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Propionate Formation by *Opitutus terrae* in Pure Culture and in Mixed Culture with a Hydrogenotrophic Methanogen and Implications for Carbon Fluxes in Anoxic Rice Paddy Soil

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Propionate-forming bacteria seem to be abundant in anoxic rice paddy soil, but biogeochemical investigations show that propionate is not a correspondingly important intermediate in carbon flux in this system. Mixed cultures of *Opitutus terrae* strain PB90-1, a representative propionate-producing bacterium from rice paddy soil, and the hydrogenotrophic *Methanospirillum hungatei* strain SK maintained hydrogen partial pressures similar to those in the soil. The associated shift away from propionate formation observed in these cultures helps to reconcile the disparity between microbiological and biogeochemical studies.

Sugar polymers form a large part of the plant residues ploughed into rice paddy soil, mainly as rice straw, and appear to be the major sources of growth substrates for the organisms of the microbial community of methanogenic bulk soil (9, 21, 29). These are fermented largely to acetate and propionate, which account for 48 to 83% and 18 to 28% of the methane production, respectively (12). The contributions of acetate and propionate to the total carbon flux do not appear to be very different in unamended bulk soil and in rice straw-amended soil (12). Previous investigations using cultivation-dependent and cultivation-independent methods have shown that the numerically dominant saccharolytic bacteria of laboratory rice paddy soil microcosms belonged to a number of different phylogenetic lineages (4, 13, 20, 30). The majority of representative isolates of these produced acetate and propionate as the major end products of sugar fermentation (4, 20). Pure cultures of propionate-producing saccharolytic bacteria generally produce 2 mol of propionate (or propionate plus succinate) per mol of acetate produced (1, 15, 24) when growing with hexoses, as did the isolates from paddy soil. Propionate and succinate are formed in a reductive pathway to reoxidize reduced electron carriers (1, 15). In a system where hexoses are fermented by such propionate-producing bacteria, the contribution of propionate to the carbon flow to methane should be 78%, assuming that the acetate and propionate formed are degraded further to methane and carbon dioxide. Since propionate was found to account for only 18 to 28% of the methane formed (12), this means either that classical propionate-forming bacteria are not those responsible for the major part of the carbon flow from sugars to organic acids or that they carry out a different metabolism in the soil. *Opitutus terrae* (5) is one of the propionate-forming species that were representatives of numerically abundant bacterial populations within the paddy soil system (4, 13). We investigated the formation of propionate by *O. terrae* in pure culture and in mixed culture with a hydrogenotrophic methanogen.

O. terrae strain PB90-1 (DSM 11246) was grown in pure and mixed cultures in sulfide-reduced, bicarbonate-buffered medium SM supplemented with vitamins (3). Inocula were grown with 4 mM glucose or 0.05% (wt/vol) pectin (Fluka, Buchs, Switzerland [25]) as the growth substrate in 50-ml volumes in completely filled screw-cap bottles. One 50-ml bottle was used to inoculate each 1-liter fermentation experiment. Fermentation experiments were carried out with 1-liter aliquots of SM containing 4 mM glucose or 0.05% (wt/vol) pectin in glass bottles with a total volume of 1.132 liters. The bottles were closed with butyl rubber stoppers secured with an aluminum screw ring, under a headspace of N_2 at 8×10^4 Pa and CO_2 at 2×10^4 Pa. These bottles were incubated lying on their sides on a shaking platform at 75 rpm. All incubations were at 30°C, in the dark. The results are the means or ranges from duplicate experiments, except where noted otherwise.

O. terrae fermented glucose mainly to propionate and acetate (Table 1). Glucose and organic fermentation end products were measured by high-performance liquid chromatography (2). The final ratio of propionate plus succinate to acetate was 2.2 mol/mol (for both replicates). Initial attempts to study the behavior of *O. terrae* in mixed culture with the hydrogen-utilizing methanogen *Methanospirillum hungatei* were not successful, because the rate of fermentation of glucose was too high to allow a steady-state partial H_2 pressure (pH_2) to be established (data not shown). We therefore grew *O. terrae* with pectin in pure culture and in mixed culture with *M. hungatei*.

M. hungatei strain SK (kindly provided by F. Widdel) was grown in 50-ml aliquots of medium SM, containing 2 mM sodium acetate, in 125-ml serum vials closed with butyl rubber stoppers and under a headspace of N_2 at 8×10^4 Pa, CO_2 at 2×10^4 Pa, and H_2 at 6×10^4 Pa. Six 50-ml cultures were harvested by centrifugation (2,000 × *g* at 5°C for 30 min) in the 125-ml serum vials in which they were grown. The supernatants were removed by introducing a stream of sterile N_2 at a pressure of about 5×10^3 Pa, via a hypodermic needle, and allowing the liquid to be expelled from the inverted vial via a second hypodermic needle, leaving the cell pellet. The six pellets were

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TABLE 1. Fermentation products of *O. terrae* grown in pure culture with glucose or pectin or with pectin in mixed culture with *M. hungatei*

Substrate or product	Amt (SD) of substrate used or product formed (mmol/liter) when grown with:		
	Glucose[a] (pure culture)	Pectin[b] (pure culture)	Pectin[b] (+ *M. hungatei*)
Glucose	4.21 (0.28)	NA[c]	NA
Propionate	5.01 (0.68)	2.71 (0.56)	1.36 (0.40)
Acetate	2.34 (0.33)	2.25 (0.39)	2.25 (0.34)
Ethanol	0.20 (0.03)	0.30 (0.03)	0.33 (0.07)
Succinate	0.18 (0.04)	0.35 (0.08)	0.21 (0.05)
Lactate	0.07 (0.01)	0.15 (0.05)	0.00
Methanol	0.00	1.05 (0.26)	1.42 (0.33)
H_2	0.0001 (0.00002)	0.016 (0.004)	0.0002 (0.00003)
CH_4	ND[d]	ND	0.12 (0.02)
Cells of *O. terrae*[e]	0.87 (0.08)	0.46 (0.08)	0.58 (0.11)
Cells of *M. hungatei*[e]	NA	NA	0.24 (0.05)

[a] The carbon balance was 106%, and the balance of available H was 108%.
[b] The amount of pectin degraded was not accurately determined.
[c] NA, not applicable.
[d] ND, not determined.
[e] The empirical formula for cell material was taken to be $C_4H_7O_3$.

resuspended in a total of 1 ml of sterile SM, and 0.5 ml was used to inoculate each 1-liter mixed-culture experiment mixture with a sterile syringe and hypodermic needle.

O. terrae grew more slowly with pectin ($\mu = 0.17$ day^{-1} [SD = 0.01]) than with glucose ($\mu = 2.8$ day^{-1} [SD = 0.04]), which allowed a long period of fermentation in mixed culture under a more or less constant pH_2 (Fig. 1). The growth rates of cultures were estimated from the rates of acetate formation. Pectin was measured colorimetrically (10) and gradually disappeared, and the overlying liquid became turbid due to an increase in cell numbers until growth and fermentation ceased after about 800 h of incubation. The extents (84.4% in the pure cultures and 86.2% in the mixed cultures) and rates of pectin utilization and the drops in culture pH (2) were almost identical in pure and mixed cultures, and the estimated specific growth rate ($\mu = 0.18$ day^{-1} [SD = 0.01]) in the mixed cultures with *M. hungatei* was similar to that in pure culture. The growth yield of *O. terrae*, estimated from cell numbers, was about 26% higher in the mixed culture with *M. hungatei* than in pure culture. Cell numbers were determined microscopically under phase contrast by using a Neubauer counting chamber (Hecht, Sondheim-Rhön, Germany), and the dry masses were calculated from conversion factors estimated from pure cultures (3): 10^{10} cells of *O. terrae* had a dry mass of 8.58 mg, and 10^{10} cells of *M. hungatei* had a dry mass of 12.89 mg.

Propionate and acetate were the major products of pectin fermentation by *O. terrae* in pure culture (Table 1). Acetate was the major product for the first 300 h, but after this propionate accumulated at higher concentrations. pH_2 was measured by gas chromatography (2); H_2 was present only at low concentrations, and after 700 h of incubation the pH_2 reached ca. 250 Pa (Fig. 1), equivalent to 5.6 μmol/liter of culture (SD = 1.4). Methanol is known to be a major end product of pectin metabolism and is formed from the methoxyl groups of pectin (23). The decrease in succinate production in the mixed culture is presumably linked to the decrease in the propionate production. Succinate is an intermediate in the pathway for propionate formation by many anaerobic bacteria, including *Opitutus* sp. strain VeGlc2 (1, 11, 15).

In mixed culture with *M. hungatei*, acetate was the major product of pectin fermentation, and smaller amounts of propionate were formed (Table 1). The production of propionate in the mixed cultures decreased noticeably in comparison to the pure cultures, and the final concentration was about half of that in pure cultures (Fig. 1 and Table 1). The pH_2 was between 2.5 and 3.5 Pa, once a quasi-steady state was reached (Fig. 1). This is similar to the pH_2, ca. 1.5 to 12 Pa, observed in experiments with rice field soil slurries and found in situ in the soil itself (6, 12, 16). pCH_4 was measured by gas chromatography (2) and increased with time, until at the end of the incubation period, the pCH_4 reached ca. 1,530 Pa, equivalent to 0.12 mmol/liter of culture (SD = 0.02). Assuming that 1 mol of CH_4 is formed by the oxidation of 4 mol of H_2, approximately 25 times as much H_2 was produced in the methanogenic cocultures as in the pure cultures.

The results indicate a strong coupling of the two organisms through hydrogen transfer. It is well documented that H_2-utilizing methanogens can be involved in interspecies hydrogen transfer reactions with H_2-producing fermentative bacteria (14, 18, 22, 31), which result in shifts in the patterns of fermentation products. However, there are very few reports of a shift to less propionate formation under decreased pH_2 by propionate-forming bacteria. The shift observed in our experiments is apparently not due to differences in the rates of pectin utilization or growth or differences in the change of pH in the cultures, as these were the same in pure and mixed cultures. Our results suggest that the production of propionate is at least in part dependent on the concentration of H_2 and that the production of propionate may be accelerated when the pH_2 is increased. This is presumably the case in pure culture, in which the rate of propionate production increased when the pH_2 exceeded ca. 20 Pa, which is higher than the pH_2 in rice field soil (ca. 1.5 to 12 Pa). In the mixed cultures of *O. terrae* with *M. hungatei*, with a low pH_2 similar to that found in the soil, less propionate was produced. Assuming further methanogenic degradation of the products produced from pectin by *O. terrae*, propionate plus succinate would be the precursors for 57 to 61% of the CH_4 formed from a pure-culture type of

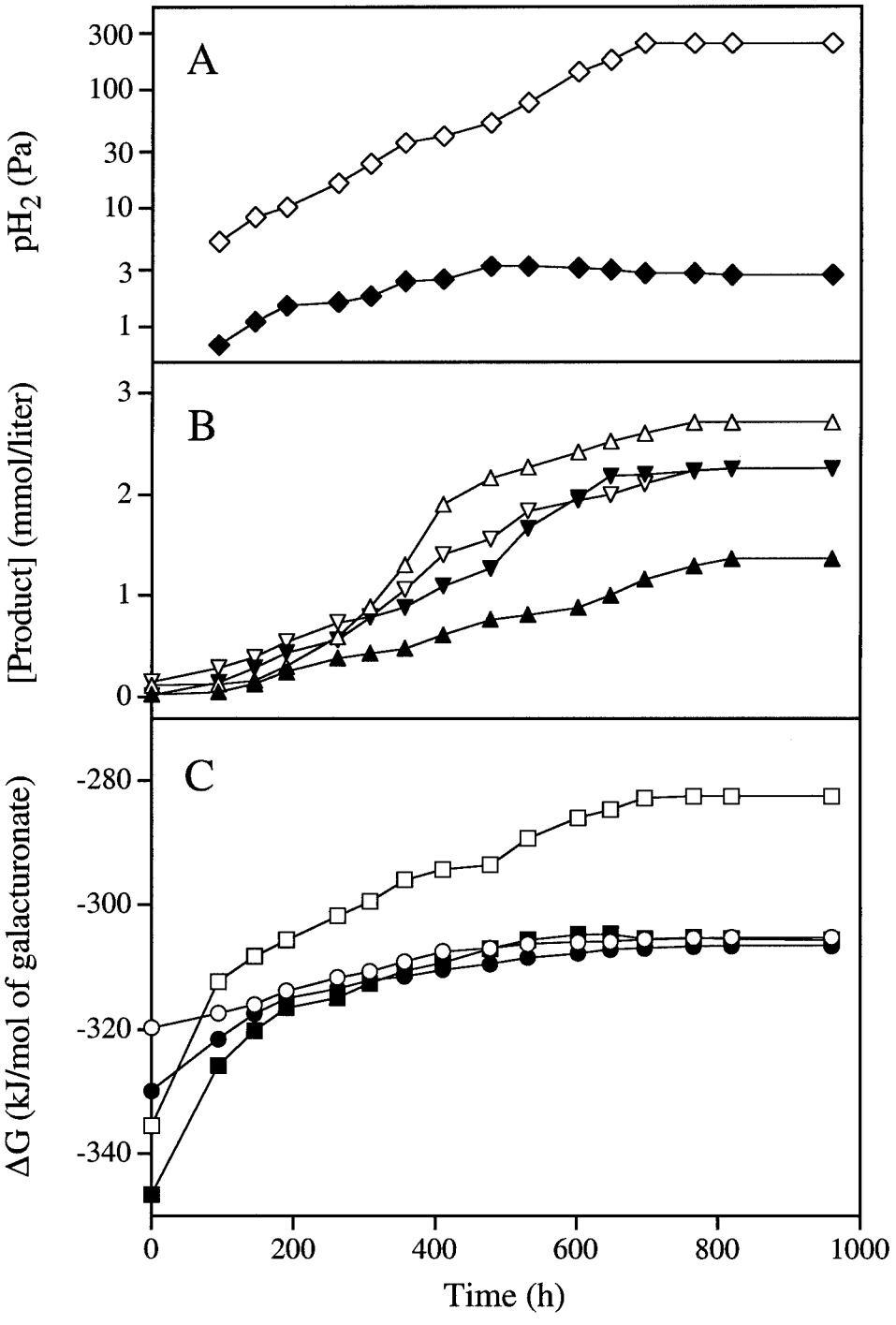

FIG. 1. (A) Changes in the pH_2 in a representative pure culture of *O. terrae* (◇) and in a representative mixed culture of *O. terrae* with *M. hungatei* (◆), both growing with pectin. (B) Acetate (▽, ▼) and propionate (△, ▲) concentrations in the same pure culture (open symbols) and mixed culture (closed symbols). (C) Temporal changes in Gibbs free energies for the formation of acetate + H_2 (□, ■) and acetate + propionate (○, ●) in the same pure culture (open symbols) and mixed culture (closed symbols).

fermentation pattern, but only for 40 to 44% from a mixed-culture (low-pH_2) type of fermentation pattern. Hydrogen production by *O. terrae* accounts for very little of the total CH_4 production; even in the mixed cultures it would account for only about 1.8 to 1.9% of the total CH_4 formed if the other fermentation products were to be metabolized further under methanogenic conditions.

The Gibbs free energies, $\Delta G_{T(30°C)}$, of substrate transformation in the pure cultures of *O. terrae* and in the mixed cultures with *M. hungatei* were calculated from the concentrations of

reactants and products and the standard Gibbs free energies (17, 27, 28), corrected for the actual temperature (30°C) by the Van't Hoff equation (7). For our calculations we estimated a galacturonate concentration of 10^{-6} M. A 10-fold change in the galacturonate concentration will result in a corresponding 5.8-kJ/mol change in the $\Delta G_{T(30°C)}$ for galacturonate fermentation, so that the absolute values but not the relative values are changed. The standard Gibbs free energy of formation for galacturonic acid was calculated to be −1,090.4 kJ/mol, by using a group contribution method (19). The enthalpy of formation for galacturonic acid was estimated from standard tables (8, 26). From these tables, the mean difference between the enthalpy of formation of a primary alcohol and that of its corresponding acid was calculated to be −208.0 kJ/mol. The estimated enthalpy of formation of galacturonic acid was −1,494.3 kJ/mol, i.e., the estimated enthalpy of formation of the corresponding acid (galactose, −1,286.3 kJ/mol [8]) less 208.0 kJ/mol. In the pure cultures, i.e., at higher pH_2, the Gibbs free energy of the fermentation of galacturonate to acetate plus propionate stayed almost constant during the incubation period and was more negative than that of the fermentation of galacturonate to acetate plus H_2 after the initial 100 h (Fig. 1). Therefore, the conversion of galacturonate to acetate plus propionate is thermodynamically more favorable at high pH_2, and this is the pathway that dominated under these conditions. In contrast, in the mixed cultures, i.e., at a lower pH_2, the Gibbs free energy of the fermentation of galacturonate to acetate plus H_2 was very similar to that of the fermentation of galacturonate to acetate plus propionate (Fig. 1). At lower pH_2s, the conversion of galacturonate to acetate plus H_2 and acetate and propionate seem equally favorable. The fermentation balances obtained with the mixed cultures under these conditions suggest that the hydrogen-forming and the propionate-forming pathways operate simultaneously. Hence, under these conditions, there is only a partial shift to acetate plus H_2 production. There therefore seems to be a thermodynamically influenced control of carbon flux through the two alternative pathways.

We hypothesize that the shift in the acetate/propionate ratio of fermentation end products of *O. terrae* to less propionate formation under a pH_2 similar to that in rice paddy soil could reconcile the apparent discrepancy between the numerical dominance of propionate-forming bacteria and the smaller-than-predicted role for propionate as a fermentation intermediate in the same system. We suggest that these numerically abundant propionate-producing bacteria may indeed play an important quantitative role in the fermentation of sugars but that they produce less propionate under the conditions prevailing in their natural soil habitat than they do in pure laboratory cultures.

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