APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1983, p. 185–190
0099-2240/83/070185-06$02.00/0
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Vol. 46, No. 1

Degradation of Ethylene Glycol and Polyethylene Glycols by Methanogenic Consortia†

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Received 1 March 1983/Accepted 2 May 1983

Methanogenic enrichments capable of degrading polyethylene glycol and ethylene glycol were obtained from sewage sludge. Ethanol, acetate, methane, and (in the case of polyethylene glycols) ethylene glycol were detected as products. The sequence of product formation suggested that the ethylene oxide unit [HO-(CH_2-CH_2-O-)$_x$H] was dismutated to acetate and ethanol; ethanol was subsequently oxidized to acetate by a syntrophic association that produced methane. The rates of degradation for ethylene, diethylene, and polyethylene glycol with molecular weights of 400, 1,000, and 20,000, respectively, were inversely related to the number of ethylene oxide monomers per molecule and ranged from 0.84 to 0.13 mM ethylene oxide units degraded per h. The enrichments were shown to best metabolize glycols close to the molecular weight of the substrate on which they were enriched. The anaerobic degradation of polyethylene glycol (molecular weight, 20,000) may be important in the light of the general resistance of polyethylene glycols to aerobic degradation.

Ethylene glycol (EG; 1,2-ethanediol) and its oligomers and polymers are used in the production of substances such as surfactants, explosives, cosmetics, heat transfer fluids, solvents, lubricants, and plastics (1, 3). Little attention has been given to their fate in anoxic habitats, such as those in waste treatment, sediments, and landfills, even though billions of pounds are manufactured and discharged into the environment annually, and the high-molecular-weight polymers (up to 25,000) are relatively resistant to aerobic biodegradation (3). In fact, the biodegradation of most polymers, especially of synthetic polymers, by obligate anaerobes is poorly understood. We felt that the general resistance of polyethylene glycols (PEGs) to rapid aerobic degradation, along with their heavy use, made it important to establish whether or not they can be degraded anaerobically and, if so, to compare the rates and products of degradation with those for aerobic systems.

Aerobic microorganisms use both EG and PEGs as sources of carbon and energy (8, 13, 20, 21). The aerobic metabolism of EG is relatively common, and the pathways of its metabolism are known (2, 7, 16, 20, 22, 23). However, the ether bond of the oligomers and polymers is comparatively resistant to microbial attack. This is especially true for the degradation of PEG with a molecular weight of 20,000 [PEG-20,000; HO-(CH_2-CH_2-O-)$_{450}$ H]. Haines and Alexander (6) reported the isolation of several monocultures of aerobic bacteria able to grow on PEG-20,000, but did not report carbon balances or demonstrate the extent of polymer degradation. The only other report of PEG-20,000 degradation involves the coculture of a *Flavobacterium* sp. and a *Pseudomonas* sp. (9), in which neither microorganism alone degraded the polymer. In this case, significant degradation of the polymer was not verified.

The anaerobic metabolism of EG has been reported. The fermentation of EG by *Clostridium glycolicum* yields equimolar amounts of acetate and ethanol (5); the metabolism of EG by a *Flavobacterium* sp. under microaerophilic conditions follows the sequence acetyl-CoA, acetylphosphate, and acetate (23). The only apparent example of anaerobic PEG degradation is for PEG-400 in anaerobic sludge reactors, where enhanced methane production was noted (11).

We were able to enrich for glycol-degrading consortia from sludge on EG, diethylene glycol (DEG), PEG-400, PEG-1000, and PEG-20,000. We report here on the degradation rates, intermediate products, specificity of enrichment for polymer length, and extent of substrate conversion to gaseous products for each of the five glycols.

MATERIALS AND METHODS

Cultures. Glycol-degrading bacterial consortia were obtained from sludge of a municipal anaerobic digestor

† Published as journal article no. 10785 of the Michigan Agricultural Experiment Station.

in Mason, Mich. Serum bottles (160 ml) were flushed with a 90% N_2-10% CO_2 gas mixture which had been passed over hot copper filings to remove traces of oxygen. During flushing, 10 ml of sludge was added with 90 ml of reduced medium. The bottles were then sealed with black butyl rubber stoppers (Bellco Glass, Inc.) and crimped with aluminum seals to maintain anaerobic conditions. Enrichments were maintained by the weekly transfer of 10 ml of enrichment to 90 ml of fresh medium. Incubation was at 37°C and static.

The basic minimal medium (D. R. Shelton, personal communication) was composed of (per liter): 0.30 g of KH_2PO_4, 0.35 g of K_2HPO_4, 0.5 g of NH_4Cl, 0.1 g $MgCl_2$, 70 mg of $CaCl_2 \cdot 2H_2O$, 20 mg of $FeCl_2 \cdot 4H_2O$, 1 ml of trace metals solution (25), 1.2 g of $NaHCO_3$, 120 mg of $Na_2S \cdot 9H_2O$, and 1 ml of vitamin B solution (18). The substrate was added to give a 0.2% final concentration. The enrichment substrates were either EG (Mallinckrodt, Inc.), DEG (J. T. Baker Chemical Co.), PEG-400 (Fisher Scientific Co.), PEG-1000, or PEG-20,000 (both from J. T. Baker Chemical Co.). Substrates at a 0.2% concentration are equivalent to 36 mM EG (36 mM ethylene oxide units), 21 mM DEG (42 mM ethylene oxide units), 5 mM PEG-400, 2 mM PEG-1000, and 0.1 mM PEG-20,000 (45 mM ethylene oxide units each). PEG-20,000, manufactured by Union Carbide Corp., is termed PEG compound 20M and is formed by joining 8,000-molecular-weight polymers with a diepoxide. It has an average approximated molecular weight of 20,000, but the molecular weight distribution is from 5,000 to 80,000, with the largest portion being unreacted 8,000-molecular-weight monomer (L. F. Theiling, Union Carbide Corp., personal communication).

Experimental procedures. Each consortia used in the experiment had undergone 30 weekly transfers after their initial establishment. The substrate range of each enrichment was determined by transferring 10 ml of actively metabolizing culture to 90 ml of medium containing one of the five substrates. Separate cultures were established to determine the rates of product formation and EG and DEG utilizaton by each bacterial consortium grown on its own substrate. All experiments were done in triplicate and repeated.

Since the polymeric substrates cannot be quantified easily, the rates of degradation were assessed as rates of product formation for DEG, PEG-400, PEG-1000, and PEG-20,000. EG was easily and accurately quantified; therefore, the degradation rate of EG was based on a gas chromatographic assay of EG. For the comparison of rates, all values are expressed as millimolar ethylene oxide units degraded, which correspond to the two-carbon products ethanol and acetate. A rate of product formation of 0.5 mM acetate and 0.5 mM ethanol per h, therefore, was presumed to indicate a rate of 1.0 mM ethylene oxide units metabolized per h.

Analytical methods. Aqueous samples (2 ml) were periodically withdrawn by syringe from the incubated bottles, filtered through a 0.45 μm filter (Millipore Corp.) into glass vials, and frozen until analyses were done. At time zero and after 126 h, we took 1-ml samples to determine protein concentration by the method of Lowry et al. (24), using bovine serum albumin as the standard. Bacterial protein was made soluble by heating the samples in 0.5 N NaOH at 90°C for 10 min. Growth yields (Y-substrate) for the percentage of substrate degraded were expressed as micrograms of protein formed per millimole of ethylene oxide unit degraded.

EG, DEG, and ethanol were measured with a Perkin-Elmer 900 gas chromatograph equipped with a 2-m Chromosorb 101 packed glass column (Anspec Co., Inc.) and a flame ionization detector. N_2 was the carrier gas at a flow rate of 50 ml/min. For DEG, the injector, column, and manifold temperatures were 250°C. For EG and ethanol, the column temperature was 150°C. Acetate was assayed with a 2-m, Carbopack C-0.3% CW 20 M-1% H_3PO_4 packed glass column (Supelco, Inc.). The samples were acidified with formic acid before injection. The injector, column, and manifold temperatures were set at 125°C. Methane was quantified by injecting 0.2 ml of culture headspace gas into a Carle model 8500 gas chromatograph equipped with a Porapak Q column (Anspec Co., Inc.) and a microthermistor detector. When methane derived from acetate oxidation was used in calculations of product accumulation, the methane value was reduced by four-fifths, and the remaining amount was used to infer the amount of acetate oxidized because of the stoichiometry $4HO\text{-}CH_2\text{-}OH + HCO_3^- + H^+ \rightarrow 5CH_4 + CO_2$.

Microscopy was by phase contrast and fluorescence with a Leitz Ortholux microscope. The fluorescence was used to identify methanogenic bacteria as described by Mink and Dugan (12).

RESULTS

Glycol degradation. Bacterial enrichments were successfully established on each substrate, EG, DEG, PEG-400, PEG-1000, and PEG-20,000, as evidenced by an increase in turbidity and the production of methane. Microscopic examination revealed that the EG, DEG, and PEG-400 consortia were dominated by two morphological types of bacteria. These were isolated from the DEG consortia and tentatively identified as a *Methanobacterium* sp. and a *Desulfovibrio* sp. The loss of the methanogen occurred sporadically in some of the cultures, which resulted in a loss of culture viability. A further characterization of this apparent syntrophic relationship is being conducted. The PEG-1000 and PEG-20,000 consortia exhibited less distinctive, more varied morphologies of bacteria. One week after transfer to fresh medium, both cultures in the high-molecular-weight substrates contained a large number of fluorescent bacteria resembling *Methanosarcina* sp.

Figure 1 depicts the formation of degradation products by the consortia enriched on EG and DEG. The parallel formation of ethanol and acetate suggests that the glycol monomer unit was dismutated. After 70 h, the conversion of ethanol to acetate with concurrent methane formation was readily apparent in the EG consortia; EG degradation appeared complete before ethanol oxidation commenced. In contrast, the DEG consortia (Fig. 1) appeared to oxidize ethanol during DEG use, and ethanol was never

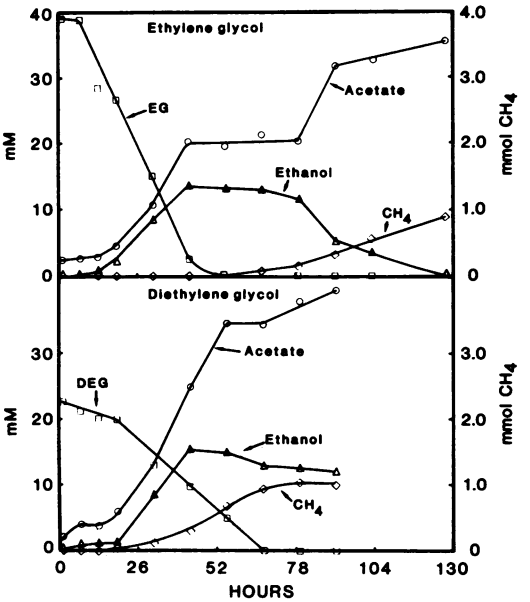

FIG 1. Temporal formation of degradation products by the consortia enriched on EG and DEG.

completely oxidized to acetate. In a 100-ml culture, the dismutation of 40 mM ethylene glycol units would produce 20 mM ethanol and 20 mM acetate; a final methane quantity of 1.0 mmol could theoretically be produced: $4HO\text{-}CH_2\text{-}CH_2\text{-}OH \rightarrow 2CH_3CH_2OH + 2CH_3COOH$; $2CH_3CH_2OH + 2H_2O \rightarrow 2CH_3COOH + 4H_2$; and $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$. As expected, the amount of methane in both consortia was one-fourth that of the final acetate concentration and, therefore, is evidence that methane was produced only as a product of ethanol oxidation.

Similar studies with the PEG-400 consortia showed only a transient, low level of ethanol accumulation (18 to 66 h). Methane and ethanol were first detected at the same sampling time; the amount of methane produced subsequently increased until ethanol disappeared (data not shown). This is further evidence that for these consortia, methane was produced only as a product of ethanol oxidation. Methane, ethanol, acetate, and EG (4 to 5 mM) were produced in the PEG-1000 and PEG-20,000 consortia. Ethanol was present only in trace quantities, probably because the rate-limiting step of PEG-1000 and PEG-20,000 degradation was polymer hydrolysis. At 126 h, the amount of methane produced was again one-fourth that of the final concentration of acetate.

Rates of PEG degradation and growth yields. A general decrease in the overall rate of degradation occurred with increases in PEG molecular weight (Table 1). The utilization of glycols as substrates is also shown by the 5-day Y-substrate values (Table 1). The reported DEG consortium growth yield may be too low since ethanol was not totally oxidized to acetate (Fig. 1), whereas in the other consortia it was. Three different DEG consortia were subsequently grown until ethanol oxidation was complete; the Y-substrate calculated was 213 μg of protein per mmol of ethylene oxide unit degraded. This is still significantly lower than the Y-substrate for the PEG-1000 and PEG-20,000 consortia.

Specificity of consortia for polymer length. Two general observations are evident from the study on substrate specificity (Table 2). First, neither the EG nor DEG consortia were able to significantly attack glycols of higher molecular weight. Second, each PEG enrichment effectively used DEG, perhaps explaining why we were unable to detect DEG as an intermediate. The PEG-20,000 consortia displayed slower degradation of the lower-molecular-weight substrates as compared with the rest of the consortia (e.g., cf. 5-day data on DEG [Table 2]). This may be due to either a lag period in substrate use or to a low relative density of bacteria able to use glycols; microscopic examination of the PEG-20,000 consortia revealed few of the bacterial types which dominated the enrichments metabolizing low-molecular-weight glycols.

The percent degradation for the PEG-1000 and PEG-20,000 consortia was based largely on the accumulation of methane, as the other products were mostly oxidized by day 12 (Table 2). As noted above, both consortia contained *Methanosarcina* sp., which may be responsible for the removal of the acetate. The EG, DEG, and PEG-400 consortia accumulated acetate with no

TABLE 1. Rates of product formation, carbon recovery, and growth yield of each enrichment on its glycol substrate

Enrichment and substrate	Rate of product formation (mM C_2 U/h)[a]	% Carbon recovered in products[b]	Y-substrate (μg of protein/mmol of C_2 U)[c]
EG	0.84 ± 0.04	86–88	144
DEG	0.73 ± 0.05	85–89	148
PEG-400	0.66 ± 0.03	69–75	182
PEG-1000	0.36 ± 0.04	59–63	437
PEG-20,000	0.13 ± 0.01	84–87	512

[a] The rates of product formation (ethanol, acetate) are expressed as the mean ± the standard deviation (n = 3) and were calculated for the time period in which a near-constant rate of product formation was observed.

[b] The percent carbon recovered in product is for the period of constant rate and is less than the total carbon recovered in product value found after a long incubation (Table 2).

[c] Y-substrate was calculated at 126 h. The mean is given (n = 3).

TABLE 2. Specificity of each consortium for polymer length

Consortium	% Substrate metabolized after 12 days				
	EG[a]	DEG[a]	PEG-400[b]	PEG-1000[b]	PEG-20,000[b]
EG	100	100 (21)[c]	7	7	7
DEG	100	100 (100)	9	0	0
PEG-400	86	100 (100)	100	62	50 (14)
PEG-1000	44	100 (83)	60	82	0
PEG-20,000	55	100 (60)	90	83	82 (33)

[a] Based on substrate disappearance.
[b] Based on the accumulation of ethanol, acetate, and methane.
[c] The data given within parentheses are the results after 5 days of incubation and show the slow adaptation of the consortia to the given substrates.

subsequent oxidation. The pH of these consortia fell to 6.0 during their growth period, whereas that of the PEG-1000 and PEG-20,000 consortia remained near 7.0 to 7.2. This may have selected against the *Methanosarcina* sp.

DISCUSSION

This study demonstrates significant rates of anaerobic biodegradation of EGs and, most importantly, of the recalcitrant PEG-20,000 polymer. Heretofore, research has demonstrated the aerobic degradation of PEGs with molecular weights of only 6,000 (16) and less (20) with activated, acclimated sludge. In addition, Cox and Conway (4) found that PEG-1540 was consumed in 2 days, wehreas Pitter (17) found that the degradation of 1% PEG-600 and PEG-800 enrichments took 30 days and that of PEG-1000 and PEG-1500 enrichments took 55 and 75 days, respectively. Haines and Alexander (6), using aerobic soil isolates, found that 1% concentrations of PEG-400 were degraded in 5 days and those of PEG-1000 were degraded in 10 days. Our study showed at least an 82% degradation of PEG-20,000 and an 83% degradation of PEG-1000 in 12 days and a 100% degradation of PEG-400 in less than 4 days. Unfortunately, the comparison of our rates of degradation with those obtained aerobically is difficult since the latter data are from biological oxygen demand studies or from the loss of total organic carbon (and thus not specific for substrate conversion), whereas ours rely upon a determination using product recovery. Nonetheless, the substantial rates of anaerobic biodegradation of PEGs by our enrichments obtained from sewage sludge demonstrate a good potential for using anaerobic organisms in PEG removal. Considering the higher cost of aerobic treatment systems, the anaerobic process may be advantageous for the removal of this and perhaps other synthetic polymers.

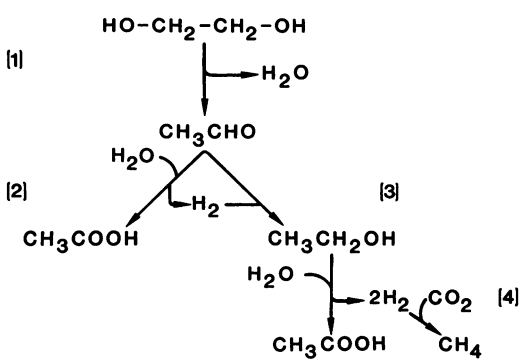

FIG. 2. Proposed pathway for degradation of EG by methanogenic consortium.

Based on the identity and sequence of the products formed, we are proposing the degradation route for EG in methanogenic consortia shown in Fig. 2. Reactions [1] and [2] have been proposed previously by Wiegant and DeBont (22) for *Mycobacterium* E44, which metabolizes EG aerobically. For our anaerobic consortia, acetaldehyde is suggested as the electron sink in a dismutation producing acetate and ethanol; reaction [3] thus would account for the early formation of ethanol. The subsequent consumption of ethanol with a concomitant production of acetate and methane is described by reaction [4]. Whereas reactions [1] through [3] are energetically favorable (19), reaction [4], the oxidation of ethanol to acetate, is only favorable if the hydrogen concentration is kept low. This presumably was accomplished by the methanogen(s) and suggests a syntrophic association. The stoichiometry and sequence of the products that we observed (Fig. 1) make any other degradative pathway unlikely, although another possibility does exist. EG could be hydrogenated to ethanol ($\Delta G^{0\prime} = -21$ kcal [ca. -87.9 kJ]) with the hydrogen derived from acetaldehyde oxidation to acetate, but this would require the presence of novel enzyme(s) and, therefore, is less likely.

The ability of our PEG consortia to accumulate EG and to use EG and DEG (Table 2)

suggests that glycol units were released from the polymer by hydrolysis before subsequent metabolism to acetate, ethanol, and methane. This is similar to the examples of the aerobic, hydrolytic microorganisms which also use EG and were studied by Haines and Alexander (6). In contrast, other aerobic depolymerizations appear to involve either an initial dehydration of the terminal glycol unit (16) or a dehydrogenation (9, 10, 15), in which case, the microorganisms appear unable to use EG. Because the glycol polymers are degraded by a rate one-sixth that of monomer degradation (Table 2), it appears that the polymer first must be hydrolyzed into fragments, from which monomers can then be hydrolytically removed. If enzymatic attack occurred only from the ends, we would expect the degradation rate of the PEG-20,000 polymer [HO-(CH_2-CH_2-O-)$_{450}$H] to be less than one-sixth that of the monomer.

Our results demonstrated an inability of the EG and DEG consortia (where selection was for non-depolymerizing bacteria) to metabolize polymers. This is comparable to earlier research with aerobic bacteria (14), which showed that the ability to grow on oligomers, but not polymers, of EG is due to an inability of appropriate enzyme(s) to reach the polymer substrate. We have also demonstrated that each consortium, although adapted to metabolizing glycols with a molecular weight similar to that of its own substrate, also was able to use DEG efficiently. The hydrolytic cleavage of polymers may explain the DEG metabolism, and a difference in the conformational structure of polymers may dictate substrate specificity. Cox (3) noted that PEG biodegradability may depend on the conformation of the molecule, which appears to be helical in solution (1), whereas low-molecular-weight PEG (<400) has a zig-zag pattern. Such conformational differences could influence the activity of an enzyme and inhibit, for example, PEG-20,000 utilization by the bacteria of the PEG-400 consortia. In this way, conformational differences could explain the relatively greater ability of the PEG-400 and PEG-1000 consortia to degrade their own respective polymer.

The relatively greater growth yields obtained for high-molecular-weight polymers, as measured by protein concentrations, were unexpected. The results may have been due to either an increase in yield from some acetate metabolism in the consortia or to a difference in metabolism between the dominate bacterial types. Pure-culture studies may help clarify this by separating other members of the consortia from the glycol degraders.

ACKNOWLEDGMENT

We thank D. R. Shelton for his early observations on methane production from the glycols by sewage sludge.

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