

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