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Metabolism of Polyethylene Glycol by Two Anaerobic Bacteria, *Desulfovibrio desulfuricans* and a *Bacteroides* sp.†

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Two anaerobic bacteria were isolated from polyethylene glycol (PEG)-degrading, methanogenic, enrichment cultures obtained from a municipal sludge digester. One isolate, identified as *Desulfovibrio desulfuricans* (strain DG2), metabolized oligomers ranging from ethylene glycol (EG) to tetraethylene glycol. The other isolate, identified as a *Bacteroides* sp. (strain PG1), metabolized diethylene glycol and polymers of PEG up to an average molecular mass of 20,000 g/mol [PEG 20000; HO-(CH_2-CH_2-O-)$_n$H]. Both strains produced acetaldehyde as an intermediate, with acetate, ethanol, and hydrogen as end products. In coculture with a *Methanobacterium* sp., the end products were acetate and methane. Polypropylene glycol [HO-(CH_2-CH_2-CH_2-O-)$_n$H] was not metabolized by either bacterium, and methanogenic enrichments could not be obtained on this substrate. Cell extracts of both bacteria dehydrogenated EG, PEGs up to PEG 400 in size, acetaldehyde, and other mono- and dihydroxylated compounds. Extracts of *Bacteroides* strain PG1 could not dehydrogenate long polymers of PEG (≥1,000 g/mol), but the bacterium grew with PEG 1000 or PEG 20000 as a substrate and therefore possesses a mechanism for PEG depolymerization not present in cell extracts. In contrast, extracts of *D. desulfuricans* DG2 dehydrogenated long polymers of PEG, but whole cells did not grow with these polymers as substrates. This indicated that the bacterium could not convert PEG to a product suitable for uptake.

Polyethylene glycol [PEG; HO-(CH_2-CH_2-O-)$_n$H] is a nonionic, water-soluble polymer of ethylene oxide and is used in large quantities in the production of surfactants, lubricants, plastics, and cosmetics (6, 12). PEG was once thought to be a recalcitrant xenobiotic with nondegradable ether bonds (6), but both aerobic and anaerobic biodegradation have been reported. Under aerobic conditions, PEG is degraded by dehydrogenation to either carboxylated intermediates (15, 16) or glycolaldehyde (26), by acetaldehyde production (20), and by extracellular hydrolytic cleavage with production of ethylene glycol (EG) and diethylene glycol (11). Monomeric EG is degraded either by oxidation to glycolic acid with further metabolism through the glycerate pathway (4, 10, 29) or by dehydration to acetaldehyde with metabolism through the tricarboxylic acid cycle (28).

Recent evidence suggested that anaerobic depolymerization of PEG results in the production of acetaldehyde, which is subsequently dismutated to acetate and ethanol or dehydrogenated to acetate under methanogenic conditions (7, 23). Under anaerobic and microaerophilic conditions, EG is metabolized by dehydration to acetaldehyde, followed by dismutation to acetate and ethanol (1, 8, 9, 29).

Anaerobic cleavage of ether bonds has been reported only for the depolymerization of PEG, the demethoxylation of methoxylated aromatics (2), and the degradation of the arylglycerol-β-aryl-ether linkage in lignin (5). The novelty of this reaction led us to study the anaerobic cleavage of ether bonds in PEGs in more detail. This paper presents evidence that the ether bonds of long-chain PEGs are cleaved by either extracellular hydrolysis or hydrogenation. Two strictly anaerobic bacteria which degrade glycols were identified, and their glycol-degrading properties were compared.

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MATERIALS AND METHODS

Abbreviations used. Compounds for which abbreviations or designations are used are ethylene glycol (EG), diethylene glycol (DEG), triethylene glycol (TREG), tetraethylene glycol (TEEG), polyethylene glycols with average molecular weights of 400 (PEG 400), 1,000 (PEG 1000), and 20,000 (PEG 20000), polyoxyethylene 23-lauryl ether (Brij 35), polyoxyethylene sorbitan monolaurate (Tween 20), polypropylene glycols with average molecular weights of 425 (PPG 425) and 2,000 (PPG 2000), and dichlorophenol indophenol (DCPIP).

Isolation and characterization of bacterial strains. Methanogenic consortia which used either EG, DEG, PEG 400, PEG 1000, or PEG 20000 as the sole carbon and energy source were obtained from the sludge of a municipal anaerobic digester in Mason, Mich. The anaerobic enrichment procedure was described previously (7). Bacterial strains DG2 and PG1 were isolated from the DEG- and PEG 1000-degrading consortia, respectively, with anaerobic roll tubes (18). The roll tubes were made by using an anaerobic mineral medium (RAMM) (24) supplemented with 0.2% substrate, 2% agar, and either 20 mM Na_2SO_4, a lawn of *Methanobacterium* strain DG1 as H_2 consumer, or no added electron acceptor. Isolated colonies were transferred from the roll tubes with sterile Pasteur pipettes to RAMM amended with 50 ml of substrate in 160-ml serum bottles which were sealed with butyl rubber stoppers. The procedure was done three more times. All transfers were made under a 90:10 gas mixture of N_2-CO_2. Culture purity was checked both by phase microscopy and growth on complex media.

The substrate ranges for both the methanogenic consortia and the isolates were assessed by measuring growth as change in the A_{560} of triplicate 10-ml cultures in 20-ml Balch tubes; a Turner model 350 spectrophotometer was used. Various substrates (0.1%) were tested as the sole carbon and

energy source for the following cultures under the indicated conditions: (i) the enriched methanogenic consortia; (ii) strain DG2 under fermentative conditions, with 20 mM Na_2SO_4, and in coculture with the methanogen *Methanobacterium* strain DG1; and (iii) strain PG1 under fermentative conditions and in coculture with the methanogen. The pH was adjusted to 7.2 when necessary. Fermentation balances for PG1 were obtained from 50-ml cultures grown in 160-ml serum bottles, and for DG2 they were obtained from 320-ml cultures grown in 4-liter flasks sealed with rubber stoppers. Both growth and fermentation balances were determined after three successive transfers of 10% inoculum grown on the substrate of interest.

Substrate dehydrogenation by cell extracts. Cell extracts of strains PG1 and DG2 were obtained by passing washed cell suspensions (in 10 mM phosphate buffer [pH 7.2]) through a French pressure cell (10,000 lb/in^2), centrifuging the resultant solution at 15,000 × *g* for 15 min, and collecting the supernatant. All transfers were made under an 80:20 gas mixture of N_2-CO_2. Substrate oxidation by the cell extract was assayed by measuring the initial rate of DCPIP reduction as a change in the A_{600} (15); a Perkin-Elmer model 320 spectrophotometer was used. The reaction mixture contained the following per ml of cell extract: 43 nmol of DCPIP, 14 nmol of flavin adenine dinucleotide, and 18 μmol of Tris buffer (pH 7.0). The reaction was started by the addition of 1 μmol of substrate. All substrates were added on an equimolar basis to provide the same number of hydroxyl groups for enzymatic action.

Analytical methods. Aqueous samples (1 ml) were periodically withdrawn with a syringe from the incubated cultures and filtered through a 0.45-μm membrane filter (Millipore Corp.). EG, DEG, methane, acetate, propionate, and ethanol were assayed by gas chromatography, as described previously (7). Acetaldehyde was assayed with a Perkin-Elmer 900 gas chromatograph equipped with a 2-m steel column packed with 10% SP-1000 on 80/100 Supelcoport (Supelco, Inc.). The injector and manifold temperatures were 180°C; the column temperature was 130°C. Succinate and lactate were measured after methylation (13) with a 2-m packed glass column (Carbopack C–0.3% Carbowax 20M–1% H_3PO_4). The injector and manifold temperatures were 180°C. The initial and final column temperatures and the program rate were 100°C, 160°C, and 16°C/min, respectively. N_2 at 50 ml/min was the carrier gas for both columns. Hydrogen was quantified by injecting 3 ml of culture headspace gas into a Carle model AGC-111 gas chromatograph equipped with a thermistor detector.

Protein concentrations in the cell extracts were determined by the method of Lowry (30), with bovine serum albumin as the standard. Bacterial protein was made soluble by heating samples in 0.5 N NaOH at 90°C for 10 min. Cytochromes were assayed in cell extracts of strain PG1 grown on glucose and strain DG2 grown on pyruvate and Na_2SO_4. The extracts were used to obtain difference spectra (27) with a Perkin-Elmer model 320 spectrophotometer. Microscopy was phase-contrast with a Leitz Ortholux microscope. Flagella were observed by transmission electron microscopy with a Philips 300 microscope. Desulfoviridin was assayed by the procedure of Postgate (21).

RESULTS

Characterization of isolates. Two bacteria able to use PEG as their sole carbon and energy source were recovered as colonies in roll tubes. Strain DG2 was isolated as a DEG-fermenting bacterium from a methanogenic enrichment fed DEG. With Na_2SO_4 provided, colonies with bacteria morphologically similar to strain DG2 were black, indicating that sulfate redution to sulfide had occurred. When a lawn of *Methanobacterium* strain DG1 was provided, colonies occurred as mixtures of the proposed DG2 and methanogen. Although colonies of strain DG2 were obtained from every enrichment, DG2 grew in pure culture by using as substrates only ethylene oxide oligomers ranging from EG through TEEG (Fig. 1a). By comparison with the substrate range for the DEG-degrading enrichment (Fig. 1a), it appeared that strain DG2 was the primary bacterium degrading oligomeric EG in the enrichments.

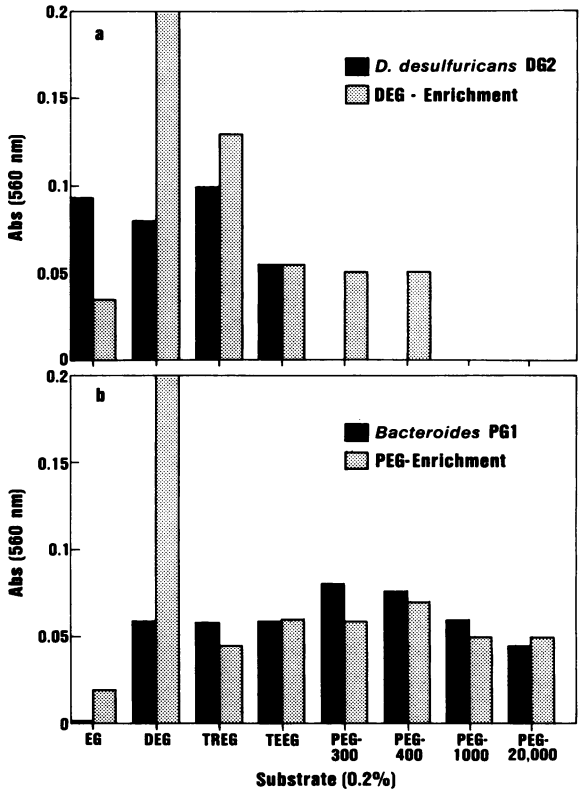

FIG. 1. Substrate ranges for the DEG-degrading enrichment and *D. desulfuricans* DG2 at 4 days (a) and for the PEG 1000-degrading enrichment and *Bacteroides* strain PG1 at 10 days (b). The ranges were obtained by growth measurements with EG or PEGs (0.2%) as the sole substrate.

Strain DG2 is a strictly anaerobic, gram-negative, nonsporeforming, curved rod (1 by 3 to 5 μm) and is motile with a single polar flagellum. The principal cytochrome is c_3; desulfoviridin is present. Growth by sulfate reduction or in coculture with the methanogen occurred with lactate, pyruvate, malate, or ethanol as the substrate. Growth by fermentation occurred with pyruvate. From these characteristics, the bacterium was identified as *Desulfovibrio desulfuricans*. *D. desulfuricans* DG2 fermented 1,2-propanediol and 1,3-propanediol with the production of H_2 and produced only the corresponding acid during growth with sulfate and in coculture with the methanogen. Substrates not supporting growth included glycolate, glyoxylate, glycerol, ethanolamine, Tween 20, Brij 35, PPG 425, and PPG 2000.

Strain PG1 was isolated as a PEG-fermenting bacterium from the methanogenic enrichment fed PEG 1000. With

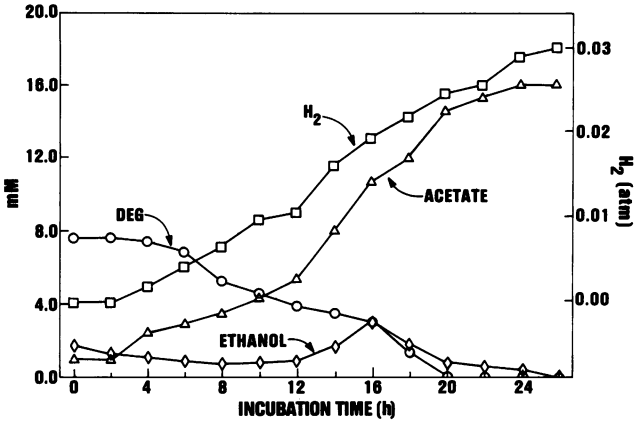

FIG. 2. Degradation of DEG by *D. desulfuricans* DG2, resulting in production of acetate, ethanol, and H_2. Ethanol was subsequently oxidized to acetate.

Na_2SO_4 provided, colonies with bacteria morphologically similar to strain PG1 occurred as mixtures also containing bacteria resembling *D. desulfuricans* DG2. When a lawn of *Methanobacterium* strain DG1 was provided, the colonies occurred as mixtures of the proposed PG1, DG2, and methanogen. Bacteria with the morphology of strain PG1 occurred only in PEG-degrading enrichments, as determined by microscopic observation of enrichments and colonies. A comparison of the substrate ranges for PG1 and the PEG 1000-degrading enrichment (Fig. 1b) indicated that strain PG1 was the primary PEG-degrading bacterium in the enrichments.

Strain PG1 is a strictly anaerobic, nonmotile, gram-negative, pleomorphic rod (1 by 2 μm) with rounded ends, occurring singly or in pairs during log-phase growth. Long chains of bacteria occurred during the late log phase. Growth occurred by fermentation of glucose and peptone, with production of succinate, lactate, and acetate. No cytochromes were detected when PG1 was grown in a non-heme-containing medium. Thus, the bacterium was presumed to be a *Bacteroides* sp. *Bacteroides* strain PG1 grew by fermentation with Tween 20, Brij 35, 1,3-propanediol, 1,2-propanediol, or ethanolamine as a substrate. Substrates not supporting growth included glycolate, glyoxylate, glycerol, PPG 425, and PPG 2000. The addition of 0.1% sodium acetate did not improve the growth of either strain with non-growth-supporting substrates.

Glycol degradation. Fermentation of DEG by pure cultures of *D. desulfuricans* DG2 resulted in the formation of acetate and H_2, with ethanol as an intermediate (Fig. 2). Acetaldehyde was produced at concentrations of 0.1 to 0.35 mM. The standard-free-energy change ($\Delta G°$) remained favorable for the overall reaction at the maximum H_2 level observed (0.025 atm [~2.53 kPa]) (standard-free-energy change at pH 7.0 [$\Delta G°'$] = −78 kJ/mol; 25). The sequence of reaction products was the same for *Bacteroides* strain PG1 with PEG 1000 as the substrate, although ethanol was not entirely oxidized (Table 1). Both *D. desulfuricans* DG2 and *Bacteroides* strain PG1 grew well in coculture with the methanogen as the electron sink and DEG or PEG 1000 as the respective substrate. The stoichiometry for glycol degradation in each case is shown in Table 1.

The cell extracts of both *Bacteroides* strain PG1 and *D. desulfuricans* DG2 dehydrogenated glycols, other hydroxylated substrates, and acetaldehyde, as determined by the substrate-dependent reduction of DCPIP (Table 2). A lag occurred between the time of substrate addition and the observed reduction of DCPIP. The lag time was shortest for acetaldehyde (4 to 5 min), and for PEGs it was at least 10 to 15 min and generally increased with increasing polymer length. This indicated that PEG was first converted to acetaldehyde, which was then dehydrogenated. PG1 extracts were not active on glycerol, a compound with three adjacent hydroxyl groups. Although strain PG1 was able to use PEG 1000 or PEG 20000 as its sole carbon and energy source (Fig. 1b), the cell extract of PG1 dehydrogenated glycols only in the range EG through PEG 400. After 200 min with no dehydrogenation of either PEG 1000 or PEG 20000, DEG was added to the reaction mixtures. Reduction of DCPIP commenced after 10 min, demonstrating that the cell extract was active.

The cell extract of *D. desulfuricans* DG2 dehydrogenated all glycols ranging from EG through PEG 1000; slightly lower rates occurred with PEG 300 through PEG 1000 (Table 2). The ability of the enzymes(s) to dehydrogenate the longer polymers was unexpected since DG2 used only EG through TEEG as carbon and energy sources (Fig. 1a).

Polypropylene glycol degradation. Since the PEG-degrading isolates did not degrade PPGs, we attempted to obtain degradation of PPG 425 and PPG 2000 by using sludge obtained from digesters in Holt and Mason, Mich. Attempts to transfer initial enrichments to PPG-amended medium and to obtain additional CH_4 with PPG additions were unsuccessful.

DISCUSSION

D. desulfuricans DG2 and *Bacteroides* strain PG1 both fermented PEGs to acetate, ethanol, and hydrogen, with acetaldehyde as an apparent intermediate. In the presence of the methanogen, acetate was the sole oxidized product; the reducing equivalents were consumed in the production of methane (Table 1). This is in agreement with previous

TABLE 1. Product recovery for degradation of DEG by *D. desulfuricans* DG2 and PEG 1000 by *Bacteroides* strain PG1

Culture	Substrate (mmol)	Amt (mmol) of product formed[a]			
		Ethanol	Acetate	H_2	CH_4
DG2	DEG (2.7)	0	5.2	5.0 (0.031[b])	0
DG2 + DG1	DEG (2.5)	0	4.8	0	1.2 (1.25[c])
DG2 + Na_2SO_4	DEG (2.7)	0	5.2	0	0
PG1	PEG 1000 (0.10)	1.0	0.73	0.002 (0.005[b])	0
PG1 + DG1	PEG 1000 (0.10)	0	1.90	0	0.62 (0.58[c])

[a] Stoichiometry of degradation was based on the following equations: for DEG, $HO\text{-}CH_2\text{-}CH_2\text{-}O\text{-}CH_2\text{-}CH_2\text{-}OH + H_2O \rightarrow 2CH_3COOH + 2H_2$; for PEG 1000, $PEG\ 1000 + 22H_2O \rightarrow 22CH_3COOH + 22H_2$.

[b] Atmospheres (1 atm = 101.3 kPa).

[c] Amount of methane expected based on the stoichiometry of H_2 production indicated in footnote *a*.

TABLE 2. Dehydrogenation of substrates by cell extracts of *Bacteroides* strain PG1 and *D. desulfuricans* DG2

Substrate	Dehydrogenation[a] by cell extract of:			
	Bacteroides strain PG1		*D. desulfuricans* DG2	
	Rate[b] (nmol/min per mg of protein)	Lag time[c] (min)	Rate (nmol/min per mg of protein)	Lag time (min)
EG	10.0	15	2.4	15
DEG	9.5	10	3.8	15
TREG	5.0	15	3.6	15
TEEG	ND[d]	ND	4.5	20
PEG 300	4.2	60	1.4	40
PEG 400	4.3	55	1.5	55
PEG 1000	0.0	>200	1.6	10
PEG 20000	0.0	>200	ND	ND
Acetaldehyde	4.4	4	4.5	5
n-Propyl alcohol	5.3	4	ND	ND
n-Butyl alcohol	4.9	4	2.7	10
Glycerol	0.0	>60	2.9	12

[a] All values are means of two experiments.
[b] Rates are for DCPIP reduction. No measurable reduction occurred without substrate addition.
[c] Time elapsed before start of DCPIP reduction.
[d] ND, Not determined.

studies of anaerobic EG metabolism (1, 8, 9, 29) and of PEG metabolism by *Pelobacter venetianus* (23) and methanogenic consortia (7)

A dehydrogenase system capable of oxidizing PEGs and other hydroxy compounds was present in cell extracts of both *Bacteroides* strain PG1 and *D. desulfuricans* DG2. Three factors suggested that dehydrogenation was preceded by the conversion of EG and PEG to acetaldehyde: (i) DCPIP reduction started 4 to 5 min after the addition of acetaldehyde to the enzyme extracts, and glycols required at least 10 to 15 min for DCPIP reduction to start (Table 2); (ii) product recoveries in this (Table 1) and other studies (1, 7, 9, 23, 29) account for a single dehydrogenation of individual ethoxy units to acetate; and (iii) acetaldehyde was detected as an intermediate, reaching quantities representing 15% of the substrate ethoxy units. A diol dehydratase which converts EG to acetaldehyde is present in some EG-grown anaerobic bacteria (8).

Extracts of *Bacteroides* strain PG1 did not dehydrogenate PEG 1000 or PEG 20000 (Table 2), although whole cells metabolized both polymers (Fig. 1b). This suggested that extracytoplasmic depolymerization of long polymers is necessary before cell uptake, in contrast to evidence that *P. venetianus* may internally depolymerize PEG 20000 (23). Two other factors suggested that depolymerization was extracytoplasmic. (i) PEG 20000 with an M_n of 20,000 and a radius of 4.90 nm (22) appears to be too large to directly enter bacteria. For example, the porin channels of *Escherichia coli* and *Paracoccus denitrificans* have radii of 0.6 and 0.9 nm, respectively (19, 31). The cell wall and protoplast of *Bacillus megaterium* have an exclusion threshold for polymers within an M_n of 1,200 and a radius of 1.1 and 0.6 nm, respectively (22). (ii) *D. desulfuricans* DG2 grew in spent medium from which *Bacteroides* strain PG1 had been removed by centrifugation and to which 0.1% (wt/vol) PEG 1000 and Na_2SO_4 were added. This indicated that the depolymerization enzyme(s) of PG1 was released from the cells, since DG2 cannot use PEG 1000 as a substrate (Fig. 1a).

The degradation of PEG by *Bacteroides* strain PG1 would thus proceed by the following sequence: (i) noncytoplasmic depolymerization, (ii) conversion of the oligomeric intermediate(s) to acetaldehyde, and (iii) internal dehydrogenation of acetaldehyde to acetate. The final step would yield energy.

EG was not detected as a depolymerization product, and since PG1 cannot use EG as a substrate, it is unlikely that polymer hydrolysis to monomeric units occurred. A cleavage of ether bonds to create oligomers is more likely, especially considering that cleavage of interior ether linkages is required for the metabolism of PEG 20000, which consists of aryl epoxide-linked polymers of PEG 6000 (L. F. Theiling, [Union Carbide Corp.], personal communication). Oligomers were not easily identifiable because of their presence in the original substrate.

Oligomeric PEGs (<400 g/mol) have a zigzag shape, whereas longer PEGs have a bond-stabilizing, helical conformation (3). Some aerobic bacteria (14, 26) and *Bacteroides* strain PG1 cell extracts (Table 2) do not dehydrogenate the longer, helical-shaped PEGs, indicating that enzymatic activity may be constrained by PEG conformation.

The anaerobic cleavage of ether bonds has only recently been observed, and the mechanism(s) has not been established. One mechanism for the anaerobic depolymerization of PEG assumes either a dehydration and rehydration of the terminal EG residue ($R\text{-}O\text{-}CH_2\text{-}CH_2\text{-}OH \rightarrow R\text{-}O\text{-}CH{=}CH_2 + H_2O \rightarrow R\text{-}O\text{-}CHOH\text{-}CH_3 + H_2O \rightarrow R\text{-}OH + CHO\text{-}CH_3$) or a coenzyme B_{12}-dependent shift of the terminal hydroxy group ($R\text{-}O\text{-}CH_2\text{-}CH_2\text{-}OH \rightarrow R\text{-}O\text{-}CHOH\text{-}CH_3 \rightarrow R\text{-}OH + CHO\text{-}CH_3$) followed in either case by hydrolytic cleavage of the resultant hemiacetal linkage to yield acetaldehyde (23). In contrast, internal ether cleavage of PEG requires either hydrolytic or reductive cleavage. Either mechanism would allow for the observed degradation of the arylglycerol-β-aryl-ether linkage in lignin (5); hydrolysis was the mechanism proposed for the demethoxylation of methoxylated aromatics (2).

The cell extracts of *D. desulfuricans* DG2 dehydrogenated EG through PEG 1000 (Table 2). Whole cells, though, did not metabolize PEGs greater in size than TEEG. This indicated that *D. desulfuricans* DG2 lacks a depolymerization mechanism. TEEG was incompletely utilized by the DEG enrichment and by *D. desulfuricans* DG2 (Fig. 1a), suggesting that not all of the compounds in the size range denoted as TEEG were used as substrate.

EG appeared to be toxic to the methanogenic enrichments, accounting for the observed low methane production (Fig. 1a and b). Metabolism of DEG was rapid, and the conversion of acetate to CH_4 by *Methanosarcina* sp. accounted for the greater productivity of the enrichments as opposed to the pure cultures.

The substrate ranges for *Bacteroides* strain PG1 and *D. desulfuricans* DG2 were limited to a few compounds other than PEGs and the characteristic growth substrates. *Bacteroides* strain PG1 used ethanolamine and the polyethoxylate moiety of the nonionic surfactants (Tween 20 and Brij 35) as substrates. Although EG and *n*-alcohols were dehydrogenated by the cell extracts, PG1 whole cells could not use these compounds for growth.

Unexpectedly, neither *D. desulfuricans* DG2 nor *Bacteroides* strain PG1 could metabolize PPG, although propanediol served as a substrate for both strains. The depolymerizing system(s) of both strains thus appears limited to ether-linked ethoxy units. Our inability to obtain PPG degradation in sludge enrichments suggested that PPGs may be recalcitrant to anaerobic degradation.

Bacteria with the morphology of *D. desulfuricans* DG2 and *Bacteroides* strain PG1 were present in PEG 20000-degrading enrichments obtained from six different digesters in Michigan. Therefore, both microorganisms may be of ecological significance in the anaerobic degradation of PEGs and nonionic polyethoxylate surfactants which are present in high concentrations in municipal waste (12, 17).

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