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Utilization of methoxylated benzoates and formation of intermediates by *Desulfotomaculum thermobenzoicum* in the presence or absence of sulfate

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Abstract. *Desulfotomaculum thermobenzoicum* strain TSB (DSM 6193) was found to utilize some methoxylated benzoates as carbon and energy source with or without sulfate. 3- or 4-Methoxybenzoate, vanillate (4-hydroxy-3-methoxybenzoate), syringate (3,5-dimethoxy-4-hydroxybenzoate) and 3,4,5-trimethoxybenzoate were converted to corresponding hydroxybenzoates. However, neither 2-methoxybenzoate nor 2,6-dimethoxybenzoate was utilized. The organism grew acetogenically on each of the methoxylated benzoates in the absence of sulfate.

3,4-Dihydroxy-5-methoxybenzoate was detected during conversion of syringate, and syringate and 3,4-dihydroxy-5-methoxybenzoate were detected during conversion of 3,4,5-trimethoxybenzoate as intermediates.

These findings indicate that 4-methoxyl-group is most readily cleaved, whereas 2-methoxyl-group is not utilized by the organism.

Key words: *Desulfotomaculum thermobenzoicum* – Intermediates of methoxylated benzoates conversion – Acetogenic growth – Methoxylated benzoates

Methoxylated aromatic compounds are known to be structural units of lignin and its derivatives. Cleavage of methoxylated benzoates by anaerobic consortia or bacteria has been studied. Healy and Young (1979) first reported the degradation of vanillate and other aromatic compounds to methane by an anaerobic consortium, and Kaiser and Hanselmann (1982) also showed the metabolism of substituted monoaromatic compounds by anaerobic sediments. Krumholz and Bryant (1986) found *Syntrophococcus sucromutans* to use methoxylated benzoates as electron acceptors with several substrates such as fructose as electron donor. *Desulfotomaculum orientis* utilizes trimethoxybenzoate in the absence of sulfate, although it grows slowly (Klemps et al. 1985). *Acetobacterium woodii* grows acetogenically on methoxylated aromatic compounds as the sole organic substrates (Bache and Pfennig 1981). Recently, DeWeerd et al. (1988) showed that anaerobic bacteria, including *D. orientis*, *A. woodii* and *S. sucromutans*, converted methoxybenzoates to hydroxybenzoates with the *O*-demethylation reaction rather than the demethoxylation reaction. However, no description is available as for the intermediary products from methoxylated aromatic compounds and the site-specificity of the cleavage reaction by those organisms.

In the present work, we found that *Desulfotomaculum thermobenzoicum*, which was first described as a thermophilic benzoate-degrading sulfate reducer (Tasaki et al. 1991), grew on methoxylated benzoates with or without sulfate and formed several intermediates during conversion of methoxylated benzoates. We discuss some characteristics of cleavage reaction of methoxylated benzoates by the organism.

Materials and methods

Organism

Desulfotomaculum thermobenzoicum strain TSB (DSM 6193) isolated from a thermophilic anaerobic digester (Tasaki et al. 1991) was used throughout the experiments.

Growth experiments on methoxylated benzoates

Basal medium was prepared as described previously (Tasaki et al. 1991). Cells grown on 5 mM benzoate with 20 mM Na_2SO_4 were inoculated into the medium containing each methoxylated benzoate (5 mM) with or without sulfate (20 mM) (Inoculum 5% vol/vol).

Preparation of cell suspension

Desulfotomaculum thermobenzoicum strain TSB was cultured on 3,4,5-trimethoxybenzoate (5 mM) in the absence of sulfate. Cells at the late exponential phase were harvested by centrifugation at 10,000 × *g* for 15 min, washed twice, and suspended in fresh medium without substrate and sulfate. Cell suspension was subsequently

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dispensed into 30-ml serum bottles, each containing 15 mg dry weight of cells in 20 ml. The head space was filled with N_2/CO_2 (4:1, vol/vol, 147 kPa overpressure). After addition of each substrate, cultivation was carried out at 55 °C.

Determinations

Growth was monitored spectrophotometrically at 600 nm. Cell dry mass was calculated based on the correlation between optical density and dry weight of cells grown on 3,4,5-trimethoxybenzoate without sulfate.

Aromatic compounds were measured by HPLC (column, GL Science Corp., Tokyo, Inertsil ODS 5 µm, 4.6 × 250 mm; eluent, methanol/water (60/40), pH adjusted to 2.6 with H_3PO_4). Fatty acids and alcohols were measured by gas chromatography (column, PEG-6000 15% on Flusin P 60/80; glass column, 1.5 mm × 3 m; column temperature, 160 °C) with an FI-detector and/or by HPLC (column, Shimadzu Corp., Tokyo, SCR-101H, 7.9 × 300 mm; eluent, 15 mM perchloric acid).

Assay of carbon monoxide dehydrogenase

Carbon monoxide dehydrogenase was measured by following the reduction of methylviologen at 578 nm under the anaerobic conditions (Spormann and Thauer 1988).

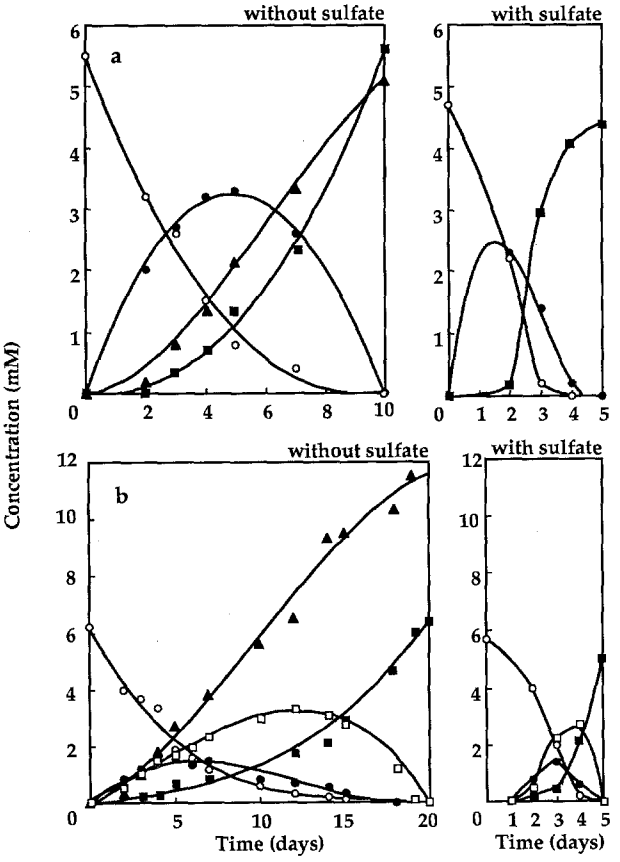

Fig. 1. Conversion of syringate (**a**) or 3,4,5-trimethoxybenzoate (**b**) by *Desulfotomaculum thermobenzoicum* strain TSB. Each *left figure* shows conversion without sulfate, *right figure* shows conversion with sulfate. a) conversion of syringate, b) conversion of 3,4,5-trimethoxybenzoate. *Symbols;* a) –○–: syringate, –●–: 3,4-dihydroxy-5-methoxybenzoate, –■–: gallate, –▲–: acetate. b) –○–: 3,4,5-trimethoxybenzoate, –●–: syringate, –□–: 3,4-dihydroxy-5-methoxybenzoate, –■–: gallate, –▲–: acetate

Results

Growth experiments on methoxylated benzoates

Desulfotomaculum thermobenzoicum strain TSB grew on five methoxylated benzoates with or without sulfate. 3-Methoxybenzoate and 4-methoxybenzoate were converted to 3-hydroxybenzoate and 4-hydroxybenzoate, respectively. 2-Methoxybenzoate was not utilized. Vanillate (4-hydroxy-3-methoxybenzoate) was converted to protocatechuate (3,4-dihydroxybenzoate). Syringate (3,5-dimethoxy-4-hydroxybenzoate) was converted to gallate (3,4,5-trihydroxybenzoate) via 3,4-dihydroxy-5-methoxybenzoate (Fig. 1a). 3,4,5-Trimethoxybenzoate was converted to gallate (Fig. 1b). In this conversion, syringate and 3,4-dihydroxybenzoate were detected as intermediates.

In the absence of sulfate, hydroxylated benzoates and acetate were produced from all the methoxylated benzoates, while only hydroxylated benzoates were detected in the presence of sulfate with H_2S formation (data not shown). With sulfate, growth yields were higher and growth was faster than without sulfate (Table 1). All methoxylated benzoates were converted to hydroxybenzoates within 5 days, although the conversion without sulfate were two or three times slower.

Fermentation by cell suspension

Stoichiometric conversion of four methoxylated benzoates to hydroxylated benzoates and acetate in the absence of sulfate was investigated using concentrated cell suspension. Degradation of methoxylated benzoates progressed linearly with acetate formation. During conversion of syringate and 3,4,5-trimethoxybenzoate, the same intermediates as those detected in growing cultures were formed. On all substrates, mol acetate per mol methoxyl-group on the average was calculated to be 0.75. The stoichiometry of conversion of each substrate was consistent with the following equations.

Table 1. Growth of *Desulfotomaculum thermobenzoicum* strain TSB on some methoxylated benzoates with or without sulfate

Substrate added (mmol/l)		Sulfate added (mmol/l)	Dry cells (mg/l)	Growth yield: g dry weight per mol substrate
4-Methoxybenzoate	(4.8)	0	17	3.5
	(4.6)	20	24	5.2
Vanillate	(3.7)	0	14	3.8
	(3.9)	20	22	5.6
Syringate	(5.5)	0	23	4.1
	(4.2)	20	36	8.6
3,4,5-Trimethoxybenzoate	(6.0)	0	28	4.7
	(5.5)	20	54	9.8

4-Methoxybenzoate

$$4\,C_8H_7O_3^- + 2\,HCO_3^- \rightarrow 4\,C_7H_5O_3^- + 3\,CH_3COO^- + H^+$$

Vanillate

$$4\,C_8H_7O_4^- + 2\,HCO_3^- \rightarrow 4\,C_7H_5O_4^- + 3\,CH_3COO^- + H^+$$

Syringate

$$2\,C_9H_9O_5^- + 2\,HCO_3^- \rightarrow 2\,C_7H_5O_5^- + 3\,CH_3COO^- + H^+$$

3,4,5-Trimethoxybenzoate

$$4\,C_{10}H_{11}O_5^- + 6\,HCO_3^- \rightarrow 4\,C_7H_5O_5^- + 9\,CH_3COO^- + 3\,H^+$$

Methanol was not utilized by the cell suspension.

Activity of carbon monoxide dehydrogenase

The activity of carbon monoxide dehydrogenase, a key enzyme of CODH pathway, was detected in the cells grown on 3,4,5-trimethoxybenzoate without sulfate (1100 nmol/min/mg protein) and with sulfate (820 nmol/min/mg protein).

Discussion

Acetogenic growth of Desulfotomaculum thermobenzoicum on methoxylated benzoate

It is known that sulfate-reducing bacteria grow by fermenting some substrates such as malate, pyruvate and lactate (Miller and Wakerley 1966; Miller and Neumann 1970; Postgate 1952). In the present work, we found several methoxylated benzoates to allow good growth of *D. thermobenzoicum*, a thermophilic sulfate-reducing bacterium, without sulfate. Until now, only a few sulfate-reducing bacteria, including *Desulfotomaculum orientis*, were known to use trimethoxybenzoate without sulfate, although they grow slowly and stoichiometry of the fermentation has not been discussed (Klemps et al. 1985). *D. thermobenzoicum* forms acetate and hydroxylated benzoates from methoxylated benzoates. The acetogenic growth on these substrates is quite similar to the growth of *Acetobacterium woodii* on methoxylated benzoates (Bache and Pfennig 1981). Both organisms produce 0.75 mol acetate from 1 mol methoxyl-group. However, *D. thermobenzoicum* is not able to utilize methanol, while *A. woodii* is. This finding indicates that acetate is formed from methoxyl-group possibly via some methyl compounds but not via methanol, and methoxylated benzoates might be *O*-demethylated to hydroxybenzoates (DeWeerd et al. 1988).

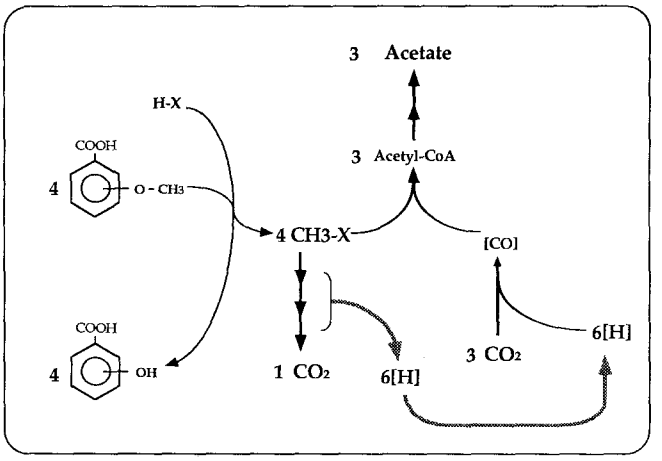

Fig. 2. Proposed pathway of acetogenesis from methoxylated benzoate

The activity of carbon monoxide dehydrogenase was detected in the cells grown on 3,4,5-trimethoxybenzoate without sulfate, suggesting that CODH pathway is involved in the conversion of methoxylated benzoates with acetogenesis. We infer that methyl-group may be introduced to CODH pathway via some methyl-carriers and converted to acetate (Fig. 2).

Conversion of methoxylated benzoates with sulfate

When *D. thermobenzoicum* grows on methoxylated benzoates in the presence of sulfate, acetate is not produced. With acetate and sulfate, the organism shows only slight growth. These results indicate that the organism appears to oxidize methoxyl-group to CO_2 directly without acetogenesis under the sulfate-sufficient conditions. The organism is able to obtain more energy by *O*-demethylation associated with sulfate reduction than by acetogenesis without sulfate, as suggested by higher cell yields and faster growth (Table 1).

Pathway of methoxylated benzoates to hydroxylated benzoates

Figure 1 shows that the first step of demethylation of 3,4,5-trimethoxybenzoate to gallate is a demethylation of the 4-methoxy-group, but not the 3- or 5-methoxyl-group. Syringate is subsequently converted to gallate via 3,4-dihydroxy-5-methoxybenzoate. Transient accumulation of such intermediates confirmed us that demethylating reactions of methoxylated benzoates proceed stepwise (Fig. 3). Kaiser and Hanselmann (1982) reported stoichiometric methane formation from some methoxylated aromatic compounds by anaerobic sediments and they speculated that 3,4,5-trimethoxybenzoate was converted to 4,5-dimethoxy-3-hydroxybenzoate. Our results were quite different from their proposed pathway.

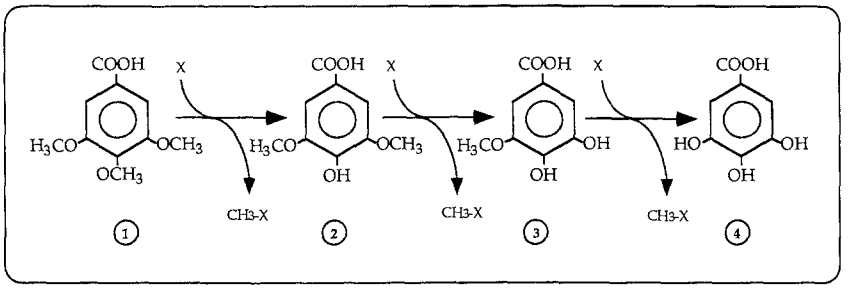

Fig. 3. Proposed pathway of conversion of methoxylated benzoate to hydroxylated benzoate. ① 3,4,5-trimethoxybenzoate, ② syringate, ③ 3,4-dihydroxy-5-methoxybenzoate, ④ gallate

The organism utilizes neither 2-methoxybenzoate nor 2,6-dimethoxybenzoate suggesting the inability of this organism to demethylate the methoxyl-group at 2-position. 2,3,4-Trimethoxybenzoate, 2,3-dimethoxybenzoate and 2,4-dimethoxybenzoate were also degraded to acetate and hydroxylated benzoates by *D. thermobenzoicum* (data not shown). However, we could not identify the fermentation products because the chemicals such as 3,4-dihydroxy-2-methoxybenzoate were not commercially available. The organism is able to utilize neither 2-methoxybenzoate nor 2,6-dimethoxybenzoate. Considering these findings and the molar ratio of acetate to methoxyl-group, 2,3,4-trimethoxybenzoate, 2,3-dimethoxybenzoate and 2,4-dimethoxybenzoate appeared to be converted to 3,4-dihydroxy-2-methoxybenzoate, 3-hydroxy-2-methoxybenzoate and 4-hydroxy-2-methoxybenzoate, respectively.

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