Note

Involvement of a Polyethylene glycol (PEG)-oxidizing Enzyme in the Bacterial Metabolism of PEG

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We have isolated many polyethylene glycol (PEG)-utilizing bacteria and confirmed that PEG is oxidatively metabolized by a synergistic mixed culture of two bacteria which are individually unable to utilize PEG as a sole carbon and energy source.^{1~3)} The PEG-oxidizing enzyme was purified from the mixed culture and characterized as 2,6-dichlorophenolindophenol (DCIP)-dependent dehydrogenase.^{3,4)} Other works also showed PEG-oxidizing activities in PEG-utilizing bacteria.^{5~8)} On the other hand, Haines and Alexander suggested that PEG 20,000 was hydrolyzed by an extracellular enzyme of a strain, *Pseudomonas aeruginosa*, into diethylene glycol and ethylene glycol which were incorporated and metabolized by washed cells.⁹⁾ In our experiments,³⁾ however, PEG 6,000 was neither hydrolyzed by an extracellular enzyme of the mixed culture nor by an intracellular one. The culture could not grow on ethylene glycol or diethylene glycol which were presumed intermediates in PEG metabolism according to the report of Haines and Alexander. Instead PEG-oxidizing activity linked with DCIP was found in sonic extracts of the mixed culture.

In the present paper we report that PEG-utilizing bacteria have PEG-oxidizing systems in their cell-free extracts and tetraethylene glycol (TEG) is oxidatively metabolized by resting cells of these bacteria.

Average molecular weights and sources of PEGs used were the same as those listed in the previous report.¹⁾ All the PEG solutions used for measuring PEG-oxidizing activity were freshly prepared before use. PEG 400-utilizing (Nos. 42 and 43), PEG 1,000-utilizing (C-1 and Nos. 101~103), PEG 4,000-utilizing (Nos. 201~204, D-1 and D-2) and PEG 20,000-utilizing (E-1) strains were isolated by the enrichment culture technique from soil and waste water and also from activated sludge samples as described previously.¹⁾ All strains were maintained on PEG slants. The two strains composing E-1 were usually maintained

on a PEG 6,000 slant as a mixed culture.²⁾ All strains were grown on a basal medium (pH 7.2) containing 5 g of PEG, 3 g of $(\text{NH}_4)_2\text{SO}_4$ or $(\text{NH}_4)_2\text{HPO}_4$, 2 g of K_2HPO_4 , 1 g of NaH_2PO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.5 g of yeast extract in one liter of tap water. The bacteria were cultured in a 2-liter shaking flask containing 500 ml of liquid medium with shaking at 28°C for 3 days (all strains except E-1) or 7 days (E-1). After cultivation, the cells were harvested by centrifugation at $8,000 \times g$ at 4°C for 20 min and washed twice with physiological saline. The washed cells were suspended in an appropriate amount of 0.05 M potassium phosphate buffer (pH 8.0), and disrupted with a 19 kHz ultrasonic oscillator (Kaijo-Denki Co., Ltd., Japan) on ice. Cell debris was removed by centrifugation at $12,000 \times g$ at 4°C for 20 min. The resultant supernatant solution was used as a cell-free extract.

PEG-oxidizing activity was measured by determining a 2,4-dinitrophenylhydrazine-positive compound according to the method of Narrod and Jakoby.¹⁰⁾ The validity of this method was confirmed by our previous study: The formation of TEG-aldehyde and valeraldehyde from substrates, TEG and *n*-pentanol, by PEG dehydrogenase was confirmed.¹¹⁾ The standard reaction mixture (2.5 ml) contained 250 μmol of potassium phosphate buffer (pH 8.0), 100 mg of PEG and an appropriate amount of the cell-free extract. The reaction was started by the addition of the cell-free extract and carried out at 30°C. As a reaction blank, 0.1 ml of 4 N HCl was added to a reaction mixture before starting the reaction. After 20 min incubation, the reaction was stopped by the addition of 0.1 ml of 4 N HCl. The reaction mixture (1.0 ml) was boiled for 10 min with an equal volume of 0.1% 2,4-dinitrophenylhydrazine solution in 2 N HCl. After cooling, 5 ml of 1 N alcoholic KOH (4 N KOH:99.5% ethanol=1:3), prepared immediately before use, was added, and the mixture was stirred vigorously. Then the absorbance at 580 nm was measured with a Hitachi double-beam spectrometer 200-10. The amount of the reaction product (PEG-aldehyde)¹¹⁾ was calculated by reference to a standard curve prepared with glycol aldehyde. The protein content of the enzyme preparation was determined by the method of Lowry *et al.*¹²⁾ One unit of the enzyme was defined as the enzyme quantity which formed 1 μmol of aldehyde per min. The specific activity of the enzyme was expressed in terms of the units of enzyme contained per milligram of protein.

As previously reported,³⁾ PEG-oxidizing activity was found in cell-free extracts of a mixed culture (E-1). To determine the mechanism by which PEG is metabolized by other PEG-utilizing bacteria, PEG-oxidizing activities of sonic extracts of PEG-utilizing bacteria grown on PEG media were investigated. The enzyme activity was found in all the PEG-utilizing bacteria (Table I). All the PEG 4,000-utilizing strains and one PEG 1,000-utilizing strain, No. 103, had strong DCIP-dependent activities similar to that of the mixed culture.³⁾ In contrast, two strains of PEG 400-

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TABLE I. PEG-OXIDIZING ACTIVITY IN CELL-FREE EXTRACTS OF PEG-UTILIZING BACTERIA

Strain ^a	Carbon source and enzyme substrate	Total protein ^b (mg)	Specific activity ^c (units/mg protein, $\times 10^3$)			
			Electron acceptor (0.1 mM)			
			None	DCIP	NAD	NADP
42	PEG 400	100	2.4	0.3	1.4	1.9
43	PEG 400	164	1.3	0.6	1.3	1.4
C-1	PEG 1,000	56	0.3	1.7	0.6	1.1
101	PEG 1,000	68	1.8	2.5	1.2	2.1
102	PEG 1,000	59	1.1	0.6	1.0	0.7
103	PEG 1,000	188	5.1	30.4	2.7	1.8
201	PEG 4,000	219	2.4	11.2	0.8	0.4
202	PEG 4,000	203	2.9	12.8	1.1	0.6
203	PEG 4,000	194	2.9	14.1	1.7	1.6
204	PEG 4,000	251	2.2	8.3	0.4	0.2
D-1	PEG 4,000	231	2.3	9.1	0.7	0.4
D-2	PEG 4,000	237	2.0	10.3	1.4	1.5
E-1	PEG 6,000	238	2.2	18.9	2.1	0.9

^a Each culture was carried out for 3 days except for E-1. E-1 was cultivated for 7 days.

^b Values are expressed as total protein of cell-free extracts prepared from 500-ml cultures.

^c Enzyme activity was measured by determining a 2,4-dinitrophenylhydrazine-positive compound as described in the text.

utilizing bacteria and PEG 1,000-utilizing strains No. 101 and 102 showed DCIP-independent activities. The enzyme activity of strain C-1 was elicited by DCIP and NADP.

While all of the bacteria possessed enzyme activities of differing substrate specificities toward PEGs, they had only slight or no activity toward ethylene glycol (Table II). These extracts extremely oxidized PEGs close to the molecular weight range of those utilized as growth substrates by the respective strains. In addition, the cell-free extract of strain No. 103 appreciably oxidized PEG 2,000~6,000 which were not utilized as sole carbon and energy sources by the bacterium.

From these results, there seem to be two types of PEG-oxidizing enzymes; DCIP-dependent PEG-oxidizing enzymes and DCIP-independent PEG-oxidizing enzymes. PEG 400-utilizing bacteria and some of the PEG 1,000-utilizing bacteria formed DCIP-independent enzymes. Other PEG 1,000-utilizing bacteria, all the PEG 4,000-utilizing bacteria and E-1 possessed DCIP-dependent enzymes. The fact that PEG-oxidizing enzymes are present in all the PEG-utilizing bacteria reveals that PEG is oxidatively metabolized by these strains.

Washed cells of PEG-oxidizing bacteria obtained from 100-ml cultures were incubated with 0.2 g of TEG in 20 ml of 0.01 M potassium phosphate buffer (pH 7.2) at 28°C for one day with shaking. The strains used were the same as those used for testing PEG-oxidizing activities of sonic extracts were. The reaction was stopped by centrifuging the mixture at $10,000 \times g$ for 20 min; intact cells were then

TABLE II. OXIDIZING ACTIVITY TOWARD SEVERAL PEGS IN CELL-FREE EXTRACTS OF TYPICAL PEG-UTILIZING BACTERIA

Substrate ^a	Specific activity (units/mg protein, $\times 10^3$) ^b				
	Strain				
	42	101	103	203	E-1
Ethylene glycol	0.2	0.0	0.2	0.2	0.0
Tetraethylene glycol	4.4	8.2	7.1	4.7	9.2
PEG 400	3.3	6.5	7.3	7.4	12.2
PEG 1,000	0.9	2.0	4.3	4.0	7.6
PEG 2,000	0.4	0.4	2.9	2.1	4.4
PEG 4,000	0.5	0.3	2.6	1.7	3.9
PEG 6,000	0.5	0.4	0.7	0.5	1.3

^a Final concentration of 5 mM.

^b Enzyme activity was measured by determining a 2,4-dinitrophenylhydrazine-positive compound as described in the text.

removed. TEG and its metabolites were separated on a 0.25 mm-thick silica gel 60 F₂₅₄ plates (E. Merck). The chromatograms were developed with 1-propanol-ethanol-water (50:30:20, v/v/v). TEG and its metabolites were detected with a K₂Cr₂O₇-H₂SO₄ reagent.¹³⁾ Authentic TEG-monocarboxylic acid and TEG-dicarboxylic acid

were prepared and identified as described previously.³⁾ TEG and triethylene glycol were commercially obtained and showed single peaks, respectively, on gas chromatography which was carried out under the same conditions as described previously.³⁾ All the strains metabolized TEG and formed metabolites with the same *R_f* values as authentic TEG-monocarboxylic acid, TEG-dicarboxylic acid and triethylene glycol. TEG-monocarboxylic acid was the common main product formed by all the strains, but fluctuations were observed in the formation of TEG-dicarboxylic acid and triethylene glycol by these strains.

From these results, we can conclude that oxidative degradation is common to the metabolism of PEG by aerobic bacteria. Our previous report showed that PEG dehydrogenase of a PEG 20,000-utilizing synergistic mixed culture was membrane-bound and was solubilized with laurylbetaine.⁴⁾ It will be interesting to see whether the other PEG-oxidizing enzymes are membrane-bound or not.

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