Journal of Industrial Microbiology & Biotechnology (2000) 24, 2–6

www.nature.com/jim

Expression of the creatininase gene from *Pseudomonas putida* RS65 in *Escherichia coli*

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The gene encoding creatininase from *Pseudomonas putida* RS65 was cloned, sequenced and expressed in *Escherichia coli*. One plasmid containing a 7.0-kb *Hin*dIII insert was selected by its ability to express creatininase activity. After deletion of the adjacent restriction fragments, a 1.1-kb *Sph*I fragment, which contained the full length of the creatininase gene, was subcloned into a pUC18 vector and the nucleotide sequence of the creatininase gene was determined. The gene consists of 771 base pairs and encodes a protein of 257 amino acids. The constitutive creatininase productivity of *E. coli* DH5α (pCRN741) cultured in broth was about 8.5-fold higher than that of *P. putida* RS65 cultured in a creatinine-containing medium. The creatininase gene was expressed efficiently in *E. coli* from its own promoter. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 2–6.

Keywords: creatininase; creatinine amidohydrolase; *Pseudomonas putida*; cloning; DNA sequencing

Introduction

Determination of creatinine and creatine in urine and serum is of significant value for diagnosis of renal, muscular and thyroid functions. Creatininase (creatinine amidohydrolase, EC 3.5.2.10) catalyzes the hydrolysis of creatinine to creatine. Creatininase in combination with creatinase (creatine amidinohydrolase, EC 3.5. 3.3) and sarcosine oxidase (EC 1.5.3.1) is practically useful for the diagnostic assessment of serum creatinine concentration [5]. Recently, much attention has been directed to the production of creatininase from microorganisms [3,4,6,8,11,13,15,17–19] and the genes coding for creatininase from *Pseudomonas* [19] and *Arthrobacter* [13] were cloned and sequenced.

To produce creatininase for development of the creatininase assay, we isolated a creatinine-assimilating bacterium, *P. putida* NTU-8, from soil [8]. A creatininase-producing mutant RS65 was isolated [9]. In the present study, the sequencing and expression of the creatininase gene was determined in *E. coli*. We found that the constitutive creatininase productivity of *E. coli* DH5α (pCRN741) was about 8.5-fold higher than that of *P. putida* RS65. The creatininase gene was expressed efficiently in *E. coli* from its own promoter.

Materials and methods

Bacterial strains and plasmids

The following strains were used: *E. coli* DH5α [16] and *P. putida* RS65 [9]. Plasmids used were pBR322 and pUC18 purchased from Boehringer Mannheim (Mannheim, Germany) and pMMB67EH [2] obtained from M Bagdasarian, Institute of Michigan Biotechnology, Lansing, MI, USA.

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Received 2 April 1999; accepted 31 July 1999

Chemicals

Restriction endonucleases, T4 DNA ligase, IPTG (isopropyl-β-D-thiogalactopyranoside) and ATP were purchased from Boehringer Mannheim. Taq DNA polymerase, polymerase chain reaction (PCR) buffer, and deoxynucleotides were purchased from Perkin-Elmer Cetus (Foster City, CA, USA). Oligonucleotides were synthesized (Quality System Inc, Taipei, Taiwan) with an automated cyanoethyl phosphoramidite chemistry on a Cruachem PS250 from Cruachem Inc (Dulles, VA, USA). Luria-Bertani (LB) medium was purchased from Difco (Detroit, MI, USA). DEAE Sepharose CL-6B and Sepharose CL-6B were purchased from Pharmacia (Uppsala, Sweden). A low molecular weight electrophoresis calibration kit and a Bio-Rad protein assay kit were obtained from Pharmacia Biotech (Milwaukee, WI, USA) and Bio-Rad Laboratories (Richmond, CA, USA), respectively. All other chemicals were purchased from Sigma (St Louis, MO, USA).

Making DNA libraries from P. putida RS65

All DNA manipulations were based on the methods of Sambrook *et al* [16]. Genomic DNA from *P. putida* RS65 was isolated, and partially digested with *Hin*dIII. Fragments of 2–9 kb were isolated using electrophoresis on a low melting agarose gel. Several clones of *Hin*dIII-digested genomic DNA were ligated with *Hin*dIII-linearized pBR322. The ligation mixtures were transformed into *E. coli* DH5α, and recombinants were selected by spreading them on LB agar plates containing 100 μg ml^{-1} ampicillin and incubating the mixture at 37°C for 24 h.

Screening of creatininase-positive clones using an indicator test

A modification of the indicator test [1,7] was used for detecting creatininase activity. *E. coli* transformants were grown for 12 h in 5 ml LB medium containing 100 μg ml^{-1} of ampicillin at 37°C. One-milliliter cultures were withdrawn, centrifuged, and the pellets were suspended in 1 ml of 10 mM potassium phosphate buffer (pH 7.5) containing

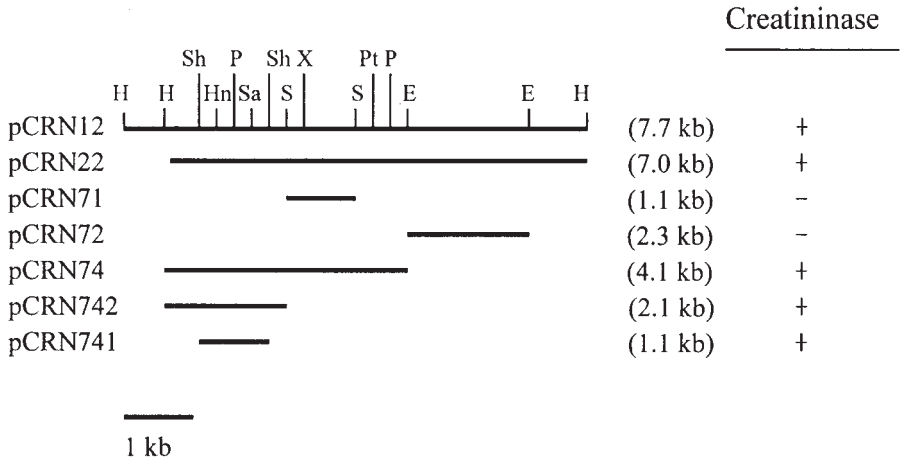

Figure 1 The restriction endonuclease mapping and subcloning of the creatininase gene from *P. putida* RS65. The creatininase activity of transformants was detected by the indicator test. The abbreviations of restriction endonuclease enzymes are: E, *Eco*RI; H, *Hin*dIII; Hn, *Hin*dII; P, *Pvu*II; Pt, *Pst*I; S, *Sma*I; Sa, *Sal*I; Sh, *Sph*I; X, *Xho*I.

1% creatinine and 20 μl toluene. After 1 h incubation at 37°C, 4 μl of 0.04 U ml^{-1} creatinase, 4 μl of 0.25 U ml^{-1} urease and 100 μl of 0.01% phenol red were added to the mixture. If the transformant possessed the creatininase activity, the solution developed an easily visible red-pink color.

Cell-free extracts

E. coli DH5α (pCRN741) was grown aerobically in 2.0 L of LB medium at 37°C for 17 h. The cells were harvested by centrifugation, and washed twice with 50 mM Tris buffer (pH 8.0). Cells were resuspended in 150 ml of 50 mM Tris buffer and disrupted with a soniprep 150 sonicator (MSE Sci Inc, Leicestershire, UK) at 50 W for 5 min with ice water cooling. Debris was removed by centrifugation at 12000 $\times$ *g* for 30 min. The supernatant was used as a cell-free extract.

Enzyme assay

Creatininase activity was measured as described in Yamamoto *et al* [19]. Each 0.5-ml enzyme solution was added to 0.5 ml 50 mM phosphate buffer (pH 7.5) containing 1 mM creatinine. After incubation at 37°C for 30 min, the residual creatinine was determined by adding 2.5 ml of 1% picric acid: 7.5% NaOH = 4 : 1 (v/v). The mixture was diluted with 10 ml water and absorbance at 520 nm was measured after 30 min. One unit of creatininase was defined as the amount of enzyme which decomposes 1 μmol of creatinine under these conditions.

DNA sequencing

The fragment of *Pseudomonas* DNA in plasmid pCRN741 was amplified by polymerase chain reaction (PCR) using Gene Amp PCR system 2400 (Perkin Elmer, Branchburg, NJ, USA). The PCR conditions were as follows: denaturation, 94°C, 15 s; annealing, 55°C, 15 s; extension, 72°C, 30 s for 30 cycles and 72°C for 6 min. PCR amplification of pCRN741 with oligonucleotides 17 mer (GTTTTCCCAGTCACGAC) and 24 mer (AGCGGATAACAATTTCACACAGGA) from pUC18 yielded a single 1.1-kb DNA fragment. After purification of the PCR product by PEG (polyethylene glycol) precipitation [16], the purified DNA was used as template and sequenced by fluorescence DyeDeoxy method (ABI PRISM™ Dye Terminator Cycle Sequencing Core kit, Applied Biosystems, USA).

Creatininase gene amplified by PCR

The structure gene of creatininase was amplified by PCR reaction using the following primers: 5′-CGGGATCCCCGATGAACGATAGCGTTGTAATTGGCG-3′ [the sequence that overlaps the *Bam*HI site (underlined) encodes the N-terminal amino acids (MNDSVVIG)] and 5′-CCAAGCTTAACTGAATGCCTCGCGGACAGCGTT-3′ (the *Hin*dIII site is underlined and lies with two bases of the stop codon; the oligonucleotide is complementary to the sequence that encodes the C-terminal amino acids NAVREAFS). The PCR product was purified and digested

Table 1 The expression of creatininase activity by *E. coli* transformants in the absence or presence of IPTG

Plasmid[a]	Creatininase activity[c] (U ml^{-1})	
	0 mM IPTG	0.1 mM IPTG[b]
PCRN12	0.002	0.002
PCRN22	0.001	0.001
PCRN74	0.022	0.024
PCRN742	0.228	0.232
pCRN741	0.678	0.678
PMMBCRN	0.057	0.258
pUCCRN	0.033	0.073

[a]Transformants were grown aerobically in an L-tube loaded with 10 ml of LB-medium containing 100 μg ml^{-1} ampicillin, and incubated at 37°C, 100 rpm for 12 h. The inoculum size was 1%.
[b]IPTG (0.1 mM) was added at the beginning of incubation.
[c]Creatininase activity was measured by the decrease in creatinine concentration by the enzyme reaction at 37°C for 30 min. The optical density was measured at 520 nm.

by *Bam*HI and *Hin*dIII, and then ligated with *Bam*HI and *Hin*dIII-digested pUC18 vector.

Results

Cloning of the creatininase gene

A genomic library of *P. putida* total DNA was constructed in pBR322 using *Hin*dIII. Approximately 2000 transformants were obtained on LB-ampicillin plates, and 500 of them were tested using the indicator test for creatininase production. The plasmids of two positive clones (Figure 1) were named pCRN12 and pCRN22, they carried a 7.7-kb and a 7.0-kb *Hin*dIII insert, respectively. The 7.0-kb *Hin*dIII fragment was selected for further studies and subcloned into the pUC18 vector. All subclones were analyzed for creatininase activity using the indicator test. Two positive subclones (Figure 1), which carried a 1.1-kb *Sph*I-*Sph*I insert and a 2.1-kb *Hin*dIII-*Sma*I insert were selected and named pCRN741 and pCRN742, respectively. The expression of creatininase activity of pCRN741 was superior to those of pCRN12, pCRN22, and pCRN742 in either the absence or presence of IPTG induction (Table 1). The results revealed that the structure gene of creatininase was localized in a 1.1-kb *Sph*I-*Sph*I insert of pCRN741 (Figure 1).

The 1.1-kb *Sph*I-*Sph*I fragment was isolated from pCRN741 and ligated with a *Sph*I-*Sph*I-cut pMMB67EH vector which contained the *Ptac* promoter leading to plasmid pMMBCRN. The expression of creatininase activity of pMMBCRN was only about one-twelfth that of pCRN741 (Table 1), however, the creatininase activity of pMMBCRN increased about 4.5-fold by IPTG induction.

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   1 GCATGCCGTGACCTACGGGATAGAGCCACGGCAGCTTTTGTCAATTCCAGGTCGGAAACTGACAATTCCAGCCGATGGCG  80
  81 ACTCGATCAAATTGGGACATCTGCTACTGCTGCAAACCGGACGAGCTTTTGCAATTACAAGAACATAGACAGACCACCCG 160
 161 ATG AAC GAT AGC GTT GTA ATT GGC GAA CTC ACC TGG CCC GAG TAC GCC CGG CGT GTT GCA  220
   1 M   N   D   S   V   V   I   G   E   L   T   W   P   E   Y   A   R   R   V   A   20
 221 TCC GGC AGC CCG ATA TTC TTG CCT GTA GGC GCC CTG GAG CAA CAC GGC CAC CAC ATG TGC  280
  21 S   G   S   P   I   F   L   P   V   G   A   L   E   Q   H   G   H   H   M   C   40
 281 ATG GAA GTG GAT GTA CTG CTA CCG ACC GCG CTG TGC AAG GCC GTA GCG CGC AAT GTC GAC  340
  41 M   E   V   D   V   L   L   P   T   A   L   C   K   A   V   A   R   N   V   D   60
 341 GGC CTG GTG CTC CCC ATT GGC CTA CGG CTA CAA GTC GCA GCA AAA ATC GGG CGG CGG AAA  400
  61 G   L   V   L   P   I   G   L   R   L   Q   V   A   A   K   I   G   R   R   K   80
 401 CCA CTT TCC CGG CAC CAC CAG CCT GGA TGG CGC AAC ACT GAC ACA TAC CAT CCA GGA CAT  460
  81 P   L   S   R   H   H   Q   P   G   W   R   N   T   D   T   Y   H   P   G   H  100
 461 CAT CAG GGA GCT GGC CCG GCA CGG CGC GCG TCA GCT GGT GAT GAT GAA CGG GCA CTA CGA  520
 101 H   Q   G   A   G   P   A   R   R   A   S   A   G   D   D   E   R   A   L   R  120
 521 GAA ACT CCA TGT TCA TCG TGG AAG GCA TCG ACC TGG CGC TGC GCG AAC TCC GTT ATG CCG  580
 121 E   T   P   C   S   S   W   K   A   S   T   W   R   C   A   N   S   V   M   P  140
 581 GCA TCA CCG ATT TCA AGG TCG TGG TCC TGT CCT ACT GGG ACT TCG TCA ACG CGC CTG AGG  640
 141 A   S   P   I   S   R   S   W   S   C   P   T   G   T   S   S   T   R   L   R  160
 641 TCA TCC AGG AAC TCT ACC CTG ACG GAT TCC TCG GTT GGG ACA TTG AAC ACG GTG GCG TCT  700
 161 S   S   R   N   S   T   L   T   D   S   S   V   G   T   L   N   T   V   A   S  180
 701 TCG AGA CTT CGG TGG ATG CTG GCC CTG CAC CCC GAG AAA GTC GAC CTG ACC CGT GCC GTC  760
 181 S   R   L   R   W   M   L   A   L   H   P   E   K   V   D   L   T   R   A   V  200
 761 GAT CAT CCG CCA GCC ACC TTC CCC CCC TAC GAT GTG TTC CCG ATC ATT GCC GAG CGC ACG  820
 201 D   H   P   P   A   T   F   P   P   Y   D   V   F   P   I   I   A   E   R   T  220
 821 CCG GCG TGT GGA ACC TTG TCA TCG CCA AAA GGC GCC AGC CGC GAG AAA GGC GAA CTG ATT  880
 221 P   A   C   G   T   L   S   S   P   K   G   A   S   R   E   K   G   E   L   I  240
 881 CTG CGT GTC TGC ACC GAA GGC ATC AGC AAC GCT GTC CGC GAG GCA TTC AGT TAG CGCCGCAA 942
 241 L   R   V   C   T   E   G   I   S   N   A   V   R   E   A   F   S   *          258
 943 CACCGCATTGATCTACCAGAGCACGATTCGCATTAGCTGTTAACCCGCCATCTATAACAACAAGGGTAATGATATTGTCC 1022
1023 AGCCATTCAACTCTGGCCCCCGGCCTCAAGCAGCGCCACGTCACCATGCTCTCGATAGCCGGCGCTATCGGTGCCGGCCT 1102
1103 TTTCATCGGATCGGGGCATGC                                                            1123
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Figure 2 Nucleotide sequence and deduced amino acid sequence of the creatininase gene from *P. putida* RS65. The Shine–Dalgarno sequence is boxed. The deduced amino acid sequence is numbered from the initiation codon (ATG) of the nucleotide sequence of the creatininase gene. *Stop codon (TGA). The accession number in GenBank is AF164677.

Analysis of the creatininase gene

The DNA sequence (Accession No: AF164677) of the creatininase-coding region in a 1.1-kb *Sph*I-*Sph*I insert of pCRN741 (Figure 2) revealed an open reading frame of 771 bp with a preceding consensus ribosomal binding site, GACAGA, eight bases upstream of the ATG initiation codon. The similarity of the creatininase gene of *P. putida* RS65 was 72.8% to that of recombinant DNA for *P. putida* PS-7 [19] and 43.2% to that of *Arthrobacter* sp TE1826 [13].

Expression of the structure gene of creatininase

The open reading frame of the creatininase gene in a 1.1-kb insert of pCRN741 was amplified by PCR. The ligation mixture was transformed into *E. coli* DH5α. One clone was selected (Table 1) and named pUCCRN. *E. coli* DH5α (pUCCRN) could constitutively express the creatininase activity when cultured in LB medium, probably because of the basal level expression of *lac* promoter in pUCCRN. On the other hand, the expression of creatininase activity of pUCCRN increased about 2.2-fold after IPTG induction (Table 1).

Purification of creatininase

The creatininase from *E. coli* DH5α (pCRN741) was purified from the cell-free extract obtained through successive purification steps involving ammonium sulfate fractionation, DEAE Sepharose CL-6B and Sepharose CL-6B column chromatographies. The purified enzyme obtained exhibited 50% total activity and a 2.5-fold increase in specific activity when compared with the cell-free extract. The molecular weight of purified enzyme estimated by SDS-polyacrylamide gel electrophoresis was 28000 (Figure 3). The optimal pH and temperature of the purified enzyme for creatininase activity were 7.0–8.0 and 45°C, respectively. At 75°C for 30 min, about 40% of the original activity still remained (data not shown).

Discussion

Much attention has been directed to the production of creatininase from microorganisms. However, only the enzymatic properties of creatininase from *Pseudomonas* [10,15] and *Alcaligenes* [3] have been reported. Creatininase from *P. putida* var *naraensis* C-83 [15] is composed of a homo-octamer with a molecular weight of 175 000. On the contrary, creatininase from *Alcaligenes* sp [3] was a dimer with a molecular weight of 160000. The molecular weight of the purified enzyme from *P. putida* RS65 determined by Sepharose CL-6B gel filtration was 224000. The molecular weight of a subunit estimated by SDS-polyacrylamide gel electrophoresis was 28 000. Thus, the creatininase from *P. putida* RS65 might be an octamer.

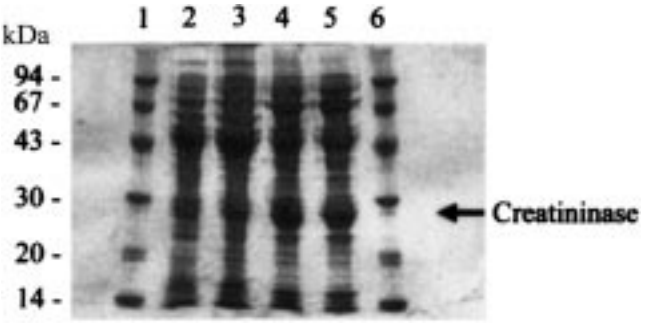

Figure 3 SDS-PAGE of protein containing creatininase activity from *E. coli* DH5α (pCRN741). Lanes 1 and 6: molecular weight standard proteins-phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa). Lane 2: crude extract. Lane 3: creatininase from ammonium sulfate fractionation. Lane 4: creatininase from DEAE-Sepharose CL-6B column chromatography. Lane 5: creatininase from Sepharose CL-6B column chromatography. Acrylamide concentration: 15%. Electrophoresis conditions: 150 V, 1 h.

The creatininase activity of pCRN741, which carried a 1.1-kb insert from *P. putida* RS65 was superior to that of pUCCRN, which carried a 0.77-kb insert from the structure gene of *Pseudomonas* creatininase in the absence of IPTG induction. However, the creatininase activity of pUCCRN increased about 2.2-fold after IPTG induction. This result suggested that the 0.77-kb insert of the structure gene of *Pseudomonas* creatininase in pUCCRN was regulated by the *lac* promoter of pUC18. On the other hand, the creatininase activity of pCRN741 was not enhanced by IPTG induction. This implied that the promoter region of the creatininase gene from *P. putida* RS65 might be contained in the 1.1-kb insert of pCRN741. Its hyperconstitutive creatininase productivity also suggested that the pCRN741 expressed the creatininase gene efficiently in *E. coli* from its own promoter instead of the *lac* promoter of pUC18 [14].

The optimal pH and temperature of purified enzyme from *E. coli* DH5α (pCRN741) were similar to those of creatininase from *P. putida* RS65 [10], *P. putida* var *naraensis* C-83 [15], and *Alcaligenes* sp [3]. The thermal stability of the purified enzyme was the same as that of creatininase from *P. putida* var *naraensis* C-83 when both enzymes were treated at 75°C for 30 min.

The creatininase gene with a calculated molecular weight of 27 927 agreed well with the molecular weights of the purified creatininase from *P. putida* RS65 and *E. coli* DH5α (pCRN741). Furthermore, the cloned gene possessing creatininase activity was also confirmed by Southern blotting analysis to have a homologous sequence with the *Hin*dIII-digested genomic DNA from *P. putida* RS65 (data not shown). The deduced amino acids from the creatininase gene of *P. putida* RS65 were 64.4% identical to those of *Pseudomonas* sp PS-7 [19] and only 31.0% homologous to those of *Arthrobacter* sp TE1826 [13].

To achieve economic viability by genetic engineering, increasing production yield is the single most important factor in the production of biotherapeutic drugs and diagnostic kits. Many efforts have been made to construct an efficient vector which can hyperexpress a foreign insert DNA in specific host cell, such as *E. coli*. From this work, the DNA sequence of the promoter region from *P. putida* RS65 might be a good reference for new expression vector construction harbored in *E. coli*.

Acknowledgements

Financial support for this study from the National Science Council of the Republic of China (NSC85-2321-B-002-002 BA) is gratefully acknowledged. We also thank TK Cheng,

Institute of Animal Science, National Taiwan University for providing the ABI 373 DNA sequencer.

References

1 Chang MC, CC Chang and JC Chang. 1992. Cloning of a creatinase gene from *Pseudomonas putida* in *Escherichia coli* by using an indicator plate. Appl Environ Microbiol 58: 3437–3440.
2 Furste JP, W Pansegrau, R Frank, H Blocker, P Scholz, M Bagdasarian and E Lanka. 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. Gene 48: 119–131.
3 Inouye Y, Y Matsuda, T Naid, S Arai, Y Hashimoto, K Asano, M Ozaki and S Nakamura. 1986. Purification and characterization of creatinine amidohydrolase of *Alcaligenes* origin. Chem Pharm Bull 34: 269–274.
4 Kaplan A and D Naugler. 1974. Creatinine hydrolase and creatine amidinohydrolase: presence in cell-free extracts of *Arthrobacter ureafaciens*. Mol Cell Biochem 3: 9–25.
5 Khan GF and W Wernet. 1997. A highly sensitive amperometric creatinine sensor. Anal Chim Acta 351: 151–158.
6 Kim JM, S Shimizu and H Yamada. 1986. Sarcosine oxidase involved in creatinine degradation in *Alcaligenes denitrificans* subsp *denitrificans* J9 and *Arthrobacter* spp J5 and J11. Agric Biol Chem 50: 2811–2816.
7 Koyama Y, S Kitao, HY Otake, M Suzuki and E Nakano. 1990. Cloning and expression of the creatinase gene from *Flavobacterium* sp U-188 in *E. coli*. Agric Biol Chem 54: 1453–1457.
8 Liu WH, JY Ho and ST Shu. 1986. Isolation and identification of a creatininase-producing bacterium. J Chin Agric Chem Soc 24: 175–183.
9 Liu WH and CF Huang. 1986. Production of creatininase by an antibiotic resistant mutant of *Pseudomonas putida* NTU-8. J Chin Agric Chem Soc 24: 288–293.
10 Liu WH and CF Huang. 1988. Purification and some properties of creatininase produced by an antibiotic resistant mutant *Pseudomonas putida* NTU-8. J Chin Agric Chem Soc 26: 398–405.
11 Moellering D and K Beaucamp. 1977. Creatinine amidohydrolase and creatine amidinohydrolase production. German Patent: 2 167 034.
12 Narayanan S and HD Appleton. 1980. Creatinine: a review. Clin Chem 26: 1119–1126.
13 Nishiya N, A Toda and T Imanaka. 1998. Gene cluster for creatinine degradation in *Arthrobacter* sp TE1826. Mol Gen Genet 257: 581–586.
14 Pflugmacher U, B Averhoff and G Gottschalk. 1996. Cloning, sequencing and expression of isopropylbenzene degradation genes from *Pseudomonas* sp strain JR1: identification of isopropylbenzene dioxygenase that mediates trichloroethene oxidation. Appl Environ Microbiol 62: 3967–3977.
15 Rikitake K, I Oka, M Ando, T Yoshimoto and D Tsuru. 1979. Creatinine amidohydrolase (creatininase) from *Pseudomonas putida*. J Biochem 16: 1109–1117.
16 Sambrook J, EF Fritsch and T Maniatis. 1989. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
17 Suzuki M and N Saito. 1976. Creatinine amidohydrolase and creatine amidinohydrolase production. German Patent: 2 614 114.
18 Tsuru D, I Oka and T Yoshimoto. 1976. Creatinine decomposing enzymes in *Pseudomonas putida*. Agric Biol Chem 40: 1011–1018.
19 Yamamoto K, M Oka, T Kikuchi and S Emi. 1995. Cloning of the creatinine amidohydrolase gene from *Pseudomonas* sp. Biosci Biotechnol Biochem 59: 1331–1332.