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Optimal Resolution of L-Carnitine from Racemic DL-Carnitine by *Enterobacter* sp. Assimilating D-Carnitine

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In order to isolate a microorganism having preferential degradation of D-carnitine from DL-carnitine, a bacterium assimilating D-carnitine as a sole carbon and energy source was isolated from soil by enrichment culture and partially identified as *Enterobacter* sp. Also, a mutant having lessened L-carnitine decomposition rates was selected with nitrosoguanidine mutagenesis, which led to decrease the specific activities of carnitine dehydrogenase (7.6-fold) and β-hydroxybutyrate dehydrogenase (9.5-fold) as compared to the wild strain. Meanwhile, optimal culture conditions for optical resolution of DL-carnitine were investigated. Under optimal conditions, 3.53 g/l L-carnitine was obtained from 20 g/l DL-carnitine, which corresponded to 35.3% L-carnitine yield and 97.9% optical purity.

Carnitine, a betaine of γ-amino-β-hydroxybutyric acid, transports activated fatty acids and activated acetate across the inner mitochondrial membrane (4). Since the publication of two papers in 1955 (5, 6), studies of the physiological role of carnitine and its biosynthesis and degradation have been steadily increased. Various strains of *Pseudomonas* can metabolize L-carnitine stereospecifically using L-carnitine dehydrogenase (EC 1.1.1.108) when the cells are grown in a DL-carnitine-containing medium (7, 8). The microorganism, *Escherichia coli*, can reduce carnitine to γ-butyrobetaine by hydrogenation after dehydration of carnitine to γ-crotonbetaine (13). *Serratia marcescens* can produce trimethylamine and malate from DL-carnitine (14). Recently, a microorganism assimilating D-carnitine preferentially, from a medium containing DL-carnitine as a sole carbon and energy source, was isolated (1).

In this paper, we isolated bacteria from soil, which assimilate D-carnitine as a sole carbon and energy source. Among isolated bacteria, a bacterium having a higher assimilating capacity for D-carnitine than L-carnitine was isolated and partially identified. Also, attempts to develop a microbe with a lessened ability to decompose L-carnitine from the racemic mixture, DL-carnitine, were performed by mutagenesis with NTG (N-methyl-N'-nitro-N-nitrosoguanidine). Optimal culture conditions for preferential degradation of D-carnitine from a medium containing DL-carnitine as a sole carbon and energy source were investigated.

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MATERIALS AND METHODS

Microorganism and Chemicals

Bacteria assimilating D-carnitine as a sole carbon and energy source were isolated from a soil sample from Jinju (Korea). DL-Carnitine, L-carnitine, D-carnitine, nicotinamideadenine dinucleotide, acetyl coenzyme A, 5,5'-dithiobis-(-2-nitrobenzoate), carnitine acetyltransferase, and nitrosoguanidine were purchased from Sigma Chemical Co. (U.S.A.). Carbopack B/4% carbowax 20 M/0.8% KOH was obtained from Supelco Chemical Co. (U.S.A.) and all other chemicals in this study were reagent grade.

Media and Culture

The isolation and culture medium consisted of 5.0 g of D-carnitine (or 10.0 g of DL-carnitine), 5.5 g of KH_2PO_4, 10.0 g of Na_2HPO_4, 2.0 g of $(NH_4)_2HPO_4$, 1.5 g of $NH_4H_2PO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 1.5 mg of $CaCl_2$, 0.06 mg of $Fe_2(SO_4)_3$, 0.02 mg of $ZnSO_4 \cdot 7H_2O$, 0.02 mg of $CuSO_4 \cdot 5H_2O$ and 0.02 mg of $MnSO_4 \cdot H_2O$ in 1 liter of distilled water with a pH of 6.8 adjusted with 4N NaOH (15). The medium for mutant selection was agar plates containing either 5 g/l D- or L-carnitine as the sole carbon and energy source. Stock cultures were grown on Luria-Bertani (LB) medium. The strains were grown for 16 h at 30°C with shaking (130 rpm/min) using a 1% inoculum cultured overnight at 30°C in 5 ml of LB broth.

Isolation of Strain and Mutant

Strains which assimilated D-carnitine as a sole carbon and energy source were isolated from the soil sample by enrichment culture, after cultivation in the isolation medi-

um. A strain having the ability to metabolize D-carnitine at a faster rate than L-carnitine was isolated by analyzing the residual amount of the L-carnitine in the medium after the logarithmic growth phase. The original medium contained 10 g/l of DL-carnitine. The mutants exhibiting a lessened L-carnitine decomposition rate were obtained by NTG mutagenesis. The bacterial culture was kept at 30°C for 30 minutes with a final concentration of 300 μg/ml of NTG and was treated with 1,000 unit/ml of penicillin G and 0.1 mg/ml of D-cycloserine for further enrichment (10). The desired mutant, which grew poorly in the medium containing 5 g/l of L-carnitine but grew rapidly on plates containing 5 g/l of D-carnitine, was selected by the replica plating method. The characterization of the isolated strain was carried out based on Bergey's Manual of Systematic Bacteriology (8th ed.) (3).

Analytical Methods

The activities of L-carnitine dehydrogenase (EC 1.1.1.108) and β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) were measured spectrophotometrically as described by Aurich *et al* (2) and Price *et al* (12), respectively. The analysis of DL-carnitine was performed by spectrophotometry and thin layer chromatography (1). The analysis of L-carnitine was carried out by high performance liquid chromatography with a Shodex C_{18} column (Shoko Co.) and UV detector at 220 nm (15) and enzymatic method using carnitine acetyltransferase (EC 2.3.1.7) specific to L-carnitine (11). Optical rotations were obtained in 10 cm cell at 589 nm (sodium line) and 25°C by using a JASCO Model DIP-370 polarimeter (Japan Spectroscopic Co.). A growth of strain was determined by measuring optical density of culture at 600 nm. Protein was measured by the method of Lowry *et al.*, using bovine serum albumin as the standard (9).

RESULTS AND DISCUSSION

Isolation of a Bacterium Assimilating D-Carnitine

For the resolution of DL-carnitine by a microorganism which is able preferentially to degrade the unnatural form, D-carnitine, microorganisms assimilating D-carnitine were primarily screened. First, 8 strains having the ability to metabolize D-carnitine at a faster rate were selected from 53 strains isolated from soil by enrichment culture, following cultivation in the isolation medium. To further select the strain which preferentially utilizes D-carnitine, above 8 strains were grown in culture medium containing 10 g/l of DL-carnitine for 16 h at 30°C, and cell growth and amount of residual L-carnitine in the medium were compared. Although the strain No. KC-006 left less L-carnitine than the strain No. KC-001 did, the strain KC-006 showed higher ability to metabolize D-carnitine based on the cell growth and optical yield (Table 1). According to optical resolution ability, the strain No. KC-006 was finally selected for breeding.

Table 1. Comparison of cell growth and residual L-carnitine in isolated microorganisms.

Strains	Cell growth (A_{660})	Residual L-carnitine (g/l)	L-Carnitine ratio (%)
KC-001	0.908	2.616	42.77
KC-002	0.882	2.357	37.09
KC-003	1.292	2.398	40.66
KC-004	1.379	1.061	54.11
KC-005	1.070	2.107	53.94
KC-006	1.387	1.768	61.07
KC-007	1.148	1.541	49.06
KC-008	0.948	1.889	54.15

Cultivations were carried out for 16 h at 30°C in the screening medium containing 10 g/l of DL-carnitine as sole carbon and energy source.

$$\text{L-Carnitine ratio (\%)} = \frac{\text{Residual L-carnitine}}{\text{Residual DL-carnitine}} \times 100.$$

Identification of the Isolated Strain No. KC-006

The isolated strain KC-006 was rod-shaped (1.3×3 μm) cell which showed single polar flagellum (Fig. 1) and was Gram-negative. It produced ivoric mucoid colonies on LB-agar medium and was facultative anaerobe. Most carbon compounds could be assimilated and the indole and oxidase tests were negative. The other physiological and biochemical characteristics of the isolated strain have been summarized in Table 2. Based on these results, the isolated strain KC-006 was partially identified as *Enterobacter* sp., referring to Bergey's Manual of Systematic Bacteriology (8th ed.).

Fig. 1. Electron micrograph of the isolated strain KC-006 by transmittance electron microscope (Sorvall TEM, 100 CX-II 80 KV, Japan) after staining with 2% phosphotungstic acid (pH 6.8).

Table 2. Physiological and biochemical characteristics of the isolated strain No. KC-006.

Assimilation of carbon compound:					
Glucose	+	Arabinose	+	N-acetylgucosamine	+
Mannitol	+	Maltose	+	Fructose	+
Citrate	+	Adonitol	+	Dulcitol	−
Mannose	+	Malate	+	Gluconate	+
Ribose	+	Glycerol	+	Glucuronic acid	+
Inositol	+	Succinate	+	Lactose	+
Lactate	−	Melibiose	−	Raffinose	+
Xylose	+	Rhamnose	+	Melezitose	+
Sucrose	+	Acetate	+		

Reduction of nitrate to nitrite	: +
Reduction of nitrite to nitrogen	: −
Indole production from tryptophan	: +
Hydrolysis of gelatin	: −
Hydrolysis of starch	: +
Arginine dihydrolase	: −
β-Galactosidase	: +
Oxidase	: −
Catalase	: +
Urease	: +
Methylred test	: −
OF test	Fermentative

*Symbols: +, positive; −, negative.

Isolation of Mutants Having Lessened L-Carnitine Decomposition Rates

To isolate mutants having lessened L-carnitine decomposition rates, mutagenesis of the strain No. KC-006 with 300 μg/ml of NTG was carried out for the reduction of the activities of L-carnitine dehydrogenase and β-hydroxybutyrate dehydrogenase in wild-type strain, *Enterobacter* sp. KC-006. The selected mutants were cultivated in medium containing 1% DL-carnitine after cultivation at 30°C for 16 h, by comparing the residual amount of L-carnitine and the L-carnitine ratio in the medium (Table 3). The mutant strain, NH-104, preferentially utilized

Table 3. Comparison of cell growth and residual L-carnitine in *Enterobacter* sp. KC-006 and mutant strains.

Strains	Cell growth (A_{660})	Residual L-carnitine (g/l)	L-Carnitine ratio (%)
Wild strain KC-006	1.425	1.853	60.89
Mutants			
NH-101	1.253	2.552	70.53
NH-102	1.537	1.924	67.27
NH-103	1.153	2.437	72.98
NH-104	1.387	2.252	81.03

Cultivations were carried out for 16 h at 30°C in the screening medium containing 10 g/l of DL-carnitine as sole carbon and energy source.

$$\text{L-Carnitine ratio (\%)} = \frac{\text{Residual L -carnitine}}{\text{Residual DL -carnitine}} \times 100.$$

Table 4. Comparision of cell growth and specific activities of L-carnitine dehydrogenase and β-hydroxybutyrate dehydrogenase in *Enterobacter* sp. KC-006 and its mutant, NH-104.

Strains	Cell growth (A_{660})	Specific activity (nmol/min·mg protein)	
		L-Carnitine dehydrogenase	β-Hydroxybutyrate dehydrogenase
Wild strain KC-006	1.458	442	115
Mutant strain NH-104	1.324	58	12

The specific activities of the enzymes were measured with cell-free extract of harvested cells after cells were cultivated for 16 h at 30°C in the screening medium containing 10 g/l of DL-carnitine as sole carbon and energy source.

D-carnitine, based on the increased 20% amount of remaining L-carnitine and the 25% L-carnitine ratio, when compared to the wild strain. To investigate the characteristics of mutant NH-104, changes of activity for L-carnitine dehyrogenase and β-hydroxybutyrate dehydrogenase were examined (Table 4). The specific activities of L-carnitine dehyrogenase and β-hydroxybutyrate dehydrogenase decreased 7.6-fold and 9.5-fold respectively, compared to those of the wild strain, which are much lower activity than those of *Pseudomonas putida* cultured in 0.5% DL-carnitine (7). Also, when mutant NH-104 was grown in medium containing 1.0% DL- or 0.5% D-carnitine, D-carnitine was preferentially assimilated without influence of L-car-

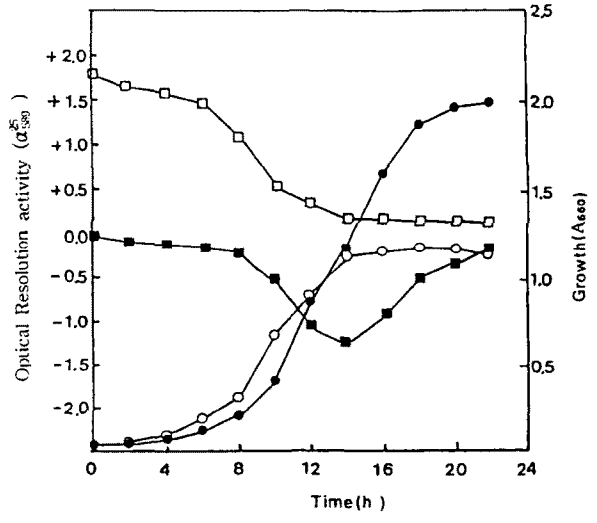

Fig. 2. Assimilation of D-carnitine by *Enterobacter* sp. NH-104.

Optical activities of D-carnitine (□—□) and DL-carnitine (■—■) were measured when cells were cultivated at 30°C in the screening medium 10 g/l of DL-carnitine (●—●) and 5 g/l of D-carnitine (○—○) as sole carbon source, respectively.

Table 5. Effect of substrate concentration on the resolution of DL-carnitine by *Enterobacter* sp. NH-104.

DL-Carnitine (g/l)	Cell growth (A_{660})	Residual L-carnitine (g/l)	L-Carnitine ratio (%)
5	0.940	0.932	96.52
10	1.360	2.275	80.17
20	1.582	6.854	79.02
30	0.783	13.544	54.21
40	0.042	19.253	51.17

Cultivations were carried out for 16 h at 30°C in the fermentation medium containing various concentrations of DL-carnitine.

$$\text{L-Carnitine ratio (\%)} = \frac{\text{Residual L -carnitine}}{\text{Residual DL -carnitine}} \times 100.$$

nitne (Fig. 2). These data indicated that the activities of the enzymes specific for the degradation of L-carnitine were reduced in mutant NH-104. This led to preferential degradation of D-carnitine from a DL-carnitine mixture, and the remaining L-carnitine had a high optical purity. Thus, the mutant, *Enterobacter* sp. NH-104 was finally selected to resolute L-carnitine from DL-carnitine by preferential degradation of D-carnitine.

Optimal Culture Conditions for Optical Resolution of DL-Carnitine

In order to optimize culture conditions for the resolution of DL-carnitine by *Enterobacter* sp. NH-104, the optimal concentration of DL-carnitine was primarily investigated. When cells of *Enterobacter* sp. NH-104 were cultivated in culture media containing DL-carnitine varied from 5.0 to 40.0 g/l, cell growth increased as DL-carnitine concentration increased up to 20 g/l (Table 5). However, the highest resolution of DL-carnitine was obtained at DL-carnitine concentration of 5.0 g/l. This implicated that D-carnitine was utilized in cell growth up to 20 g/l based on the L-carnitine ratio. 20 g/l of DL-carnitine was most effective for the resolution without inhibiting cell growth. It was reported that inorganic nitrogens were utilized in the metabolism of D-carnitine by *Psedomonas* sp. (8). The different inorganic nitrogens were added to the culture media at a C/N ratio of 42.5. The result is summerized in Table 6. *Enterobacter* sp. NH-104 utilized the ammonium form rather than the nitrate and nitrite form of nitrogen sources. $NH_4H_2PO_4$ was optimal nitrogen source among the ammonium forms for growth and resolution. The growth was proportional to the increase in C/N ratio to 25 and the optical purity was high at this ratio. However, the L-carnitine yield was high at the C/N ratio of 42.5 based on the cell growth and residual L-carnitine. For better growth in minimal medium, the various organic nitrogens as growth factors were added to the medium at 0.05% (w/v). Table 7 shows that yeast extract promotes 40% of the growth without change of optical purity compared to the control. In the case of casamino acid, the growth was high but the L-carnitine yield and ratio were reduced by 61.82%

Table 6. Effect of inorganic nitrogens on the resolution of DL-carnitine by *Enterobacter* sp. NH-104.

Nitrogen sources	C/N ratio	Cell growth (A_{660})	Residual L-carnitine (g/l)	L-Carnitine ratio (%)
$NH_4H_2PO_4$	10	1.375	6.431	75.61
	25	1.613	6.372	81.57
	42.5	1.532	6.987	78.53
	50	1.471	7.363	71.36
	100	0.986	7.674	72.89
$(NH_4)_2SO_4$	42.5	1.201	7.418	71.03
NH_4NO_3	42.5	1.237	7.123	72.58
NH_4Cl	42.5	1.018	7.382	64.41
$(NH_2)_2CO$	42.5	1.497	7.008	73.04
KNO_3	42.5	0.692	8.347	69.27
KNO_2	42.5	0.237	9.752	54.25

Cultivations were carried out for 16 h at 30°C in the fermentation medium containing 20 g/l of DL-carnitine and the other components.

$$\text{L-Carnitine ratio (\%)} = \frac{\text{Residual L -carnitine}}{\text{Residual DL -carnitine}} \times 100.$$

Table 7. Effect of organic nitrogens on the resolution of DL-carnitine by *Enterobacter* sp. NH-104.

Organic nitrogens (0.5 g/l)	Cell growth (A_{660})	Residual L-carnitine (g/l)	L-Carnitine ratio (%)
None	1.585	6.440	81.08
Yeast extract	2.305	6.654	78.52
Bactopeptone	2.089	4.745	67.61
Beef extract	2.256	5.064	75.66
Casamino acid	3.283	2.455	61.18
Corn steep liquor	2.499	6.134	64.43

Cultivations were carried out for 16 h at 30°C in the fermentation medium containing various growth factors, 20 g/l of DL-carnitine, 3.40 g/l of $NH_4H_2PO_4$ and the other components.

$$\text{L-Carnitine ratio (\%)} = \frac{\text{Residual L -carnitine}}{\text{Residual DL -carnitine}} \times 100.$$

Table 8. Effect of temperature on the resolution of DL-carnitine by *Enterobacter* sp. NH-104.

Temperature (°C)	Cell growth (A_{660})	Residual L-carnitine (g/l)	L-Carnitine ratio (%)
24	1.975	8.049	76.48
27	2.654	7.321	81.47
30	2.978	6.325	87.27
33	3.857	5.561	92.45
36	3.176	6.194	85.27

Cultivations were carried out for 16 h at 30°C in the fermentation medium (pH 6.4) containing 20 g/l of DL-carnitine, 3.40 g/l of $NH_4H_2PO_4$, 0.5 g/l of yeast extract, 400 μg/l of Ca^{2+} and the other components.

$$\text{L-Carnitine ratio (\%)} = \frac{\text{Residual L -carnitine}}{\text{Residual DL -carnitine}} \times 100.$$

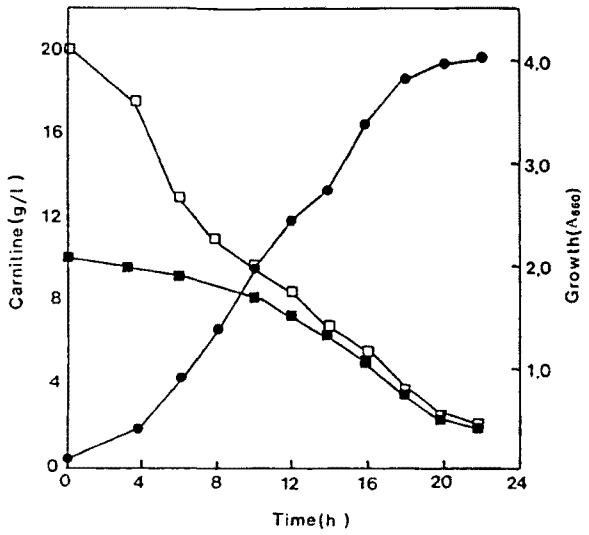

Fig. 3. Time course in the resolution of DL-carnitine by *Enterobacter* sp. NH-104.

Cultivation was carried out for 18 h at 33°C in the fermentation medium (pH 6.4) containing 20 g/l of DL-carnitine, 3.40 g/l of $NH_4H_2PO_4$, 0.5 g/l of yeast extract, 400 μg/l of Ca^{2+} and the other components. ●—●, Growth; □—□, DL-Carnitine; ■—■, L-Carnitine.

and 24.54%, respectively. Thus, yeast extract was used to obtain cells having the ability to decompose D-carnitine. To determine the optimal culture temperature, the temperature varied from 24°C to 36°C (Table 8). The growth increased as the temperature raised. At 36°C, the growth was high and the optical purity was considerably increased to 92.45, although the L-carnitine yield was slightly reduced. Under above optimal culture conditions, the change in optical resolution in the media was examined during cultivation. Fig. 3 shows that D-carnitine was rapidly metabolized in 10 h and then L-carnitine was degraded. When growth proceeded to the stationary phase, changes in optical resolution were minimal. After 18 h in culture, residual L-carnitine and optical purity were 3.530 g/l and 97.88%, respectively. It is noted that D-carnitine was preferentially metabolized for the cell growth up to stationary phase and then L-carnitine was utilized as a carbon and energy source.

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