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Isolation of (S)-2,3-dichloro-1-propanol assimilating bacterium, its characterization, and its use in preparation of (R)-2,3-dichloro-1-propanol and (S)-epichlorohydrin

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SUMMARY

A bacterium that stereospecifically assimilates (S)-2,3-dichloro-1-propanol was isolated from soil by enrichment culture. By taxonomic studies, the strain was identified as *Alcaligenes* sp. The bacterium could degrade and assimilate some chlorohydrins. Its cell-free extracts, which had dehalogenase and epoxyhydrolase activities, converted various halohydrins to the dehalogenated alcohols, and epoxides to the diols. This strain was similar to (R)-2-3-dichloro-1-propanol assimilating bacterium in degradation of 2,3-dichloro-1-propanol, but had a somewhat different character. Optically pure (R)-2,3-dichloro-1-propanol (100% e.e.) was isolated from the racemate using the stereospecific assimilation by the bacterium. Highly pure optically active (S)-epichlorohydrin (99.5% e.e.) was obtained by treatment with aqueous NaOH. The new isolate was also compared to the (R)-2,3-dichloro-1-propanol assimilating bacterium.

INTRODUCTION

Optically active epichlorohydrin (EP) [1] is an important C3 chiral building block for the synthesis [2] of chiral pharmaceuticals such as β-adrenergic blockers [3–5], vitamins [6], pheromones [7], natural products [8], and new materials such as ferro electric crystals [9]. We have been studying the use of microorganisms and enzymes for the preparation of halogenated aliphatic compounds and have isolated a (R)-2,3-dichloro-1-propanol ((R)-DCP) assimilating bacterium [10]. This stereospecific bacterial degradation was a very effective, useful and practical method for the preparation of (S)-DCP and (R)-EP [11] (Fig. 1). However, for the synthesis of chiral compounds, or pharmaceuticals, both optically pure enantiomers are generally required because various synthesis strategies of chiral target compounds use both enantiomers. Optically active epichlorohydrin is also not an exception. Therefore, for the purpose of obtaining the reverse optical isomers: (R)-DCP and (S)-EP, we have been seeking an (S)-DCP assimilating microorganism. In this paper, we describe the screening, isolation of the bacterium, its characterization, comparison with (R)-DCP assimilating bacterium [10], and preparation of (R)-DCP and (S)-EP using this bacterium.

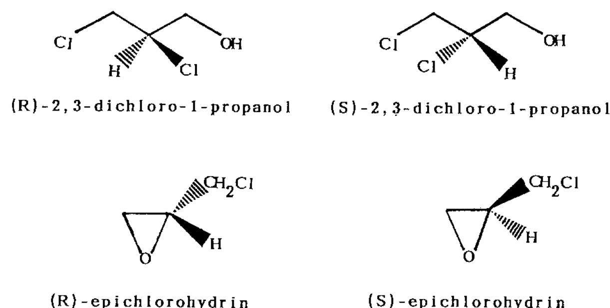

Fig. 1. Both enantiomers of 2,3-dichloro-1-propanol and epichlorohydrin.

MATERIALS AND METHODS

(S)- and (RS)-DCP

Optically pure (S)-DCP was prepared by using an (R)-DCP assimilating bacterium [10]. 3-Chloro-1,2-propanediol (CPD), 2,3-dibromo-1-propanol (DBP), (RS)-DCP and other halogenated compounds were purchased from

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Tokyo Kasei Kogyo (Tokyo, Japan). Other reagents used were of analytical grade.

Screening method

The soil samples were collected from petrochemical plant grounds. The enrichment culture was carried out using a synthetic medium containing (RS)- or (S)-DCP as a single source of carbon. Three grams of sample soils were suspended in 50 ml tubes containing 10 ml of sterile synthetic medium, consisting of 0.2% (v/v) (RS)- or (S)-DCP, 0.1% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) K_2HPO_4, 0.1% (w/v) $Na_2HPO_4 \cdot 2H_2O$, 0.2% (w/v) $NaH_2PO_4 \cdot 12H_2O$, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, and 0.001% (w/v) $FeSO_4 \cdot 7H_2O$, 0.0001% (w/v) $CuSO_4 \cdot 5H_2O$, $MnSO_4 \cdot 5H_2O$, 0.008% (w/v) bromothymol blue (pH indicator), pH 6.8. The test tubes were shaken at 30 °C for 2 weeks. When the color of bromothymol blue (BTB) changed from green to yellow, an aliquot of the soil suspension was spread on a nutrient agar plate consisting of 1% (w/v) glucose, 1% (w/v) peptone, 1% (w/v) yeast extracts, and incubated at 30 °C for 3 days. An assessment for the assimilation of (RS)-DCP and (S)-DCP was done as follows: Grown microorganisms were transferred to the agar plate of the synthetic medium, incubated at 30 °C for 7 days, and color change of BTB was observed. The degradation test was done in a 20-ml test tube containing 5 ml of the synthetic medium ((RS)-DCP concentration was 0.2% (v/v) and 0.1% (v/v)). The degradation ratio was estimated by gas chromatography [10].

Identification of DS-K-S38

Characterization and identification employed 'Bergey's Manual of Systematic Bacteriology' [12]. Tests for physiological properties were carried out as previously described [10].

Cultivation

A 40 ml seed culture of the organism was inoculated into a 2.5-l jar fermentor of the synthetic medium containing 0.2% (v/v) (RS)-DCP, which was incubated at 30 °C with 500 rpm agitation, 500 ml/min aeration, and at a controlled pH of 7.0 with 1 N NaOH. The growth of the cells was followed turbidimetrically at 660 nm. Degradation was estimated by gas chromatography [10]. Chloride ion was measured by the method of Iwasaki et al. [13]. A 100-l scale cultivation to obtain a large amount of (R)-DCP was carried out in a similar manner.

Preparation of cell-free extracts

Cell-free extracts were obtained by French press disruption of harvested cells in the synthetic medium described above. Protein was assayed with the Lowry method [14].

Degradation of halogenated compounds

The method of the degradation test, estimation of the degradation ratio, and identification of metabolites were carried out using gas chromatography (Shimadzu, GC-9A system, Kyoto, Japan) as was previously described [10].

Enzymatic conversion of various halohydrins and epoxides by the cell-free extracts was measured at 30 °C in 1 ml of 50 mM phosphate buffer (pH 7.0), containing 0.1% (v/v) substrates and cell-free extracts [10]. One unit of enzyme activity was defined as the conversion of 1 μmol of substrates per min and the specific activity was indicated as units per mg protein.

Isolation of (R)-DCP

Residual (R)-DCP in the culture filtrate was obtained by extraction with diethyl ether, drying, evaporation and distillation in vacuo (bp 75–76 °C/20 mmHg). In a 100-l scale cultivation, the culture filtrate (80 l) was passed through a charcoal column (10 l) to absorb (R)-DCP and was then eluted with acetone (25 l), evaporated and distilled in vacuo (yield 40%).

Conversion to (S)-EP

Epoxidation from (R)-DCP to (S)-EP (bp 118 °C) was carried out by treatment with aqueous NaOH. The formed (S)-EP was dried with anhydrous $MgSO_4$ and distilled using a Wittomer distillation column (bp 118 °C/760 mmHg; total yield, 74%) [11].

Optical purity

Specific rotation was measured with a high speed automatic digital polarimeter (Jasco, model DIP-360, Tokyo, Japan). The optical purities of purified (R)-DCP and (S)-EP were measured by HPLC analysis (Hitachi L-6000 system, Tokyo, Japan) of Mosher's ester [15] of the (R)-DCP and complexation gas chromatography (Shimadzu GC-14A system, Kyoto, Japan) of (S)-EP [16], respectively.

RESULTS

Result of screening

Table 1 summarizes the result of the screening in the final selection. From about 1000 soil samples from petrochemical plant grounds, six (RS)-DCP assimilating bacteria and six (R)-DCP assimilating bacteria were found, while only one bacterial strain, DS-K-S38, which preferentially assimilated (S)-DCP as a sole source of carbon was finally isolated. The desired strain was found to belong to *Alcaligenes* sp. according to the morphological, cultural, and physiological characteristics of the strain DS-K-S38 shown in Table 2.

TABLE 1

Result of screening in isolation of (S)-DCP assimilating bacterium

Strains	Degradation ratio (%)				Assimilation test on the synthetic medium plate[a]		Predictable assimilation type	Specific rotation values
	0.2% (v/v) of (RS)-DCP		0.1% (v/v) of (RS)-DCP		(S)-DCP	(RS)-DCP		
	24 h	48 h	24 h	48 h				
DS-K-18	1.5	4.5	38.5	85.1	+	++	RS	
DS-K-25	41.4	60.0	59.8	64.2	+	+	RS or S	−9.2
DS-K-31	0.6	6.8	71.9	94.2	+	++	RS	
DS-K-33	12.3	51.5	49.1	58.3	+	++	RS or S	−10.5
DS-K-34	13.8	45.5	52.4	49.8	+	++	S	+1.3
DS-K-35	7.5	45.0	89.8	100	+	+	RS	
DS-K-S38	2.5	32.7	41.4	51.7	+	+	S	+10.7
DS-K-40	22.2	51.4	56.9	88.1	+	++	RS	
DS-K-41	0.5	0.8	39.1	100	+	++	RS	
DS-K-42	50.7	55.5	52.0	56.5	−	++	R	
DS-K-45	42.1	53.6	45.7	53.1	−	++	R	
DS-K-48	46.4	53.2	48.7	58.0	−	++	R	
DS-K-49	43.3	51.0	48.3	56.1	−	++	R	

[a] Symbols: +, the color of bromothymol blue (BTB) changed from green to yellow; ++, the color of BTB changed from green to orange. −, the color did not change.

TABLE 2

Some morphological, physiological properties of strain DS-K-S38

Morphology	rod
Length	1.8–2.2 μm
Width	0.4–0.6 μm
Colony morphology	circular, smooth surphase
Pigment	−
Flagellation	peripheral flagella
Motility	+
Gram stain	negative
Endospores formed	−
Acid-fast staining	not detective
Oxidase	+
Catalase	+
Urease	−
Nitrate reduction	−
OF test (Hugh Leifson method)	O (glycerine)
PHB accumulation	+
Arginine dihydrolase	−
Utilization of ammonium	+
nitrate	+

Cultivation

Fig. 2 shows the growth of the strain DS-K-S38 on (RS)-DCP. (S)-DCP was degraded with liberation of chloride ion. After 48 h, cell growth ceased and the final degradation was estimated to be 52%. A trace amount of CPD [10] and glycerol was detected in the logarithmic

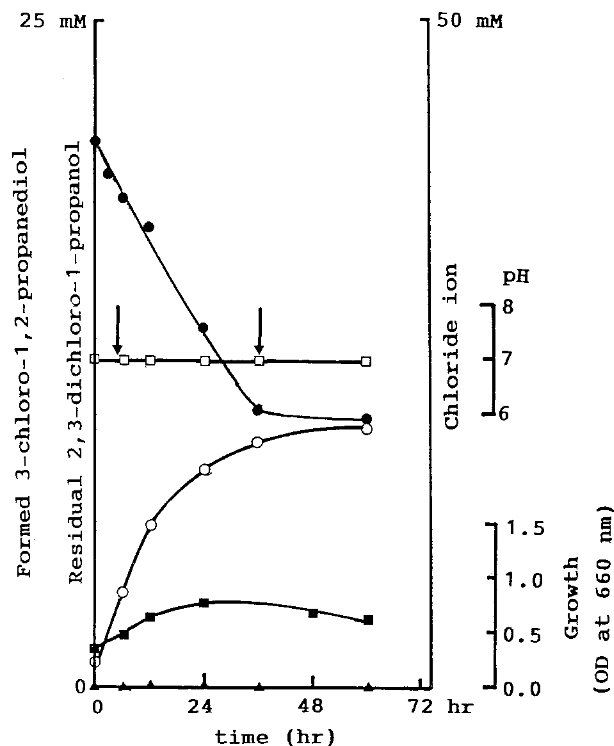

Fig. 2. Time-course of cultivation of the strain DS-K-S38 ■, growth; ●, residual 2,3-dichloro-1-propanol; ▲, formed 3-chloro-1,2-propanediol; ○, chloride ion; □, pH; pH was controlled during the period indicated by arrows.

phase. These results were similar to that of (R)-DCP assimilating strain [10]. The specific rotation of residual (R)-DCP gave $[\alpha]_D^{25} = +10.7$ ($c = 1.25$, in CH_2Cl_2) and the value was in good accord with the opposite one of (S)-DCP (ref. (S)-DCP; $[\alpha]_D^{25} = -10.5$, $c = 1.36$, in CH_2Cl_2) [11].

Degradation of halogenated compounds

Table 3 shows the degradation activity of DS-K-S38. Various low molecular weight chlorinated aliphatic hydrocarbons and related epoxides (mainly C3 chlorinated compounds) were tested as to whether or not they were assimilated. DS-K-S38 could degrade chlorohydrins such as CPD, DCP, propylene chlorohydrin, and the epoxide of glycidol, but it could not degrade 1,3-dichloro-2-propanol, DBP, EP, propylene oxide, chloroacetone, and chloropropionic acid. CPD was a good carbon source for growth, but the other compounds were poor carbon sources. Fig. 3 shows a comparison of degradation activity between strain DS-K-S38 ((S)-type) and strain OS-K-29 ((R)-type). A remarkable point was that the kind of assimilatable and degradable compounds were less those of the (R)-type. 1,3-Dichloro-2-propanol and EP were good carbon sources for (R)-type growth, however, the (S)-type could not degrade and assimilate them, regardless of the concentration.

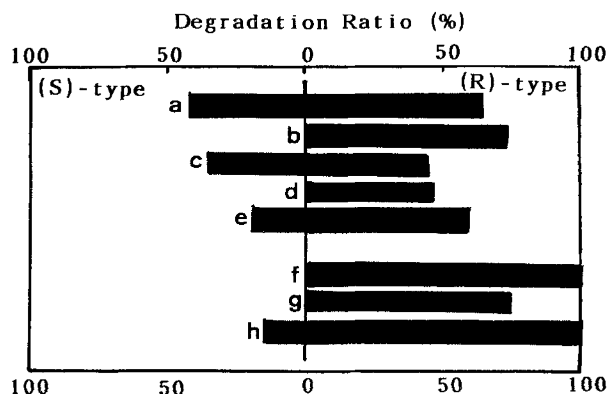

Fig. 3. Comparison of degradation for halohydrins and epoxides between (S)-type and (R)-type. In both of the (S)- and (R)-type [10], the degradation for halohydrins and epoxides by cell are summarized and expressed. Substrates: a, CPD; b, 1,3-dichloro-2-propanol; c, DCP; d, DBP; e, propylene chlorohydrin; f, EP; g, epibromohydrin; h, glycidol.

Enzymatic conversion

Table 4 shows the conversion activity of cell-free extracts of strain DS-K-S38. C3 halohydrins were dehalogenated, yielding dehalogenated alcohols. Epoxides, except propylene oxide, were hydrolyzed and converted to

TABLE 3

Degradation for various halogenated compounds by strain DS-K-S38

Carbon sources	Growth (OD at 660 nm)	Final pH	Degradation (%)
Halohydrins			
3-Chloro-1,2-propanediol	0.85	6.16	56.8
1,3-Dichloro-2-propanol	0.04	7.00	0.0
2,3-Dichloro-1-propanol	0.17	5.64	36.7
2,3-Dibromo-1-propanol	0.00	6.83	0.0
Ethylene chlorohydrin	0.08	7.01	3.1
Propylene chlorohydrin	0.07	6.94	20.7
Butylene chlorohydrin	0.07	6.95	24.2
Epoxides			
Epichlorohydrin	0.00	6.87	0.0
Epibromohydrin	0.00	6.83	0.0
Glycidol	0.20	7.03	16.2
Propylene oxide	0.00	6.86	0.0
Haloacids and others			
Chloroacetone	0.00	6.89	0.0
n-Propylchloride	0.00	6.94	0.0
α-Chloropropionic acid	0.00	6.90	0.0
β-Chloropropionic acid	0.00	6.90	0.0

TABLE 4

Degradation for various halohydrins and epoxides by cell-free extracts

Substrates	Specific activity (mU/mg)	Detectable compounds
Halogenated alcohols		
3-Chloro-1,2-propanediol	3.4	glycerol
1,3-Dichloro-2-propanol	3.2	3-chloro-1,2-propanediol, glycerol
2,3-Dichloro-1-propanol	1.5	3-chloro-1,2-propanediol, glycerol
2,3-Dibromo-1-propanol	1.8	glycerol
Propylene chlorohydrin	1.5	1,2-propanediol
Epoxides		
Epichlorohydrin	8.1	3-chloro-1,2-propanediol, glycerol
Epibromohydrin	0.8	
Propyleneoxide	0.0	
Glycidol	0.3	glycerol

Reaction time was 2–24 h.

the diols. Although EP was not assimilated, the epoxyhydrolase activity for EP was the highest. Fig. 4 shows a comparison of relative activity of cell-free extracts between the (S)-type and the (R)-type. Some notable differences were found in the activities of the cell -free extracts. In the case of the (R)-type, the whole conversion activity was strong and broad; especially, strong epoxyhydrolase activity was found. On the other hand, in the case of the (S)-type, the pattern of the relative activity for halohydrins was similar to that of the (R)-type, however, the activity was weak. The epoxyhydrolase activity of the (S)-type was very weak except for EP.

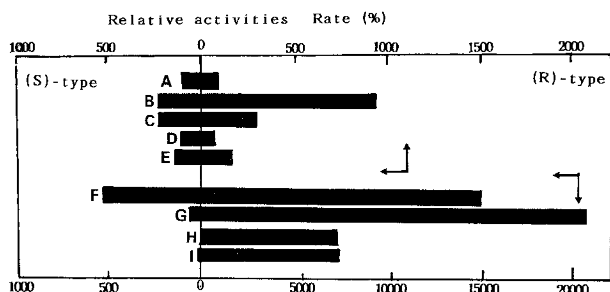

Fig. 4. Comparison of relative activities of cell-free extracts for halohydrins and epoxides between (S)-type and (R)-type. In both of the (S)- and (R)-type [10], the conversion activities were expressed as percentages of the rate found with DCP ((S)-type, 1.5 mU/mg protein; (R)-type, 1.1 mU/mg protein). Substrates: A, DCP; B, 1,3-dichloro-2-propanol; C, CPD; D, propylene chlorohydrin; E, DBP; F, EP; G, epibromohydrin; H, propylene oxide; I, glycidol.

Optical purity of (R)-DCP and prepared (S)-EP

The obtained (R)-DCP was converted to Mosher's ester [17], and the optical purity was estimated as 100% e.e. by HPLC analysis [11]. The optical purity of (S)-EP prepared from the (R)-DCP was estimated as 99.5% e.e. by complexation gas chromatography [16] (Fig. 5). The isolation of pure (R)-DCP was first.

DISCUSSION

A bacterium preferentially assimilating (S)-DCP ((S)-type) was isolated from soil. The isolated bacterium was considered to be the reverse type of (R)-DCP assimilating bacterium (*Pseudomonas* sp. OS-K-29), previously reported [10]. During the cultivation, in which (RS)-DCP was the single source of carbon, the bacterium degraded and assimilated half of the DCP such as the (R)-type [10] shown in Fig. 2. Both the (S)-type and (R)-type were isolated from the same petrochemical plant grounds as stereospecific DCP assimilating bacterium, CPD and glycerol were detected in each degradation of DCP, and the pattern of relative activity for halohydrins in the cell-free extracts also resembled each other. Therefore, we considered that the possible degradation pattern was similar to that of the (R)-type [10]; DCP was dehalogenated and degraded to glycerol via CPD. Bacterial degradation of (RS)-DBP, in which the Cl of DCP is substituted for Br, was reported by Castro and Bartnicki [19], DBP was converted to epibromohydrin by strong halohydrin epoxidase, resulting in the accumulation of epibromohydrin. In the degradation of (S)-DCP it was not determined whether or not dechlorination occurred via EP, because epoxyhy-

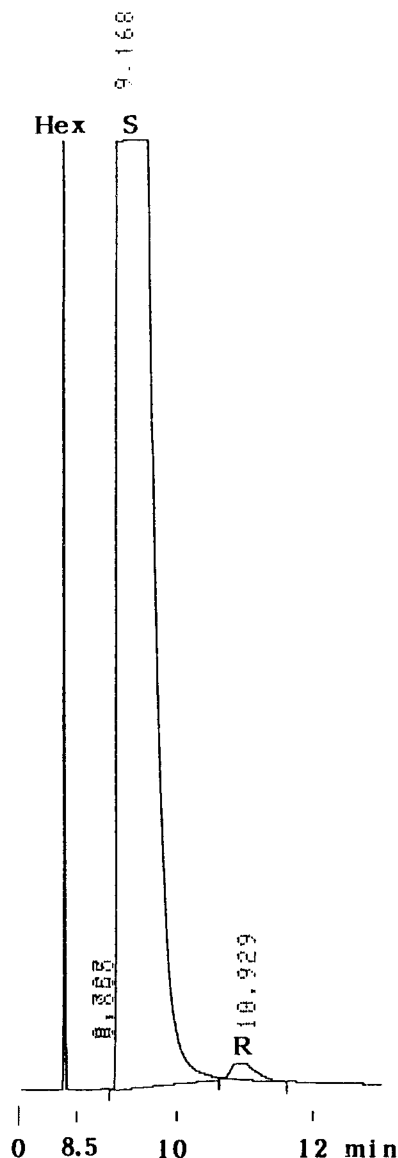

Fig. 5. Estimation of optical purity of (S)-epichlorohydrin by complexation gas chromatography. Symbols: Hex, Hexane; S, (S)-epichlorohydrin; R, (R)-epichlorohydrin. Analysis conditions: column, 0.25 mm × 30 m (coated with bis-3-(heptafluorobutyryl)-1R-camphorates of cobalt (II) in SE-54 by a dynamic method); sample 0.6 μl of 5% (v/v) hexane solution; column temp., 40 °C; injection temp. and detector temp. (FID). 150 °C; carrier gas (nitrogen), 1 ml/min; split ratio, 1:50.

drolase activity for EP was strong compared to the dechlorinating activity for DCP, while EP was not accumulated. This result was similar to the case of the (R)-type [10]. The dehalogenating (S)-DCP enzyme in the (S)-type seemed to be novel because of its high stereospecificity, in addition, convertible DCP was the (S)-form. Stereospecific dehalogenation for DCP was not known except for the (R)-DCP assimilating bacterium [10].

The degradation activity for CPD was stronger than that for glycidol, although glycidol was not accumulated. this indicated the possibility that the chlorine atom of CPD was directly eliminated without the action of halohydrin epoxidase.

Janssen et al. reported (RS)-CPD [17] and bromoethanol degrading bacteria [18]. They stated that the chlorine atom of CPD was eliminated via glycidol, and the bromine atom of bromo-ethanol was directly released. Dechlorination of the (S)-type for CPD seemed to be similar to that of the bromo-ethanol degrading bacteria.

This microbial resolution was effective and useful for preparing (R)-DCP and (S)-EP; residual (R)-DCP in cultivation of the (S)-type was found to be optically pure, and highly pure (S)-EP was prepared from this product. (RS)-DCP is produced economically by the petroleum industry, therefore, the method is considered practical. Habets-Crutzen et al. reported the formation of (S)-EP from allylchloride by microbial stereospecific epoxidation; the method was stereospecific epoxidation by alkene assimilating bacteria with an optical purity of 80–98% e.e. [20]. However, in view of production, we consider that our method has advantages such as DCP has a higher solubility in water, lower toxicity, and a higher boiling point than allylchloride. Asymmetric syntheses have also been developed, but these methods are not simple [1,2]. The present microbial resolution is now in progress on a pilot plant scale. Currently, the (S)-EP is being used in the synthesis of chiral drugs or natural products [5,8]. Both enantiomers of DCP and EP could be obtained in a highly enantiomerically pure state by using previous reported (R)-DCP assimilating bacterium [10] and this (S)-DCP one together. It should also be possible to use these optically active compounds for easily obtaining C3 chiral building blocks.

Further studies for enzymes of the (R)- and (S)-type will be published elsewhere.

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