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Novel Metabolic Transformation Pathway for Cyclic Imides in *Blastobacter* sp. Strain A17p-4

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The metabolic transformation pathway for cyclic imides in microorganisms was studied in *Blastobacter* sp. strain A17p-4. This novel pathway involves, in turn, hydrolytic ring opening of a cyclic imide to yield a monoamidated dicarboxylate, hydrolytic deamidation of the monoamidated dicarboxylate to yield a dicarboxylate, and dicarboxylate transformation similar to that in the tricarboxylic acid cycle. The initial step is catalyzed by a novel enzyme, imidase. Imidase and subsequent enzymes involved in this metabolic pathway are induced by some cyclic imides, such as succinimide and glutarimide. Induced cells metabolize various cyclic imides.

Two cyclic-ureide transformation pathways have been well characterized. One is dihydropyrimidine transformation (Fig. 1A) (11, 15), and the other is hydantoin transformation (Fig. 1B) (13). Cyclic imides have structures similar to those of cyclic ureides and are known to be hydrolyzed by mammalian dihydropyrimidinase, which functions in pyrimidine metabolism (3–5). However, there have been no reports on the microbial transformation of cyclic imides.

The microbial transformation of cyclic ureides involves reactions useful for the production of optically active compounds. The best-known example is optically active amino acid production by hydantoin-transforming microorganisms (16). *Blastobacter* sp. strain A17p-4, which is a gram-negative, nonmotile, non-spore-forming, obligatorily aerobic, nonfermentative rod, was isolated by us as a competent strain for D-amino acid production from 5-monosubstituted hydantoin (6). The strain is good material for the analysis of enzymes involved in cyclic-ureide transformation because of its high activity.

During the course of enzymatic analysis of hydantoin transformation in this bacterium (8), we found the transformation of not only diverse cyclic ureides but also cyclic imides in the bacterium (8). That was the first report on the microbial transformation of cyclic imides, and we discovered that the initial step of the transformation is catalyzed by a novel enzyme, imidase, which is different from known cyclic-ureide-transforming enzymes (7). Imidase catalyzes the hydrolysis of a cyclic imide to a monoamidated dicarboxylate.

We investigated the metabolic fate of cyclic imides in *Blastobacter* sp. strain A17p-4 and found that monoamidated dicarboxylates were further transformed. In this report, we describe the presence of a novel metabolic transformation pathway for cyclic imides in this bacterium.

MATERIALS AND METHODS

Chemicals. 2-Methylsuccinimide was synthesized from methylsuccinimide and aqueous ammonia (1). All of the other chemicals used were of analytical grade and are commercially available.

Microorganism and media. *Blastobacter* sp. strain A17p-4 (6) was used for all experiments. The nutrient medium used comprised 1.5 g of uracil, 1 g of KH_2PO_4, 1 g of K_2HPO_4, 0.3 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $FeSO_4 \cdot 7H_2O$, 3 g of yeast extract, 3 g of meat extract, 2 g of peptone, and 10 g of glycerol in 1 liter of tap water, pH 7.0. The minimum liquid medium used comprised 1 g of KH_2PO_4, 1 g of K_2HPO_4, 0.3 g of $MgSO_4 \cdot 7H_2O$, 2 g of NH_4Cl, 1 mg of thiamine hydrochloride, 2 mg of riboflavin, 2 mg of nicotinic acid, 2 mg of pantothenic acid, 2 mg of pyridoxine hydrochloride, 0.1 mg of biotin, 1 mg of *p*-aminobenzoate, and 0.1 mg of folic acid in 1 liter of deionized water, pH 7.0.

Investigation of the succinimide metabolic pathway. Cells were cultured in the nutrient medium at 28°C for 4 days and then harvested by centrifugation at 14,000 × *g* for 10 min. The cells were washed with physiological saline and then centrifuged. The reaction mixture comprised 1% (wt/vol) washed cells, 20 μmol of Tris/HCl (pH 7.5), and 4 μmol of succinimide, as a substrate, in a total volume of 200 μl. The reaction was carried out at 30°C with shaking for 4 h. The reaction mixture was sampled at 1-h intervals, and the samples were analyzed by two independent high-performance liquid chromatography (HPLC) systems: (i) at 210 nm on a Cosmosil $5C_{18}$-packed column (4.6 by 250 mm; Nacalai Tesque, Kyoto, Japan) with 25 mM potassium phosphate buffer (pH 7.0) containing 1 mM *n*-octylamine as the mobile phase at a flow rate of 0.3 ml/min and at 22°C (14) and (ii) at 210 nm on a Cosmosil $5C_{18}$-packed column (4.6 by 250 mm; Nacalai Tesque) with 250 mM KH_2PO_4 (pH 4.2) at a flow rate of 1.0 ml/min and at 30°C. Quantification was based on the peak area.

Induction of succinimide-transforming enzymes. *Blastobacter* sp. strain A17p-4 was cultured in minimum liquid medium containing 20 mM sucrose or succinimide as the sole carbon source at 28°C for 4 days. The reaction conditions and analytical methods were the same as those described above, except that succinimide, succinamic acid, succinate, or fumarate was used as the substrate.

Cultivation with various cyclic imides as the sole carbon source. *Blastobacter* sp. strain A17p-4 was cultured in minimum liquid medium containing various cyclic imides or ureides at 20 mM as the sole carbon source at 28°C. Minimum

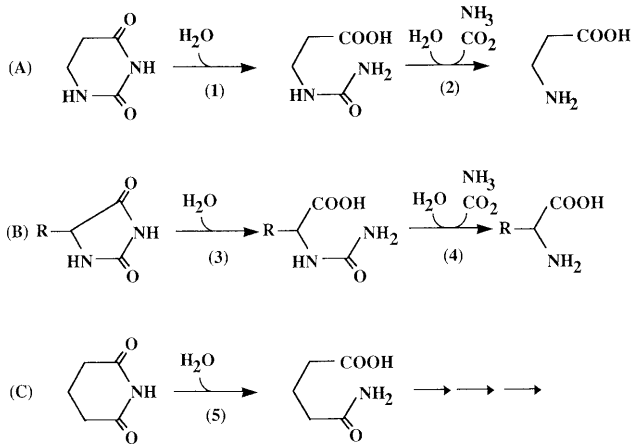

FIG. 1. Metabolic transformation pathways for dihydropyrimidine (A), hydantoin (B), and cyclic imides (C). Enzymes: 1, dihydropyrimidinase; 2, β-ureidopropionase; 3, hydantoinase; 4, *N*-carbamoyl amino acid amidohydrolase; 5, imidase.

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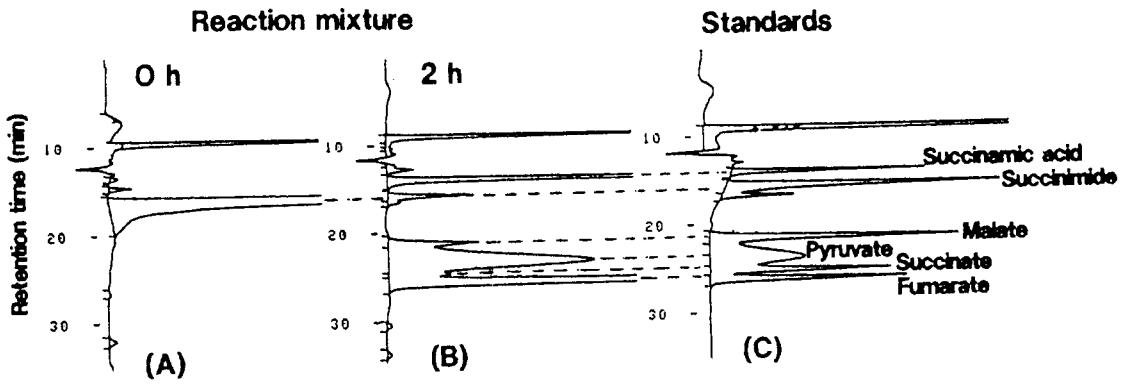

FIG. 2. Identification of intermediates involved in the succinimide metabolic transformation pathway in *Blastobacter* sp. strain A17p-4. The HPLC profiles of a 0-h reaction mixture (A), a 2-h reaction mixture (B), and standards (C) are shown. The reaction and analysis (method i) were carried out as described in Materials and Methods.

liquid medium without any supplements was used for control experiments. Growth was monitored by measuring the optical density at 610 nm of the culture broth.

Decomposition of various cyclic imides. Cells cultivated in minimum liquid medium supplemented with 20 mM succinimide at 28°C for 4 days were used. The reaction conditions were the same as those described above, except that various cyclic imides and ureides were used as the substrate. The reaction mixtures were analyzed by HPLC at 210 nm on a Cosmosil $5C_{18}$-packed column (4.6 by 250 mm) with CH_3CN-H_2O-H_3PO_4 (80:20:0.01, by volume) as the mobile phase for phthalimide, and with 250 mM KH_2PO_4 as the mobile phase for the others, at a flow rate of 1.0 ml/min.

RESULTS

A novel degradation pathway for cyclic imides in *Blastobacter* sp. strain A17p-4. By using washed cells of *Blastobacter* sp. strain A17p-4 grown in nutrient medium, the metabolic fate of succinimide, a model cyclic imide, was investigated. During the course of the reaction, several metabolites were detected. These metabolites were identified as succinamic acid, succinate, fumarate, malate, and pyruvate by comparison of their retention times with those of various standards by two independent HPLC systems. Figure 2 shows an example of an elution profile obtained with one system (method i). Following the consumption of succinimide, the production of these compounds increased. Therefore, these compounds derived from succinimide (Fig. 3). These results indicate the presence of a novel transformation pathway of succinimide in this bacterium (Fig. 4). Some parts of this pathway are the same as those involved in the tricarboxylic acid cycle.

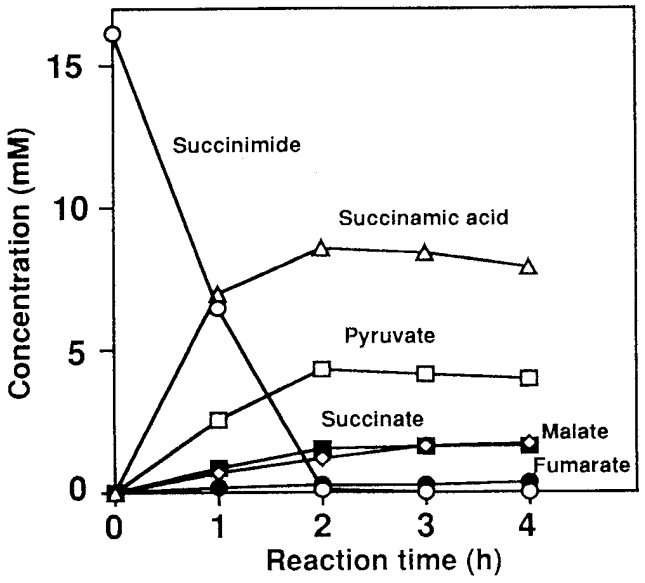

FIG. 3. Time course of succinimide metabolic transformation by *Blastobacter* sp. strain A17p-4. The concentrations are averages of two separate determinations by HPLC assay method i that were reproducible within ±10%.

Induction of succinimide-degrading enzymes. *Blastobacter* sp. strain A17p-4 grows in minimum liquid medium supplemented with sucrose or succinimide as the sole carbon source. After 4 days of cultivation in sucrose or succinimide medium at 28°C, the cells were used for the reaction with succinimide, or one of its degradation intermediates, as the substrate (Table 1). All activities in the succinimide-grown cells were higher than those in the sucrose-grown cells; the enzymes involved in cyclic-imide transformation were induced by succinimide. In particular, succinamic acid-hydrolyzing (amidase) activity was significantly induced by succinimide. This result showed that not only imidase (7) but also amidase plays an important role in succinimide degradation. The induction of successive enzymes contributes to the utilization of cyclic imides for cell growth.

Utilization of various cyclic imides as a sole carbon source. *Blastobacter* sp. strain A17p-4 was cultured in a minimum liquid medium containing a cyclic imide or ureide, such as succinimide, glutarimide, maleimide, hydantoin, dihydrouracil, 2,4-thiazolidinedione, rhodanine, parabanic acid, thiohydantoin, or pseudohydantoin, as the sole carbon source. The bacterium grew only in medium containing succinimide or glutarimide (Fig. 5). These results suggest that among the

TABLE 1. Induction of cyclic-imide-transforming enzymes in *Blastobacter* sp. strain A17p-4

Substrate	Sp act of resting cells[a] (μmol/min/g of wet cells)	
	Sucrose-grown cells	Succinimide-grown cells
Succinimide	11	86
Succinamic acid	0.12	9.4
Succinate	0.24	2.8
Fumarate	1.3	6.5

[a] Specific activities of cells are averages of three separate determinations that were reproducible within ±10%.

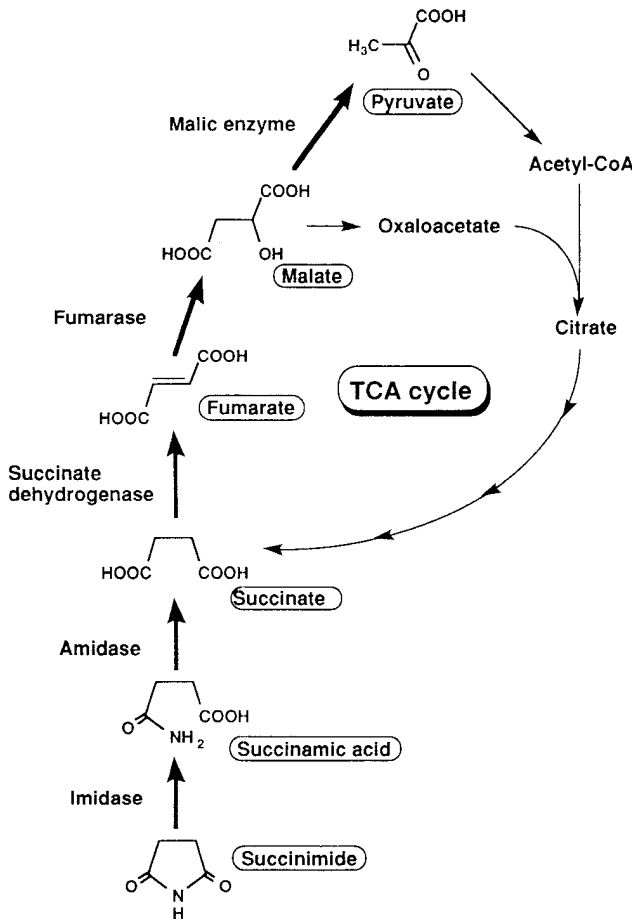

FIG. 4. Proposed pathway for succinimide metabolic transformation in *Blastobacter* sp. strain A17p-4. TCA, tricarboxylic acid; CoA, coenzyme A.

compounds examined, the successive enzymes can be induced only by these two cyclic imides or that if they are induced, the substrate specificities of the successive enzymes are restricted to these two imides for full decomposition corresponding to cell growth.

Metabolism of various cyclic imides by succinimide-grown cells. The metabolism of various cyclic imides other than succinimide by succinimide-grown cells was investigated (Table 2 and Fig. 6). The cells metabolized cyclic imides with structures similar to that of succinimide, such as maleimide, 2-methylsuccinimide, and glutarimide. In maleimide transformation, fumarate, malate, and pyruvate were detected as metabolites. In 2-methylsuccinimide transformation, 2-methylsuccinate and mesaconate were detected as metabolites. In glutarimide transformation, glutaramic acid and glutarate were detected as metabolites. These results indicate that the same metabolic pathway as for succinimide transformation is involved in the transformation of structurally related imides. On the other hand, phthalimide, which has an aromatic group in its ring structure, was metabolized at a very low rate.

In addition to simple cyclic imides, sulfur-containing imides (e.g., 2,4-thiazolidinedione and rhodanine) and cyclic ureides (e.g., hydantoin and dihydrouracil) are also metabolized.

DISCUSSION

The metabolism of cyclic imides has been studied in mammals in relation to the detoxication of the antiepileptic agents ethotoin and phensuximide (2). Dihydropyrimidinase, which is involved in pyrimidine metabolism, functions in cyclic-imide hydrolysis in mammals (17). However, there had been no reports on the microbial transformation of cyclic imides until our work. We first reported the hydrolysis of cyclic imides in microorganisms and discovered that it is catalyzed by a novel enzyme, imidase. In this study, we further investigated the metabolic fate of cyclic imides in a bacterium, *Blastobacter* sp. strain A17p-4, and showed a novel metabolic pathway. This pathway involves the hydrolytic ring opening of a cyclic imide to yield a monoamidated dicarboxylate, hydrolytic deamidation of the monoamidated dicarboxylate to yield a dicarboxylate, and dicarboxylate transformation similar to that in the tricarboxylic acid cycle. For example, succinimide is first hydrolyzed by imidase to succinamic acid and the succinamic acid is deamidated by amidase to succinate. The succinate then enters the tricarboxylic acid cycle and is transformed to fumarate, malate, and pyruvate, in that order (Fig. 4). Some related cyclic imides are also transformed through this pathway. The initial step of this pathway is catalyzed by a novel enzyme, imidase (7), and the next step is catalyzed by amidase. These two enzymes and successive enzymes are induced by some cyclic imides. This suggests that the bacterium produces these enzymes to use cyclic imides as energy sources.

The novel cyclic-imide metabolic pathway is very similar to dihydropyrimidine transformation and also hydantoin transformation. All of these pathways involve ring-opening hydrolysis and successive amide hydrolysis. We have already purified some enzymes involved in these pathways (8–13) and proved that specific enzymes contribute to each transformation. The enzymes catalyzing the initial step of each pathway, i.e., dihy-

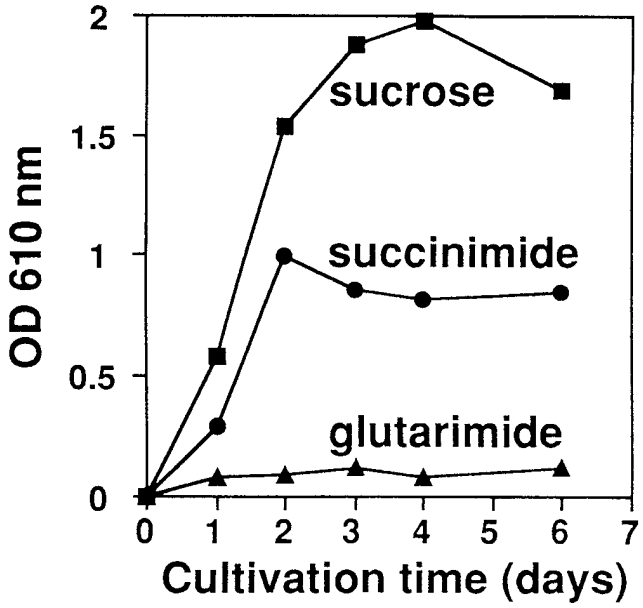

FIG. 5. Growth of *Blastobacter* sp. strain A17p-4 cultivated in minimum liquid medium supplemented with sucrose, succinimide, or glutarimide as the sole carbon source. The optical density (OD) values are averages of three separate determinations that were reproducible within ±10%.

TABLE 2. Metabolic transformation of various cyclic imides by *Blastobacter* sp. strain A17p-4

Compound[a]	Relative consumption rate (%)[b]
a, succinimide	100
b, maleimide	95
c, 2-methylsuccinimide	20
d, glutarimide	99
e, phthalimide	2.7
f, 2,4-thiazolidinedione	110
g, rhodanine	69
h, hydantoin	93
i, dihydrouracil	29

[a] The structures of the compounds are shown in Fig. 6.

[b] The rate of succinimide consumption (86 μmol/min/g of wet cells) was taken as 100%. The relative rates shown are averages of three separate determinations that were reproducible within ±10%.

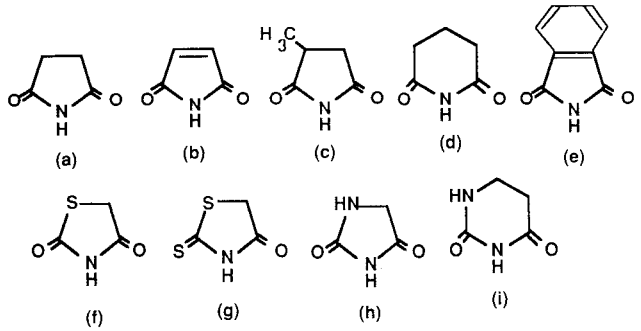

FIG. 6. Structures of compounds a to i in Table 2.

dropyrimidinase for cyclic ureides of dihydropyrimidine and hydantoin and imidase for cyclic imides, are distinct from each other in *Blastobacter* sp. strain A17p-4 (7). Further analysis of the successive enzymes involved in microbial cyclic-imide transformation will make clear the differences of cyclic-imide and cyclic-ureide metabolic transformations.

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