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Determination of the G + C Content of Two *Syntrophus buswellii* Strains by Ultracentrifugation Techniques

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Abstract. An anaerobic syntrophic bacterial culture degrading benzoate was isolated from a river sediment. The syntrophic organism was grown in coculture in the presence of a hydrogenotrophic strain, *Desulfovibrio fructosovorans* or *Methanospirillum hungatei*. The G + C content of the syntrophic benzoate degrader determined by density gradient ultracentrifugation was similar to that of *Syntrophus buswellii* (54.3%). A method ensuring the G + C% determination of syntrophic bacteria is presented.

Several syntrophic anaerobic bacteria have been described: *Syntrophomonas wolfei* [6, 9], *Syntrophomonas sapovorans* [15], *Syntrophobacter wolinii* [17], *Syntrophospora bryantii* [7, 21], and *Syntrophus buswellii* [11, 12]. Among these strains, the guanine + cytosine content of the DNA (G + C%) has been determined only for *S. bryantii* when grown as pure culture on crotonate [21] and for *S. wolinii* [17] after the lysis of its partner in coculture. Usually, determination of G + C% in syntrophic bacteria is inaccurate when the partners have G + C% of the same range [6, 9, 11, 12, 15].

We report on the isolation and characterization of a syntrophic benzoate degrader, and we determine the G + C% content of the DNA of *S. buswellii* and our isolate by ultracentrifugation techniques. Furthermore, a pure coculture of the syntrophic organism associated with *Methanospirillum hungatei* was obtained.

Materials and Methods

Organisms. *Methanospirillum hungatei* (DSM 864) [2] and the coculture *S. buswellii–Desulfovibrio desulfuricans* (DSM 2612 A) were obtained from the German collection of microorganisms. *Desulfovibrio fructosovorans* (DSM 3604) was from our culture collection [13]. *D. desulfuricans* was isolated from the coculture *S. buswellii–D. desulfuricans*. Strain S was isolated from sediments of Huveaune river, near Marseilles, France. It has been deposited with the DSM, under numbers 4156A and 4156B, when cocultured with the sulfate reducer or the methanogen, respectively.

Media and conditions of growth. Media were prepared by the techniques of Hungate [4]. The organisms were cultivated under strict anaerobiosis at 37°C. The basal medium, for isolation and stock culture maintenance, contained the following constituents in distilled water (g per liter): NH_4Cl, 1.0; $CaCl_2$; $2H_2O$, 0.15; NaCl, 1; KCl, 0.5; $MgCl_2$; $6H_2O$, 0.4; KH_2PO_4, 0.2; CH_3COONa; $3H_2O$; 1; resazurin, 0.001; sodium selenite solution [14], 1 ml; Imhoff-Stuckle mineral solution [5], 1.5 ml; pH 7.0. The medium was boiled under a stream of O_2-free N_2, cooled at room temperature, and 5 ml of medium was distributed into Hungate tubes which were outgassed with $N_2 : CO_2$ (80 : 20%). After autoclaving (110°C for 30 min), 0.05 ml of 2% $Na_2S \cdot 9H_2O$, 0.2 ml of 10% $NaHCO_3$ (sterile, anaerobic solutions), 0.05 ml of filter-sterilized vitamin solution [20], and 0.1% dithionite solution were added to the tubes before inoculation.

Solid medium for roll-tubes was prepared by adding 2% (w/v) agar (Difco Laboratories, Detroit, Michigan) in basal medium.

The basal medium was supplemented with sodium benzoate or Na_2SO_4 at appropriate concentrations from sterile stock solutions or by including these compounds in the medium prior to sterilization.

The enrichment cultures contained the basal medium with 7 mM sodium benzoate and 20 mM Na_2SO_4. They were maintained by 10% transfer of the culture every 2 weeks. *D. fructosovorans* and *D. desulfuricans* were grown in liquid cultures containing 20 mM Na_2SO_4, 20 mM fructose, and 20 mM sodium formate, respectively. *M. hungatei* was cultivated under an 80 : 20% $H_2 : CO_2$ gas phase (2 atm.).

Liquid cultures of the benzoate-degrading bacterium associated with *Desulfovibrio* sp. were grown in tubes containing 20 mM sodium benzoate and 20 mM Na_2SO_4.

Cocultures containing the benzoate degrader and *M. hungatei* were maintained as described above except that sulfate was deleted from the medium. Cocultures were transferred every two weeks. Cultures were routinely checked for purity by microscopic

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Table 1. Substrates tested for growth of strain SF. Substrate concentrations (in mmol/L) in the test cultures are given in parentheses. All growth tests were performed in duplicates.

Substrates utilized:
Benzoate (5, 20), hydrocinnamate (5, 20)
Substrates tested, but not utilized:
Fructose (20), glucose (20), proprionate (20), methanol (20), valerate (10), butyrate (10), succinate (10), adipate (10), phenylacetate (10), phenol (10), cellobiose (10), heptanoate (10), *p*-amino benzoate (10), cyclohexanone (10), hexanoate (10), *p*-hydroxybenzoate (10), pimelate (10), ferulate (10), crotonate (10)

examination and by inoculating liquid medium containing 20 mM glucose and 0.2% yeast extract. Test tubes were incubated under both anaerobic and aerobic conditions.

Substrate utilization. The use of substrates was determined in medium prepared under 80 : 20% N_2 : CO_2 gas phase, in duplicate vessels. The substrates (Table 1) were autoclaved or filter sterilized, stored under N_2, and added to autoclaved media by syringe injection. Initial substrate concentration was 20 mM except for cellobiose and phenol (10 mM each), hydrocinnamate (5 and 20 mM), and benzoate (13.8 and 20 mM). Growth was determined by measuring optical density and sulfide production after one month of incubation.

Analytical methods. Optical density was determined at 580 nm with a UV-160A Shimadzu spectrophotometer (Shimadzu Corporation, Kioto, Japan). Sulfide was determined by the method of Cord-Ruwisch [1]. Acetate production was measured by gas chromatography at 190°C, on a Porapak Q column, connected to the flame-ionization detector of a Chrompack CP 9000 gas chromatograph (Chrompack France, Les Ulis, France), with N_2 as the carrier gas. Prior to analysis, culture supernatant was acidified to pH 1.0 with 50% (v/v) H_3PO_4. Benzoate was quantified by HPLC on a Spherisorb column (C18-3 μm; Touzart et Matignon, Vitry-sur-Seine, France) of 50-mm length and 4.1-mm inner diameter.

DNA base composition. Cells were lysed with sodium dodecyl sulfate, and DNA was purified from the cell lysate by the method of Marmur [8]. The buoyant density of the DNA was determined by cesium chloride density gradient centrifugation in a Beckman model E ultracentrifuge by the methods of Meselson et al. [10] and Szybalski [19]. The cesium chloride density was measured in a digital precision densitometer (DMA 02C of Anton Paar) by the method of Stabineer [16]. DNA from *Micrococcus luteus* (G + C content, 71.0 mol%) was used as a standard.

Results

Enrichment of the benzoate-degrading mixed culture Huv. After four transfers of the first enrichment culture on benzoate medium, a stable bacterial population (Huv) was obtained. Benzoate was degraded to acetate and methane. At least four morphologically dominant species were observed. Under epifluorescence microscopy at 420 nm, the H_2-consuming methanogen was identified as *M. hungatei*; the acetate-utilizing methanogens were large filamentous *Methanothrix*, rod-like. The predominantly nonmethanogenic bacteria were motile coccoid forms, responsible for benzoate degradation. The fourth strain was a spore-forming rod, which reduced sulfate in lactate-containing medium; its role in the culture is unexplained.

Isolation of the syntrophic benzoate degrader. A benzoate degrader was isolated in coculture with *D. fructosovorans* by diluting the Huv culture into benzoate sulfate medium containing agar; each dilution was inoculated with a pure culture of *D. fructosovorans*. Separate colonies developed from the last dilution tube after 2 months of incubation, but the coculture remained contaminated with a small *Desulfovibrio*-like form. Purification was obtained with fumarate as electron acceptor. The isolated coculture (SF) was routinely cultivated on a benzoate–sulfate medium. Strain SF, containing the syntrophic bacterium (S) and *D. fructosovorans* (F), was further characterized.

Isolation of an anoxic coculture of the benzoate degrader with *M. hungatei*. The coculture SF was transferred several times in a liquid medium without sulfate and inoculated with *M. hungatei*. However, *D. fructosovorans* cells were still present. This triculture was diluted to 10^{-12}, and *M. hungatei* was inoculated in each dilution tube. After 2 months, growth appeared in the 10^{-6} dilution tube, which consisted only of the benzoate degrader and *M. hungatei*. The purity was tested in a medium containing sulfate and fructose. This coculture was named SH (Fig. 1).

Using the same techniques, we isolated *S. buswellii* [11, 12] with *M. hungatei* (BH).

Physiology. Strain SF grew on synthetic medium. The only substrates degraded by strain SF were benzoate and hydrocinnamate (Table 1). The growth of SF was tested with different concentrations of benzoate (Table 2). Growth increased until the concentration was 30 mM benzoate. With initial benzoate concentrations up to 10 mM, all the substrate was degraded, and the acetate produced was in agreement with the stoichiometric reaction relative to benzoate degradation proposed by Mountfort and Bryant (1982) [11]:

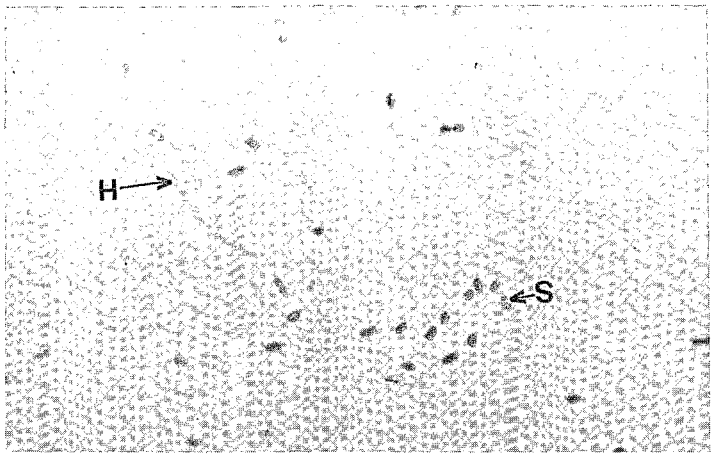

Fig. 1. Light micrograph of cells of the coculture SH.

$$C_7H_6O_2 + 6\ H_2O \rightarrow 3\ C_2H_4O_2 + CO_2 + 6\ H^+ + 6\ e^- \quad \text{(eq 1)}$$

At concentrations above 10 mM, benzoate was not entirely degraded, and the ratio of acetate/benzoate degraded decreased greatly. Moreover, another product was detected by HPLC, which was not cyclohexanecarboxylate, 1-cyclohexen-carboxylate, 3-cyclohexen-carboxylate, adipate, or cyclohexanone.

Experiments with resting cells of SF (10^{10} cells/ml), in the presence of sulfate, showed that benzoate (10 mM) was degraded in acetate. The same experiments were realized with other substrates, which are intermediates proposed in the methanogenic fementation of benzoate by Grbic-Galic and Young [3]: cyclohexane carboxylate (10 mM, 10^{10} cells/ml), methylcyclohexane (10 mM, 10^{11} cells/ml), and caproate (25 mM, 10^{13} cells/ml). None of these subrates was degraded.

Table 2. Growth of strain SF at different benzoate concentrations

Initial benzoate (mM)	Acetate formed (mM)	H_2S found (mM)	Benzoate recovered (mM)	Acetate/ benzoate degraded
5	15	2.4	0.05	3
10	27.5	4.5	0.06	2.8
15	41	6	0.09	2.8
20	48	6.5	3	2.8
30	56	7.2	8	2.5
40	50	7.6	14	1.9
50	45	6.8	24	1.7

DNA base composition. Mixtures of pure DNA of *Micrococcus luteus* with DNA of *D. frutosovorans*, *D. desulfuricans*, or *M. hungatei* were centrifuged to check the procedure. In each case, two distinct peaks were obtained at equilibrium, in agreement with results obtained earlier with *D. frutosovorans* [13] or *M. hungatei* [2]. The mol% G + C of *D. desulfuricans* was 54.8. In a second set of determinations, the DNA of *M. luteus* was added to the DNA of the four cocultures (Table 3, Fig. 2).

The syntroph S cocultured with *D. fructosovorans* (SF) or the methanogen (SH) has a mean G + C% of 54.3 ± 1.0 mol%. The G + C% of *S. buswellii* cannot be obtained in the presence of *D. desulfuricans*, since the two bacteria present only one peak by ultracentrifugation. In the presence of *M. hungatei*, the estimated G + C% of *S. buswellii* was 54.1 ± 1.0 mol%.

Discussion

A new strain of anaerobic degrader was isolated in coculture with *D. fructosovorans*. It was morphologically similar to *S. buswellii*, isolated by Mountfort et al. [11, 12], and degraded the same substrates: benzoate and hydrocinnamate. Although *S. buswellii* was primarily cultivated in a medium containing rumen fluid [11], we have shown that it can be grown in a simple mineral medium as our isolate and does not require any growth factors contained in rumen fluid.

For low initial benzoate concentrations (up to 10 mM), benzoate was stoichiometrically degraded in acetate by coculture SF. When the benzoate concentration was greater than 10 mM, the acetate quan-

Table 3. G + C% values of the different cultures and cocultures examined

Culture	Syntroph	*D. fructosovorans*	*M. hungatei*	*D. desulfuricans*
SF	54.4	64.0		
SH	54.2		44.8	
BD	54.6			54.6
BH	54.1		44.8	
F		63.8		
D				54.8
H			45.0	

tities recovered from the medium were lower than the theoretical values. An unknown cumulative product was detected by HPLC, and benzoate was not completely degraded. Benzoate fermentation may be partially inhibited by high substrate concentration. Despite the phenotypic homologies between the strain SF and *S. buswellii,* to our knowledge, we report for the first time the isolation of a pure culture of *S. buswellii* strain associated with *M. hungatei.* The two syntrophic strains were further studied for their G + C%.

Several authors attempted to estimate the G + G% of syntrophs. Zhao et al. [21] succeeded with *S. bryantii* because this strain can grow axenically on crotonate. Mah et al. [7] isolated the DNA of *S. wolinii,* strain LX-2. The G + C% of the culture was determined by using a coculture with a methanogen, which was then submitted to freezing-thawing and digestion with lysozyme to eliminate the DNA of the methanogen. Thus the G + C% of the syntroph was detectable in a CsCl gradient. With the other syntrophic bacteria, G + C% determination was not performed [6, 15, 17] or was unsuccessful, since the hydrogenotrophic sulfate-reducing partner (*D. desulfuricans*) had a G + C% in the same range as the syntrophic degrader [9, 11, 12].

The high G + C% of *D. fructosovorans,* compared with that of *D. desulfuricans,* allowed us to determine precisely the G + C% of the syntroph. DNA from the syntrophic strain cocultured with *D. fructosovorans* or *M. hungatei* was separated into two peaks by ultracentrifugation, corresponding to the G + C% of the benzoate degrader (54.3%) and *D. fructosovorans* (64%) or *M. hungatei* (48%). We found that *S. buswellii* had a G + C% of 54.1%, close to that of the syntroph.

Our results show that to estimate the G + C% of a syntroph, it is advisable to use two cocultures with hydrogenotrophic sulfate-reducing bacteria having different G + C%. Use of sulfate reducers rather than methanogenic bacteria is preferred, since cultivation after several replications [6] and axenization [11, 12] with methanogens appears to be difficult. This will ensure that separated DNA peaks will be obtained from the coculture by the ultracentrifugation technique, as well as purified DNA of the syntrophic bacterium. It will, therefore, also allow DNA–DNA hybridization between syntrophic bacteria or other microorganisms with similar phenotypic characteristics.

Szewzyk and Schink [18] determined the G + C% of the strain HQGö1 (53.2 ± 1.0%), which grows syntrophically on benzoate. They could not decide whether their isolate was related to *S. buswellii,* in spite of its resemblance to it. With regard to the G + C% of their isolate, they classified it in the genus *Pelobacter* (G + C: 51–59), since it fermented

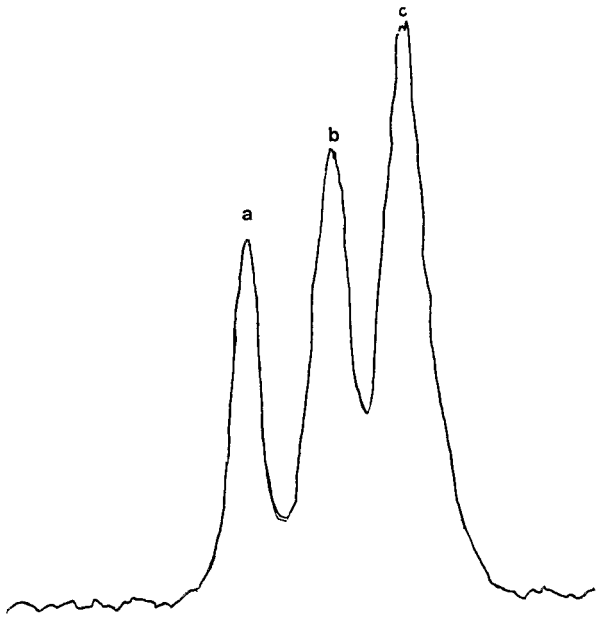

Fig. 2. Absorption diagram obtained during differential ultracentrifugation in $CsCl_2$ gradient of a mixture of DNA of *M. luteus* (a), *D. fructosovorans* (b) and the syntrophic benzoate degrader (c).

some subtrates without mineral or bacterial electron acceptor. Our results show that oxydation of aromatic compounds via H_2 interspecies transfer as well as the G + C% content in the DNA are in favor of phylogenetic proximity between some species of the genus *Pelobacter* and the syntrophs.

Before definite conclusions on the relationships between the syntrophs and the degrading benzoate strains described in the genus *Pelobacter* are drawn. DNA–DNA hybridization of the different bacteria involved should be done.

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