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Distribution of hydantoinase activity in bacterial isolates from geographically distinct environmental sources

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Abstract

Hydantoin cleaving bacterial isolates were recovered from terrestrial soil samples originating from different geographic sources (Antarctica, South Africa and China) using culture-based screening methods (selective agar plates and shake flask cultures supplemented with hydantoins). Thirty-two bacterial isolates possessing the capability to transform the model substrates benzylhydantoin and dihydrouracil to the corresponding *N*-carbamoyl-amino acids were successfully cultured. Amplification and sequencing of the 16S rDNA revealed that the isolates belonged to the genera *Arthrobacter*, *Burkholderia*, *Bacillus*, *Delftia*, *Enterobacter*, *Flavobacterium*, *Ochrobactrum*, *Pseudomonas* and *Stenotrophomonas*, with one isolate assigned to the family Microbacteriacae. We have shown that microorganisms with hydantoinase activity are: (i) distributed in various geographically distinct environmental habitats, (ii) distributed worldwide and (iii) found in certain bacterial genera. Furthermore, we have demonstrated the presence of hydantoinase activity in genera in which hydantoinase activity has not previously been reported.

Keywords: Hydantoinase; Screening; Extreme environment

1. Introduction

Enzymes classed as "hydantoinases" catalyse the hydrolysis of hydantoins in a ring-opening step after which a further enzymatic or chemical hydrolytic step can lead to the formation of amino acids. These enzymes may have different substrate specificities and in general are selective in forming either L- or D-*N*-carbamoyl amino acids. Hydantoinases are usually grouped, according to their stereospecificity, as D-, L- or non-selective hydantoinases. D-Hydantoinase, together with *N*-carbamoyl-D-amino acid hydrolase [1] is used in the production of D-amino acids for the synthesis of semi-synthetic antibiotics, peptide hormones, pyrethroids and pesticides [2].

Hydantoinases are classed as cyclic amidases (EC 3.5.2). In this study, in accordance with Syldatk et al. [3], the name hydantoinase will be employed for all enzymes that hydrolyse hydantoin and/or 5′-monosubstituted hydantoin derivatives and not as a synonym for dihydropyrimidinases, as stated in the EC-nomenclature. It has been shown that dihydropyrimidinases and hydantoinases are not necessarily the same enzyme [3]. The function of dihydropyrimidinases is the hydrolysis of dihydrouracil derivatives, a reaction involved in the reductive pathway of pyrimidine degradation. The in vivo metabolic function of many hydantoinases is still unknown. For example, the hydantoinase from *Agrobacterium* sp. [4] is not able to hydrolyse dihydropyrimidines, but can hydrolyse hydantoin and 5-monosubstituted hydantoins.

On the basis of amino acid sequence determinations and phylogenetic analyses, it has been shown that the L-hydantoinase from *Arthrobacter aurescens* DSM 3745 belongs to a protein superfamily, which includes dihydropyrimidinases, collapsin response mediator proteins, allantoinase, dihydroorotase and ureases. [5]. It is suggested that hydantoinases are members of a very old protein family and have evolved from a common ancestor, and that these first primitive, ancient microorganisms were

Abbreviations: BnH, benzylhydantoin; CA, *corynebacterium* medium; CA-plates, *corynebacterium* agar plates; C-Ala, *N*-carbamoyl-β-alanine; D-/L-C-Phe, D-/L-carbamoyl-phenylalanin; DU, dihydrouracil; GM, growth medium; HMH, D,L-5-(hydroxy-methyl) hydantoin; IMH, D,L-5-(3-indolylmethyl) hydantoin; RBC, rotating biological contactor; SA, South Africa; TBE, Tris–borate buffer

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able to use abiotically synthesised hydantoins and *N*-carbamoyl-α-amino acids as C- and/or N-sources [3]. Gojkovic et al. [6] demonstrated that dihydropyrimidinases, and thus the reductive catabolism of pyrimidines, are present in all major eukaryotic kingdoms; a comparison of dihydropyrimidinase-like enzymes showed that the majority of bacterial hydantoinases belong to the same group as the eukaryotic dihydropyrimidinases, and the authors suggest that the ancient progenitor of this group was likely to be a catabolic enzyme.

The question of the natural function, and origins, of hydantoinases remains. This study will not resolve this question, but will give more detailed information on the occurrence of hydantoin cleaving enzymes in nature. Hydantoinase activity has been found in a wide spectrum of microorganisms belonging to, amongst others, the genera *Arthrobacter*, *Pseudomonas*, *Bacillus* and *Flavobacterium* [7,8]. However, no precise data are available of the exact origins and nature of the environmental sample used for the isolation of microorganisms with hydantoinase activity. Thus, we have conducted a screening program for bacteria with hydantoinase activity from terrestrial soil samples of different geographic regions, including extreme habitats. This study has focused on the questions: (i) are microorganisms with hydantoinase activity only found in a small range of similar environmental habitats; (ii) are they distributed worldwide; (iii) are hydantoinases limited to certain bacterial genera?

2. Experimental procedures

2.1. Chemicals

Hydantoins were kindly supplied by Degussa AG, Germany. All other chemicals used were obtained from commercial sources and were of reagent grade.

2.2. Soil samples

Soil and sediment samples for enrichment experiments were collected in South Africa, Lesotho, Swaziland; China (Yunnan Province, and the Inner Mongolian Autonomous Region), and the Miers Valley, McMurdo Dry Valleys, Eastern Antarctica. Soil samples from South African National Parks were collected with the permission of the South African National Parks (SANP). Soil samples from China and Antarctica were provided under the auspices of the EU-MGATech and UWC-Waikato University-Antarctica NZ research programs, respectively (DAC).

2.3. Media

The following media were used: *Medium I*, according to reference [9], used for the screening experiments, contained: 10.0 g/l HMH or IMH, 0.2 g/l fructose, 3.9 g/l $(NH_4)_2SO_4$, 0.95 g KH_2PO_4, 2 g $K_2HPO_4{\cdot}3H_2O$, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.02 g $CaCl_2{\cdot}2H_2O$ and 10 ml of a trace element solution at pH 7.0. The trace element solution contained: 50 mg/l H_3BO_3, 40 mg/l $MnSO_4{\cdot}2H_2O$, 40 mg/l $ZnSO_4{\cdot}7H_2O$, 20 mg/l $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 20 mg/l $FeCl_3$, 10 mg/l KI and 4 mg $CuSO_4{\cdot}5H_2O$.

CA-plates, used for the isolation and/or storage of bacterial isolates, contained: 10 g/l casein peptone, 5 g/l yeast extract, 5 g/l glucose, 5 g/l NaCl, 15 g/l agar, pH 7.2 (Media 53, German Collection of Microorganisms and Cell Cultures). *CA*, used for the storage of bacterial isolates and for the starter cultures, contained: 10 g/l casein peptone, 5 g/l yeast extract, 5 g/l glucose and 5 g/l NaCl.

GM, according to [9], used as growth medium for biotransformation assays, contained: 10 g/l glucose, 6.5 g/l $(NH_2)_4SO_2$, 0.2 g/l $MgSO_4$, 0.02 g/l $MnCl_2.4H_2O$, 0.02 g/l $FeSO_4$, 0.02 g/l $CaCl_2{\cdot}2H_2O$, 5.54 g/l KH_2PO_4, 7.6 g/l $K_2HPO_4{\cdot}3H_2O$, 0.32 g/l citrate-1-hydrate and 1 g/l IMH at pH 6.8.

TBE (10×), used for agarose gel electrophoresis, was prepared as follows: 108 g Tris and 55 g boric acid were dissolved in 900 ml water. Forty milliliters 0.5 M Na_2EDTA (pH 8.0) was added and the volume adjusted to 1 l.

2.4. Isolation of hydantoin cleaving isolates

Two methods for the isolation of microorganisms possessing hydantoin-cleaving enzymes were used. First, soil samples were enriched aerobically in shake flasks, at 30 °C, 160 rpm on medium I supplemented with either IMH or HMH. Chinese soil samples were also incubated at 40 and 50 °C. Bacterial isolates were obtained by spreading the enriched medium on agar plates (selective plates) based on medium I, containing the enrichment substrate with addition of 15 g/l agar. Pure isolates were obtained by picking single colonies and replating onto fresh agar plates. Finally, isolates were transferred onto CA-plates. In a second screening method, approximately 1 g of soil sample was added to 10 ml sterile water, containing 0.0005 g nystatin (as fungicide) and 10 μl Triton®X100, mixed well and 100 μl of the resulting suspension was spread on selective plates (see above). Further isolation steps were conducted as described above. Chinese thermal pool sediment samples were also incubated at 40 °C and Antarctic soil samples at 4 °C. Isolates were stored for short periods at 4 °C on CA-plates or long term at −70 °C in a sterile solution of 20% glycerol in CA.

2.5. Assay of enzyme activity

For the detection of hydantoinase activity in the bacterial isolates, biotransformation experiments were conducted as follows: a loopfull of bacterial biomass was inoculated into 5 ml CA at 30 °C and incubated over night as a starter culture. Two milliliter starter culture was added to 50 ml of GM (supplemented with IMH as inducer for enzyme expression) and incubated at 30 °C and 160 rpm. Cells were harvested after 48 h by centrifugation (10 min, 8000 rpm and 4 °C; Beckman, AvantiTM J-25). Resting cells were obtained by washing twice with 100 mM K-phosphate buffer (pH 8), followed by centrifugation and resuspension in the same buffer. The substrate BnH was dissolved in 100 mM K-phosphate buffer (pH 8.0), assisted by a 30-min sonication. The assay substrates (50 mM DU or 10 mM BnH, dissolved in 100 mM K-phosphate buffer (pH 8.0)) were pre-incubated

at 40 °C. The reaction was started by addition of 1.5 ml resting cells to 1.5 ml pre-incubated substrate solution and was conducted at 40 °C. The reaction was stopped by addition of 100 μl 33% trifluoroacetic acid to 500 μl of the reaction solution and centrifugation at 13,000 rpm for 7 min (Heraeus, Biofuge pico).

2.6. Analysis

The concentrations of BnH and L- and/or D-C-Phe were determined by HPLC analysis (Merck Hitachi, La Chrom) according to [10], using a Nucleodex β-PM-column (Macherey-Nagel, Germany). The mobile phase contained 20% MeOH/80% (0.1% H_3PO_4) solution, pH 3.7 (NaOH). The flow rate was 0.2 ml/min. Detection was carried out at 210 nm. The product of DU-biotransformations, *N*-carbamoyl-β-alanine, was determined photometrically after derivatization. Three hundred microliters of the cell free biotransformation supernatant were added to 800 μl Ehrlich Reagent (1 g 4-dimethylaminobenzaldehyde, 5 ml H_2O, 5 ml 6 M HCl) and 900 μl water. The quantification of the yellow product was performed photometrically at 430 nm (Unicam, Heλos α).

2.7. 16S rDNA preparation and sequencing

Genomic DNA from the bacterial isolates was extracted and purified using the Quiagen DNeasy Tissue Kit (Quiagen), following the manufacturers instructions for Gram-positive bacteria. DNA was checked for quality and quantity using 1% agarose gel electrophoresis in TBE buffer stained with ethidium bromide (10 μl/100 ml) and quantified under UV-light in comparison to standard gene fragments of 10 and 25 ng. Polymerase chain reaction (PCR) was used for the amplification of the 16S rRNA gene and performed using a ThermoHybaid PCRsprint Thermocycler under the following conditions: 0.2 mM dNTPs (Promega, USA), 5 μl *Taq*-Polymerase 10×-reaction buffer (Promega), 0.5 mM forward primer, 0.5 mM reverse primer, 1.5 mM $MgCl_2$ (Promega), 0.5 μl *Taq*-Polymerase (Promega), 1 μl genomic DNA, and PCR water combined to a total volume of 50 μl. The following Universal Bacterial oligonucleotide primer sequences were used: forward primer E9F (5′-GAG TTT GAT CCT GGC TAG [11]) and reverse primer U151OR (5′-GGT TAC CTT GTT ACG ACTT [12]). For the amplification the following cycles were used: Initial denaturation at 94 °C for 1 min followed by 30 amplification cycles with template DNA denaturation at 94 °C for 1 min, primer annealing at 51 °C for 1 min and primer extension at 72 °C for 1 min and final extension at 72 °C for 10 min.

Amplified DNA was purified from the reaction mixture by agarose gel purification using the GFX™ PCR-DNA and gel band purification kit (Amersham Bioscience, New York). PCR products were sequenced by the Department of Molecular and Cell Biology, University of Cape Town, South Africa, using the oligonucleotide primer E9F. The resulting nucleotide sequences were analysed using the BioEdit Sequence Alignment Editor software (Copyright® 1997–2001 Tom Hall, Department of Microbiology, North Carolina State University). Nucleotide sequence homology searches were carried out using the BlastN electronic mail server from the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/).

3. Results and discussion

Bacterial isolates with the ability to grow on IMH as a nutritional source were recovered from enrichment experiments, but interestingly, no hydantoinase-positive bacteria were found in enrichment cultures using HMH. Since all bacterial isolates were isolated using IMH as an enrichment substrate, this hydantoin was routinely used as an inducer for hydantoinase expression in bioconversion experiments. Isolates with activity towards BnH and DU were characterized using 16S rRNA gene amplification, sequencing and database comparisons. The isolates were assigned according to the closest 16S rDNA match (Table 1). Further characterization via classical bacterial identification is in progress.

In total, 32 bacterial strains were isolated from terrestrial soil samples of different environmental sources (see Table 1). *Pseudomonas putida* strains and an *Agrobacterium tumefaciens* strain have previously been isolated from South Africa and characterized [13]. Pseudomonads have been isolated from a wide variety of sources, including soils, fresh or sea water, sewage, foodstuffs and food industry wastes [14].

Various members of the genus *Pseudomonas* with the ability to produce carbamoyl-amino acids and/or amino acids have been reported, including a *Pseudomonas desmolyticum* isolate with the capability to produce D-phenylglycine [15] and various *P. putida* strains [16–18]. In this study, bacterial isolates with hydantoinase activity, belonging to the genus *Pseudomonas*, were found in a variety of soil samples including dry alpine soils, compost and oligotrophic gravels from Antarctica. All isolates showed degradation of the test substrates DU and BnH, but at different levels of conversion (Table 1).

Of particular interest are two *Pseudomonas* strains isolated from Antarctic soil samples, one (isolate G7) from Bratina Island and the other (isolate N7) from the Miers Valley, Antarctica. These dry valley deserts of Eastern Antarctica are generally accepted to be some of the harshest arid environments on earth and have formerly been considered to be highly unfavourable to life [19]. No Antarctic bacterial isolates have been reported previously as having hydantoinase activity. The discovery of *Pseudomonas* strains from this environment is not unexpected; previous literature reports describe 32 psychrophilic bacteria belonging to the genus *Pseudomonas* having been isolated from an Antarctic cyanobacterial mat. Three novel species were named (*Pseudomonas antarctica* sp. nov., *Pseudomonas meridiana* sp. nov and *Pseudomonas proteolytica* sp. nov. [20]). Psychrophiles are defined as microorganisms possessing cardinal growth temperatures of 15 °C (optimal), 20 °C (maximal) and 0 °C (minimal) [21]. Recent growth experiments showed that the *Pseudomonas* strains G7 and N7 were able to grow well under low temperature and had higher growth rate at 20 than at 30 °C (data not shown). The ability of all isolates to grow at approximately 30 °C is fully consistent with the growth characteristics of psychrotrophic organisms.

Table 1
Hydantoinase positive bacterial isolates

Description of soil sample	Isolate no.	Genus	%Homology	DU-biotr	BnH-biotr	
			bp	C-Ala	L-C-Phe	D-C-Phe
SA, Table mountain, 700 m a.s.l, soil	N1	*Stenotrophomonas* sp.	99%/472	+	−	++
SA, Stellenbosch, pinewood forest, soil	K3	Unclassified Microbacteriacae	100%/445	++	+	+
	M3	*Burkholderia* sp.	98%/513	+	+	−
SA, Worchester, Distell Brandy Distillery, biofilm of a RBC for wine distillery wastewater	D24	*Ochrobactrum* sp.	98%/749	+++	−	+
SA, Stellenbosch, Reliance Compost Company, compost of wine yards	G6	*Pseudomonas* sp.	98%/692	++	−	+
	C15	*Ochrobactrum* sp.	99%/530	+++	−	++
	H7	*Staphylococcus* sp.	99%/592	++	−	+
SA, Stellenbosch, Reliance Compost Company, compost of fiber material	F8	*Flavobacterium* sp.	98%/563	++	−	+
SA, Stellenbosch, Reliance Compost Company, shredder material (5 weeks old)	K5	*Acinetobacter* sp.	98%/356	++	−	+
	I21	*Ochrobactrum* sp.	99%/585	+++	−	+
	J24	*Ochrobactrum* sp.	100%/586	++	−	+
SA, Stellenbosch, Reliance Compost Company, shredder material (12 weeks old)	J7	*Enterobacter* sp.	97%/384	+	−	+
	N8	*Pseudomonas* sp.	99%/492	+++	−	+
	P8	*Pseudomonas* sp.	99%/754	++	−	+
SA, West Coast N. P., guano from a small pond at Langebaan lagoon	A16	*Bacillus* sp.	99%/727	+++	−	++
SA, Veldrift, "guano" from a salt lake with bird dung	F16	*Bacillus* sp.	100%/520	++	−	++
SA, West Coast N.P., algal material of Langebaan lagoon	D17	*Bacillus* sp.	100%/688	++	−	+
	F21	*Ochrobactrum* sp.	100%/735	+++	−	+
SA, Cederberg mountains, 1100 m a.s.l., dry soil	L9	*Pseudomonas* sp.	99%/584	++	−	+
SA, Tsitsikamma N.P., soil of an Afromontane forest	M18	*Pseudomonas* sp.	99%/445	++	−	+
	H20	*Bacillus* sp.	100%/565	++	−	++
Swaziland, Ezulweni valley, red soil	G18	*Bacillus* sp.	100%/501	++	−	++
Lesotho, Blue Mountain Pass, soil, 2500 m a.s.l	K20	*Arthrobacter* sp.	100%/587	+	−	+
Inner Mongolia, IHX	F18	*Bacillus* sp.	99%/736	++	−	++
Inner Mongolia, BJ1	K18	*Bacillus* sp.	99%/633	++	−	++
Inner Mongolia, hypersaline lake; GHI	I20	Probably *Streptomyces*	−	+	−	+
	G21	*Ochrobactrum* sp.	95%/351	+++	−	+
Antarctic, Dry Valley, soil	N7	*Pseudomonas* sp.	98%/524	+++	−	+
Antarctic, Bratina Island, sediment of a saline pond	E7	*Arthrobacter* sp.	99%/764	++	−	+
	F7	*Arthrobacter* sp.	98%/593	++	−	++
	G7	*Pseudomonas* sp.	99%/711	+++	−	+++
Long Pu, Kunming, SW China, algal mat, 60–65 °C (screening at 50 °C)	I24	*Delftia* sp.	99%/588	+++	−	+

%Homology/bp: homology to the best match in the database, and the number of nucleotides used in the sequence comparison using E9F as sequencing primer. Conversion data are shown as: (DU to *N*-C-Ala + <1 mM; ++ >1 mM < 10 mM; +++ >10 mM) and BnH-biotransformation D/L-C-Phe (− no product; + <0.5 mM; ++ >0.5 mM).

Bacterial isolates from the genus *Pseudomonas* and *Bacillus* represent the major groups in this screening approach. Both genera were found in South African compost samples and dry soil samples (Fig. 1), whereas two *Bacillus* strains (isolates F18 and K18) where isolated from a saline environment. Both strains, as well as *Ochrobactrum* G21 and strain I20, were able to grow under saline conditions (GM supplemented with 10% NaCl), but not at a higher salt content (data not shown). All four strains were therefore designated as moderate halophilic. Different *Bacillus* strains with the ability to cleave hydantoins have been reported in literature, and most possess thermostable hydantoinases [22–24].

Only one *Ochrobactrum anthropi* strain has been reported previously. This strain, isolated from a soil sample from Spain, showed the ability to release methionine from D,L-(2-methylthioethyl) hydantoin [25]. The hydantoinase of this strain was inducible with D,L-(2-methylthioethyl) hydantoin and was alkalostable, with a pH optimum of 9.0. In the present study, a number of *Ochrobactrum* strains were isolated, mostly from compost samples (see Table 1; Fig. 1) and from a biofilm of a

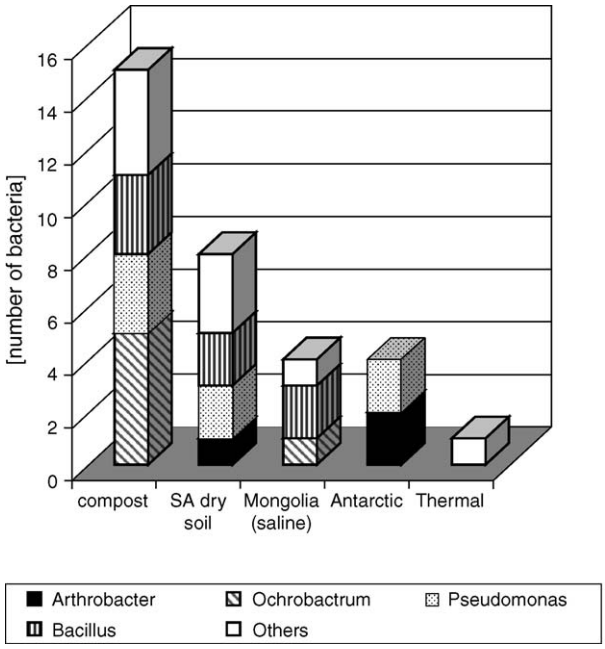

Fig. 1. Distribution of isolated bacterial genera in relation to environmental type. Soil samples (described in Table 1) are grouped by the dominant property. *Compost:* derived from compost or from habitats with a high amount of degrading material. *SA dry soil:* samples from South African desert and montane sites. *Mongolia (saline):* all saline Mongolian sediment samples. *Antarctic:* all Antarctic soil samples. *Thermal:* Sediment samples from habitats with elevated temperature (more than 50 °C).

rotating biological contactor (RBC) used for the treatment of a wine distillery wastewater. In addition, one *Ochrobactrum* strain has been isolated from a hypersaline salt lake from Inner Mongolia. To the authors' knowledge, only one hydantoinase has been reported from a halophilic organism; a halophilic *Pseudomonas* sp. NCIM5109 isolated from sea water was shown to have the ability to produce D-*N*-carbamoylphenylglycine [26].

The genus *Arthrobacter* has previously been shown to be an important source of hydantoinase activities. These Gram-positive organisms are found in a wide variety of ecological habitats: in soils and sewage, and associated with fish and plants [27]. The hydantoinases of the *A. aurescens* strains DSM3745 and 3747 have been well-described [28] and both produce α-amino acids from 5-monosubstituted hydantoins [28]. The hydantoinase from *A. aurescens* DSM 3745 belongs to the amidohydrolase superfamily [5] and has been shown to be a Zn^{2+}-metalloenzyme [29,30] with L-selectivity in the conversion of D,L-5-(3′-indolylmethyl)-hydantoin. The hydantoinase and carbamoylase genes from *A. aurescens* DSM 3747 and DSM 3745 have each been cloned and their nucleotide sequences determined. These two enzymes show a high degree of nucleotide and amino acid sequence identity (96–98%) [31,32].

In this study, *Arthrobacter* strains were found both in hot desert (African) and cold desert (Antarctic) soils.

Two other *Arthrobacter* strains (F7 and G7) were found in sediment of a saline pond from Bratina Island, Antarctica. These bacterial isolates showed the same growth characteristics as the *Pseudomonas* strains N7 and G7 (data not shown), and have been designated as psychrotrophic. The isolation of *Arthrobacter* strains from this environment is not unexpected, since psychrophilic and psychrotrophic *Arthrobacter* strains have been reported from samples of subterranean cave silts [33], glacier silts [34] and the soils of Antarctica [35–39]. Psychrotrophic bacteria isolated from cyanobacterial mats in the McMurdo Dry Valleys, Antarctica have been recently characterized as *Arthrobacter flavus* sp. nov. [40] and *Arthrobacter roseus* sp. nov. [41]. Two isolates from penguin rookery soil samples in Antarctica have been proposed as *Arthrobacter gangotriensis* sp. nov. and *Arthrobacter kerguelensis* sp. nov. [42].

We also report the isolation at a growth temperature of 50 °C of a *Delftia* sp. strain from a hot spring algal mat (Long Pu, China) with activity towards BnH and DU. To our knowledge, no hydantoinase from this genus has been reported previously. However, thermophilic hydantoinases have been described. A thermophilic D-hydantoinase from the moderate thermophile *Bacillus stearothermophilus* SD-1 was shown to have pH and temperature optima of approximately 8.0 and 65 °C, respectively [22]. The most thermophilic hydantoinase reported to date (T_{opt} = 80 °C) is derived from the hyperthermophilic archaeon, *Methanococcus jannaschii;* isolated from a submarine hydrothermal vent [43]. Thermostable hydantoinases have also been isolated from a mesophilic *Bacillus* sp. AR9 [23] and a moderate thermophilic *B. stearothermophilus* NS1122A, with optimal temperatures between 60 and 70 °C [24].

D-Hydantoinase activity has been reported in *Burkholderia pickettii* [44], towards D,L-5-mercaptoethyl-hydantoin in *Enterobacter cloacae* [45], and in several different *Flavobacterium* species [46,47]. Bacterial isolates recovered from South African soil and belonging to these genera were also found in our study.

We also report the detection of hydantoinase activity in bacterial genera where this enzyme has not been reported previously. These include *Stenotrophomonas* sp. N1, *Microbacteriacae* sp. K3, *Staphylococcus* sp. H7, *Acinetobacter* sp. K5, *Delftia* sp. I24 and an unidentified isolate I20 (possibly *Streptomyces*; Dr. Eberspächer, personal communication).

The aim of this study was the recovery of microorganisms with the ability to cleave hydantoins. The standard condition for principal isolation of microorganisms was medium I (pH 7.0) at 30 °C. Apart from *Delftia* sp. I24, all bacterial isolates were recovered under standard conditions. However, isolates originating from saline and cold environments (Mongolia and Antarctica, respectively) were not isolated under conditions exactly reflecting their environmental origins. After subsequent tests for their ability to grow under saline and/or cold conditions, these strains were designated as psychrotrophic or moderately halophilic, as appropriate.

4. Conclusion

We conclude, on the basis of our isolation data and published literature that: (i) hydantoinase-positive bacterial isolates are very widely dispersed, both geographically and with respect to environmental conditions, and (ii) among culturable aerobes, hydantoinases are predominantly found in certain genera (*Pseudomonas*, *Ochrobactrum*, *Bacillus*, *Arthrobacter*). We have also

described microorganisms for which no hydantoinase activity was reported previously.

With respect to the natural function of hydantoinases, it may be significant that the majority of the isolates recovered were derived from samples with a high organic load, such as compost soil and guano. These findings support the hypothesis that hydantoinases are involved in catabolic pathways to access hydantoins and hydantoin-like molecules as metabolic substrates.

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References

[1] A. Louwrier, C.J. Knowles, Enzyme Microb. Technol. 19 (1996) 562–571.
[2] C. Syldatk, A. Läufer, R. Müller, H. Höke, Adv. Biochem. Eng. Biotechnol. 41 (1990) 29–75.
[3] C. Syldatk, O. May, J. Altenbuchner, R. Mattes, M. Siemann, Appl. Microb. Biotechnol. 51 (1999) 293–309.
[4] S. Runser, P.C. Meyer, Eur. J. Biochem. 213 (1993) 1315–1324.
[5] O. May, A. Habenicht, R. Mattes, C. Syldatk, M. Siemann, Biol. Chem. 379 (1998) 743–747.
[6] Z. Gojkovic, L. Rislund, B. Andersen, M.P.B. Sandrini, P.F. Cook, K.D. Schnackerz, J. Piškur, Nucleic Acids Res. 31 (2003) 1683–1692.
[7] C. Syldatk, D. Cotoras, G. Dombach, C. Gross, H. Kallwass, F. Wagner, Biotechnol. Lett. 9 (1987) 25–30.
[8] R. Tsugawa, S. Okumura, T. Ito, N. Katsuga, Agric. Biol. Chem. 30 (1966) 27–34.
[9] C. Syldatk, V. Mackowiak, H. Höke, C. Gross, G. Dombach, F. Wagner, J. Biotech. 14 (1990) 345–362.
[10] H. Teves, T. Waniek, M. Pietzsch, C. Syldatk, M. Reuss, J. Fresenius, Anal. Chem. 363 (1999) 738–743.
[11] V. Farrelly, F.A. Rainey, E. Stackebrandt, Appl. Environ. Microbiol. 61 (1995) 2798–2801.
[12] A.L. Reysenbach, N.R. Pace, Archaea. A Laboratory Manual. Thermophiles, Cold Spring Harbour, 1995.
[13] S.G. Burton, R.A. Dorrington, C. Hartley, S. Kirchmann, G. Matcher, V. Phehane, J. Mol. Catal. B: Enzymatic 5 (1998) 301–305.
[14] A. Balows, H.G. Trüper, M. Dworkin, W. Harder, K.H. Schleifer, The Prokaryotes, second ed., Springer Verlag, New York, 1992, p. 3074.
[15] D.V. Gokhale, K.B. Bastawde, S.G. Patil, U.R. Kalkote, R.A. Joshi, T. Ravindranathan, B.G. Gaikwad, V.V. Jogdand, S. Nene, Enzyme Microb. Technol. 18 (1996) 353–357.
[16] H.R. Chien, W.-H. Hsu, Biotechnol. Tech. 10 (1996) 879–882.
[17] H.R. Chien, Y.L. Jih, W.Y. Yang, W.H. Has, Biochim. Biophys. Acta 1395 (1998) 68–77.
[18] G. La Pointe, S. Viau, D. LeBlanc, N. Robert, A. Morin, Appl. Environ. Microbiol. 60 (1994) 888–895.
[19] W.L. Boyd, J.T. Staley, J.W. Boyd, Antarctic Res. Ser. 8 (1996) 125–159.
[20] G.S.N. Reddy, G.I. Matsumoto, P. Schumann, E. Stackebrandt, S. Shivaji, Int. J. Syst. Evol. Microbiol. 54 (2004) 713–719.
[21] R.Y. Morita, Bacteriol. Rev. 39 (1975) 144–167.
[22] S.G. Lee, D.C. Lee, S.P. Hong, M.H. Sung, H.S. Kim, Appl. Microb. Biotechnol. 43 (1995) 270–276.
[23] A. Sharma, R.M. Vohra, Biochem. Biophys. Res. Commun. 234 (1997) 485–488.
[24] T. Ishikawa, Y. Mukohara, K. Watabe, S. Kobayaschi, H. Nakamura, Biosci. Biotechnol. Biochem. 58 (1994) 265–270.
[25] C. Pozo, B. Rodelas, S. de la Escalera, J. González-López, J. Appl. Microbiol. 92 (2002) 1028–1034.
[26] S.S. Sudge, K.B. Bastawde, D.V. Gokhale, U.R. Kalkote, T. Ravindranathan, Appl. Microbiol. Biotechnol. 49 (1998) 594–599.
[27] A. Balows, H.G. Trüper, M. Dworkin, W. Harder, K.H. Schleifer, The Prokaryotes, second ed., Springer Verlag, New York, 1992, pp. 1285–1286.
[28] C. Gross, C. Syladtk, F. Wagner, Biotechnol. Tech. 1 (1987) 85–90.
[29] O. May, M. Siemann, M.G. Siemann, C. Syldatk, J. Mol. Catal. B: Enzym. 5 (1998) 367–370.
[30] O. May, M. Siemann, C. Syldatk, J. Mol. Catal. B: Enzym. 4 (1998) 211–218.
[31] B. Wilms, A. Wiese, C. Syldatk, R. Mattes, J. Altenbuchner, M. Pietsch, J. Biotechnol. 68 (1999) 101–113.
[32] A. Wiese, B. Wilms, C. Syldatk, R. Mattes, J. Altenbuchner, Appl. Microbiol. Biotechnol. 55 (2001) 750–757.
[33] A.M. Gounot, Ann. Inst. Pasteur Paris 113 (1967) 923–945.
[34] A. Moiroud, A.M. Gounot, Hebd. Seances Acad. Sci. Ser. D Sci. Nat. 269 (1969) 2150–2152.
[35] R.M. Johnson, M. Inai, S. McCarthy, J. Ariz-Nev. Acad. Sci. 16 (1981) 51–60.
[36] R.M. Johnson, R.D. Bellinoff, Antarct. Res. Ser. 30 (1981) 169–184.
[37] J.M. Madden, S.K. Siegel, R.M. Johnson, Antarct. Res. Ser. 30 (1979) 77–103.
[38] J. Siebert, P. Hirscht, Polar Biol. 9 (1988) 37–44.
[39] S. Shivaji, N.S. Rao, L. Saisree, G.S.N. Reddy, G. Seshu Kumar, P.M. Bhargava, Polar Biol. 10 (1989) 225–229.
[40] G.S.N. Reddy, R.K. Aggarwal, G.I. Matsumoto, S. Shivaji, Int. J. Syst. Evol. Microbiol. 50 (2000) 1553–1561.
[41] G.S.N. Reddy, J.S.S. Prakash, G.I. Matsumoto, E. Stackebrandt, S. Shivaji, Int. J. Syst. Evol. Microbiol. 52 (2002) 1017–1021.
[42] P. Gupta, G.S.N. Reddy, D. Delille, S. Shivaji, Int. J. Syst. Evol. Microbiol. 54 (2004) 2375–2378.
[43] J.-H. Chung, J.-H. Back, J.-H. Lim, Y.I. Park, Y.S. Han, Enzyme Microb. Technol. 30 (2002) 867–874.
[44] Z. Xu, Y. Liu, Y. Yang, W. Jiang, E. Arnold, J. Ding, J. Bacteriol. 185 (2003) 4038–4049.
[45] G. LaPointe, D. Leblanc, A. Morin, Appl. Microbiol. Biotechnol. 42 (1995) 895–900.
[46] K. Yokozeki, K. Sano, C. Eguchi, K. Yamada, K. Mitsugi, Agric. Biol. Chem. 51 (1987) 363–369.
[47] Y. Nishida, K. Nakamichi, K. Nabe, T. Tosa, Enzyme Microb. Technol. 9 (1987) 721–725.