

## Coaggregation Facilitates Interspecies Hydrogen Transfer between *Pelotomaculum thermopropionicum* and *Methanothermobacter thermautotrophicus*

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A thermophilic syntrophic bacterium, *Pelotomaculum thermopropionicum* strain SI, was grown in a monoculture or coculture with a hydrogenotrophic methanogen, *Methanothermobacter thermautotrophicus* strain ΔH. Microscopic observation revealed that cells of each organism were dispersed in a monoculture independent of the growth substrate. In a coculture, however, these organisms coaggregated to different degrees depending on the substrate; namely, a large fraction of the cells coaggregated when they were grown on propionate, but relatively few cells coaggregated when they were grown on ethanol or 1-propanol. Field emission-scanning electron microscopy revealed that flagellum-like filaments of SI cells played a role in making contact with ΔH cells. Microscopic observation of aggregates also showed that extracellular polymeric substance-like structures were present in intercellular spaces. In order to evaluate the importance of coaggregation for syntrophic propionate oxidation, allowable average distances between SI and ΔH cells for accomplishing efficient interspecies hydrogen transfer were calculated by using Fick's diffusion law. The allowable distance for syntrophic propionate oxidation was estimated to be approximately 2 μm, while the allowable distances for ethanol and propanol oxidation were 16 μm and 32 μm, respectively. Considering that the mean cell-to-cell distance in the randomly dispersed culture was approximately 30 μm (at a concentration in the mid-exponential growth phase of the coculture of 5 × 10<sup>7</sup> cells ml<sup>-1</sup>), it is obvious that close physical contact of these organisms by coaggregation is indispensable for efficient syntrophic propionate oxidation.

In anaerobic digestors, organic matter is converted to methane and CO<sub>2</sub> via various intermediates (1, 27). Among the most important intermediate metabolites are volatile fatty acids (VFAs), such as acetate, propionate, and butyrate, and it has been reported that accumulation of VFAs results in a significant decrease in the methane production efficiency in such digestors (16, 17, 37, 38). VFAs are, however, unfavorable substrates for anaerobes, since oxidation of these substrates to H<sub>2</sub> and CO<sub>2</sub> (or formate) is endoergonic under standard conditions (i.e., the changes in the Gibbs free energy are positive [ $\Delta G^{\circ} > 0$ ]) (Table 1) and is thermodynamically feasible only when the H<sub>2</sub> partial pressure (or formate concentration) is kept low (3, 11, 29, 34). For instance, thermodynamic estimation has predicted that H<sub>2</sub> partial pressures as low as 10 Pa and 100 Pa are necessary for the oxidation of propionate and butyrate, respectively (29, 34). Since H<sub>2</sub> and formate are scavenged mainly by the carbonate respiration of methanogenic archaea, syntrophic association of VFA-oxidizing bacteria (called syntrophs) and methanogenic archaea is considered indispensable for efficient VFA oxidation (27, 36).

As described previously, syntrophic VFA oxidation depends on interspecies electron (as H<sub>2</sub> and formate) transfer. Re-

searchers have suggested that close physical contact between syntrophs and methanogens is important for efficient interspecies electron transfer (5, 28, 34). Using a diffusion theory, they have elegantly simulated the inverse relationship between the average distance from syntrophs to methanogens and the hydrogen flux between the organisms (5, 28). Fluorescence in situ hybridization (FISH) has shown that syntrophic bacteria occurred close to methanogenic archaea in granular sludge taken from highly efficient upflow anaerobic sludge blanket (UASB) reactors (5, 10, 13), which also suggests that close physical contact is important.

Despite the recognition of the importance of physical contact between syntrophs and methanogens in aggregated biomass (e.g., UASB granules) (5, 28), it is not yet clear if these organisms also make physical contact in suspended cultures. In addition, the effects of growth substrates on the physical contact have not been examined yet. We also considered the possibility that defined coculture experiments would provide valuable information about how the organisms establish close physical contact. In the present study, therefore, we investigated coaggregation of a syntrophic propionate-oxidizing bacterium, *Pelotomaculum thermopropionicum* strain SI (13, 14), and an H<sub>2</sub>-consuming methanogen, *Methanothermobacter thermautotrophicus* strain ΔH (33), in cocultures. These organisms were chosen because previous studies have shown that they represent important populations in high-temperature anaerobic digestors (1, 13, 19, 31). Emphasis was placed on

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TABLE 1. Standard Gibbs free energy changes ( $\Delta G^\circ$ ) for anaerobic reactions (pH 7)<sup>a</sup>

Compound	Reaction	$\Delta G^\circ$ (kJ mol <sup>-1</sup> )
<b>Hydrogen-releasing reactions</b>		
Propionate	$\text{CH}_3\text{CH}_2\text{COO}^- + 3\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{HCO}_3^- + 3\text{H}_2 + \text{H}^+$	76.1
Ethanol	$\text{CH}_3\text{CH}_2\text{OH} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + 2\text{H}_2 + \text{H}^+$	9.6
1-Propanol	$\text{CH}_3\text{CH}_2\text{CH}_2\text{OH} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{CH}_2\text{COO}^- + 2\text{H}_2 + \text{H}^+$	3.0
<b>Hydrogen-consuming reaction</b>		
Hydrogen	$\text{H}_2 + 1/4\text{HCO}_3^- + 1/4\text{H}^+ \rightarrow 1/4\text{CH}_4 + 3/4\text{H}_2\text{O}$	-33.9
<b>Syntrophic methanogenesis</b>		
Propionate	$\text{CH}_3\text{CH}_2\text{COO}^- + 3/4\text{H}_2\text{O} \rightarrow 3/4\text{CH}_4 + \text{CH}_3\text{COO}^- + 1/4\text{HCO}_3^- + 1/4\text{H}^+$	-25.6
Ethanol	$\text{CH}_3\text{CH}_2\text{OH} + 1/2\text{HCO}_3^- \rightarrow 1/2\text{CH}_4 + \text{CH}_3\text{COO}^- + 1/2\text{H}_2\text{O} + 1/2\text{H}^+$	-58.2
1-Propanol	$\text{CH}_3\text{CH}_2\text{CH}_2\text{OH} + 1/2\text{HCO}_3^- \rightarrow 1/2\text{CH}_4 + \text{CH}_3\text{CH}_2\text{COO}^- + 1/2\text{H}_2\text{O} + 1/2\text{H}^+$	-64.8

<sup>a</sup>  $\Delta G^\circ$  values were obtained from references 27 and 36.

the conditions and processes for coaggregation and the theoretical background.

#### MATERIALS AND METHODS

**Strains and culture conditions.** *P. thermopropionicum* SI (=DSM 13744) was grown in a monoculture or coculture with *M. thermautotrophicus*  $\Delta\text{H}$  (=DSM 1053) in a 100-ml serum vial containing 50 ml of a culture medium described elsewhere (32). Strain SI was kindly provided by Hiroyuki Imachi (Nagaoka University of Technology, Nagaoka, Japan), while strain  $\Delta\text{H}$  was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The medium was supplemented with 0.1% Bacto yeast extract (Difco) and a growth substrate at a concentration of 17 mM. Cultivation was conducted at 55°C under an atmosphere consisting of N<sub>2</sub> plus CO<sub>2</sub> (80:20, vol/vol) without shaking. A monoculture of strain  $\Delta\text{H}$  was grown as described above except that the gas phase was replaced with H<sub>2</sub>-CO<sub>2</sub> (80:20, vol/vol). In order to examine microbial growth and hydrogen release, cultivation was initiated by inoculating the medium with 5 ml of a preculture in the same medium. To inhibit the H<sub>2</sub> consumption by  $\Delta\text{H}$  cells, 2-bromoethanesulfonate (2-BES) was added at a concentration of 6 mM immediately after cultivation was begun (9).

**Microscopic methods.** Phase-contrast micrographs were taken using an E600 optical microscope (Nikon). Cells in a culture were counted using a BX60 fluorescence microscope (Olympus), after the cells were stained with 2 mg liter<sup>-1</sup> of 4',6-diamidino-2-phenylindole (DAPI) for 2 min and collected on an Isopore membrane (0.22  $\mu\text{m}$ ; Millipore). SI cells could be distinguished from  $\Delta\text{H}$  cells by color, shape, and fluorescence intensity. SI cells were elliptical and white and exhibited relatively strong fluorescence due to their high permeability to DAPI. By contrast,  $\Delta\text{H}$  cells were rod shaped and green and exhibited relatively weak fluorescence due to their stiff cell wall made of pseudomurein.  $\Delta\text{H}$  cells could also be identified by F<sub>420</sub> autofluorescence (13).

In order to determine the ratio of the number of SI cells adhering to  $\Delta\text{H}$  cells ( $R_{\text{SI-}\Delta\text{H}}$ ) to the total number of SI cells (single cells plus adhering cells), more than 1,000 DAPI-stained cells from more than six different samples were analyzed with a fluorescence microscope, and the ratio was calculated with the following equation:

$$R_{\text{SI-}\Delta\text{H}} (\%) = \frac{\text{number of SI cells adhering to } \Delta\text{H cells}}{\text{total number of SI cells}} \times 100 \quad (1)$$

Cells in aggregates were not considered for this calculation.

**Electron microscopy.** Field emission-scanning electron microscopy (FE-SEM) was performed as described previously (15). Cells were grown in the presence of glass plates, each glass plate was carefully removed from the culture, and cells on the plate were fixed with 1.25% glutaraldehyde and 1.3% osmium tetroxide. After cells were dehydrated using a graded series of ethanol solutions, they were dried using an HCP-2 drier (Hitachi). The resulting specimen was coated with osmium using a CVD coating device (Hitachi). The specimen was observed with an S4500 FE-SEM (Hitachi) at 5 kV.

Transmission electron microscopy (TEM) was also performed as described previously (15). Cells were negatively stained with phosphotungstic acid and observed with an H7000 TEM (Hitachi) at 75 kV.

**AI.** The aggregation index (AI) was determined by methods described previously (6, 20). After the optical density at 600 nm ( $\text{OD}_{600}$ ) of a culture was

measured using a DU640 spectrophotometer (Beckman), aggregated cells were dispersed by gentle homogenization using a tissue grinder (PTFE type; Asahi Techno Glass), until the  $\text{OD}_{600}$  no longer increased. The AI was calculated using the following equation:

$$\text{AI} (\%) = \frac{\text{OD}_{600} \text{ after dispersion} - \text{OD}_{600} \text{ before dispersion}}{\text{OD}_{600} \text{ before dispersion}} \times 100 \quad (2)$$

**Estimation of interspecies distance.** A theoretical average interspecies distance between SI and  $\Delta\text{H}$  cells was estimated by using Fick's diffusion law (5, 28), which is expressed by the following equation:

$$J_{\text{H}_2} = A_{\text{SI}} \cdot D_{\text{H}_2} \frac{C_{\text{H}_2\text{-SI}} - C_{\text{H}_2\text{-}\Delta\text{H}}}{d_{\text{SI-}\Delta\text{H}}} \quad (3)$$

where  $J_{\text{H}_2}$  is an H<sub>2</sub> flux between SI and  $\Delta\text{H}$  cells,  $A_{\text{SI}}$  is the surface area of an SI cell,  $D_{\text{H}_2}$  is the H<sub>2</sub> diffusion constant in water,  $C_{\text{H}_2\text{-SI}}$  is the H<sub>2</sub> concentration immediately outside an SI cell,  $C_{\text{H}_2\text{-}\Delta\text{H}}$  is the H<sub>2</sub> concentration immediately outside a  $\Delta\text{H}$  cell, and  $d_{\text{SI-}\Delta\text{H}}$  is the average distance between SI and  $\Delta\text{H}$  cells.

**Gas chromatography.** After a liquid sample was passed through a 0.22- $\mu\text{m}$  membrane (type GV; Millipore) and acidified with concentrated HCl, volatile fatty acids and short-chain alcohols were analyzed using a gas chromatograph (GC-2010; Shimadzu) with a flame ionization detector and a DB-FFAP column (Shimadzu). The operation temperatures were as follows: injection temperature, 250°C; column temperature, 40 to 240°C (increased at a rate of 10°C min<sup>-1</sup>); and detector temperature, 300°C. The methane, hydrogen, nitrogen, and carbon dioxide in the headspace of a serum vial were measured using a gas chromatograph (GC-14A; Shimadzu) equipped with a thermal conductivity detector and a molecular sieve 5A 60-80/Porapak Q 80-100 column (Shimadzu). The column, injection, and detector temperatures were 50°C, 100°C, and 80°C, respectively.

#### RESULTS

**Influence of culture conditions on aggregation.** *P. thermopropionicum* SI was grown in cocultures with *M. thermautotrophicus*  $\Delta\text{H}$  on propionate, ethanol, or 1-propanol (Fig. 1) and also in monocultures on fumarate or pyruvate (data not shown). It has been reported that strain SI produces acetate, H<sub>2</sub>, and CO<sub>2</sub> from ethanol and propionate, while formate did not contribute significantly to the syntrophic growth with the methanogen (14). Figure 1 also shows the production of relevant products and intermediate metabolites. The data show that growth on propionate was much slower than growth on ethanol or 1-propanol. The amounts of methane produced in these cultures were in good agreement with the amounts estimated according to the equations in Table 1. As shown in Fig. 1C, the propanol culture exhibited two phases of growth, a first phase in which 1-propanol was transformed to propionate and a second phase in which propionate was oxidized. The amount of propionate that accumulated after the first phase was equiv-

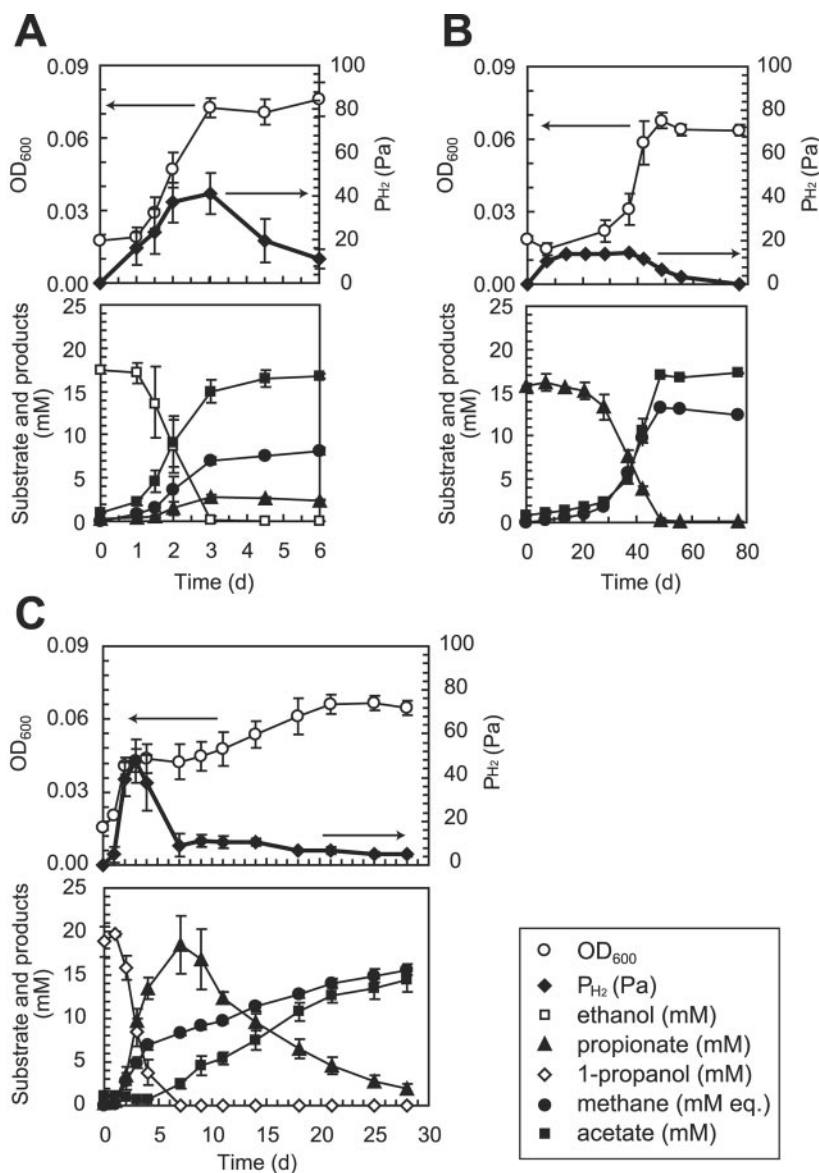


FIG. 1. Syntrophic oxidation of ethanol (A), propionate (B), and 1-propanol (C) by *P. thermopropionicum* SI in coculture with *M. thermautotrophicus*  $\Delta$ H. The methane concentration was expressed as “mM equivalents” (mM eq.) by assuming that all methane was present in the aqueous phase. The data are means  $\pm$  standard deviations obtained from three independent cultures.

alent to the amount of 1-propanol added when cultivation was begun.

When strain SI was grown alone on fumarate or pyruvate, the cells were fully dispersed, and no aggregates were observed even in the stationary phase (Fig. 2A). Strain  $\Delta$ H was also completely dispersed in a monoculture (Fig. 2B). By contrast, when the organisms were grown in a coculture, large cell aggregates were observed in the stationary phase (Fig. 2C). Aggregates were observed in all cocultures, albeit to different degrees. AI values were determined using equation 1 to more quantitatively compare aggregation trends under different culture conditions (Table 2). It was found that aggregation was most substantial when organisms were grown on propionate and less substantial when organisms were grown on ethanol. The aggregation trend in the first phase of the propanol culture

was similar to that in the ethanol culture, while the second phase and the propionate culture exhibited similar trends (Table 2).

In the mid-exponential growth phase (OD<sub>600</sub>: approximately 0.03), cell aggregates were also observed in cocultures, although the sizes were relatively small compared to the aggregates observed in the stationary phase (data not shown). In these aggregates,  $\Delta$ H cells were shown to be the major components, while many SI cells were also incorporated into the aggregates, indicating that SI and  $\Delta$ H cells coaggregated. When dispersed cells in coculture were observed with a microscope, many SI cells were found to adhere to  $\Delta$ H cells to form SI- $\Delta$ H pairs in the mid-exponential growth phase; in contrast, pairs of SI cells or pairs of  $\Delta$ H cells were not frequent (data not shown). Pairs of SI- $\Delta$ H cells were, however, rarely observed in

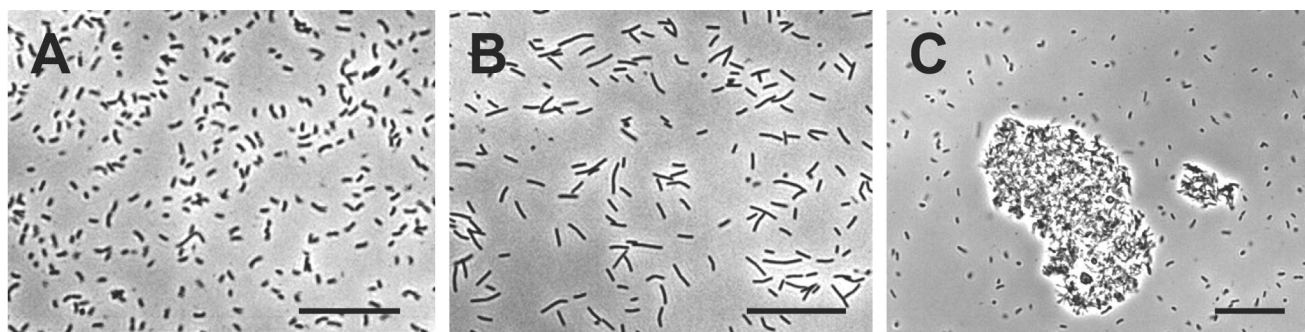


FIG. 2. Phase-contrast micrographs of an SI monoculture grown on fumarate (A), a  $\Delta$ H monoculture grown on  $H_2$  plus  $CO_2$  (B), and a coculture of strains SI and  $\Delta$ H grown on propionate (C). All micrographs were taken in the stationary phase (a phase in which the  $OD_{600}$  was no longer elevated). Bars, 25  $\mu$ m.

the stationary-phase cultures. We estimated  $R_{SI-\Delta H}$  values for dispersed SI cells in the mid-exponential growth phase under different growth conditions using equation 2 (Table 2). The  $R_{SI-\Delta H}$  values show that pairs of SI- $\Delta$ H cells were most abundant in the propionate coculture.

**FE-SEM observation of coculture.** FE-SEM was used to gain insight into mechanisms underlying the connection and coaggregation of SI and  $\Delta$ H cells. Compared to conventional scanning electron microscopy, FE-SEM improves the spatial resolution by using a field emission electron source, minimizes sample charge-up and damage by operating at lower electron-accelerating voltages, and gives better images for immediate biological surfaces by reducing electron penetration (25). For FE-SEM, cells were grown in the presence of glass plates at the bottom of a culture vial, which resulted in many cells being deposited (loosely attached) onto each glass plate.

Electron micrographs showed clear cell morphology, cell-cell contact, and the formation of large cell aggregates (Fig. 3). In these micrographs, SI cells are relatively thick and sausage shaped, while  $\Delta$ H cells are relatively thin, straight, and rod shaped. It was found that SI cells produced long flagellum-like filaments (length,  $\sim 10 \mu$ m) in a monoculture (Fig. 3A). These filaments were also observed by TEM (data not shown), although a previous report indicated that strain SI was nonmotile (14). In contrast, strain  $\Delta$ H produced no filaments (Fig. 3B). When a coculture of SI and  $\Delta$ H was observed by FE-SEM, the cells were found to be connected with the flagellum-like fila-

ments of SI cells (Fig. 3C); it is likely that this is the initial step for SI cells to make contact with  $\Delta$ H cells. When SI cells approached  $\Delta$ H cells, flagellum-like filaments coiled around  $\Delta$ H cells (Fig. 3D and E). In relatively small aggregates, the filaments were observed to intertwine with each other (Fig. 3E). In large aggregates, extracellular polymer substance (EPS)-like structures were seen in intercellular spaces (Fig. 3F). These results suggest that flagellum-like filaments of strain SI play roles in coaggregation of SI and  $\Delta$ H cells. In addition, we need to consider the possibility that EPS-like material is involved in coaggregation.

**Estimation of interspecies distances.** We were interested in examining how coaggregation contributed to interspecies electron transfer between SI and  $\Delta$ H cells. In this case,  $H_2$  was the only compound that contributed to the interspecies electron transfer (14). A relationship between the  $H_2$  flux between SI and  $\Delta$ H and the average distance between the cells was described by using Fick's diffusion law (equation 3). In the present study, in order to estimate " $d_{SI-\Delta H}$ " values for syntrophic oxidation of propionate, ethanol, and 1-propanol, the other parameters were determined as follows. The  $J_{H_2}$  value was calculated from a substrate oxidation rate in the coculture experiment (Fig. 1) by using the chemical equation in Table 1, and the value was equivalent to that calculated from the methane production rate. The  $A_{SI}$  value was estimated by assuming that an SI cell was cylindrical with a diameter of 0.4  $\mu$ m and a length of 0.9  $\mu$ m. The  $D_{H_2}$  value at 55°C was obtained from reference 40. For  $C_{H_2-SI}$ , we used the maximum  $H_2$  concentration below which substrate oxidation could proceed; this value was determined by experiments in which  $H_2$  consumption and methane production by  $\Delta$ H cells were completely inhibited by adding 2-BES (Fig. 4). A partial pressure (in Pa) was converted to an aqueous concentration (in  $\mu$ M) by using Henry's constant for  $H_2$  at 55°C (7,722 Pa) (41). The  $C_{H_2-SI}$  values determined (i.e., the maximum  $H_2$  concentrations) were equivalent to the values predicted thermodynamically. For  $C_{H_2-\Delta H}$ , we used the theoretical minimum  $H_2$  concentration above which an  $H_2$ -consuming methanogen can gain energy by carbonate respiration (11). Since the maximum and minimum  $H_2$  concentrations were used as  $C_{H_2-SI}$  and  $C_{H_2-\Delta H}$ , respectively, the " $d_{SI-\Delta H}$ " value was defined as the allowable average interspecies distance for accomplishing syntrophic oxidation at

TABLE 2. AI and ratio of the number of SI- $\Delta$ H pairs to the total number of dispersed SI cells under different culture conditions<sup>a</sup>

Cells	Substrate	AI (%) <sup>b</sup>	$R_{SI-\Delta H}$ (%) <sup>c</sup>
SI	Fumarate	0.2 $\pm$ 0.3	NA <sup>d</sup>
$\Delta$ H	$H_2$ - $CO_2$	0.4 $\pm$ 0.3	NA
SI + $\Delta$ H	Ethanol	5.6 $\pm$ 0.5	27.4 $\pm$ 7.4
	Propionate	19.1 $\pm$ 0.4	49.6 $\pm$ 7.5
	1-Propanol	6.3 $\pm$ 1.4	30.4 $\pm$ 6.3
	Propionate <sup>e</sup>	11.2 $\pm$ 2.8	50.4 $\pm$ 8.3

<sup>a</sup> The data are means  $\pm$  standard deviations ( $n > 3$ ).

<sup>b</sup> The values were estimated by using equation 1 for stationary-phase cultures.

<sup>c</sup> The values were estimated by using equation 2 for exponential-phase cultures.

<sup>d</sup> NA, not analyzed.

<sup>e</sup> Data for cells at the propionate oxidation phase of the 1-propanol culture (Fig. 1C).

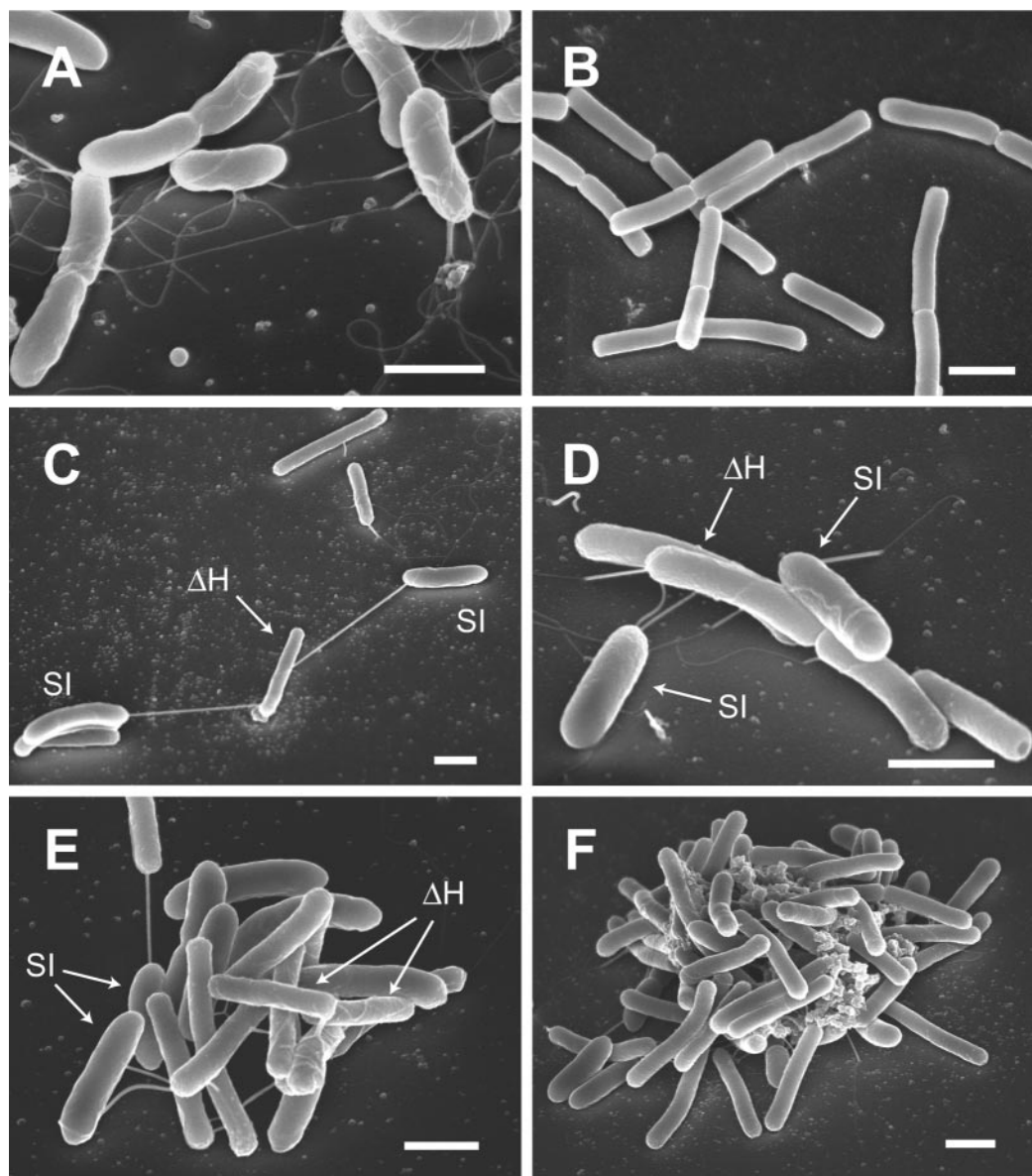


FIG. 3. Representative FE-SEM images of a monoculture of strain SI grown on fumarate (A), a monoculture of strain  $\Delta H$  grown on  $H_2$  plus  $CO_2$  (B), and a coculture of SI and  $\Delta H$  cells grown on propionate (C to F). Bars, 1  $\mu m$ .

an observed substrate oxidation rate. The values determined are summarized in Table 3.

Table 3 shows that the  $d_{SI-\Delta H}$  value for propionate is much less than the  $d_{SI-\Delta H}$  values for ethanol and 1-propanol. The  $OD_{600}$  in the mid-exponential growth phase of an SI- $\Delta H$  coculture was approximately 0.04 (Fig. 1), which corresponded to approximately  $5 \times 10^7$  cells  $ml^{-1}$ . In this case, the theoretical mean distance of randomly dispersed cells was calculated to be 30.4  $\mu m$ . Clearly, in order to accomplish syntrophic propionate oxidation at the rate observed, the average interspecies distance should be much less than the distance between randomly dispersed cells (2  $\mu m$  versus 30  $\mu m$ ). The allowable average interspecies distances for ethanol and propanol oxidation were, however, much greater than the distance for propionate oxidation. These estimates clearly show that close physical

contact was particularly important for syntrophic propionate oxidation.

## DISCUSSION

In the present study we found that *P. thermopropionicum* SI and *M. thermautotrophicus*  $\Delta H$  coaggregated when they were grown in a syntrophic coculture (Fig. 2 and 3). This coaggregation was dependent on the growth substrate; thus, coaggregation was more significant when cells were grown on propionate than when cells were grown on ethanol or 1-propanol (Table 2). We considered the possibility that this coaggregation trend was correlated with the energetics of the oxidation reaction; i.e., coaggregation was particularly important for energetically unfavorable syntrophic propionate oxidation. Ther-

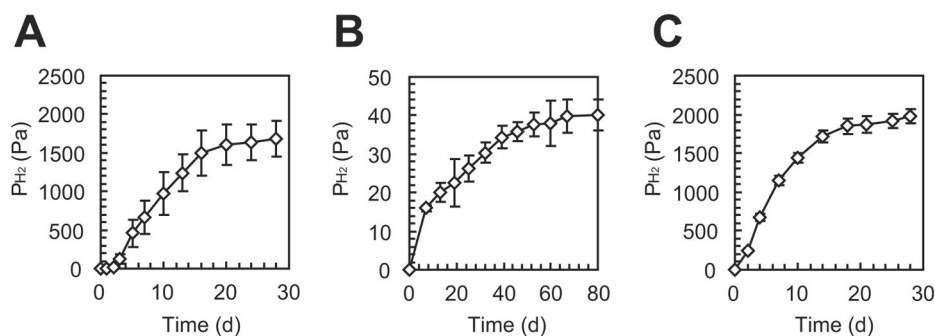


FIG. 4. Determination of the maximum  $H_2$  partial pressures for ethanol (A), propionate (B), and 1-propanol (C) oxidation. Hydrogen consumption by  $\Delta H$  cells was inhibited by adding 2-BES. The data are means  $\pm$  standard deviations obtained from three independent cultures.

modynamic analyses have estimated that syntrophic propionate oxidation is energetically feasible only when the circumstantial  $H_2$  partial pressure is very low (27, 28, 34); in the present study, propionate oxidation by SI cells stopped when the  $H_2$  partial pressure was approximately 40 Pa (Fig. 4). The diffusion theory has suggested that at such a low  $H_2$  concentration, the distance between cells of an  $H_2$  producer and cells of an  $H_2$  consumer should be very small to obtain a sufficient rate of  $H_2$  flux between them (34); actually, we estimated that the interspecies distance should be less than 2  $\mu m$  for propionate oxidation at the rate observed (Table 3). We concluded that strains SI and  $\Delta H$  coaggregated in order to achieve efficient interspecies  $H_2$  flux.

The FE-SEM data suggest that flagellum-like filaments produced by strain SI are involved in coaggregation of SI and  $\Delta H$  cells. Flagella have been known to play important roles in adhesion of bacteria to a variety of materials (23). O'Toole and Kolter showed that flagella were essential for the initial attachment of bacteria to abiotic surfaces (24). Tasteyre et al. have shown that flagellum proteins play a role in adherence of *Clostridium difficile* to mouse cecal mucus (35). They demonstrated that the flagellum cap protein FliD was highly adhesive to mouse cells (35). It has also been suggested that the flagellum cap protein is important for adhesion of *Pseudomonas aeruginosa* to mucin (2). Although involvement of flagella in prokaryotic interspecies attachment has not been well described, in the present study we found that many SI and  $\Delta H$  cells were connected with flagellum-like filaments of SI cells (Fig. 3C, D, and E). In particular, the frequent long-distance

connection of these cells with the filaments suggests that a tip structure of the filaments may play a role in initiating the contact. In addition to flagella, some bacterial strains (e.g., clostridia) (18, 21) have been known to produce unidentified thick filaments that connect cells of these strains. In order to identify the filament of strain SI, molecular characterization of component proteins is necessary.

Microorganisms utilize EPS materials for creating networks of cells in biofilms (7). In addition, EPS materials have also been found in dense granular assemblages of microorganisms in aerobic and anaerobic wastewater treatment facilities (4, 8, 22, 30). McSwain et al. have suggested that the formation and stability of granules are dependent on noncellular protein cores (22). In the present study, we found that EPS-like structures were present in intercellular spaces of SI- $\Delta H$  aggregates (Fig. 3F). We stained coaggregates of SI and  $\Delta H$  cells with calcofluor (a dye for staining polysaccharides) and found that intercellular EPS-like structures contained polysaccharides (unpublished data). Since a genome analysis of strain  $\Delta H$  revealed that  $\Delta H$  possesses genes for polysaccharide metabolism (33), it is possible that strain  $\Delta H$  was responsible for the production of EPS; however, there has been no report describing production of polysaccharides by strain  $\Delta H$ . It would be interesting if  $\Delta H$  produces polysaccharides only in the presence of a syntrophic partner. We also need to examine whether EPS-like structures really contribute to coaggregation.

The diffusion theory (i.e., Fick's law) has been applied to simulation of the importance of close physical contact of syntrophs and methanogens for syntrophic VFA oxidation (5, 34),

TABLE 3. Parameters in Fick's equation and allowable interspecies distances

Parameter <sup>a</sup>	Value	Value for:			
		Ethanol	Propionate	1-Propanol	Propionate <sup>b</sup>
$A_{SI}$ ( $\mu m^2$ cell <sup>-1</sup> )	1.38				
$D_{H_2}$ ( $m^2$ s <sup>-1</sup> )	$10.6 \times 10^{-9}$				
$C_{H_2-\Delta H}$ ( $\mu M$ )	0.0018				
$C_{H_2-SI}$ ( $\mu M$ ) <sup>c</sup>		$12.1 \pm 1.7$	$0.29 \pm 0.02$	$14.2 \pm 0.1$	NA <sup>d</sup>
$J_{H_2}$ ( $pmol$ cell <sup>-1</sup> day <sup>-1</sup> ) <sup>c</sup>		$0.987 \pm 0.129$	$0.155 \pm 0.033$	$0.567 \pm 0.080$	$0.192 \pm 0.046$
$d_{SI-\Delta H}$ ( $\mu m$ )		15.5	2.3	31.6	1.9 <sup>e</sup>

<sup>a</sup> See equation 3.

<sup>b</sup> Data for the propionate oxidation phase of the 1-propanol culture (Fig. 1C).

<sup>c</sup> The data are means  $\pm$  standard deviations ( $n = 3$ ).

<sup>d</sup> NA, not analyzed.

<sup>e</sup> The value was estimated by using the  $C_{H_2-SI}$  value for the propionate culture (0.29  $\mu M$ ).

although to our knowledge there has been no example that practically applied this theory to analysis of syntrophic VFA-oxidizing consortia. In the present study we introduced the available experimental data into Fick's equation to estimate the distances between SI and  $\Delta$ H cells allowable for accomplishing substrate oxidation at the rates observed (Table 3). As a result, the estimated values reasonably explained why SI and  $\Delta$ H cells should coaggregate, demonstrating the utility of this theory. An interesting feature of this theory is the relationship among an  $H_2$  flux (corresponding to substrate oxidation kinetics), an  $H_2$  concentration gradient (reflecting the thermodynamics of substrate oxidation), and an average distance between an  $H_2$  producer and a consumer. Accordingly, we are able to correlate thermodynamics with kinetics for syntrophic substrate oxidation. In order to further improve the applicability of the diffusion theory for analysis of syntrophic consortia, a radial diffusion model (3) may be useful. In addition, for fine-scale analyses, functional heterogeneity within a culture needs to be considered.

*P. thermopropionicum* strain SI was isolated from a UASB reactor as a representative propionate-oxidizing bacterium (13, 14), and FISH has shown that *Pelotomaculum* group bacteria are abundant populations in UASB granules (14). For *M. thermotrophicus*, abundant closely related organisms have been detected in high-temperature anaerobic digestors by 16S rRNA gene analyses (19, 31) and immunological detection (1). The results of these studies have suggested that strains SI and  $\Delta$ H represent important organisms in high-temperature methanogenic digestors. We therefore believe that the findings obtained in the present study have profound implications for understanding the formation of UASB granules. Several different mechanisms have been proposed so far for the granulation of anaerobic organisms (12). Weigant has proposed the "spaghetti theory" (39), in which *Methanosaeta* (formerly *Methanothrix*) filaments play primary roles in the granulation. Another idea is the "Cape Town hypothesis," which proposes involvement of extracellular polypeptides from *Methanobacterium* (26). Based on the results of the present study, we propose an alternative possibility, that coaggregation of VFA-oxidizing syntrophs and  $H_2$ -consuming methanogens facilitates initiation of granulation. To address this possibility, coaggregation of these organisms will be analyzed in the initial phase of granulation in an UASB reactor.

In conclusion, the present study provides data that can bridge the gap between the ecological process for granulation and the molecular mechanisms for cell adhesion. In order to better understand the granulation process, studies should move toward molecular and genomic analyses of the relevant organisms. We are currently conducting genome sequencing of *P. thermopropionicum* strain SI with the hope of gaining insight into mechanisms that underlie the syntrophism of this organism with its methanogenic partner.

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