Efficient conversion of 5-substituted hydantoins to D- α -amino acids using recombinant *Escherichia coli* strains

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D-Amino acids, important intermediates in the production of semisynthetic penicillins and cephalosporins, are currently prepared from the corresponding hydantoins using bacterial biomass containing two enzymes, hydantoinase and carbamylase. These enzymes convert the hydantoins first into carbamyl derivatives and then into the corresponding D-amino acids. In an attempt to select more efficient biocatalysts, the hydantoinase and carbamylase genes from *Agrobacterium tumefaciens* (formerly *A. radiobacter*) were cloned in *Escherichia coli*. The genes were assembled to give two operon-type structures, one having the carbamylase gene preceding the hydantoinase gene and the other with the carbamylase gene following the hydantoinase gene. The recombinant strains stably and constitutively produced the two enzymes and efficiently converted the corresponding hydantoins into *p*-hydroxyphenylglycine and phenylglycine. The order of the genes within the operon and the growth temperature of the strains turned out to be important for both enzyme and D-amino acid production. The configuration with the carbamylase gene preceding the hydantoinase gene was the most efficient one when the biomass was grown at 25 °C rather than 37 °C. This biomass produced D-amino acid twice as efficiently as the industrial strain of *A. tumefaciens*. The efficiency was found to be correlated with the level of carbamylase produced, indicating that the concentration of this enzyme is the rate-limiting factor in D-amino acid production under the conditions used on an industrial scale.

Keywords: hydantoinase, carbamylase, D-amino acids, gene expression

INTRODUCTION

Among the many optically active compounds industrially utilized as fine chemical intermediates, the D-amino acids D-phenylglycine and D-*p*-hydroxyphenylglycine are particularly important for their use in the production of semisynthetic penicillins and cephalosporins (Takahashi *et al.*, 1979; Runser *et al.*, 1990; Runser & Ohleyer, 1990; Lee *et al.*, 1994).

These D-amino acids are currently synthesized in a two-step reaction process using as substrates DL-5-mono-

substituted hydantoins (Olivieri *et al.*, 1981; Takahashi *et al.*, 1979). During this two-step process, the substrate is first hydrolysed to the D-carbamyl derivative by a D-specific hydantoinase and then further converted into the corresponding D-amino acid by an *N*-carbamyl-D-amino acid amidohydrolase (hereinafter carbamylase). Several micro-organisms that express both hydantoinase and carbamylase have been isolated, making the current industrial process for the production of these two D-amino acids particularly simple. Bacterial biomass and substrate are incubated in the same reaction tank until the reaction reaches its completion. Interestingly, since these hydantoins spontaneously racemize in the reaction conditions used, the specificity of hydantoinase for the D configuration allows the quantitative conversion of the DL-hydantoins into the corresponding D-amino acids.

The D-amino acid production process which makes use

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Abbreviation: CPHPG, *N*-carbamyl-D-*p*-hydroxyphenylglycine.

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of a bacterial strain, *Agrobacterium tumefaciens* (formerly *A. radiobacter*), was first developed in the 1980s (Olivieri *et al.*, 1981) and has since then been used on an industrial scale for the commercial manufacture of these important intermediates. Despite its simplicity, the process has margins for further optimization. In particular, the efficiency of the process could be increased by (i) improving the chemical stability of carbamylase, (ii) improving the thermal stability of carbamylase, and (iii) improving the bacterial strain used in the process.

As far as chemical stability is concerned, carbamylase is particularly sensitive to oxidizing conditions (Kim & Kim, 1995), rapidly losing its activity when exposed to oxygen and hydrogen peroxide (Grifantini *et al.*, 1996). Recently, we have established the role of the five cysteines present in the molecule in determining the type and rate of oxidation and we have indicated a way to lessen the sensitivity of the enzyme to the oxidizing agents (Grifantini *et al.*, 1996).

In addition to chemical instability, carbamylase is also thermally inactivated during the reaction (Ogawa *et al.*, 1994b) and therefore the use of an enzyme mutant with a higher T_{50} value would improve the D-amino acid production process (T_{50} is defined as the temperature at which the enzyme loses 50% of its activity).

Finally, as far as biomass efficiency is concerned, the *A. tumefaciens* strain currently used has two main drawbacks. First, it is particularly susceptible to bacteriophage infection. Secondly, high levels of hydantoinase and carbamylase activities are only achieved by subculturing the strain in the presence of inducers such as hydantoin derivatives or pyrimidines, and retaining the inducer throughout fermentation (Meyer & Runser, 1993). However, even in the presence of such inducers, the levels of enzymes can vary from batch to batch. Therefore, the replacement of *A. tumefaciens* with a more robust strain able to guarantee high and reproducible levels of enzyme activities would be desirable.

In the present work we report the isolation of both hydantoinase and carbamylase genes from *A. tumefaciens* and their cloning in *Escherichia coli*. The two genes were inserted into an expression plasmid in such a way that a polycistronic expression cassette was created. High levels of stable, constitutive expression of both enzymes were obtained and the recombinant *E. coli* strain turned out to be more efficient than *A. tumefaciens* when assayed for the production of D-amino acids.

METHODS

Plasmids and strains. *A. tumefaciens* (*A. radiobacter*) NRRL B11291 was obtained from the NRRL culture collection. For chromosomal library preparation, plasmid pUC18 and *E. coli* JM109 were used; for the expression of D-hydantoinase and carbamylase, *E. coli* 71/18 (Sambrook *et al.*, 1989) and plasmid pSM671 were used. pSM671 is a derivative of pSM214 (Velati Bellini *et al.*, 1991) in which the ampicillin-resistance gene has been replaced with a pUC-derived multi-cloning site. Phage M13 mp8 and *E. coli* TG1 (Sambrook *et al.*, 1989) were used

for site-directed mutagenesis. Unless otherwise stated, *E. coli* strains were grown under aerobic conditions at 37 °C in LB or 2 × YT medium (Sambrook *et al.*, 1989) and transformed by either CaCl_2 treatment (Dagert & Ehrlich, 1979) or electroporation (Dower *et al.*, 1988).

Enzymes and reagents. Enzymes were purchased from Boehringer Mannheim and from New England Biolabs and used according to the manufacturers' specifications. PCR reactions were performed with Taq Polymerase (Boehringer) and Pfu Polymerase (Stratagene) using standard protocols. Primers and mutagenic oligodeoxynucleotides were synthesized using a Beckman P1000 DNA synthesizer. Sequencing was performed by the dideoxynucleotide termination method (Sanger *et al.*, 1977) using a Sequenase sequencing kit (US Biochemical). [^{35}S]dATP- αS was from Dupont. Chromatographic material was from Pharmacia. N-Carbamyl-D-p-hydroxyphenylglycine (CPHPG) and DL-p-hydroxyphenylhydantoin were prepared from D-p-hydroxyphenylglycine (Fluka) (Stark & Smith, 1963; Suzuki *et al.*, 1973).

Preparation and screening of the *A. tumefaciens* chromosomal libraries. *A. tumefaciens* NRRL B11291 was grown at 28–30 °C for 16–20 h in a medium containing 1% glucose, 0.3% yeast extract, 1.36% KH_2PO_4 , 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (pH 7). Cells were collected by centrifugation and chromosomal DNA was purified with CsCl (Sambrook *et al.*, 1989). Chromosomal DNA was partially cleaved with either *Bam*HI or *Sac*I and the fragments with a mean size of 3000–4000 bp were electroeluted from 0.8% agarose gels (Sambrook *et al.*, 1989) and subsequently ligated to *Bam*HI- or *Sac*I-linearized pUC18. Both ligation mixtures were used to electroporate JM109 cells (Bio-Rad gene pulser) and transformants were selected on 2 × YT plates containing 0.04 µg X-Gal ml⁻¹, 0.06 mM IPTG, 100 µg ampicillin ml⁻¹. The white colonies were transferred to LB plates supplemented with 100 µg ampicillin ml⁻¹ and the clones carrying the hydantoinase genes were screened by colony hybridization using the non-radioactive DIG-System kit (Boehringer). The *Bam*HI library was screened with the following degenerated oligodeoxynucleotides: 5'-C/AGN ATT/C/A GTN AAT/C TAT/C GCN GCN GC-3', 5'-GGN CCN ATT/C/A CAA/G CGN GC-3', 5'-C/AGN GCN GAT/C GTN ATG GA-3'. For the screening of the *Sac*I library, the following oligodeoxynucleotide was used as probe: 5'-ATC GTA ACC GCG GAC GGG ATT TCT CCC-3'.

Protein analysis and enzyme assay on cellular extracts. Cells were grown overnight at room temperature or at 37 °C in LB medium supplemented with the appropriate antibiotic and collected by centrifugation. Unless otherwise stated, cells from 5 ml cultures were suspended in 300 µl 20 mM sodium phosphate, 20% (v/v) glycerol, pH 8.0, and sonicated to homogeneity. The homogenized suspension was clarified in an Eppendorf centrifuge for 10 min at 4 °C and the pellets were resuspended in an equal volume of buffer. Supernatants and pellets were analysed by SDS-PAGE (Laemmli, 1979). Hydantoinase activity in supernatants was assayed at 40 °C in 500 µl 0.2 M sodium phosphate buffer, pH 8.0, containing 20 mM DL-p-hydroxyphenylhydantoin. Aliquots were taken at various times and the reaction was stopped by adding trichloroacetic acid to a final concentration of 4%. After centrifugation, 600 µl supernatant was added to 300 µl Ehrlich's reagent (10% 4-dimethylaminobenzaldehyde in conc. HCl) and the carbamyl derivative was spectrophotometrically determined at 438 nm. N-Carbamylase activity was tested in the same buffer at pH 7.0 using 0.12 M CPHPG as substrate and the concentration of NH_4^+ ions determined as

described by Weatherburn (1967). When D-hydantoinase was assayed in the presence of N-carbamylase, 20 mM DL-*p*-hydroxyphenylhydantoin was used as substrate and D-hydantoinase activity was calculated by adding to the μmol CPHPG produced min^{-1} the μmol NH_4^+ released during the same period of time. One unit (U) of enzyme activity is defined as the amount of enzyme giving 1 μmol product min^{-1} , for both carbamylase and hydantoinase.

D-Amino acid production. For D-amino acid production using either CPHPG or DL-*p*-hydroxyphenylhydantoin as substrate, whole cells from *A. tumefaciens* cultures were used, grown in 20-litre fermenters according to the procedure described for the industrial process (Olivieri *et al.*, 1981). However, when *E. coli* cells were tested as catalyst, bacteria were grown in flasks, as described above. D-Amino acid was produced at 40 °C under a nitrogen atmosphere in 15 ml vials sealed with rubber caps. Each vial was filled with 10 ml 0.2 M sodium phosphate buffer, pH 8.0, containing 80 mg (wet wt) *E. coli* cells and 8% *p*-hydroxyphenylhydantoin. The concentrations of hydantoin, carbamyl derivative and amino acid in the reaction mixture were determined by HPLC.

HPLC analysis. *p*-Hydroxyphenylglycine, CPHPG and *p*-hydroxyphenylhydantoin were separated in 5% acetonitrile, 0.01% phosphoric acid in water under isocratic conditions at a flow rate of 1 ml min^{-1} , using a Beckman System Gold HPLC and a Beckman Ultrasphere ODS column (4.6 × 250 mm). Samples were diluted in the mobile phase and elution was monitored at 272 nm.

RESULTS AND DISCUSSION

Cloning of hydantoinase and carbamylase genes from *A. tumefaciens*

Ogawa *et al.* (1994a) reported the isolation, biochemical characterization and N-terminal sequencing of carbamylase from *Comamonas* sp. E222c. On the basis of the N-terminal sequence of the *Comamonas* enzyme, we designed degenerate oligodeoxynucleotides which were used to screen an *A. tumefaciens* genomic library. The library was prepared by ligating the *Bam*HI-linearized

pUC18 to chromosomal fragments obtained by partial digestion of DNA with *Bam*HI. The library screening allowed the isolation of a few positive clones among which one harboured a plasmid (pSM652) with a 4000 bp insert. The sequence analysis of the insert revealed an open reading frame (ORF1) encoding a polypeptide of 304 amino acids having molecular mass of 34 kDa (GenBank accession number X91070). ORF1 is preceded by a putative ribosome-binding site (SD1) and, further upstream, by sequences similar to the -10 and -35 regions of the *E. coli* σ^{70} promoter. The comparison of the deduced N-terminal sequence of ORF1 with the 30 amino acid N-terminal sequence of the *Comomonas* carbamylase showed a 43% identity and a 63% similarity between the two proteins, thus suggesting that ORF1 encodes a carbamylase-type enzyme. This was further supported by the fact that ORF1 was found to be homologous to the aliphatic amydases from *Rhodococcus erythropolis* and *Pseudomonas aeruginosa* (Soubrier *et al.*, 1992; Ambler *et al.*, 1987), the homology being particularly high in the region encompassing the catalytic site and including Cys 172, the residue which is strictly required in carbamylase for activity (Grifantini *et al.*, 1996).

Further sequence analysis of the 4000 bp fragment allowed the identification, upstream from ORF1, of an additional ORF (ORF2), going backward with respect to ORF1. ORF2, which appeared to be truncated in plasmid pSM652, was also preceded by a putative ribosome-binding site (SD2) and by a σ^{70} -like promoter sequence which overlaps the putative ORF1 promoter. Interestingly enough, when ORF2 was compared with the SWISS-PROT protein sequence database, homologies to enzymes belonging to the amydohydrolase family were found. In particular, 26% and 27% identities were found with the yeast allantoinase (Buckholz & Cooper, 1991) and with the β subunit of *Helicobacter pylori* urease (Labigne *et al.*, 1991), re-

Fig. 1. Schematic representation of the recombinant plasmids in the regions encompassing the carbamylase and/or hydantoinase genes. The carbamylase gene was amplified from pSM652 using the primers GGGGAATTCTTATGACACGTCAGATGATA (forward) and GGGGAAGCTTCAGAATTCGCGATCAG (reverse). The PCR product was cloned in pSM671 as an *Eco*RI/*Hind*III fragment, to give plasmid pSM637. The hydantoinase gene was amplified from pSM653 with the primers GGGGAATTCTTATGGATATCATCAAG and GGGGAATTCCTTATTGCTGTATTGCGGCGC (forward and reverse, respectively). The gene was cloned as an *Eco*RI/*Eco*RI fragment in pSM671, to give plasmid pSM638. Plasmid pSM651 was obtained by subcloning the carbamylase gene in pSM638 as an *Eco*RI/*Hind*III fragment. Finally, plasmid pSM700 was obtained by inserting the hydantoinase gene, adapted with a synthetic linker at its 5' end, in the *Hind*III-PstI sites of pSM637. In the scheme the synthetic promoter (P) and the genes are boxed, the Shine-Dalgarno sequences are underlined and the restriction sites used for the cloning are shown in bold letters.

Table 1. Level of carbamylase and hydantoinase expressed in *E. coli* strains 71/18(pSM637) and 71/18(pSM638)

Cells were collected by centrifugation from 5 ml of each culture (OD_{600} 4.0) and resuspended in 0.3 ml 0.2 M sodium phosphate buffer, pH 8. After sonication, the enzyme activities were determined in the soluble fractions, as described in Methods.

Strain	Carbamylase activity (U mg ⁻¹)	Hydantoinase activity (U mg ⁻¹)
71/18	0	0
71/18(pSM637)	1.25	0
71/18(pSM638)	0	0.27

Table 2. Level of carbamylase and hydantoinase expressed in *E. coli* strains 71/18(pSM651) and 71/18(pSM700)

Cells were collected by centrifugation from 5 ml of each culture (OD_{600} 4.0) and resuspended in 0.3 ml 0.2 M sodium phosphate buffer, pH 8. After sonication, the enzyme activities were determined in the soluble fractions, as described in Methods.

Plasmid	Growth temp. (°C)	Protein concn (mg ml ⁻¹)	Carbamylase activity (U mg ⁻¹)	Hydantoinase activity (U mg ⁻¹)
pSM651	37	9.4	0.13	0.03
pSM700	37	5.4	0.32	0.01
pSM651	25	10.2	0.04	0.01
pSM700	25	6.8	0.30	0.04

spectively, and 52% similarity with the *Pseudomonas putida* hydantoinase (LaPointe *et al.*, 1994). These observations prompted us to hypothesize that in *A. tumefaciens*, like the situation in *Pseudomonas* sp. (Watabe *et al.*, 1992), the hydantoinase and carbamylase genes are clustered and that their expression might be regulated by an as yet uncharacterized mechanism which utilizes overlapping promoters.

To isolate the entire ORF2 gene, a second library was prepared by partially digesting the *A. tumefaciens* chromosomal DNA with *SacI* and by ligating the restriction fragments to *SacI*-linearized pUC18. The positive clones were selected upon hybridization with a synthetic oligodeoxynucleotide designed on the basis of the available partial sequence of ORF2 gene (see Methods). One positive clone harboured a plasmid (pSM653) carrying the full-length ORF2 gene. The sequence analysis of pSM653 revealed that ORF2 encodes a peptide of 457 amino acids having a calculated molecular mass of 50 kDa (GenBank accession number X91070).

To confirm that ORF1 and ORF2 code for *A. tumefaciens* carbamylase and hydantoinase, respectively, the two coding regions were amplified from pSM652 and pSM653 and independently cloned in the expression vector pSM671, giving plasmids pSM637 and pSM638, respectively (Fig. 1). When crude extracts from *E. coli* 71/18(pSM637) and *E. coli* 71/18(pSM638) were

analysed by SDS-PAGE, the overexpression of two protein species with molecular masses in agreement with the theoretical ones and with those expected for D-hydantoinase and carbamylase, respectively, was detected. In addition, using the enzyme assays described in Methods, it was possible to establish that *E. coli* cells harbouring pSM637 had a strong carbamylase activity, being able to convert D-CPPHG into optically active D-*p*-hydroxyphenylglycine, while *E. coli* transformed with pSM638 produced CPPHG from racemic *p*-hydroxyphenylhydantoin (Table 1).

Construction of recombinant *E. coli* strains expressing both D-hydantoinase and carbamylase

E. coli strains expressing both D-hydantoinase and carbamylase activities were constructed by inserting the two genes into plasmid pSM671 in such a way that a polycistronic-type of structure under the control of a strong constitutive promoter was generated. A synthetic linker was placed between the two genes to provide the necessary translational signals for the promoter-distal gene. Two constructs were produced, one having the hydantoinase gene preceding the carbamylase gene (pSM651) and the other with the carbamylase gene in front of the hydantoinase gene (pSM700) (Fig. 1). The two plasmids were used to transform *E. coli* 71/18, and one clone from each transformation was selected and further characterized. Table 2 summarizes the amount

Fig. 2. Expression of hydantoinase and carbamylase in *E. coli* 71/18(pSM700). (a) *E. coli* 71/18(pSM700) was grown either at 37 °C or 25 °C and 5 ml portions from overnight cultures were collected by centrifugation, resuspended in 0.3 ml sodium phosphate buffer (20 mM, pH 8) and sonicated. After centrifugation the insoluble material (Ins) was dissolved in 0.3 ml loading buffer (Laemmli, 1970) and 4 µl of both soluble (Sol) and insoluble fractions were analysed by SDS-PAGE (10%, w/v, acrylamide), under reducing conditions. (b) *E. coli* 71/18(pSM700) was grown at 25 °C to OD₆₀₀ 0.6 (exponential phase) or 5.1 (stationary phase). Equal amounts of cells were resuspended in 0.3 ml 20 mM sodium phosphate buffer, pH 8, and sonicated. The soluble and insoluble fractions were prepared as described above and 4 µl of each sample analysed by SDS-PAGE under reducing conditions. H, hydantoinase; C, carbamylase.

of hydantoinase and carbamylase expressed by the two clones grown at either 37 °C or 25 °C. From the data reported in Table 2 it appears that the optimal growth temperature for the production of the two enzymes is different for the two strains, 37 °C being the optimal temperature for 71/18(pSM651) and 25 °C for 71/18(pSM700). The increase in both enzyme activities observed when the growth temperature of 71/18(pSM700) was shifted from 37 °C to 25 °C was due to

the partial partitioning of the two proteins in the soluble fraction. In fact, when total soluble and insoluble cellular proteins from a culture of 71/18(pSM700) grown at 37 °C were analysed by SDS-PAGE, both hydantoinase and carbamylase were found to be highly expressed but predominantly compartmentalized in the insoluble fraction (Fig. 2a).

Table 2 also shows that in 71/18(pSM700) grown at 25 °C the level of hydantoinase was comparable to that found in 71/18(pSM651) grown at 37 °C, whereas carbamylase activity was almost twice as high.

To study the stability of enzyme production, the enzyme activities and plasmid content were analysed at the stationary phase of ten serial subcultures, corresponding to approximately 80 generations. In both recombinant strains the hydantoinase and carbamylase levels were comparable among the different subcultures and neither segregational nor structural plasmid instability were observed (data not shown).

An unexpected observation was that the expression ratio of the hydantoinase and carbamylase genes was influenced by the culture time. As shown in Table 3, when 71/18(pSM700) cells were collected during the exponential phase at OD₆₀₀ 0.6, the ratio between carbamylase and hydantoinase units was approximately 1 whereas this value dropped to 0.1 during the stationary phase (OD₆₀₀ 5.1). Since, as judged by SDS-PAGE, the ratio between the total amount of hydantoinase and carbamylase appeared to be comparable in the two cell extracts (Fig. 2b), the differences in the enzyme activities could be ascribed to either differential aggregation or oxidation. In both cases a loss in specific activity is expected (Grifantini *et al.*, 1996).

Conversion efficiency of DL-monosubstituted hydantoines to D-amino acids by recombinant *E. coli* strains

D-Amino acid production in *E. coli* 71/18(pSM651) and 71/18(pSM700) strains, grown at 37 °C and 25 °C respectively, was compared with that in *A. tumefaciens* NRRL B11291 grown under industrial conditions (Olivieri *et al.*, 1981). As shown in Table 4, *E. coli* 71/18(pSM651) performed in approximately the same way as *A. tumefaciens*, both strains being able to convert 30% of the substrate into *p*-hydroxyphenylglycine after 22 h under the conditions used. On the other hand, *E. coli* 71/18(pSM700) was able to convert more than 60% of the substrate into the corresponding amino acid after the same period of time. When amino acid production efficiency of *E. coli* 71/18(pSM700) biomass from an exponential culture was compared to the efficiency of the same biomass collected at late stationary phase, a similar production rate was observed (Table 5).

From these data it appears that, under the particular experimental conditions used, the rate-limiting factor in D-amino acid production is the amount of carbamylase present in the biomass. This is particularly evident in the experiment in which *E. coli* 71/18(pSM700) cells were

Table 3. Influence of growth phase on expression of carbamylase and hydantoinase in *E. coli* 71/18(pSM700)

Cells were grown at 25 °C and aliquots of the culture collected at OD₆₀₀ 0.6 (exponential phase) and 5.1 (stationary phase). Cells were centrifuged, resuspended in 0.2 M sodium phosphate buffer, pH 8.0, at a final concentration of 100 mg ml⁻¹ and sonicated. After centrifugation (20000 g) total protein and enzyme activities were determined in the supernatant.

Growth phase	Protein concn (mg ml ⁻¹)	Hydantoinase activity (U mg ⁻¹)	Carbamylase activity (U mg ⁻¹)
Exponential	8.7	0.32	0.28
Stationary	11.6	0.45	0.04

Table 4. D-Amino acid production using *A. tumefaciens* NRRL B11291 and *E. coli* strains 71/18(pSM651) and 71/18(pSM700)

Cells (80 mg) were resuspended in 0.2 M sodium phosphate buffer, pH 8.0, containing 8% *p*-hydroxyphenylhydantoin (4.16 mmol) and incubated in sealed vials at 40 °C under a nitrogen atmosphere. At given times aliquots were syringed out and the concentrations of D-amino acid and carbamyl derivatives determined. *E. coli* strain 71/18(pSM651) was grown at 37 °C and strain 71/18(pSM700) was grown at 25 °C; *A. tumefaciens* was grown at 28 °C.

Reaction time (h)	Percentage of substrate converted to <i>p</i> -hydroxyphenylglycine (A) and <i>N</i> -carbamyl- <i>p</i> -hydroxyphenylglycine (B) by:					
	<i>E. coli</i> 71/18(pSM651)		<i>E. coli</i> 71/18(pSM700)		<i>A. tumefaciens</i>	
	A	B	A	B	A	B
2.5	6.7	9.3	14.0	9.6	7.0	2.7
15.0	28.8	11.5	48.0	11.7	27.9	8.7
22.0	36.7	12.2	61.0	12.7	35.1	9.3

Table 5. Effect of growth phase on D-amino acid production in *E. coli* 71/18(pSM700)

Cultures were grown at 25 °C and equal quantities of cells were collected at OD₆₀₀ 0.6 (exponential phase) and 5.1 (stationary phase). After centrifugation, 80 mg cells were resuspended in 0.2 M sodium phosphate buffer, pH 8.0, containing 8% *p*-hydroxyphenylhydantoin. The concentrations of D-amino acids and carbamyl derivatives were determined.

Reaction time (h)	Percentage of substrate converted to <i>p</i> -hydroxyphenylglycine (A) and <i>N</i> -carbamyl- <i>p</i> -hydroxyphenylglycine (B) during:			
	Exponential phase		Stationary phase	
	A	B	A	B
0.25	1.4	4.1	1.1	1.2
2.5	8.9	9.6	11.1	9.4
5	15.7	10.7	19.9	11.0
22	42.6	13.2	46.9	13.2

collected at two different times during growth. Even if approximately sevenfold less hydantoinase was present in stationary-phase cells, the *p*-hydroxyphenylglycine production rate was similar, if not higher, than the rate

observed with the exponentially growing cells. The levels of carbamylase were comparable between the two cultures, with a slightly higher concentration in the case of the stationary-phase culture.

Fig. 3. Enzymic hydrolysis of DL-hydantoin. (a) Effect of the buffer concentration on the kinetics of hydantoin hydrolysis. DL-*p*-Hydroxyphenylhydantoin (50 mM) was incubated at 40 °C in a 5 ml reaction volume and the kinetics of conversion was analysed with different concentrations of sodium phosphate buffer (pH 8.0) and hydantoinase. Δ , 200 mM sodium phosphate buffer, 0.6 U hydantoinase; $\square$, 100 mM sodium phosphate buffer, 1.2 U hydantoinase; $\circ$, 5 mM sodium phosphate buffer, 1.2 U hydantoinase. (b) Effect of the buffering capacity of carbamylase on hydantoin hydrolysis. DL-*p*-Hydroxyphenylhydantoin (50 mM) was incubated at 40 °C in a 5 ml reaction volume containing 0.6 U hydantoinase and the kinetics of hydrolysis was analysed with different concentrations of carbamylase. Δ , 0.6 U carbamylase; $\square$, 0.3 U carbamylase; $\circ$, no carbamylase.

Although a large number of parameters, such as substrate concentrations and solubility, cell permeability and enzyme kinetic constants, can influence the overall reaction rate, the direct correlation between the levels of carbamylase and the D-amino acid production can be explained by the pH range at which the hydantoinase works. Hydantoinase is active between pH 10.5 and 7.0 (Runser *et al.*, 1990; Kim & Kim, 1995) and since the conversion of hydantoins gives acidic carbamyl derivatives, in a non-buffered system the rate of hydrolysis decreases. On the other hand, the carbamylase-driven reaction produces ammonia from the carbamyl derivative and therefore when the two enzymes work together carbamylase acts as a buffering agent. The buffering capacity of carbamylase becomes the true limiting factor of the reaction. This model is supported by the experimental evidence showing that the concentration of the carbamyl derivative which accumulates as a reaction intermediate is practically unaffected by the amount of hydantoinase present in the cells (Tables 4 and 5).

To further support this model, sets of experiments were conducted in which the kinetics of D-CPPHG production was followed by incubating hydantoinase in different buffers solutions (5, 100 and 200 mM sodium phosphate, pH 8.0). As shown in Fig. 3(a), the production of CPPHG was fully inhibited after 2 h reaction in 5 mM sodium phosphate buffer, whereas much longer reaction times were observed at higher sodium phosphate buffer concentrations. However, the hydantoinase-driven reaction could be prolonged in 5 mM sodium phosphate buffer by adding carbamylase; the higher the carbamylase concentration was, the more the amount of hydantoin hydrolysed (Fig. 3b). In other words, carbamylase appeared to exert the same effect on hydantoinase as the sodium phosphate buffer does. Finally, increasing the amount of hydantoinase while maintaining constant the concentration of carbamylase in low buffering conditions (5 mM sodium phosphate buffer, pH 8.0) not only does not improve the kinetics of

Fig. 4. Effect of hydantoinase concentration on the kinetics of DL-hydantoin hydrolysis. DL-*p*-Hydroxyphenylhydantoin (50 mM) was incubated at 40 °C in a 5 ml reaction volume containing 0.3 U carbamylase and the kinetics of hydrolysis was analysed with different concentrations of hydantoinase. The amount of DL-*p*-hydroxyphenylhydantoin hydrolysed was calculated as the sum of the carbamyl derivative and the D-amino acid produced. $\square$, 0.15 U hydantoinase; Δ , 0.3 U hydantoinase; $\circ$, 0.6 U hydantoinase.

hydantoin hydrolysis but is even detrimental to the reaction (Fig. 4).

Concluding remarks

In conclusion, by cloning the hydantoinase and carbamylase genes in *E. coli*, a recombinant strain useful in the production of D-amino acids was obtained. In the particular expression vector used, a strong constitutive promoter and the two genes were assembled in a cassette which simulates the prokaryotic operons. The order in which the two genes were positioned within the vector turned out to be important, the construct with the carbamylase gene preceding the hydantoinase gene

giving the best amino acid production rates. This recombinant *E. coli* strain stably and constitutively produced the two enzymes and performed as a better catalyst when compared to the industrial strain of *A. tumefaciens* in D-amino acid production.

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