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Efficient Biodegradation of High-Molecular-Weight Polyethylene Glycols by Pure Cultures of *Pseudomonas stutzeri*

NURIA OBRADORS AND JUAN AGUILAR*

Department of Biochemistry, School of Pharmacy, University of Barcelona, 08028 Barcelona, Spain

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Biodegradation of polyethylene glycols (PEGs) of up to 13,000 to 14,000 molecular weight has been shown to be performed by a river water bacterial isolate (strain JA1001) identified as *Pseudomonas stutzeri*. A pure culture of strain JA1001 grew on PEG 1000 or PEG 10000 at 0.2% (wt/vol) as a sole source of carbon and energy with a doubling time of 135 or 150 min, respectively. Cultures metabolized 2 g of polymer per liter in less than 24 h and 10 g/liter in less than 72 h. The limit of 13,500 molecular weight in the size of the PEG sustaining growth and the presence of a PEG-oxidative activity in the periplasmic space indicated that PEGs cross the outer membrane and are subsequently metabolized in the periplasm. PEG oxidation was found to be catalyzed by PEG dehydrogenase, an enzyme that has been shown to be a single polypeptide. Characterization of PEG dehydrogenase revealed glyoxylic acid as the product of the PEG-oxidative cleavage. Glyoxylate supported growth by entering the cell and introducing its carbons in the general metabolism via the dicarboxylic acid cycle, as indicated by the ability of strain JA1001 to grow on this compound and the presence of malate synthase, the first enzyme in the pathway, in extracts of PEG-grown cells.

With the widespread industrial and domestic use of polyethylene glycols (PEGs), concern has been expressed in recent years about the fate of these polymers released to the environment. While several reports (3, 7, 11, 20, 22) showed that monoethylene glycol and low-molecular-weight PEGs could be degraded by pure bacterial cultures, high-molecular-weight compounds were found to be very inefficiently utilized, even by different microbial consortia (3, 7, 13, 23, 24). Consequently, these poorly metabolized PEGs may persist in the natural environment.

Haines and Alexander (7) have described a soil strain of *Pseudomonas aeruginosa* that excreted a PEG-hydrolyzing, extracellular enzyme permitting the aerobic degradation of PEGs of up to 20,000 molecular weight. The cells did not directly degrade the polymer but rather degraded the fragments generated extracellularly. The system, although not very well characterized, required more than 20 days to degrade a 1% concentration of PEGs of over 4,000 molecular weight.

Jenkins et al. (11) have obtained from different origins several unidentified bacterial isolates able to grow aerobically on PEGs of up to 4,000 molecular weight. Total degradation of 0.1% PEG 4000 by the most efficient isolate required 7 days.

A consortium of a *Pseudomonas* sp. and a *Flavobacterium* sp. capable of degrading PEGs of up to 6,000 molecular weight, also aerobically, has been reported by Kawai and colleagues (13). This cometabolism has been reported to be mediated by a membrane-bound PEG-oxidizing enzyme described as PEG dehydrogenase (14), an aldehyde dehydrogenase, and an ether-cleaving enzyme called diglycolic acid dehydrogenase (15). These authors assigned the role of PEG oxidation to the *Flavobacterium* sp. and the role of removal of the glyoxylic generated, whose excess inhibits the *Flavobacterium* sp., to the *Pseudomonas* sp.

In another context, several bacteria capable of anaerobically degrading PEG have been isolated from sewage sludge samples. A strain of *Desulfovibrio desulfuricans* able to metabolize oligomers up to tetraethylene glycol and a strain of a *Bacteroides* sp. able to degrade PEG 20000 have been reported (4). Both produced acetaldehyde as an intermediate, with acetate, ethanol, and hydrogen as end products. It has also been described that some strains of an *Acetobacterium* sp. and *Pelobacter propionicus* metabolize PEG 1000, forming acetate, in the second case together with propionate (22).

In this report we present a strain of *Pseudomonas stutzeri* that degrades PEG of up to 13,000 to 14,000 molecular weight more efficiently than previously described strains, as well as some aspects of the mechanism involved in the polymer utilization.

MATERIALS AND METHODS

Chemicals. Silica gel 60 plates, 2,6-dichlorophenol-indophenol (DCPIP), and KCN were from Merck, Darmstadt, Germany; PEGs except PEG 4000 were also from Merck. PEG 4000, Nitro Blue Tetrazolium, glyoxylic acid, and glycolic acid were from Sigma, St. Louis, Mo. Diglycolic acid was purchased from Aldrich-Chemie, Steinheim/Albuch, Germany. [^{14}C]PEG 4000 (11.0 mCi/g) was obtained from NEN Research Products, Dupont. The polydispersity of the PEGs used, as indicated by the vendor, was as follows: for PEG 200, from 190 to 210; for PEG 400, from 380 to 420; for PEG 1000, from 950 to 1,050; for PEG 2000, from 1,900 to 2,200; for PEG 4000, from 3,500 to 4,500; for PEG 6000, from 5,000 to 7,000; for PEG 10000, from 9,000 to 12,500; and for PEG 15000, from 12,500 to 16,500. No information was available for PEG 20000.

Media and growth conditions. The inorganic basal medium used consisted of 34 mM NaH_2PO_4, 64 mM K_2HPO_4, 20 mM $(NH_4)_2SO_4$, 1 μM $FeSO_4$, 0.1 mM $MgSO_4$, and 10 μM $CaCl_2$. The pH was adjusted to 7.2. Carbon sources were added to the basal inorganic medium in the following concentrations: 0.01 M glucose, 0.015 M succinate, 0.5% (wt/vol) casein acid hydrolysate, and 0.2% (wt/vol) PEG. For solid media, agar was added at a concentration of 1.5% (wt/vol).

* Corresponding author.

The medium used for induction of alkaline phosphatase activity consisted of 20 mM NH_4Cl, 20 mM KCl, 1.6 mM $MgSO_4 \cdot 7H_2O$, 120 mM Tris-HCl (pH 8.0), 0.5% proteose peptone (as an organic phosphate source), and 0.2% (wt/vol) PEG 4000.

The bacteria were cultured with continuous shaking at 30°C in 500-ml Erlenmeyer flasks partially filled with the medium. Growth was monitored at 420 nm.

Isolation of bacteria. A water sample (200 ml) containing approximately 10^6 microorganisms was taken from the River Tenes (Barcelona, Spain) and filtered through a 0.45-μm-pore-size Millipore filter. The filter was incubated aerobically at 30°C in a 100-ml Erlenmeyer flask containing 20 ml of mineral medium with 0.2% PEG 4000. After 3 days of growth, an aliquot was inoculated into a new Erlenmeyer flask containing the same medium and incubated for 3 more days. A third transfer was performed, and incubation was conducted under the same conditions. Samples from each of the three liquid cultures were plated on solid media containing PEG 4000 at 0.2% concentration.

Electron microscopy. For negative staining, a drop of the sample was applied to a copper grid coated with Formvar and carbon and stained with 2% (vol/vol) phosphotungstic acid (pH 7) or 4% (wt/vol) uranyl acetate (pH 4.8). For thin sections, preparations were fixed in 3% (wt/vol) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2), treated with 1% (wt/vol) osmium tetroxide, dehydrated, embedded in Spurr resin, sectioned on an LKB Ultratome UM III ultramicrotome, and double stained with uranyl acetate and lead citrate. The grids were examined in a Hitachi H-800 MT electron microscope.

Preparation of cell extract. Cells were harvested at the end of the logarithmic phase by centrifugation, washed in 10 mM potassium phosphate buffer (pH 7.2), and suspended in four times their wet weight of the same buffer. The suspension was sonically disrupted in an MSE sonicator set at an amplitude of 18 to 24 μm for periods of 30 s/ml of cell suspension in a tube chilled to 0°C. The disrupted cells were centrifuged at 12,000 × *g* for 30 min at 4°C, and the resultant supernatant was used as the cell extract.

Separation of cytoplasm, periplasm, and membrane fractions. The procedure used for separation of the fractions was a modification of the method described by Cheng et al. (1). Washed cells from 300 ml of culture were suspended in 54 ml of 20% (wt/vol) sucrose–10 mM Tris-HCl (pH 8.0) and incubated at room temperature for 15 min with constant stirring. The cell suspension was centrifuged at 15,000 × *g* for 30 min, and the pellet was suspended in 8.6 ml of cold (0 to 4°C) 10 mM Tris-HCl (pH 8.0) buffer containing 10 mM $MgCl_2$. The suspension was incubated for 15 min in an ice bath with constant stirring and centrifuged at 15,000 × *g* for 30 min at 4°C, and the resultant supernatant was used as the periplasm fraction.

The resultant pellet was suspended in four times its wet weight of 10 mM potassium phosphate buffer (pH 7.2) and sonically disrupted as indicated above for the preparation of cell extract. The disrupted cells were centrifuged at 100,000 × *g* for 2 h at 4°C, and the supernatant was used as the cytoplasm fraction. The pellet was suspended in the original volume of 10 mM potassium phosphate buffer (pH 7.2) and used as the membrane fraction.

Concentration of extracellular media for enzyme assay. An assay of enzyme activity was performed in the culture medium after centrifugation of the cells and their concentration (10 times) through Amicon filtration with a Diaflo (PM-10) membrane. Alternatively, proteins in the culture medium were concentrated by ammonium sulfate precipitation, centrifugation, and resuspension of the pellet in 1/10 of the original volume of the same medium.

Enzyme assays. PEG dehydrogenase activity was assayed by measuring the initial rate of DCPIP reduction at 600 nm in a reaction performed at 30°C. The enzyme preparation was incubated with an assay mixture (1 ml) that consisted of 100 mM potassium phosphate (pH 8.0), 1 mM KCN, 0.1 mM DCPIP, and 20 mM PEG 200 or 75 mM diglycolic acid. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the reduction of 1 μmol of DCPIP per min under standard assay conditions. Glyoxylate inhibition experiments were performed by determining enzyme activity at 10 and 20 mM PEG 200 in the presence of increasing concentrations of inhibitor. The K_i was calculated from the corresponding Dixon plot.

Alkaline phosphatase activity was assayed by the method of Garen and Levinthal (6). Glucose-6-phosphate dehydrogenase activity was determined by the method of Malamy and Horecker (17). NADH oxidase activity was measured according to the method of Cheng et al. (1). Malate synthase was assayed by the method of Maloy et al. (18). In all cases, 1 U of enzyme activity was defined as the amount of enzyme that catalyzed the conversion of 1 μmol of substrate per min.

The concentration of protein was determined by the method of Lowry et al. (16) with bovine serum albumin as the standard.

Gel electrophoresis. Acrylamide gel electrophoresis was performed in 7.5% acrylamide gels at pH 8.3 under nondissociating conditions at 4°C according to the method of Hames (8). Gels were stained for PEG dehydrogenase activity by incubation at 30°C in a reaction mixture that consisted of 100 mM potassium phosphate buffer (pH 8.0), 1 mM KCN, 0.3 mg of Nitro Blue Tetrazolium per ml, and the indicated substrate.

Chromatographic analysis and quantification of PEGs. PEGs were extracted by a modification of the procedure described by Jenkins et al. (11). Culture samples (10 ml) were centrifuged at 20,000 × *g* for 10 min, and the PEGs were extracted by evaporation under reduced pressure at 42°C, followed by sequential extraction of the residual solids with aliquots of chloroform (three extractions, 10 ml each) and "acid" chloroform (acetic acid-chloroform, 1:100, vol/vol). Solvent was removed by evaporation under reduced pressure at room temperature, and final traces of solvent were removed by vacuum desiccation over silica gel for a period of 12 h. Finally, the residue was suspended in 1 ml of distilled water.

Concentrated extracts were chromatographed on a 0.25-mm-thick silica gel 60 plate with methyl ethyl ketone-methanol-water-ammonia (65:20:5:10, vol/vol) as a solvent (11), and PEGs were detected with a modified version of Dragendorff reagent (19).

For PEG quantification, serial dilutions of samples of culture medium were applied to the silica gel plates and were stained with the above-indicated Dragendorff reagent. Concentrations were obtained by comparison of the spots with those developed by increasing standard concentrations made with the same-size PEG.

Other assays. 2-Keto-3-deoxyoctanoic acid was measured by the method of Hanson and Phillips (10). The determination of glyoxylic acid was performed by the method of Gamar and Gaunt (5).

TABLE 1. Yields of strain JA1001 grown on different-molecular-weight PEGs

PEG	Yield (A_{420}) at:	
	0.2%	1%
200	2.3	7.0
400	2.5	6.1
1000	2.9	6.0
2000	2.6	5.6
4000	3.2	5.6
6000	3.2	7.1
10000	2.3	5.6
15000	0.8	2.9
20000	0.1	0.1

RESULTS

Isolation and characterization of PEG-utilizing bacteria. Several fungi and actinomycetes were observed in the plates inoculated with the liquid medium of the first culture transfer derived from the river water sample. In the plates of the third transfer of the enrichment procedure some bacterial colonies appeared, and the one showing the best growth on liquid and solid media containing different-molecular-weight PEGs as a sole source of carbon and energy was isolated and labeled strain JA1001.

This strain was a gram-negative rod, strictly aerobic with a respiratory metabolism, and was catalase and oxidase positive. When screened with the API 20 E test kit (Analytical Products, Plainview, N.Y.) it was classified as a *Pseudomonas* sp. Further tests were performed with strain JA1001, which was found to be lipase and amylase positive, nonfluorescent, capable of liberating nitrogen gas from nitrate in the presence of glycerol or tartrate, and able to use glucose, maltose, glycerol, or succinate. On the basis of these phenotypic properties, strain JA1001 was identified as *P. stutzeri* (21). Electron microscopy of strain JA1001 (not shown) grown on 0.2% PEG 4000 showed the structure of a gram-negative cell wall with a clear periplasmic space, a distinguishable outer membrane, and a polar flagellum. Consistently, a 2-keto-3-deoxyoctanoic acid determination yielded a value close to that obtained with *Pseudomonas fluorescens* ATCC 12842 as a standard.

Characterization of growth on PEG. Strain JA1001 was used for the characterization of the PEG utilization. This strain grew on solid media with PEGs of molecular weights ranging from 200 to 15,000 at concentrations of 0.2 to 1% as a sole source of carbon and energy. In all cases, nonpigmented smooth colonies of 2- to 4-mm diameter appeared after 2 to 3 days of incubation at 30°C.

Cultures could always be reproduced in liquid medium, obtaining an average generation time of 140 min and the yields indicated in Table 1. It is worth pointing out that no significant difference in the generation time could be determined, irrespective of the size or the concentration of PEG used. The yields, although not strictly proportional to the carbon concentration, were always higher for the 1% concentration than for the 0.2% concentration, in general by a factor between 2 and 3.

Clearly there was a limit to the size of PEG sustaining growth of these cells. Thus, PEG 15000 presented a diminished yield compared with that of PEG 6000 or PEG 10000 while only a negligible growth was detected on PEG 20000.

Growth curves are shown for strain JA1001 in PEG 1000 (Fig. 1A) and in PEG 10000 (Fig. 1B) at 0.2% concentration.

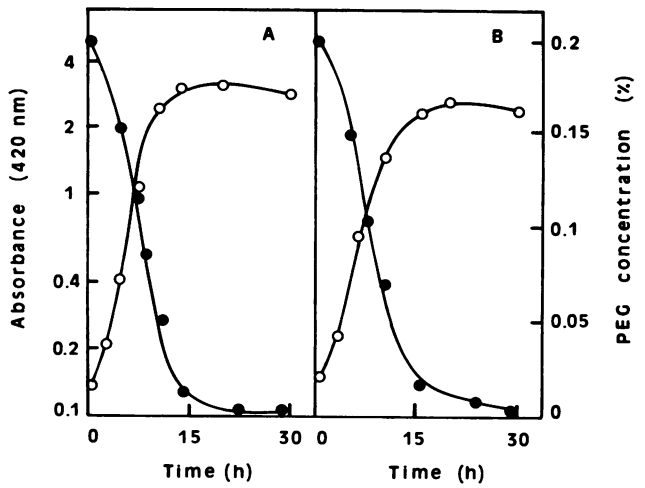

FIG. 1. Growth curve of strain JA1001 on 0.2% PEG 1000 (A) or PEG 10000 (B). Growth monitored by measurement of the A_{420} is indicated by the open symbols, and the concentration of the substrate remaining in the culture is indicated by the closed symbols.

Polymer disappearance from the medium as the culture grew was consistent with the use of PEGs as the sole source of carbon and energy and displayed total consumption of the polymer. The normal growth curve observed at 0.2% concentration changed in some cases to a biphasic curve when the concentration was raised to 1% (data not shown). This seemed not to correspond to diauxy caused by other carbon sources in the PEG preparation, since no growth was detected when PEG-negative strains of *P. fluorescens* or *Escherichia coli* were used in control experiments. In addition, the PEG disappearance time course matched the growth curve exactly (data not shown).

Oxidative cleavage of PEG. The search for an extracellular cleaving activity acting on PEG and rendering the carbons of the polymers more suitable to transport processes was unsuccessful, showing that this activity must be intracellular. Different extracellular media concentrated by ultrafiltration or by ammonium sulfate precipitation were incubated with different PEGs, radioactive and nonradioactive. To measure PEG-generated fragments after incubation, PEGs were analyzed by thin-layer chromatography and were always found not to be degraded. The same results were obtained when this kind of experiment was performed with the membrane fraction of the cells obtained in a culture of strain JA1001 in 0.2% PEG 4000. NADH oxidase was used as a membrane marker in these experiments.

The possible presence of an intracellular enzyme activity responsible for PEG degradation led us to analyze in our cells the PEG dehydrogenase activity previously described in a mixed culture by Kawai et al. (13). The activity of PEG oxidation linked to the reduction of DCPIP was found in extracts of strain JA1001 with PEG 200 as a substrate. The activity was found to be five times higher in PEG-grown cells than in succinate-, glucose-, or casein acid hydrolysate-grown cells. Glycolate and glyoxylate yielded intermediate induction values (Table 2).

Characterization of PEG dehydrogenase. Under the assay conditions described in Materials and Methods the enzyme of strain JA1001 oxidized PEGs of up to 20,000 molecular weight as well as diethylene glycol and diglycolic acid.

TABLE 2. PEG dehydrogenase activity of strain JA1001 grown under different conditions

Carbon source	PEG dehydrogenase activity (mU/mg of protein)
PEG 4000	40.0
PEG 6000	39.5
Glycolate	15.0
Glyoxylate	20.0
Glucose	9.2
Succinate	10.0
CAA[a]	8.5

[a] CAA, Casein acid hydrolysate.

Analysis of the effect of substrate concentration on PEG dehydrogenase activity revealed enzyme inhibition at a high substrate concentration (Fig. 2). The pH dependence of the enzyme activity was studied with 100 mM potassium phosphate buffer for pHs ranging from 6.0 to 8.5 and with glycine-KOH for pHs up to 10.0. An optimum pH of 8.0 was thus determined.

The product of the PEG dehydrogenase-catalyzed reaction was found to be glyoxylic acid. The product was identified after 10 min of incubation of a reaction mixture, without KCN, by the glyoxylic-specific method of Gamar and Gaunt (5).

PEG dehydrogenase activity was 50% inhibited by 3 mM glyoxylate (Fig. 3). Determination of the K_i for this inhibition with a Dixon plot yielded a value of 2.8 mM.

Localization of PEG dehydrogenase. Subcellular fractionation with glucose-6-phosphate dehydrogenase as the cytoplasmic marker, alkaline phosphatase as the periplasmic marker, and NADH oxidase as the membrane marker permitted us to locate the PEG dehydrogenase in the periplasmic fraction that was found not to be contaminated at all with the membrane or cytoplasmic enzyme marker. As much as 83% of PEG dehydrogenase was in the periplasmic fraction, while only 16% was found in the cytoplasmic fraction (Table 3). Further evidence for the periplasmic location of the PEG dehydrogenase was obtained in an electrophoretic separation of the periplasmic proteins stained for PEG dehydrogenase activity. A single band was apparent in the lane corresponding to the periplasmic fraction when staining was performed with PEG 200, PEG 4000, or diglycolic acid. As expected, no band was detected in the lane corresponding to the cytoplasmic fraction (Fig. 4).

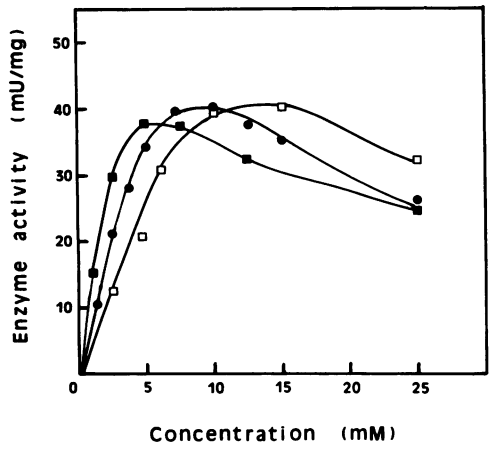

FIG. 2. Effect of substrate concentration on the activity of PEG dehydrogenase of strain JA1001. PEG dehydrogenase activity of crude extract was determined by the initial velocity of the catalyzed reaction obtained with the indicated concentrations of PEG 200 (□), PEG 400 (■), or PEG 4000 (●).

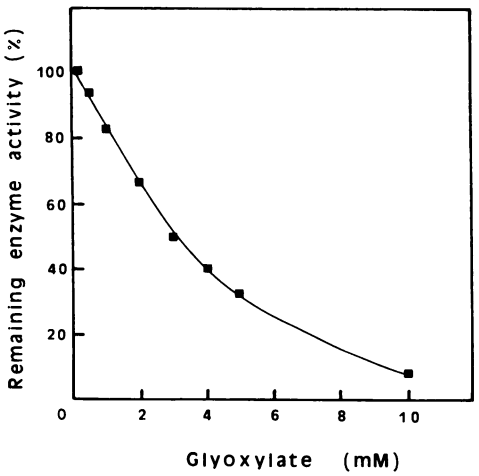

FIG. 3. Inhibition of PEG dehydrogenase by glyoxylate. PEG dehydrogenase activity of a crude extract of strain JA1001 was measured in the presence of increasing concentrations of glyoxylate that were added to the assay mixture.

Growth of strain JA1001 on glyoxylate. Strain JA1001 was able to grow on glyoxylate as a carbon and energy source. The cells grew with a doubling time of 150 min and a yield of 2.0 optical density units. In addition, a specific activity of 200 mU/mg of protein was determined for malate synthase, the enzyme responsible for the incorporation of the glyoxylate carbons into the general metabolism through the dicarboxylic acid cycle. The activity of malate synthase was very similar in cultures grown without PEG: cells of strain JA1001 grown on glucose or casein hydrolysate displayed activities of 160 or 245 mU/min/mg of protein, respectively. In a subcellular fractionation experiment, malate synthase activity was undetectable in the membrane or periplasmic fraction, the whole activity being detected in the cytoplasm.

DISCUSSION

In this report we present a strain of *P. stutzeri* that grew on PEGs of up to 13,000 to 14,000 molecular weight and totally degraded them in aerobic pure culture. Our results indicate that the efficiency of degradation in terms of the time required to reach total utilization and disappearance of the

TABLE 3. Localization of PEG dehydrogenase activity in subcellular fractionation of strain JA1001

Fraction	Enzyme activity of:							
	PEG dehydrogenase		Alkaline phosphatase		Glucose-6-phosphate dehydrogenase		NADH oxidase	
	Total U	%	Total U	%	Total U	%	Total U	%
Cytoplasm	0.225	16.0	0.700	26.2	1.09	84.1	0.11	6.8
Membrane	0.005	0.36	1.470	55.2	0.21	15.9	1.57	93.2
Periplasm	1.150	83.34	0.500	18.6	0.0	0.0	0.0	0.0

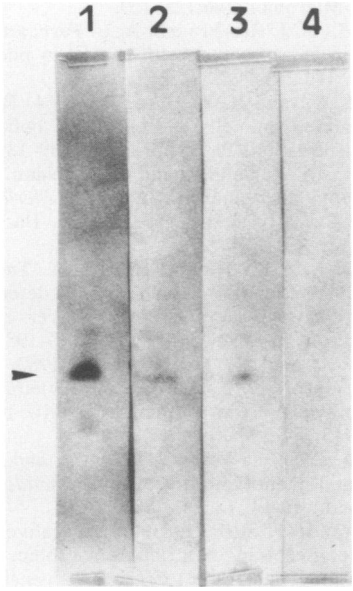

FIG. 4. Polyacrylamide gel electrophoresis of periplasmic and cytoplasmic proteins of strain JA1001 stained for PEG dehydrogenase activity. The periplasmic fraction of strain JA1001 grown on PEG 4000 at 0.2% concentration was electrophoresed and stained for PEG dehydrogenase activity by using as the substrate PEG 200 at 0.8% concentration (lane 1), PEG 4000 at 0.8% concentration (lane 2), or diglycolic acid at 150 mM concentration (lane 3). The cytoplasmic fraction of the same cells was electrophoresed and stained for activity with PEG 200 at 0.8% concentration as the substrate (lane 4). Arrow, PEG dehydrogenase.

polymer was much higher than that described by Haines and Alexander (7). These authors reported a PEG biodegradation system by a *P. aeruginosa* pure culture that required more than 20 days to totally degrade PEG when used at 1% concentration, while in our case only one-fifth of this time was required for the complete disappearance of the substrate. Interestingly, the strain isolated by Haines and Alexander degraded PEGs by means of an extracellular enzyme, in contrast to the intracellular PEG dehydrogenase described for our isolate. The differences in efficiency could rely on this different location of the degrading enzyme.

In their studies on microbial PEG growth and biodegradation, Kawai et al. (13) reported that utilization of PEGs of over 6,000 molecular weight required a cometabolism between a *Pseudomonas* sp. and a *Flavobacterium* sp. with an upper limit of polymer size of PEG 20000. We have found strain JA1001 to be able to utilize PEGs of up to 13,000 to 14,000 molecular weight in pure cultures. Although the consortium of a *Pseudomonas* sp. and a *Flavobacterium* sp. of Kawai et al. was able to degrade PEG 20000, its degradation of PEGs of below 13,000 to 14,000 molecular weight, that are also degraded by strain JA1001, is much slower: the consortium required more than 8 days to degrade 0.5% PEG 6000.

The inability of strain JA1001 to efficiently metabolize PEGs of over 14,000 molecular weight strongly suggests a permeability barrier. The localization of PEG dehydrogenase oxidative activity in the periplasmic space places this barrier in the outer membrane. It has to be stressed that PEG molecular weight indicates an average value for the molecular species found in a specific PEG preparation. Thus, a minor presence of PEGs of 13,000 to 14,000 molecular weight found in the PEG 15000 preparation may account for the partial growth observed in it. Consistently PEG 20000 supports no growth at all because no molecular species of PEG present in its preparation is under the molecular weight exclusion limit.

The periplasmic PEG-oxidative activity and the suggested entry of PEGs to the periplasmic space preclude the requirement for an extracellular enzyme digesting the polymer in smaller fragments. Permeability through porins of the outer membrane of molecules of 8,000 to 10,000 molecular weight has already been described by Hancock and Nikaido (9). Localization of PEG dehydrogenase in the periplasmic space has been proved not only by tracking the enzyme activity in the subcellular fractionation process, but also by showing in an electrophoretic development of these subcellular fractions that only the periplasm displayed the presence of a single protein accounting for the PEG oxidation. It may be pointed out that although the release of alkaline phosphatase as the periplasmic marker was incomplete, which is not a rare finding in the use of this marker in *Pseudomonas* species (2), the subcellular fractionation was reliable, as indicated by the absence of cross contamination of markers in each fraction.

A mechanism of PEG digestion could be proposed for strain JA1001 in which the polymer would enter the periplasmic space through porins of the outer membrane and subsequently be oxidized by the PEG dehydrogenase. The finding of glyoxylic acid as the product of the enzyme reaction indicates that a catalytic mechanism such as that proposed by Kawai (12) would be the one most likely to occur in our cells. However, the single band observed when PEG or diglycolic acid was used as a substrate in the electrophoresis experiments suggests that in our case one enzyme accounts for the catalytic mechanism, in contrast to the three enzymes proposed by Kawai.

To our knowledge, no information on the mechanism of entry of glyoxylic acid has been reported. However, the growth of strain JA1001 on this acid indicates that glyoxylic acid enters the cell, supporting growth, probably through the well-known dicarboxylic acid cycle pathway present in our cells.

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