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Cyclic ureide and imide metabolism in microorganisms producing a D-hydantoinase useful for D-amino acid production

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Abstract

The microbial transformation of DL-5-monosubstituted hydantoins has been applied to industrial scale production of optically active amino acids. Hydantoinase and *N*-carbamoyl amino acid amidohydrolase, which are the key enzymes in this transformation, from various microorganisms have been studied extensively. *Blastobacter* sp. A17p-4, which was isolated for D-amino acid production through hydantoin transformation, shows not only diverse cyclic ureide-metabolizing activities including those of D-hydantoinase and *N*-carbamoyl-D-amino acid amidohydrolase, but also cyclic imide-metabolizing activities. A recent study revealed the participation of D-hydantoinase in the metabolism of cyclic imides and the existence of novel enzymes, imidase and half-amidase, in this bacterium. D-hydantoinase functions in the metabolism of bulky cyclic imides, while imidase functions in that of simple cyclic imides in combination with half-amidase, which functions in the hydrolysis of the imidase reaction products, half-amides. Imidase and half-amidase are different from reported cyclic-amide-metabolizing enzymes, and are widely found in bacteria, yeasts and molds.

Keywords: Cyclic ureide; Cyclic imide; Hydantoinase; Imidase; Half-amidase

1. Introduction

In nature, cyclic ureide transformation is generally related to nucleic acid base-transformation, which comprises the catabolism and anabolism of pyrimidines and purines [1]. As examples of enzymes, the transformations involve dihydropyrimidinase in reductive pyrimidine metabolism [1,2], barbiturase in oxidative pyrimidine metabolism [3,4], dihydroorotate in pyrimidine biosynthesis [5,6], and allantoinase in purine metabolism [7]. Among these enzymatic activities, the cyclic ureide hydrolysis of five-membered ring hydantoins catalyzed by dihydropyrimidinase has gained enormous attention due to attractive industrial applications for the production of optically active amino acids, especially D-amino acids [8–10].

Blastobacter sp. A17p-4 was screened from soil for the purpose of D-amino acid production from DL-5-monosubstituted hydantoins. During the course of studies on the cyclic ureide metabolism including that of hydantoins in this bacterium, it was found that the bacterium showed not only cyclic ureide but also cyclic imide metabolizing activities [11]. The study of the metabolism of cyclic imides, which are structurally similar to cyclic ureides, is relatively new and it is not fully understood yet. Efforts have been made to clarify cyclic imide metabolism and to elucidate the physiological function of this metabolic activity in comparison with other known cyclic amide metabolisms. This review focuses on the finding of cyclic imide

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metabolism in a D-hydantoinase-producing strain, *Blastobacter* sp., and the enzymes involved in cyclic imide metabolism.

2. D-hydantoinase, its cyclic ureide- and cyclic imide-hydrolyzing activities

Owing to their ability of stereospecific resolution, microbial hydantoinases have been successfully applied to the commercial production of optically active amino acids from racemic 5-monosubstituted hydantoins [12–15]. Three types of enzymes, i.e. D-enantiomer-specific [13,16,17], L-enantiomer-specific [18], and non-specific [19] hydantoinases, are involved in hydantoin hydrolysis. This process is very similar to dihydropyrimidine hydrolysis. It has been proposed that D-enantiomer specific hydantoin hydrolysis and dihydropyrimidine hydrolysis are catalyzed by the same enzyme [13,20], while some exceptions of specific enzymes for hydantoin hydrolysis have been reported [21,22].

Blastobacter sp. A17p-4, which is a gram-negative, non-motile, non-spore-forming, obligatorily aerobic, nonfermentative rod, shows high D-enantiomer-specific hydantoinase (D-hydantoinase) activity [23]. The physicochemical properties suggested that the D-hydantoinase of this bacterium are identical to those of dihydropyrimidinase, which functions in reductive pyrimidine metabolism (Table 1).

The D-hydantoinase was purified and its substrate specificity was studied in detail [24]. Besides its conventional substrates, cyclic ureides, the D-hydantoinase also hydrolyzed cyclic imides (Table 2). The enzyme also catalyzed the reverse reaction, i.e. the cyclization of half-amides to cyclic imides [24]. This newly found catalytic activity suggested that the D-hydantoinase may also be involved in cyclic imide metabolism [24]. Mammalian dihydropyrimidinases have been reported to hydrolyze cyclic imides, and to show a wide substrate spectrum including simple and bulky cyclic imides and cyclic ureides [25–27]. These mammalian enzymes are tetramers and their NH_2-terminal amino acid sequences show good homology with those of bacterial D-hydantoinases including the *Blastobacter* D-hydantoinase. The similarities in structure, genomic sequence and catalytic function of these two groups of enzymes suggest that they are phylogenetically related and that they form a gene superfamily related to ureases [28–30].

Table 1
Properties of D-hydantoinase, imidase and half-amidase from *Blastobacter* sp. A17p-4

Properties	D-hydantoinase	Imidase	Half-amidase
Native Mr	200000	105000	48000
Subunit Mr (SDS-PAGE)	53000	35000	46000
Number of subunits	4	3	1
Substrate specificity			
Most active	Cyclic ureides	Simple cyclic imides	Half-amides
Mildly active	Bulky cyclic imides	Sulfur-containing cyclic imides	Middle chain amides
		Non-substituted cyclic ureides	Aromatic amides
Optimum pH for			
Hydrolysis	9.0–10.0	7.5–8.0	9.4–10.0
Cyclizing dehydration	5.0	6.5	–
Optimum temperature	60°C	60°C	35°C
pH stability	5.0–8.5	6.0–9.0	7.5–10.5
Thermal stability	<60°C	<60°C	<35°C
Metal ion requirement	Activation (Ni^{2+}, Co^{2+}, Mn^{2+})	(Co^{2+})	None
Inhibitor	SH-inhibitors	SH-inhibitors	SH-inhibitors
	Hg^{2+}	Cu^{2+}, Zn^{2+}, Ag^{+}, Hg^{2+}	Cu^{2+}, Zn^{2+}, Ag^{+}, Hg^{2+}
		Serine protease inhibitor	Serine protease inhibitors
		Metal ion chelators	

Table 2
Substrate specificity of D-hydantoinase from *Blastobacter* sp. A17p-4

Substrate	Relative activity (%)	K_m (mM)	V_{max} (μmol/min/mg)	V_{max}/K_m
Cyclic ureides				
Dihydrouracil	100	2.6	2.5	0.95
Dihydrothymine	73	8.8	2.2	0.25
Hydantoin	140	3.2	1.1	0.36
DL-5-methylhydantoin	54	11	1.8	0.16
DL-5-(2-methylthioethylene)-hydantoin	48	9.2	0.58	0.063
DL-5-phenylhydantoin	29	11	1.2	0.12
DL-5-(*p*-hydroxyphenyl)-hydantoin	1.6	0.62	0.020	0.032
Cyclic imides				
2-Methylsuccinimide	9.6	5.0	0.23	0.046
2-Phenylsuccinimide	5.2	6.7	0.15	0.023
Phthalimide	2.1	2.1	0.077	0.037
3,4-Pyridine dicarboximide	9.6	9.4	1.02	0.11

3. Cyclic imide transformation pathway in *Blastobacter* sp. A17p-4

Based on the finding of cyclic imide-hydrolyzing activity of the D-hydantoinase, the metabolism of various cyclic imides by *Blastobacter* sp. was investigated. The fact that *Blastobacter* sp. grows well in a synthetic minimum medium containing succinimide as a sole source of carbon indicates that the bacterium has a metabolic system for the assimilation of simple cyclic imides as energy sources and nutrients [31]. The bacterium can metabolize various simple cyclic imides with structures similar to that of succinimide such as maleimide, 2-methylsuccinimide and glutarimide, and sulfur-containing cyclic imides such as 2,4-thiazolidinedione and rhodanine, other than the bulky cyclic imide of phthalimide, which is a substrate of the D-hydantoinase [24,31]. These results indicated that the bacterium produces a simple cyclic imide-hydrolyzing enzyme other than the D-hydantoinase. Further investigation of the metabolic fate of these cyclic imides showed that they were metabolized through a novel metabolic pathway (Fig. 1). This pathway involves in turn the hydrolytic ring-opening of cyclic imides to half-amides, hydrolytic deamidation of the half-amides to dicarboxylates, and dicarboxylate transformation similar to that in the tricarboxylic acid (TCA) cycle. Two novel enzymes, imidase and half-amidase, and D-hydantoinase were found to function in this pathway. In the first step of this metabolic pathway, imidase is involved in the hydrolysis of simple cyclic imides, and D-hydantoinase is involved in the hydrolysis of bulky cyclic imides. The half-amide products were successively hydrolyzed by half-amidase in the second step of the pathway, followed by TCA cycle-like transformation [31]. The cyclic imide metabolism has practical potential for stereo- and regiospecific production of half-amides and dicarboxylates, and also the production of high-value organic acids such as pyruvate from cyclic imides.

4. Enzymes involved in cyclic imide metabolism

4.1. Imidase

Imidase, which was named according to its high activity toward cyclic imides, especially simple ones, was first purified and characterized from *Blastobacter* sp. (Table 1) [32]. This enzyme is involved in the first step of cyclic imide metabolism, catalyzing the ring-opening hydrolysis of cyclic imides to half-amides and also the reverse reaction, i.e. the cyclization of half-amides to cyclic imides [32]. It is a trimer with a relative molecular mass of 105,000, and its NH_2-terminal amino acid sequence shows no homology with other known cyclic amide-metabolizing enzymes. Imidase exhibited higher activity and affinity toward simple cyclic imides, which are not

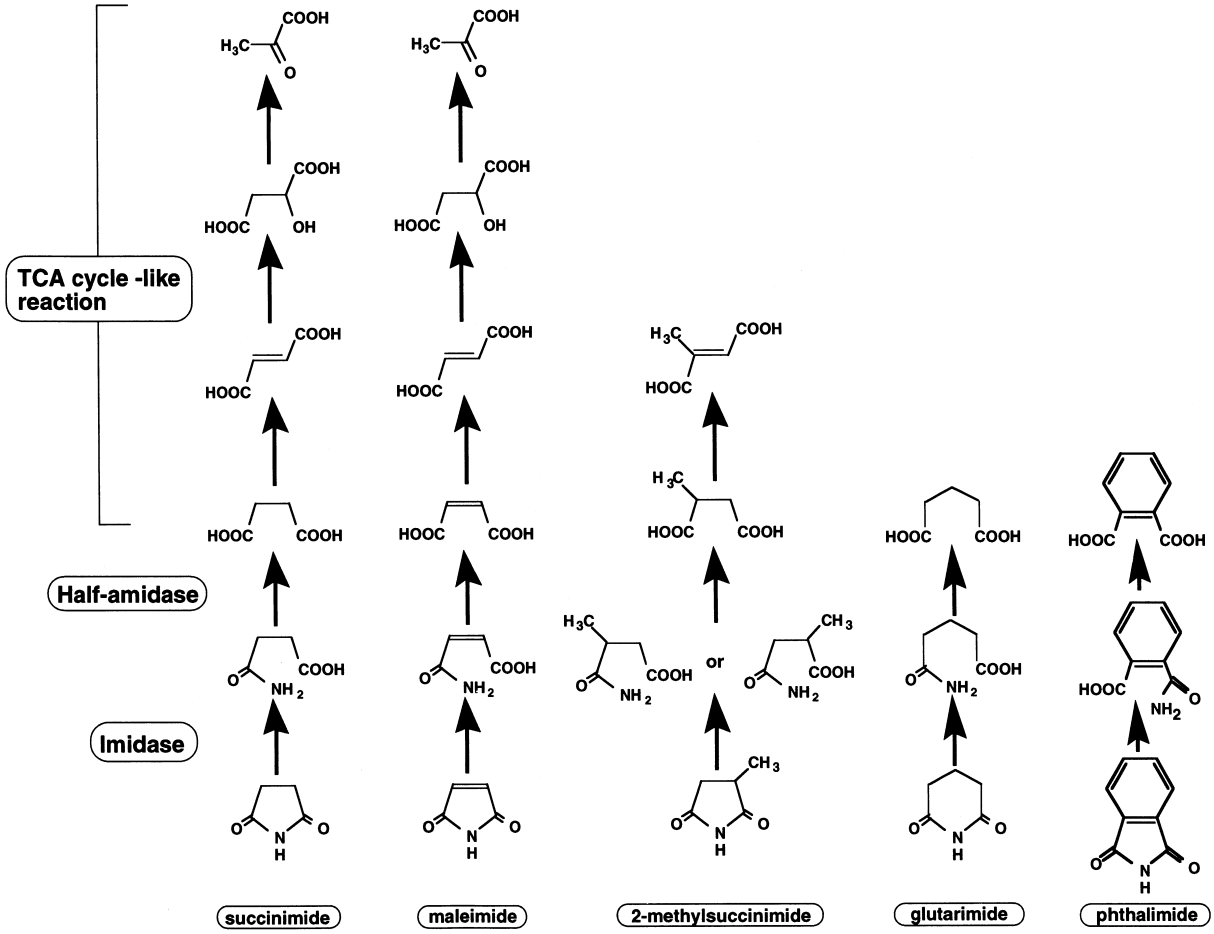

Fig. 1. Novel metabolic pathway for cyclic imides.

hydrolyzed by the D-hydantoinase, than toward the cyclic ureides of dihydrouracil and hydantoin (Table 3). Imidase is also active toward sulfur-containing cyclic imides such as 2,4-thiazolidinedione and rhodanine (Table 3). However, bulky cyclic imides or monosubstituted cyclic ureides, which are the substrates of D-hydantoinase or dihydropyrimidinase, were not hydrolyzed. Imidase is, therefore, different from bacterial D-hydantoinases or mammalian dihydropyrimidinases in structure and substrate specificity, and seems to have a specific function in cyclic imide metabolism.

Imidase activity is inhibited non-competitively by succinate, an interlinkage compound between cyclic imide metabolism and the TCA cycle, with a K_i value of 1.44 mM, suggesting that the enzyme activity is down-regulated by the metabolite of the pathway in relation with the TCA cycle [31,32].

4.2. Half-amidase

Half-amidase, which was named according to its high activity toward half-amides, was first purified and characterized from *Blastobacter* sp. (Table 1) [33]. This enzyme is involved in the second step of cyclic imide metabolism, catalyzing the irreversible hydrolytic deamidation of half-amides to dicarboxylates [33]. It is a monomer with a relative molecular mass of 48,000, and its NH_2-terminal amino acid sequence shows no homology to known cyclic

Table 3
Substrate specificity of imidase from *Blastobacter* sp. A17p-4[a]

Substrate	Relative activity (%)	K_m (mM)	V_{max} (μmol/min/mg)	V_{max}/K_m
Cyclic ureides				
Dihydrouracil	100	52	240	4.7
Hydantoin	110	5.7	34	6.0
Parabanic acid	100	62	370	6.0
Cyclic imides				
Succinimide	530	0.94	910	970
Glutarimide	630	4.5	1000	220
Maleimide	5800	0.34	5800	17000
2-Methylsuccinimide	150	nd	nd	nd
Sulfur-containing cyclic amides				
2,4-Thiazolidinedione	79	33	160	4.7
Rhodanine	680	31	1700	55
Thiohydantoin	68	23	130	5.8
Pseudothiohydantoin	7.4	nd	nd	nd

[a] nd = not determined.

amide-metabolizing enzymes or amidases. The enzyme exhibited the highest catalytic efficiency toward half-amides (Table 4), and did not act on amides, which are conventional substrates of known amidases, e.g. acyl amidase, aryl amidase, amino peptidase, asparaginase, glutaminase, *N*-carbamoyl amino acid amidohydrolase, urea amidohydrolase, allatoinase and ω-amidase. The narrow catalytic spectrum of half-amidase suggested that the enzyme specifically functions in the metabolism of cyclic imides together with imidase.

5. Physiological functions of imidase and half-amidase in cyclic imide metabolism

The production of imidase and half-amidase was enhanced in cyclic imide-grown cells compared to in sucrose-grown cells [31]. The enhancement of imidase and half-amidase production was further investigated at the gene expression level using an RNA synthesis inhibitor, actinomycin D (Fig. 2) [33]. The addition of a cyclic imide enhanced the expression of imidase and half-amidase, but when actinomycin D was added

Table 4
Substrate specificity of half-amidase from *Blastobacter* sp. A17p-4

Substrate	Relative activity (%)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\,mM^{-1}$)
Half-amides				
Succinamic acid	100	6.2	5.76	0.93
Glutaramic acid	64	2.8	2.23	1.62
Adipinamic acid	41	8.0	0.36	0.046
Middle chain amides				
Lactamide	12	3.7	0.11	0.033
n-Valeramide	11	5.1	0.18	0.037
n-Caproamide	25	17.0	0.18	0.011
Crotoamide	8	8.8	0.10	0.012
Aromatic amides				
Benzamide	8	6.5	0.13	0.020
2-Phenyl propioamide	11	3.1	0.078	0.026

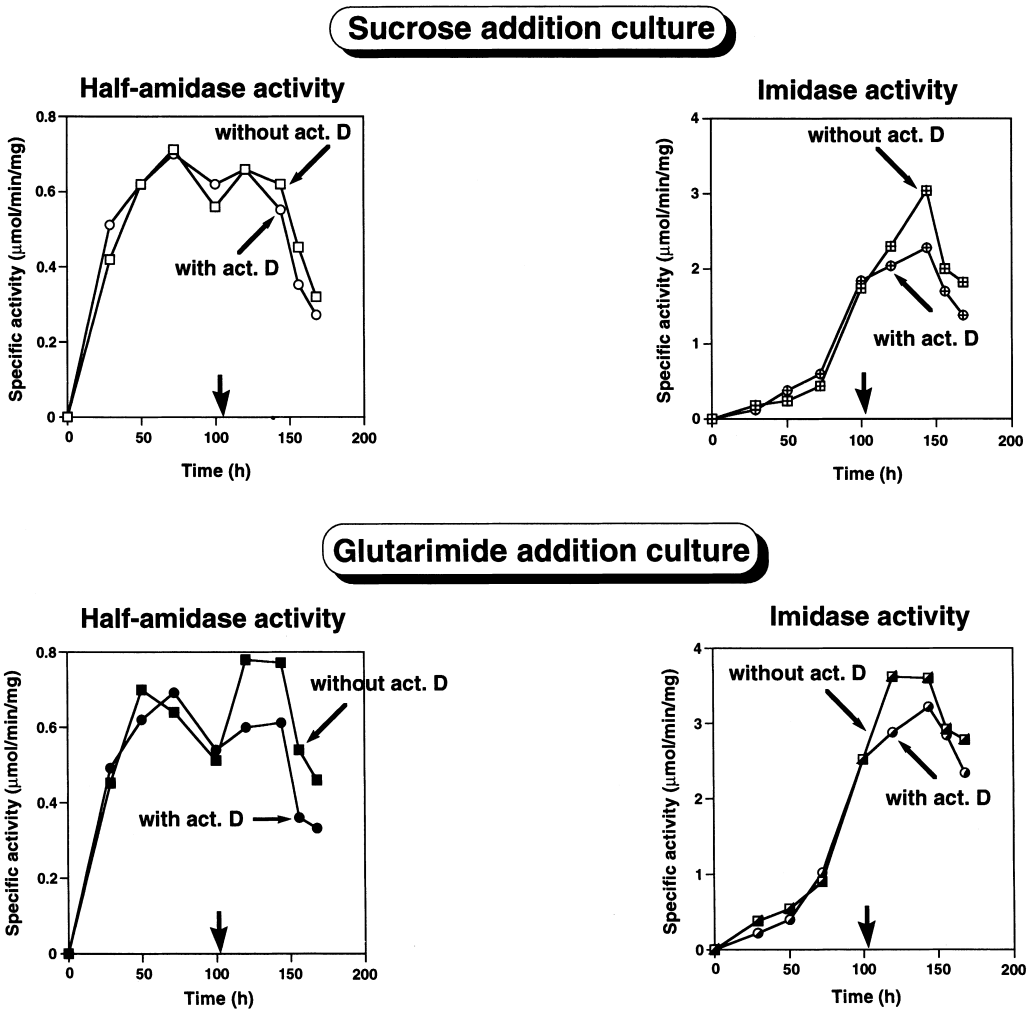

Fig. 2. Effects of a cyclic imide (glutarimide) and an RNA synthesis inhibitor, actinomycin D, on the expression of imidase and half-amidase. Four sets of cultivations, A–D, were carried out with the synthetic minimum liquid medium supplemented with 20 mM sucrose as the sole source of carbon. At the end of exponential growth (107 h), cultures A and C were supplemented with 20 mM sucrose or 20 mM glutarimide, respectively, while cultures B and D were supplemented with 20 mM sucrose or 20 mM glutarimide, respectively, together with the addition of actinomycin D to a final concentration of 1 μg/ml. At intervals, the half-amidase and imidase activities were assayed with a cell free-extract, as described previously [33]. The diagrams represent the time-courses of the half-amidase activity and imidase activity of a sucrose addition culture and a glutarimide addition culture, respectively. Addition of sucrose or glutarimide without or with actinomycin D are indicated by thick-arrow. Set A (sucrose addition without actinomycin D), (□) and (⊞); Set B (sucrose addition with actinomycin D), (○) and (⊕); Set C (glutarimide addition without actinomycin D), (■) and (◪); Set D (glutarimide addition with actinomycin D), (●) and (◒).

together with the cyclic imide, no enhancement of the production of these enzymes was observed. This was due to the inhibition of RNA synthesis, prevented the gene expression of imidase and half-amidase (Fig. 2, glutarimide addition culture). No significant enhancement of the production of these enzymes was observed on the addition of sucrose in a control experiment (Fig. 2, sucrose addition culture). These results showed that imidase gene expression and particularly half-amidase gene expression are up-regulated by

cyclic imides. All these results imply that imidase and half-amidase are produced for cyclic imide utilization [31–33].

6. Distribution of cyclic imide-metabolizing activities in microorganisms

The distribution of cyclic imide and cyclic ureide metabolism was investigated in microorganisms [34]. Besides the well-known cyclic ureide metabolism, cyclic imide metabolism is also common and widely distributed among bacteria, yeasts and molds (Fig. 3) [34]. In bacteria, the distributions of these two metabolizing activities showed no relation with each other, while they are apparently related in yeasts and molds. Yeasts and molds show lower cyclic imide-metabolizing activity than bacteria. Besides *Blastobacter* sp., some bacteria (*Bacillus*, *Arthrobacter* and *Pseudomonas*), yeasts (*Saccharomyces*), and molds (*Penicillium* and *Fusarium*) are able to grow on cyclic imides as sole sources of carbon, suggesting

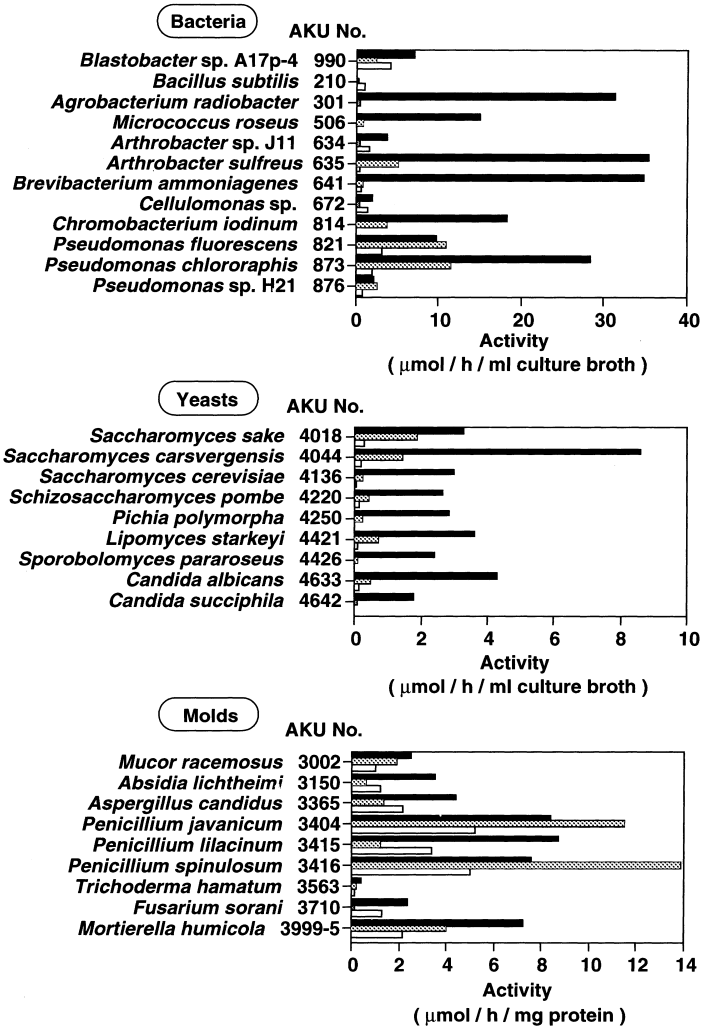

Fig. 3. Cyclic imide- and cyclic ureide-metabolizing activities in bacteria, yeasts and molds. Cyclic imide hydrolyzing activity with succinimide as a substrate (■); cyclic ureide hydrolyzing activity with dihydrouracil as a substrate (▨); cyclic ureide hydrolyzing activity with DL-5-methylhydantoin as a substrate (□).

that a cyclic imide-metabolizing system exists in various microorganisms [34].

7. Discussion: overview of cyclic amide transformation in *Blastobacter* sp.

Blastobacter sp. A17p-4 shows diverse cyclic amide-transforming activities including toward cyclic ureides and imides (Fig. 4). Ogawa et al. reported that the bacterium produces three *N*-carbamoyl amino acid amidohydrolases, i.e. *N*-carbamoyl-D-amino acid amidohydrolase, *N*-carbamoyl-L-amino acid amidohydrolase and β-ureidopropionase [23,35]. β-ureidopropionase has a broad substrate specificity, utilizing not only *N*-carbamoyl-β-amino acids but also *N*-carbamoyl-γ-amino acids and several *N*-carbamoyl-α-amino acids [36]. The hydrolysis of *N*-carbamoyl-α-amino acids by β-ureidopropionase is strictly L-enantiomer specific [36]. This strict stereospecificity makes these enzymes applicable to the production of optically active amino acids (Fig. 4). In cyclic ureide hydrolysis, the bacterium produces D-hydantoinase, which was determined to be identical to dihydropyrimidinase. D-hydantoinase is useful for the production of D-amino acids from DL-5-monosubstituted hydantoins in combination with *N*-carbamoyl-D-amino acid amidohydrolase. D-hydantoinase also hydrolyzes bulky cyclic imides other than cyclic ureides.

Cyclic imide metabolism is categorized differently from cyclic ureide metabolism (Fig. 4). Cyclic imide metabolism, especially simple cyclic imides metabolism, involves two unique enzymes, imidase and half-amidase. Imidase and half-amidase show high activity toward cyclic imides and the ring-opened products of half-amides, respectively, suggesting that the two enzymes act cooperatively in the metabolism of cyclic imides. The production of both enzymes was enhanced by cyclic imides, indicating that these enzymes were produced for cyclic imide assimilation. At the gene expression level, half-amidase was more sensitively up-regulated by cyclic imides than imidase, which is constitutively produced to some extent. On the other hand, the catalytic activity of imidase was down-regulated by succinate, while the half-amidase activity was not affected. The coordination between the catalytic and genetic regulation of imidase and half-amidase confirms the physiological importance of these enzymes in cyclic imide metabolism.

Cyclic imide hydrolysis has been studied for mammalian dihydropyrimidinases in relation to the detoxification of antiepileptic agents [25–27]. Our studies showed that microorganisms also hydrolyze cyclic imides, which involves two distinct enzymes, D-hydantoinase and imidase. The differences in substrate range exhibited by D-hydantoinase and imidase from *Blastobacter* sp., and mammalian dihydropyrimidinases are illustrated in Fig. 5. The catalytic action

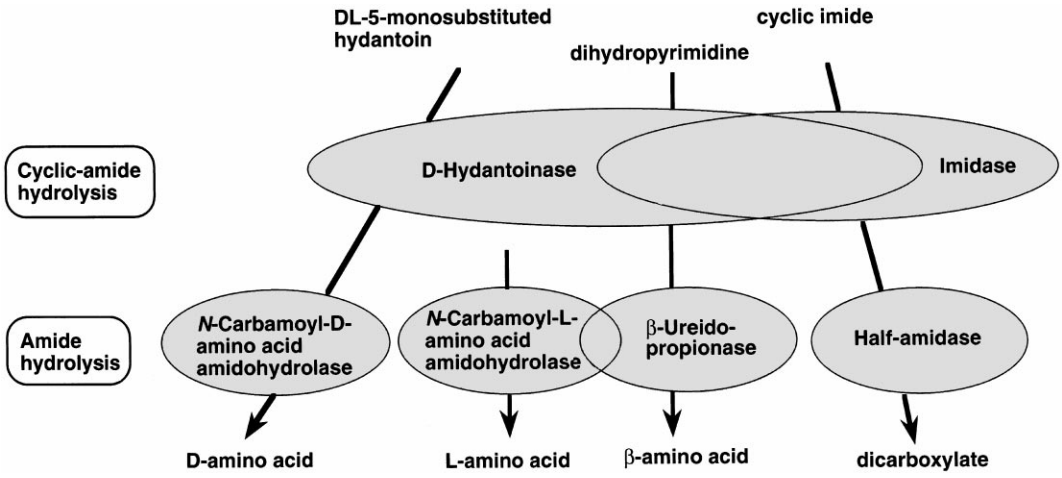

Fig. 4. Diversity of cyclic ureide and imide metabolism in *Blastobacter* sp. A17p-4.

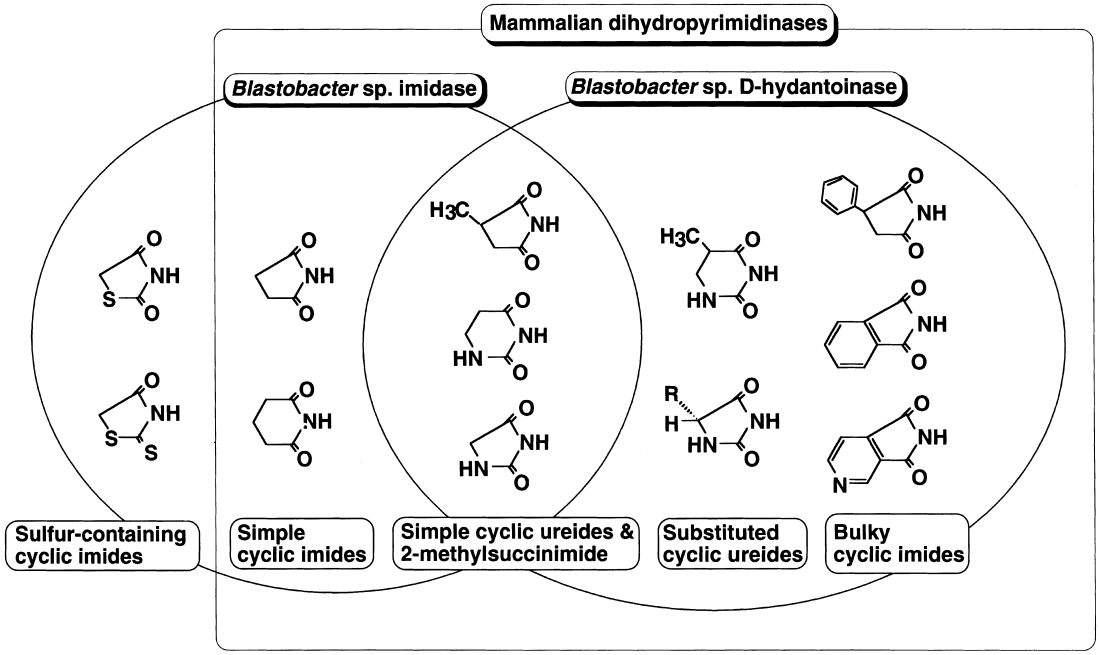

Fig. 5. An overview of the substrate range exhibited by D-hydantoinase and imidase from *Blastobacter* sp. A17p-4, and mammalian dihydropyrimidinases.

of mammalian dihydropyrimidinases is fully complemented by the combined activities of D-hydantoinase and imidase in the *Blastobacter* system; suggesting that the functions of single eukaryotic dihydropyrimidinases are found in two distinct enzymes in prokaryotes. These proposals were supported by the distributions of cyclic ureide and cyclic imide metabolism in microorganisms. In prokaryotic bacteria, there is no correlation between cyclic imide and cyclic ureide metabolism suggesting that it involves different enzyme systems. In eukaryotic fungi, however, these two activities show some similarity [34].

Above all, bacterial cyclic ureide and imide metabolism involves a diverse group of enzymes with different metabolic functions. Besides the practical application of D-hydantoinase to D-amino acid production, the practical potential of cyclic imide-transforming enzymes is now under investigation for fine enzymatic synthesis of useful compounds, and valuable organic acid production from cyclic imides and metabolic intermediates.

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