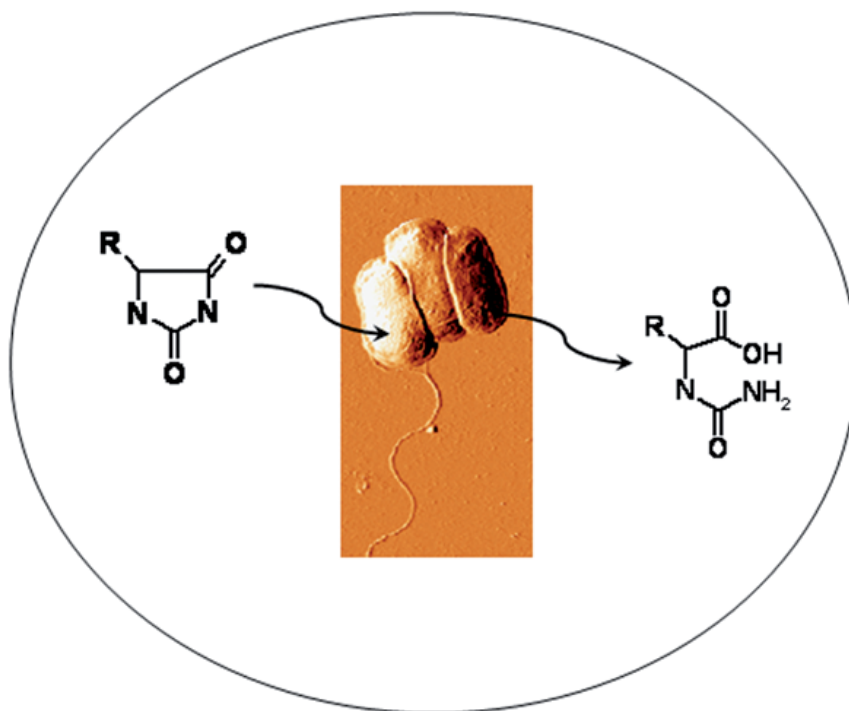


Ralph Dürr

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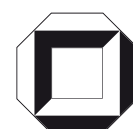


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von
Ralph Dürr



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SCREENING AND DESCRIPTION OF NOVEL HYDANTOINASES FROM DISTINCT ENVIRONMENTAL SOURCES

Von der Fakultät für Chemieingenieurwesen der Universität Karlsruhe
zur Erlangung der Würde eines Doktors der Ingenieurwissenschaften (Dr.-Ing.)
Genehmigte Dissertation

Vorgelegt von **Ralph Dürr**
(Biologe t.o.)
aus Ulm/Donau

Referent: Prof. Dr. Christoph Syldatk
Koreferent: Prof. Dr. Stephanie Burton
Tag des Kolloquiums: 05.07.2007

"Immer, wenn Dir eine Theorie als die wirklich einzig mögliche erscheint, nimm das als Zeichen, dass Du weder die Theorie noch das zu lösende Problem verstanden hast."

Karl Popper in *Objective Knowledge - an evolutionary approach* (1972)

Acknowledgment

I would like to thank the following persons for supporting the making of this Ph.D. thesis: Prof. Dr. C. Syldatk for the provision and guidance through this Ph.D. project, for the opportunity to conduct the work at his department, as well as for his help and the opportunity to realise my research visit in South Africa.

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Abstract

A screening program was conducted in order to find bacteria with "novel" hydantoinase properties. Bacteria with hydantoinase activity were recovered from terrestrial soil samples of different geographic origins, including extreme environments using culture-based screening methods. All these isolates possessed the capability to transform the model substrates D,L-5-benzylhydantoin and 5,6-dihydrouracil to the corresponding *N*-carbamoyl amino acids. The recovered bacteria were identified based on 16S rRNA gene amplification and were shown to belong to the genera *Acinetobacter*, *Arthrobacter*, *Burkholderia*, *Bacillus*, *Delftia*, *Enterobacter*, *Flavobacterium*, *Microbacteriaceae*, *Ochrobactrum*, *Pseudomonas*, *Staphylococcus*, *Stenotrophomonas* and *Streptomyces*.

These findings showed that microorganisms with hydantoinase activity are (i) distributed in various geographically distinct environmental habitats; (ii) distributed worldwide; (iii) found in certain bacterial genera. Furthermore, the presence of hydantoinase activity was shown in genera in which hydantoinase activity has not previously been reported.

A greater selected number of the recovered bacterial isolates was used for further studies. The hydantoinases of these isolates showed a broad substrate spectrum in whole-cell activity assays. No correlation between the hydantoinase specificity and a certain bacterial genus and/ or a habitat could be obtained. Polymerase chain reaction (PCR) using degenerate primer was applied for the amplification of a partial hydantoinase gene fragment. A successful hydantoinase amplification was not achieved for all strains tested, probably due to the low sequence homology of hydantoinases. Phylogenetic analysis revealed that hydantoinases and dihydropyrimidinases can be clustered on the DNA-level according to their bacterial lineage. Two DIG-labelled DNA probes were used for the search of hydantoinase genes in genomic DNA of the strains mentioned above and an *Arthrobacter* strain collection. A positive hybridisation signal was obtained amongst others for *A. ilicis* DSM20138, *A. psychrolactophilus* DSM15612 and *A. methylotrophus* DSM14008 under very stringent hybridisation conditions. All three strains showed no hydantoinase activity in biotransformations. Cryptic hydantoinase genes are assumed. They can be considered as silent/ cryptic because the hydantoinase is not expressed during growth, possibly due to the absence of an appropriate inducer.

The wild-type strains *Delftia* sp. I24, *Ochrobactrum* sp. G21 and *Bacillus* sp. F18 were chosen for further studies because all three strains were isolated from extreme habitats, either thermal spots or hypersaline lakes. The strains were described in detail using classical and molecular biological identification methods as *Delftia* sp. I24, *Ochrobactrum* sp. G21 and *Bacillus megaterium* F18. The methods used did not allow to identify the first two strains to the genus level.

The influence of bacterial growth on hydantoinase activity was investigated: Addition of

NaCl to the growth media turned out to be favourable to achieve high enzyme activity for the two halophilic strains *Ochrobactrum* sp. G21 and *Bacillus megaterium* F18. Salt stress is proposed as an activator for degradative enzymes and a co-regulation of the hydantoinase system, probably to get access to amino acids under hindered conditions. The hydantoinase from *Ochrobactrum* sp. G21 showed maximal induction by D,L-2-naphthylmethylhydantoin, from *Bacillus megaterium* F18 by D,L-5-*tert*-butylhydantoin and from *Delftia* sp. I24 with D,L-6-phenyl-5,6-dihydrouracil. Hydantoinase activity was highest in the late exponential growth phase of *Delftia* sp. I24 when grown on growth medium (GM) supplemented with or without 6-phenyl-5,6-dihydrouracil and for *Bacillus megaterium* F18 when grown in GM supplemented with 5% NaCl and D,L-5-*tert*-butylhydantoin. Highest activity of *Ochrobactrum* sp. G21 was obtained in GM supplemented with 5% NaCl and D,L-2-naphthylmethylhydantoin, but growth was very slow.

The biochemical properties of the hydantoinases were determined: The hydantoinase of *Delftia* sp. I24 had a temperature and pH optimum of 30°C and pH 9.0 and showed cofactor requirement for Ni²⁺ and Co²⁺. The enzyme was stable at 4°C and 20°C for four days. The hydantoinase of *Ochrobactrum* sp. G21 showed highest activity at 40°C and pH 8.0–8.5. The hydantoinase was cofactor dependent, but no preference for one of the ions tested was indicated. The enzyme lost activity during storage at all temperatures tested. Most activity remained after storage at 4°C. The hydantoinase of *Bacillus megaterium* F18 had a temperature and pH optimum of 45–50°C and pH 7.5–8.0. The hydantoinase showed cofactor dependency with preference to Mn²⁺ and Co²⁺. All three hydantoinases showed a very broad substrate range but with different activities regarding to the respective substrate and compared to each other.

This is the first report on characterisation of a hydantoinase from the genus *Delftia*. Additionally, no hydantoinase activity has been reported yet for halophilic *Ochrobactrum* and/or *Bacillus* species.

Of special interest was the elucidation of the genes being responsible for hydantoin and dihydropyrimidine degradation of the wild-type strains *Delftia* sp. I24 and *Ochrobactrum* sp. G21. The putative gene cluster of *Delftia* sp. I24 included four genes: an incomplete NADPH-dependent glutamate synthase (*gltB*), dihydropyrimidine dehydrogenase (*pydA*), permease (*hyuP*) and a D-hydantoinase (*hyuH*). The hydantoinase gene was expressed in *E. coli* and hydantoinase activity shown for D,L-5-(3-indolylmethyl) hydantoin and D,L-5-benzylhydantoin. The presence of a dihydropyrimidine dehydrogenase and the preference in whole-cell biotransformations for 5,6-dihydrouracil rather than hydantoin indicates that these enzymes are involved in the pyrimidine reduction pathway. The putative gene cluster of *Ochrobactrum* sp. G21 comprised nine ORFs, six being potentially involved in hydantoin-hydrolysis: carbamoylase (*hyuC*), D-hydantoinase (*hyuH*), two transporters (*OrfS1* and *OrfS2*) and two permeases (*hyuP1* and *hyuP2*). The hydantoinase was as well expressed in *E.*

coli and activity shown for the same substrates as for the cloned hydantoinase of *Delftia* sp. I24. This is the first report on the genetical organisation of hydantoin degradation for members of the genus *Delftia* and *Ochrobactrum*. Both, but especially the hydantoin-hydrolysing gene cluster of *Delftia* sp. I24 showed a high similarity with putative gene clusters from closely related bacteria. This included the presence of certain genes, gene pattern and gene orientation.

Phylogenetic analysis on a protein level of the two "novel" hydantoinases, known hydantoinases and dihydropyrimidinases, including putative protein sequences, revealed that these enzymes can be clustered, with some exceptions, in the following groups: *Rhizobiales* family (Rhizo-Fam), *Comamonadaceae* family (Com-Fam), *Pseudomonas* family (Pseud-Fam), *Bacilli* family (Bac-Fam) and *Agrobacterium* family (Agro-Fam). The highly conserved "histidine motif" of the superfamily of amidohydrolases as well as the conserved amino acids forming the active site can be found for all protein sequences used in this study. A difference was found in the substrate recognition sites, whereas some of the groups mentioned above showed to possess the same recognition sites as known hydantoinases. All these findings strongly support the hypothesis of a common ancestor for all members of the superfamily of amidohydrolases.

Zusammenfassung

Um Bakterien mit "neuen" Hydantoinaseeigenschaften zu finden, wurde ein Screening durchgeführt. Bakterien mit Hydantoinaseaktivität wurden mittels klassischer Anreicherungsverfahren aus Bodenproben von geographisch verschiedenen Ökosystemen isoliert, inklusive extremer Habitate. Die gewonnenen bakteriellen Isolate besaßen die Fähigkeit, die Modellsubstrate D,L-5-Benzylhydantoin und 5,6-Dihydrouracil zu der jeweils korrespondierenden Carbamoylaminosäure umzuwandeln. Die Analyse der 16S rRNA Gensequenz zeigte, dass die gewonnenen Wildstämme den folgenden Gattungen zuzuordnen sind: *Acinetobacter*, *Arthrobacter*, *Burkholderia*, *Bacillus*, *Delftia*, *Enterobacter*, *Flavobacterium*, *Microbacteriaceae*, *Ochrobactrum*, *Pseudomonas*, *Staphylococcus*, *Stenotrophomonas* und *Streptomyces*.

Hieraus konnte gefolgert werden, dass Mikroorganismen mit Hydantoinaseaktivität (i) in diversen geographisch verschiedenen Ökosystemen verteilt sind; (ii) weltweit vorkommen; (iii) hauptsächlich in gewissen Gattungen vorkommen. Dennoch konnten neue Gattungen gefunden werden, für die bisher noch keine Hydantoinaseaktivität beschrieben wurde.

Für weiter führende Studien wurde eine größere Anzahl der isolierten Bakterienstämme ausgewählt. Die Mehrzahl der Hydantoinasen dieser Stämme zeigte in Ganzzell-Aktivitätstests ein breites Substratspektrum. Jedoch konnte kein Zusammenhang zwischen der Substratspezifität der Hydantoinase und der Artzugehörigkeit und/ oder des Habitats des jeweiligen Vertreters gefunden werden. Zur Amplifizierung von bestimmten Hydantoinasegenabschnitten wurde die Polymerase Kettenreaktion (PCR) mit degenerierten Primern angewandt. Es konnte nicht für alle getesteten Bakterien ein Genabschnitt amplifiziert werden. Phylogenetische Studien mit den gefundenen partiellen Hydantoinasegenen und einigen in Datenbanken abgelegten Hydantoinasegenen zeigte, dass Hydantoinasen und Dihydropyrimidinasen in Abhängigkeit Ihrer Abstammung gruppiert werden können. Zwei DNA-Sonden wurden für die Suche nach Hydantoinasegenen in genomischer DNA der oben genannten Bakterien und einer *Arthrobacter* Stammsammlung verwendet. U.a. wurde ein positives Hybridisierungssignal, das die Präsenz einer möglichen Hydantoinase andeutet, für die Stämme *A. ilicis* DSM20138, *A. psychrolactophilus* DSM15612 und *A. methylotrophus* DSM14008 gefunden. In Aktivitätstests mit ganzen Zellen zeigten alle drei Stämme keine Aktivität für verschiedene Hydantoine und Dihydropyrimidine. So genannte "kryptische" oder "stille" Hydantoinasegene werden vermutet. Diese Gene werden möglicherweise während des Zellwachstums nicht abgelesen, da ein geeigneter Induktor für die jeweilige Hydantoinase nicht vorhanden war.

Die Wildstämme *Delftia* sp. I24, *Ochrobactrum* sp. G21 und *Bacillus* sp. F18 wurden für weitere Untersuchungen herangezogen, da diese drei Stämme aus extremen Habitaten (heißen Quellen oder hypersalinen Seen) isoliert worden waren. Die drei Wildstämme konnten mittels klassischer und molekularbiologischer Bestimmungsmethoden den fol-

genden Spezies zugeordnet werden: *Delftia* sp. I24, *Ochrobactrum* sp. G21 und *Bacillus megaterium* sp. F18. Es war mit den angewandten Methoden nicht möglich, die Art der beiden erstgenannten Stämme zu identifizieren.

Weiterhin wurde der Einfluss von verschiedenen Wachstumsfaktoren auf die Hydantoinaseaktivität untersucht: Die Zugabe von Salz zum Medium führte zu einer signifikanten Erhöhung der Hydantoinaseaktivität der halophilen Stämme *Ochrobactrum* sp. G21 und *Bacillus megaterium* sp. F18. Möglicherweise veranlasst Salzstress die Expression abbauender Enzyme und des Hydantoinasesystems, letzteres um Zugang zu Aminosäuren für den Metabolismus unter erschwerten Bedingungen zu erhalten. Einen großer Effekt auf die Hydantoinaseaktivität hatte die Zugabe von Induktoren zum Wachstumsmedium. Die höchste Aktivität wurden jeweils mit D,L-6-Phenyl-5,6-Dihydrouracil für die Hydantoinase von *Delftia* sp. I24, mit D,L-2-Naphtylmethylhydantoin für *Ochrobactrum* sp. G21 und D,L-5-*tert*-Butylhydantoin für *Bacillus megaterium* sp. F18 erzielt. Die besten Medien bezüglich Hydantoinaseaktivität waren: Wachstumsmedium (GM) mit und ohne D,L-6-Phenyl-5,6-Dihydrouracil für die Hydantoinase von *Delftia* sp. I24, GM ergänzt mit 5% NaCl und D,L-5-*tert*-Butylhydantoin für *Bacillus megaterium* sp. F18 und GM ergänzt mit 5% NaCl und D,L-2-Naphtylmethylhydantoin für *Ochrobactrum* sp. G21. Für die beiden erstgenannten Stämme wurde die maximale Hydantoinaseaktivität in der späten exponentiellen Phase festgestellt. Dahingegen erreichte *Ochrobactrum* sp. G21 bei geringem Zellwachstum die maximale Hydantoinaseaktivität erst nach einer Wachstumszeit von 96 Stunden.

Die Hydantoinasen der einzelnen Stämme besaßen die folgenden biochemischen Eigenschaften: Die Hydantoinase von *Delftia* sp. I24 zeigte ein Temperatur- und pH-Optimum von 30°C und pH 9.0. Biochemische Studien weisen darauf hin, dass diese Hydantoinase abhängig von den Cofaktoren Ni²⁺ und Co²⁺ ist. Stabilitätstests zeigten, dass diese Hydantoinase bei 4°C und 20°C stabil ist. Die Hydantoinase von *Ochrobactrum* sp. G21 wies die höchste Aktivität bei 40°C und pH 8.0–8.5 auf. Cofaktor-Abhängigkeit konnte nachgewiesen werden, jedoch konnte keine Präferenz für eines der getesteten Ionen festgestellt werden. Die Hydantoinase war bei keiner Lagertemperatur über vier Tage hin stabil. Die höchste Aktivität blieb bei einer Lagertemperatur von 4°C erhalten. Die Hydantoinase von *Bacillus megaterium* F18 wies ein Temperatur- und pH-Optimum von 45–50°C und pH 7.5–8.0 auf. Als benötigte Cofaktoren werden Mn²⁺ oder Co²⁺ angenommen, da bei Zugabe dieser Ionen in biochemischen Studien die höchste Aktivität erzielt wurde. Alle Hydantoinasen zeigten ein breites Substratspektrum, jeweils mit unterschiedlichen Aktivitäten.

Dies ist die erste Beschreibung und Charakterisierung einer Hydantoinase eines Vertreters der Gattung *Delftia*. Ebenso wurde in der Literatur bisher noch nicht über Hydantoinaseaktivität von halophilen *Ochrobactrum*- und *Bacillus*-Spezies berichtet.

Von speziellem Interesse war die Aufklärung der für den Hydantoinabbau verantwortlichen Gene der Stämme *Delftia* sp. I24 und *Ochrobactrum* sp. G21. Das putative Gencluster von *Delftia* sp. I24 beinhaltet vier Gene: eine unvollständige NADPH-abhängige Glutaminsynthase (*gltB*), eine Dihydropyrimidin-Dehydrogenase (*pydA*), eine Permease (*hyuP*) und eine D-Hydantoinase (*hyuH*). Das Hydantoinasegen wurde in *E. coli* exprimiert und die Umsetzung von D,L-5-(3-Indolylmethyl) Hydantoin und D,L-5-Benzylhydantoin konnte nachgewiesen werden. Das Vorhandensein einer Dihydropyrimidin-Dehydrogenase und die bevorzugte Umsetzung von 5,6-Dihydrouracil vor Hydantoin in Ganzzell-Biotransformationen zeigt an, dass diese Enzyme eine Funktion im Pyrimidineabbau haben. Das Gencluster von *Ochrobactrum* sp. G21 umfasste neun offene Leserahmen, wobei sechs davon eventuell am Hydantoinabbau beteiligt sind: Carbamoylase (*hyuC*), D-Hydantoinase (*hyuH*), zwei Transporter (*OrfS1* und *OrfS2*) und zwei Permeasen (*hyuP1* und *hyuP2*). Das Hydantoinasegen wurde ebenfalls in *E. coli* exprimiert und Aktivität wurde für dieselben Hydantoine wie für die Hydantoinase aus *Delftia* sp. I24 gezeigt. In der Literatur wurden noch keine für den Hydantoinabbau verantwortlichen Gencluster für beide oben genannten Gattungen dargestellt. Beide, aber hauptsächlich das Gencluster von *Delftia* sp. I24, zeigten eine sehr hohe Ähnlichkeit mit putativen Genclustern nahe verwandter Arten. Dies zeigte sich in der Abfolge und Orientierung von Genen, die vermutlich im Hydantoinabbau beteiligt sind.

Phylogenetische Studien mittels der Aminosäuresequenzen der in dieser Arbeit gewonnen "neuen" Hydantoinasen, bekannten Hydantoinasen und Dihydropyrimidinasen, zeigten, dass diese Enzyme mit wenigen Ausnahmen gattungs- oder ordnungsspezifisch in die folgenden Gruppen eingeteilt werden konnten: *Rhizobiales* Familie (Rhizo-Fam), *Comamonadaceae* Familie (Com-Fam), *Pseudomonas* Familie (Pseud-Fam), *Bacilli* Familie (Bac-Fam) und *Agrobacterium* Familie (Agro-Fam). Alle verwendeten Enzyme wiesen hierbei das konservierte "Histidin-Motif" der Superfamilie der Amidohydrolasen auf. Ebenso wurden für alle Vertreter die konservierten Aminosäuren gefunden, die für die Bildung des aktiven Zentrums verantwortlich sind. Ein großer Unterschied wurde für die Aminosäuren gezeigt, die vermutlich für die Substraterkennung verantwortlich sind. Bei manchen der oben genannten Familien zeigte sich hierbei eine hohe Übereinstimmung mit Substraterkennungsmotiven von beschriebenen Hydantoinasen. Die Gesamtheit dieser Ergebnisse bestätigt die Hypothese, dass alle Mitglieder der Superfamilie der Amidohydrolasen, und damit ebenso der Hydantoinasen, von einem gemeinsamen Vorfahren abstammen.

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1 List of Publications

This Ph.D. is based on the following publications and manuscripts (see section 7.1–7.4), being summarized in section 4.1–4.4:

Chapter I:

Running title: Distribution of Hydantoinases

Dürr R., Vielhauer O., Burton S.G., Cowan D.A., A. Puñal, Brandão P.F.B., Bull A.T. and Syl-
datk C. (2006) Distribution of hydantoinase activity in bacterial isolates from geographically
distinct environmental sources.

Journal of Molecular Catalysis B: Enzymatic **39**: 160-165.

Chapter II:

Running title: Biodiversity of Hydantoinases

Dürr R., Biodiversity of hydantoin cleaving enzymes.
(not published)

Chapter III:

Running title: Properties of Selected Hydantoinases

Dürr R., Brucher B., Eberspächer J., Vielhauer O., Burton S.G., Cowan D.A. and Syl-
datk C. Description and characterisation of the D-hydantoinases from three microorganisms
isolated from extreme environments.

(Submitted to *Applied and Environmental Microbiology*)

(Part of the experimental work was done within the diploma thesis of Birgit Brucher.)

Chapter IV:

Running title: Gene Clusters for Hydantoin Degradation

Dürr R., Neumann A., Vielhauer O., Altenbuchner J., Burton S.G., Cowan D.A. and Syl-
datk C. Genes responsible for hydantoin degradation of a halophilic *Ochrobactrum* sp. G21 and
Delftia sp. I24 – new insight into relation of D-hydantoinases and dihydropyrimidinases.

(Submitted to *Journal of Molecular Catalysis B: Enzymatic*)

2 Introduction

2.1 Hydantoin Cleaving Enzymes

Concerning the IUPAC-nomenclature the chemical compound hydantoin is declared as "imidazolidin-2,4-dione", a 5-membered ring bearing two nitrogen atoms and two carbonyl groups (Fig. 1).

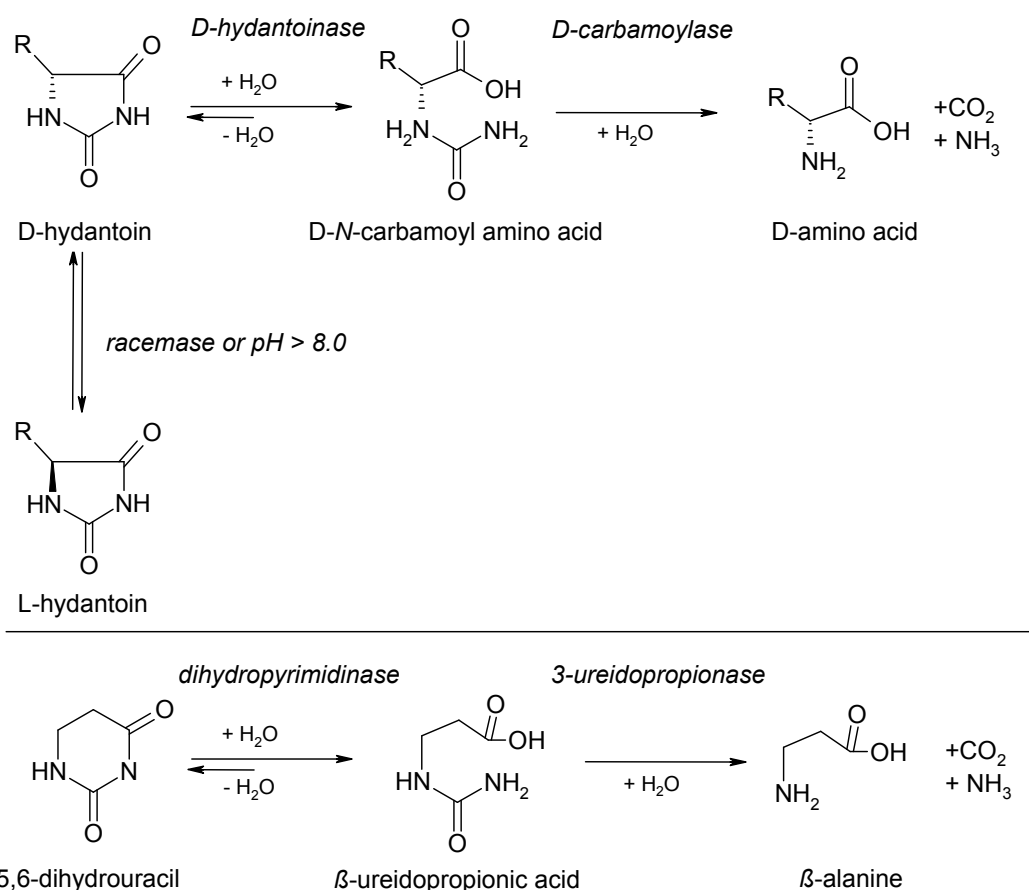


Figure 1: Reaction scheme of enzymes involved in hydantoin and dihydropyrimidine conversion.

Microbial enzymes are known to cleave hydantoin as follows (for details see: Syldatk *et al.*, 1992; Syldatk and Pietsch 1995; Ogawa and Shimizu 1997; Syldatk *et al.*, 1999): First, the hydantoin is hydrolysed by a hydantoinase by a ring opening step to form the corresponding *N*-carbamoyl amino acid. Second, a so-called "*N*-carbamoylase" (*N*-carbamoyl amino acid amidohydrolases) is able to cleave this intermediary product to the respective amino acid. Additionally, a racemase is able to racemise 5-monosubstituted hydantoin. This can also occur chemically under basic conditions (Fig. 1). In Fig. 1 only the D-specific cleavage is shown since almost all hydantoinases obtained in the present work are D-selective. Obviously, the L-route of hydantoin cleavage is analogous to the D-route shown. Hydantoinases can be grouped due to their stereospecificity in D-, L- and non-selective hydantoinases.

Hydantoinases together with dihydropyrimidinases belong to the EC group of hydrolases, in detail to the EC 3.5.2 group of cyclic amidases. In the EC nomenclature hydantoinase is an alternative name for dihydropyrimidinase (EC 3.5.2.2). Various enzymes with the ability to catalyse the cleavage of dihydropyrimidines and hydantoins are known: e.g. *Arthrobacter crystallopoietes* DSM20117 (Siemann *et al.*, 1999), *Bacillus* sp. AR9 (Sharma and Vohra, 1997), *Bacillus stearothermophilus* SD-1 (Lee *et al.*, 1994; Lee *et al.*, 1995), *Pseudomonas* sp. NCIM5109 (Sudge *et al.*, 1998). The function of dihydropyrimidinases is the hydrolysis of dihydrouracil derivatives (Fig. 1), a reaction involved in the reductive pathway of pyrimidine degradation. Dihydropyrimidinase and hydantoinase are not necessarily the same enzyme (Syldatk *et al.*, 1999). Thus, the name hydantoinase should 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 (Syldatk *et al.*, 1999). This was suggested since an *Agrobacterium* sp. and *Arthrobacter aureescens* DSM3745 were able to cleave hydantoin and 5-monosubstituted hydantoin derivatives but not dihydropyrimidines (Runser and Meyer, 1993; May *et al.*, 1998d). Additionally, an imidase from *Blastobacter* sp. was able to convert dihydropyrimidines as well as hydantoin, but here the metabolic function was different compared to dihydropyrimidinases. Nevertheless, in this study we will not strictly distinguish between these two enzymes because only a few real hydantoinases are known and, except of biochemical data, no further differentiation is known.

Besides dihydropyrimidinases and hydantoinases different hydantoin cleaving enzymes are known however with other metabolic functions (see Syldatk *et al.*, 1999):

- **Allantoinase** (allantoin amidohydrolase, EC 3.5.2.5): Allantoin is part of the purine degradation pathway of plants and microorganisms. This enzyme hydrolyses allantoin. Most allantoinases have a high substrate specificity and low enantioselectivity.
- **Carboxymethylhydantoinase** (L-carboxymethylhydantoin amidohydrolase, EC 3.5.2.4): Carboxymethylhydantoinase enables the cleavage of L-carboxymethylhydantoin to N-carbamoyl-L-aspartic acid, a reaction involved in the pyrimidine degradation pathway.
- **Carboxyethylhydantoinase** (not included in the EC nomenclature): This enzyme catalyses the hydrolysis of hydantoin propionic acid to N-carbamoyl-L-glutamic acid. It is involved in histidine degradation.
- **N-Methylhydantoinase** (N-methylhydantoin amidohydrolase, EC 3.5.2.14): This enzyme is involved in the microbial degradation of creatinine, hydrolyses N-methylhydantoin to N-carbamoylsarcosine and is ATP-dependent.

- **Imidase** (not included in the EC nomenclature): This enzyme is probably involved in the degradation of succinimide to succinamic acid. An imidase from *Blastobacter* sp. A17p-4 had a broad substrate spectrum including imides, hydantoin, dihydropyrimidine but not 5-monosubstituted hydantoins.

From recent investigations on DNA and amino acid sequences of different amidohydrolases and cyclic amidases with subsequent phylogenetic analysis it is known today that most enzymes of that group not only share a number of highly conserved regions and invariant amino acid residues but are a product of a divergent evolution (May *et al.*, 1998e; Holm and Sander, 1997). Using structure and sequence homology, hydantoinases can be grouped in the following two families.

The superfamily of "amidases involved in nucleotide metabolism" including the L-hydantoinase from *A. aurescens* DSM3745, dihydropyrimidinase, allantoinase, dihydroorotase, urease and others (like adenine deaminase, phosphotriesterase, not being found to be hydantoinases) and the family of "ATP - dependent cyclic amidases" including *N*-methylhydantoinase and L-oxoprolinase. Not included are imidase, carboxymethylhydantoinase and carboxyethylhydantoinase because there is no sequence information available for these enzymes. It is suggested that the urease-related amidases have evolved from a common ancestor. This theory is confirmed by the findings of Taillades *et al.* (1998): in the primitive hydrosphere the formation of *N*-carbamoyl amino acids and hydantoins was more effective than of α -amino acids, assuming a higher carbon dioxide concentration than that of formaldehyde. It implements that the microorganisms of that earlier times had to use *N*-carbamoyl amino acids and hydantoins as C- and/ or N-source generating the hydantoinase and *N*-carbamoylase system.

2.2 Screening for Hydantoinases

It is only possible to cultivate a limited number of bacteria whereas the total amount of bacteria is much higher. The extent of microbial and molecular diversity was shown by Torsvik *et al.* (2002) and Venter *et al.* (2004). By using a "whole-genome shotgun sequencing approach" to microbial populations from seawater samples from the Sarragosa Sea near Bermuda the second group found DNA sequences attributed to 1800 genomic species with 148 previously unknown bacterial phylotypes. More than 1.2 million unknown genes were found.

Hydantoinases are known to be present in certain microorganisms. Two alternative ways can be used to access hydantoinases. One possibility is to isolate microorganisms with hydantoinase activity and another to access directly the hydantoinase gene followed by cloning and expression of the enzyme. The first question is how it is possible to access microorganisms with the ability to cleave hydantoins from this great number of bacteria as

described above.

Up to now, only culture based methods are described in literature with exception of a few other approaches. There are two main ways of screening for bacteria with hydantoinase/ carbamoylase activity: The most common method is the use of enrichment cultures in which an environmental sample (soil, sediment, water) is inoculated in a special media containing the desired hydantoin as sole C- and/or N-source. Only bacteria with the ability to use hydantoins are able to grow and can be isolated on agar plates in the next step. The other method is the direct use of bacterial isolates from culture collections. For both ways the hydantoinase activity of pure cultures needs to be proven by activity assays and therefore the following methods can be used.

Overlay assays in which bacterial colonies are grown on agar plates and hydantoinase activity is detected by a spot-test based on the direct detection of carbamoyl amino acids in agar using the Ehrlich Reagent (Morin *et al.*, 1986a). A simple method is the addition of the unsubstituted hydantoin and phenol red to the media for agar plates. The conversion of hydantoin leads to a decrease of the pH initiating a colour change in the agar. This was used to isolate hydantoinase positive *E. coli* transformants and for the screening of hydantoinase-producing bacteria from soil (Kim *et al.*, 1997b). The disadvantage of this method is that it is not sensitive enough for L-selective hydantoinases which can not cleave unsubstituted hydantoins (May *et al.*, 1998d). Chien and Hsu (1996) developed a microtiter plate assay based on the detection of the produced carbamoyl amino acid by the addition of dimethylaminobenzaldehyde to the reaction. This reagent is also used in the so-called Ehrlich Test. The authors stated that this method is simple, rapid and highly sensitive in comparison to other methods. An analogous detection method was used to show hydantoinase activity in crude extracts of microorganisms run on polyacrylamide gels under non-denaturing conditions.

Nevertheless, these methods do not give evidence on the selectivity of the occurring reaction. A sensitive method for the differentiation of D- and L-selective hydantoinases is the use of acrylamide gels with crude extracts from microorganisms. The backward enzyme reaction is used for *in situ* product precipitation, which allows the location of the hydantoinase on a polyacrylamide gel. This is possible because the soluble substrate *N*-carbamoyl tryptophan will be converted to the poorly soluble indolylmethylhydantoin. The selectivity of hydantoinases can be detected by reactions either with D- or L-*N*-carbamoyl tryptophan (May *et al.*, 1998c).

Other detection methods are thin-layer chromatography (Lee *et al.*, 1994), a spectrophotography assay (Chevalier *et al.*, 1989) and polarimetric methods for enzyme catalysed enantioselective reactions (Teves *et al.*, 1999). The most commonly used analytical method is HPLC (High Performance Liquid Chromatography). Whole cells, resting cells or the pure enzymes can be used in activity assays. The advantage of HPLC-analytics is that both the substrate (hydantoins) and the corresponding products (carbamoyl amino acids and

amino acids) can be detected in a single run. As well the chiral separation of enantiomers is feasible.

Besides the detection methods mentioned above immunological and molecular detection methods were reported as well. Especially molecular detection methods will gain more interest since during the last decade much more genetical information of hydantoinases and the corresponding enzymes is reported. From particular interest would be the development of genetical methods to access hydantoinase genes directly from strain collections, soil or genomic libraries. Advantages are cost reduction and the loss of time intensive isolation methods.

Polyclonal antibodies were successfully developed for the detection of the L-hydantoinase of *Arthrobacter aurescens* but they were not specific for the detection of the D-hydantoinase of *Agrobacterium* sp. (Siemann *et al.*, 1993a and b). LaPointe *et al.* (1994) developed a 1.5 kb DIG-labelled DNA probe derived from a recombinant hydantoinase gene of *Pseudomonas putida* DSM 84. The probe only hybridised with total DNA from *Pseudomonas* strains of rRNA group I showing hydantoinase activity. More successful was the development of a 122 bp DNA probe from the same organism as mentioned above to detect D-hydantoinase genes in other bacterial genera by DNA and by colony hybridisation. The probe was specific while allowing 32% mismatch to detect D-hydantoinase activity in a range of bacteria (La Pointe *et al.*, 1995).

Recently, degenerate primers were reported for the amplification of a 330 bp dihydropyrimidinase gene fragment of *Bacillus* sp. TS-23 (Lin *et al.*, 2005). The amplified 330 bp DNA fragment was used as a probe to find the dihydropyrimidinase gene within a genomic library of the same strain. These degenerate primers were as well used in this study to amplify hydantoinase gene sequences from different environmental bacterial isolates. One problem of the development of universal primers for a successful amplification of the hydantoinase gene is the low sequence homology of hydantoinases.

2.3 Hydantoinases – Classification and Properties

Hydantoinases can be grouped according to their stereospecificity into D-, L- and non-selective hydantoinases. However, some hydantoinases showed a change in stereoselectivity for different substrates. The L-hydantoinase from *A. aurescens* DSM3745 showed to be L-selective for D,L-benzylhydantoin whilst conversion of D,L-methylthioethylhydantoin was D-selective (May, 1998f). Similar observations were obtained for *Flavobacterium* sp. AJ-3912. D-specificity was only obtained for benzyloxymethylhydantoin, whereas the other substrates tested were cleaved L-specific (Yokozeki *et al.*, 1987). Since almost all of the hydantoinases obtained in this study were D-selective the main focus was on D-hydantoinases. L- and non-selective hydantoinases were less reported than D-hydantoinases and found within the following species: *Arthrobacter aurescens* DSM3745

and DSM3747 (May, 1998f; Gross *et al.*, 1990), *Arthrobacter* sp. DSM7330 (Völkel and Wagner, 1995), *Arthrobacter* sp. DSM9771 (Wagner *et al.*, 1996), *Microbacterium liquefaciens* (formerly *Flavobacterium* sp.) AJ-3912 (Yokozeki *et al.*, 1987; cited in Nozaki *et al.*, 2005), a hyperthermophilic archeon *Methanococcus jannaschii* DSM2661 (Chung *et al.*, 2002), *Pseudomonas* sp. NS671 (Ishikawa *et al.*, 1993, 1997; Watabe *et al.*, 1992a) and *Pseudomonas* sp. RU-KM3_s (Burton *et al.*, 1998; Buchanan *et al.*, 2001; Matcher *et al.*, 2004).

Hydantoinase activity from *Ochrobactrum anthropi* (Pozo *et al.*, 2002) has to be mentioned. Unfortunately the authors do not report on the stereospecificity of this enzyme. The strain was able to cleave D,L-(2-methylthioethyl)-hydantoin to methionine and was induced by this hydantoin. The enzyme had a pH optimum of 9.0 and activity was significantly increased by the addition of Ca²⁺, Na⁺, Cu²⁺, Co²⁺, Mg²⁺, Zn²⁺ and Fe³⁺.

D-hydantoinases were reported for numerous microorganisms. A selection is shown in Table 1.

Table 1: Biochemical properties of selected D-hydantoinase enzymes.

(**opt.**: optimal; **metal ions**: increased activity in the presence of the respective metal ion observed; **BnH**: 5-benzylhydantoin; **BuH**: 5-(sec)-butylhydantoin; **DU**: 5,6-dihydrouacil; **HPH**: 5-hydroxyphenylhydantoin; **IBH**: 5-isobutylhydantoin; **IPH**: 5-iso-propylhydantoin; **MH**: 5-methylhydantoin; **MPH**: 5-methoxyphenylhydantoin; **MTH**: 5-(2-methylthioethyl) hydantoin; **PH**: 5-phenylhydantoin; **PrH**: 5-propylhydantoin; **THE**: 5-thienylhydantoin)

origin	optimal pH	optimal T	metal ions	selectivity	Reference
<i>Agrobacterium</i> sp. IP I-671	10.0	60°C	Ni ²⁺ , Mg ²⁺	BnH>HPH>IPH >MTH>BuH> IBH IBH >MH>Hyd (No DU)	Runser and Ohleyer 1990; Runser and Meyer, 1993
<i>Agrobacterium tumefaciens</i> NRRLB11291	9.0	60°C	Mn ²⁺	THE>MPH>PH >BuH>HPG >MH	Grifantini <i>et al.</i> , 1998; Olivieri <i>et al.</i> , 1981; Achary <i>et al.</i> , 1997
<i>Agrobacterium tumefaciens</i> RU-OR	9.0	40 – 60°C	-	MH>HPH>Hyd	Hartley <i>et al.</i> , 1998; Burton <i>et al.</i> , 1998
<i>Arthrobacter crystallopoietes</i> DSM20117	8.0	50°C	Zn ²⁺	Hyd>PH>MTH >HPH>BnH>DU	Siemann <i>et al.</i> , 1999
<i>Bacillus</i> sp. AR9	9.5	65°C	Mg ²⁺ , Ni ²⁺ , Mn ²⁺ , Co ²⁺	Hyd>PH>DU >HPH	Sharma and Vohra 1997
<i>Bacillus circulans</i>	8.0 – 10.0	75°C	Mn ²⁺ , Ni ²⁺ , Co ²⁺	Hyd>PH>MTH > PrH>IBH	Luksa <i>et al.</i> , 1997
<i>Bacillus stearothermophilus</i> SD-1	8.0	65°C	Mn ²⁺	Hyd>DU>PH >HPH	Kim <i>et al.</i> , 1997a; Lee <i>et al.</i> , 1994, 1995
<i>Bacillus stearothermophilus</i> NS1122A	9.5	60°C	Mn ²⁺ , Co ²⁺ , Ni ²⁺	MH>MTH>IBH > IPH >BnH	Mukohara <i>et al.</i> , 1994; Ishikawa <i>et al.</i> , 1994

origin	optimal pH	optimal T	metal ions	selectivity	Reference
<i>Burkholderia pickettii</i>	8.0	50°C	Co ²⁺ , Mn ²⁺ , Zn ²⁺ , Fe ²⁺ , Ni ²⁺	-	Xu <i>et al.</i> , 2003a
<i>Flavobacterium</i> sp. AJ11199	7.5–9.0	60°C	Fe ²⁺ , Mg ²⁺ , Mn ²⁺	PH>BnH>IMH > IBH > PrH>Hyd >(low DU)	Nozaki <i>et al.</i> , 2005
<i>Pasteurella</i> sp. AJ11211	7.5–9.0	55°C	Mg ²⁺ , Mn ²⁺	IMH>PH>BnH >IBH>PrH>DU >(low Hyd)	Nozaki <i>et al.</i> , 2005
<i>Pseudomonas putida</i> DSM84	9.0	55°C	Mn ²⁺ , Fe ²⁺	DU>IPH>Hyd	Morin <i>et al.</i> , 1986b,c; LaPointe <i>et al.</i> , 1994
<i>Pseudomonas</i> sp. NCIM5109	9.0–9.5	30°C	-	DU>Hyd>PH >HPH	Sudge <i>et al.</i> , 1998

D-selective hydantoinses are predominantly found in bacteria belonging to the genera *Arthrobacter*, *Agrobacterium*, *Bacillus*, *Flavobacterium* and *Pseudomonas* (Table 1). This does not mean that hydantoinses are special or unique enzymes of these genera. An indication that hydantoinses are widespread bacterial enzymes is the observation that within the last years the number of putative hydantoinses increased significantly due to the complete sequencing of bacterial genomes. Hydantoinses are predominantly known for the genera mentioned above probably because these genera can be cultured best in the screening and growth media used.

Regarding the biochemical properties, the pH-optimum of hydantoinses is found at an alkaline pH 7.5–10.0 (Table 1) and the optimal temperature lies in most cases between 50–65°C. Exceptions are the temperature optima of the hydantoinase from a halophilic *Pseudomonas* sp. NCIM5109 (30°C) and from *Bacillus circulans* (75°C, see Table 1). For industrial use, a higher temperature for the hydantoinase used and therefore the process temperature is desirable since the solubility of hydantoins increases at higher temperatures.

Hydantoinses require as well divalent metal ions as cofactor (Table 1), preferred ions are Mg^{2+} , Zn^{2+} and Mn^{2+} . Metal ion requirements can be shown by biochemical sensitivity tests using ion metal chelators like EDTA. It has to be mentioned that biochemical studies do not provide a definite evidence of the metal ions associated with the enzyme. This was reported for the L-hydantoinase of *A. aurescens* DSM3745 which showed in first reactivation experiments activation with Mn^{2+} - or Co^{2+} -ions. In contrast, by atom absorption spectroscopy (AAS) and inductive coupled plasma-atomic emission spectrometry (ICP-AES) it was shown that the enzyme contained 2.5 mol Zn^{2+} /mol subunit (May *et al.*, 1998a and b). This was confirmed by crystallographic analysis (Abendroth *et al.*, 2002b). Additionally, the presence of metal ions for the D-hydantoinses from *Thermus* sp. (Abendroth *et al.*, 2002a), *Burkholderia pickettii* (Xu *et al.*, 2003), and *B. stearothersophilus* SD-1 (Cheon *et al.*, 2002) were shown by crystallographic analysis.

2.4 The Hydantoin Hydrolysing Gene Cluster

The genes encoding for the proteins involved in hydantoin and/or dihydropyrimidine conversion have been described for different microorganisms (Fig. 2). All of the obtained gene cluster have in common genes encoding for hydantoinase/ dihydropyrimidinase and carbamoylase/ β -ureidopropionase. A pattern was obtained in the orientation of these genes being homogeneous in certain bacterial families. In the case of *Arthrobacter aurescens* DSM3747, reported to be L-specific, the genes for hydantoin conversion are transcribed in the same direction and were as follows: *hyuP* encoding for a putative transport protein; *hyuA* for the hydantoin racemase; *hyuH* for the hydantoinase gene; *hyuC* for the carbamoylase (Wiese *et al.*, 2001). The name *hyu* stands for hydantoin utalization. Interestingly, in the hydantoin gene cluster of *Arthrobacter crystallopoietes* DSM20117, the genes show as well one orientation and consist of genes encoding for a hydantoinase, one D-carbamoylase

and one putative L-carbamoylase and two other proteins showing similarity to permeases and repressors. No racemase gene was found (Werner *et al.*, 2001). The first gene cluster reported for pyrimidine reductive catabolism (*pyd*) was from a moderate thermophile bacterium *Brevibacillus agri* NCHU1002 (Kao and Hsu, 2003). The orientation of the genes was similar as reported for *Arthrobacter aureescens* DSM3747 and *Arthrobacter crystallopoietes* DSM20117. This gene cluster comprises a dihydropyrimidine dehydrogenase (*pydA*), a dihydropyrimidinase (*pydB/ dhp*) and a β -alanine synthase (*pydC*). Dihydrouracil acted as inducer and the genes were regulated under the control of σ^{54} recognized promoter at transcriptional level as polycistronic operon.

<i>Microorganism</i>	<i>Stereospecificity</i>	<i>Gene Organization</i>	<i>Reference</i>
<i>Arthrobacter aureescens</i> 3747	L		Wiese <i>et al.</i> , 2001
<i>Arthrobacter crystallopoietes</i> 20117	D		Werner <i>et al.</i> , 2004
<i>Agrobacterium sp.</i> IP I-671	D		Hils <i>et al.</i> , 2001
<i>Agrobacterium sp.</i> KNK712	D		Namba <i>et al.</i> , 1998
<i>Agrobacterium tumefaciens</i> NRRL B11291	D		Griffantini <i>et al.</i> , 1998
<i>Brevibacillus agri</i> NCHU 1002	D		Kao and Hsu, 2003
<i>Pseudomonas putida</i> RU-KM3 _s	L		Matcher <i>et al.</i> , 2004
<i>Pseudomonas sp.</i> NS671	L		Watabe <i>et al.</i> , 1992
<i>Microbacterium liquefaciens</i> AJ 3912	D,L		Suzuki <i>et al.</i> , 2005

Figure 2: Gene clusters of different microorganisms for the conversion of hydantoins and dihydropyrimidines. (**hyuP**: permease; **hyuA**: racemase; **hyuH**: hydantoinase; **hyuC**: carbamoylase; **hyuN**: NADP - flavin oxidoreductase; **hyuD**: amino acid dehydrogenase; **dhp**: dihydropyrimidinase; **gltB**: glutamate synthase; **pydA**: dihydropyrimidine dehydrogenase; **pydC**: β -alanine synthase; **bup**: β -ureidopropionase)

The genes of other gene clusters are orientated in a different way. Hils *et al.* (2001) showed for *Agrobacterium sp.* IP I-671, being D-selective, the presence of a D-hydantoinase being in the opposite direction as carbamoylase, amino acid dehydrogenase and racemase. This *hyu* gene cluster is located on a plasmid with a size of 190 kb. The same opposite direction of the hydantoinase/dihydropyrimidinase genes related to the carbamoylase were obtained for *Agrobacterium sp.* KNK712 and *Agrobacterium tumefaciens* NRRL B11291 (Fig. 2). A detailed study on the regulation of genes involved in hydantoin conversion of *Pseudomonas putida* RU-KM3_s showed an opposite orientation of the dihydropyrimidinase gene (*dhp*) to the β -ureidopropionase gene (*bup*). An open reading frame was found between these two genes, encoding a putative transport protein which shares a promoter with *bup* and are transcribed on a polycistronic mRNA. In contrast, the dihydropyrimidinase is expressed by another promoter. Growth studies using different carbon sources indicate that the expression of the

hydantoin-hydrolysing enzymes *dhp* and *bup* are regulated by carbon catabolite repression (Matcher *et al.*, 2004).

Watabe *et al.* (2002) described the hydantoin converting system of *Pseudomonas* sp. NS671, whereas the genes encoding for the hydantoinase (*hydH*), carbamoylase (*hyuC*) and racemase (*hyuA*) were located on a 172 kb plasmid and were orientated in the same direction. The classification of the ATP-dependent hydantoinase of *Pseudomonas* sp. NS671 is still unclear because the enzyme shows no enantioselectivity and therefore seemed to be different to known L-specific *N*-methylhydantoinases (Ishikawa *et al.*, 1993, 1997). It was not tested, whether *N*-methylhydantoin could be cleaved by the enzyme.

2.5 Industrial Applications

Within the last 20 years the market for the so called feed amino acids L-lysine, D,L-methionine, L-threonine and L-tryptophane increased dramatically. The food sector is of high importance with the demand for the flavor enhancer L-glutamic acid from sodium glutamate and L-aspartic acid and the sweetener L-aspartyl L-phenylalanine methyl ester (Aspartam) from L-phenylalanine. Other proteinogenic amino acids are needed in the pharmaceutical and cosmetic industry (Leuchtenberger *et al.*, 2005). A brief summary of the use of amino acids is given in Table 2.

Table 2: Selected examples for applications of enantiomerically pure amino acids.

amino acid	product	application	reference
L-aspartate/ L-phenylalanine	Aspartam	Sweetener	Calton, 1992
L- <i>tert</i> -leucine	HIV Protease Inhibitor Sandoz BB - 2516	AIDS Chirales antiviral antitumor	Bommarius <i>et. al.</i> , 1998 Drauz, 1997 Drauz, 1997
L-methionine	Docarpamine, Tanadopa Ademetionine, Gumbaral	cardiovascular, diuretic antiarthritic	Drauz, 1997 Drauz, 1997
L-leucine	Ubestatin, Bestatin Lexipafant	Immunostimulant PAF-antagonist, antiallergic	Drauz, 1997 Drauz, 1997
L-valine	Valaciclovir	Reverse-Transcriptase Inhibitor	Drauz, 1997
L-tryptophane	L - Dopa	Parkinson	Rote Liste, 1994
D-phenylglycine	Ampicillin	antibiotic	Bommarius <i>et. al.</i> , 1998
D-hydroxy- phenylglycine	Amoxicillin	antibiotic	Bommarius <i>et. al.</i> , 1998
D-phenylalanine	D-Phe-Pro-Arg-H	Thrombosis	Bajusz <i>et. al.</i> , 1990
D-valine	Fluvalinate	Pyrethroid insectizide	Drauz, 1997
e.g. D-citruline D-alanine 3-(2-naphtyl)- D-alanine	Cetrorelix	cancer therapy	Drauz, 1997 Müller <i>et. al.</i> , 1994

Production methods of amino acids are extraction, chemical synthesis, fermentation and enzymatic catalysis. The latter two processes contributed most to the growth of the amino acid market since the 1980s due to their economical and ecological advantages (Leuchtenberger *et al.*, 2005). An overview of the different production methods is given in Figure 3.

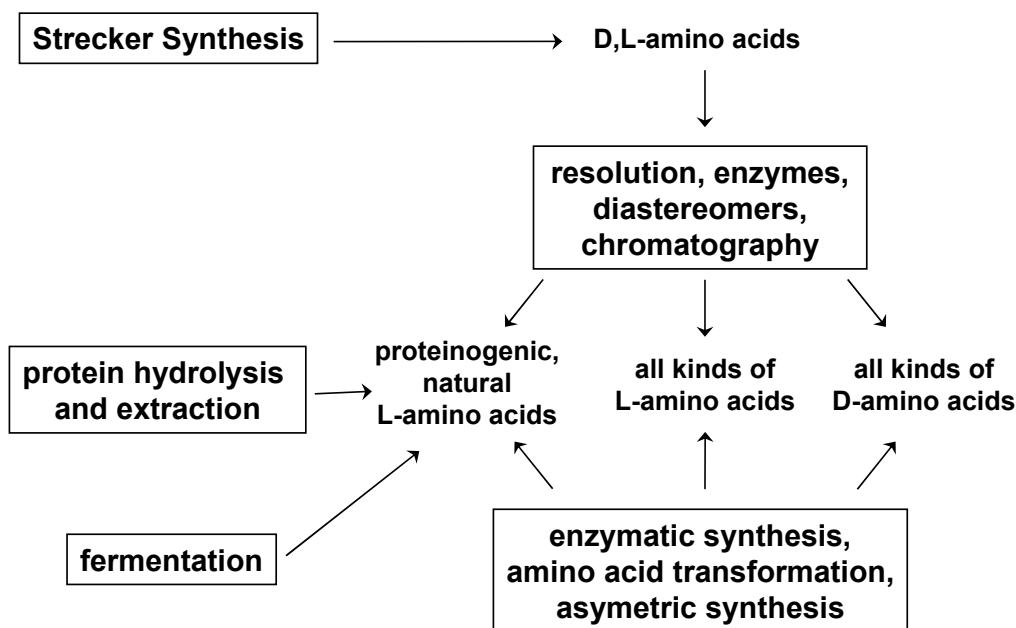


Figure 3: Basic methods for amino acid synthesis (from Drauz, 1997).

For the production of amino acids by means of the hydantoinase process amino acids are formed by an enzymatic step using hydantoins as starting material. Hydantoins can be synthesised comparatively easily by chemical methods (Bucher and Steiner, 1934). Different ways can be employed to get access to hydantoins as a starting material (Fig. 4).

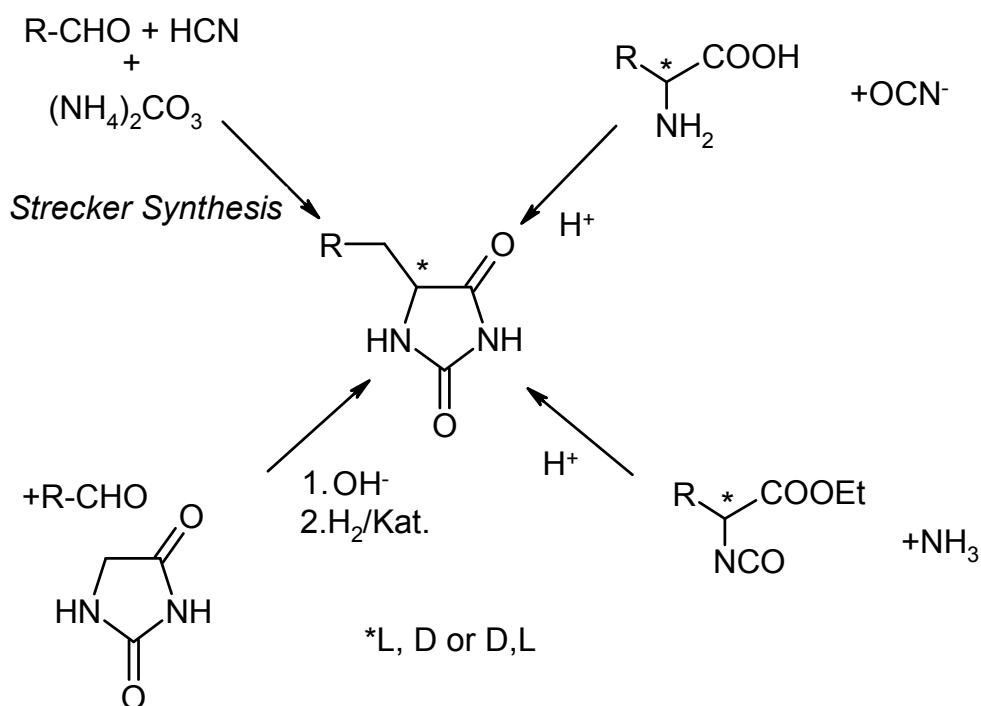


Figure 4: Methods for the chemical synthesis of hydantoins.

An advantage of the hydantoinase process is that hydantoins can racemise under certain chemical conditions (see Fig. 1) or enzymatically by a so-called hydantoin racemase. Hence, the substrate can theoretically be converted with 100% yield. Another advantage of the hydantoinase process is the broad substrate spectrum of hydantoinases and carbamoylases (Hils, 1998).

A problem of the hydantoin process is the instability of the carbamoylase which can lower the space-time yield in the process (Kim *et al.*, 1994). Recently the development of new molecular methods and extended insight in substrate accessibility helped to overcome this problem and may improve the industrial processes of amino acid production by hydantoinases and carbamoylases:

1. Directed evolution was used to convert the hydantoinase from *Arthrobacter* sp. DSM9771 from D-specificity to L-specificity with a five-fold increase in total activity (May *et al.*, 2000).
2. The oxidative stability and thermostability of a *N*-carbamoylase from *Agrobacterium tumefaciens* NRRL B11291 was improved by directed evolution using DNA shuffling (Oh *et al.*, 2002).
3. The construction of whole-cell tailor-made biocatalysts by co-expression of the genes encoding for the L-hydantoinase, the L-*N*-carbamoylase and the hydantoin racemase from *Arthrobacter aurescens* DSM3747 in *E. coli* by using vectors with different copy numbers (Wilms *et al.*, 2001).
4. The development of bifunctional hydantoinase/ carbamoylase fusion proteins with enhanced performance by the use of DNA-shuffling (Kim *et al.*, 2000).
5. The use of two hydantoinases and one carbamoylase made it possible to produce highly lipophilic, silicon-containing amino acids (Smith *et al.*, 2001). This is an example of the broad substrate range of hydantoinases and carbamoylases and for their versatility in industrial application.

Economical important targets being produced using hydantoinases and carbamoylases include aromatic D-amino acids (D-phenylglycine, *p*-hydroxy-D-phenylglycine), D-serine, L-methionine or L-phosphinotricine (Leuchtenberger *et al.*, 2005). The precursor of amoxicillin, *p*-hydroxy-D-phenylglycine is one of the most important compounds produced via the hydantoinase process. There are three different approaches for the industrial production starting with D,L-5-*p*-hydroxyphenylhydantoin (from Drauz *et al.*, 1991):

Snamprogetti-process

Immobilised dihydropyrimidinase from calf liver at pH 8.0, 30°C. The intermediary product *N*-carbamoyl-*D-p*-hydroxyphenylglycin is chemically decarboxylated with HNO₂ to the corresponding amino acid.

Kaneka-process

Immobilised, resting cells from *Bacillus brevis* with *D*-hydantoinase activity at pH 9.0, 30°C. Chemical decarboxylation is employed as well.

Recordati-process

Immobilised, resting cells from *Agrobacterium radiobacter* with *D*-hydantoinase and *D*-carbamoylase activity at pH 9.0, 30°C.

An industrial process was established by Rütgers-Biotech in the early 1990ies for the production of non-natural aromatic L-amino acids by whole cells of *Arthrobacter aurescens* DSM3745 and DSM3747 containing a racemase, L-hydantoinase and L-carbamoylase. The L-amino acids produced were L-tryptophan, L-phenylalanine, L-*O*-benzylserine, L-*p*-chloro-phenylalanine, L-*p*-fluoro-phenylalanine, L-*p*-nitro-phenylalanine, L-1-naphtylalanine, L-2-naphtylalanine, L-3,4-dimethoxy-phenylalanine and L-2-thienylalanine (Syl-datk and Pietsch, 1995).

3 Research Proposal

Hydantoin-hydrolysing enzymes have been studied for a long time and were shown to be present in various microorganisms. Much is known about the biochemical properties of different hydantoinases, the reaction mechanism of the enzyme; crystal structures were determined and hydantoin-hydrolysing gene clusters elucidated. As well, hydantoinases are used in industry for the production of various amino acids. Nevertheless, there are still many questions remaining. For example, the question of the "natural function" is not solved, including the distribution of microorganisms with hydantoinase activity in nature, and little is known about the phylogenetic relationship between hydantoinases themselves. Moreover, due to the increasing biotechnological market new enzymes with special substrate specificities are needed. Therefore the present work focused on the following main questions:

- Is it possible to find hydantoinases in bacteria in which hydantoinase activity was not shown before?
- Is there a preferred environmental natural habitat for bacteria with the ability to cleave hydantoins?
- If there are bacteria with non-described hydantoinases, do they exhibit special biochemical properties, probably due to adaption to a certain environmental habitat?
- What are the differences of "novel" hydantoinases on a molecular level?

To solve these questions the present work is concentrated on the screening, characterisation and comparison of "novel" hydantoinases as follows:

- Recovery of microorganisms with the ability to cleave hydantoins to the corresponding carbamoyl amino acids from geographically distinct environmental habitats including extreme habitats.
- Phylogenetic characterisation of the bacterial isolates.
- Characterisation of growth conditions of selected strains from extreme environments.
- Biochemical characterisation of hydantoinases.
- Investigation of the phylogenetic relationship of hydantoinases.
- Isolation of the gene clusters responsible for hydantoin conversion of selected strains.

4 Summary Chapters I–IV

4.1 Summary Chapter I: Distribution of Hydantoinases

Complete title:

DISTRIBUTION OF HYDANTOINASE ACTIVITY IN BACTERIAL ISOLATES FROM GEOGRAPHICALLY DISTINCT ENVIRONMENTAL SOURCES

A screening program was conducted with the aim to find novel hydantoinases from microorganisms of different geographical origins and to investigate their distribution in nature. This included terrestrial soil samples from various sites in South Africa, from hot springs and salt-lakes in China and environmental samples from the cold Antarctica. Thirty-two bacterial strains were isolated from these samples by enrichment techniques using D,L-5-(3-indolylmethyl) hydantoin as carbon- and nitrogen source. All microorganisms showed the ability to cleave the model substrates D,L-5-benzylhydantoin and 5,6-dihydrouracil. In biotransformations with D,L-5-benzylhydantoin all of them showed formation of *N*-carbamoyl-D-phenylalanin. Therefore these hydantoinases were classified as D-hydantoinases, except for the unclassified *Microbacteriaceae* sp. K3 and *Burkholderia* sp. M3. They were either non-selective or L-specific. All bacterial isolates were identified at the genus level by amplification and sequencing of the 16S rRNA gene. Figure 5 and Figure 6 show the geographical origin of all strains being recovered.

China, Inner Mongolia, saline lakes

Bacillus sp. F18 and K18,
Ochrobactrum sp. G21,
Streptomyces sp. I20

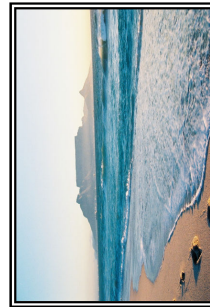
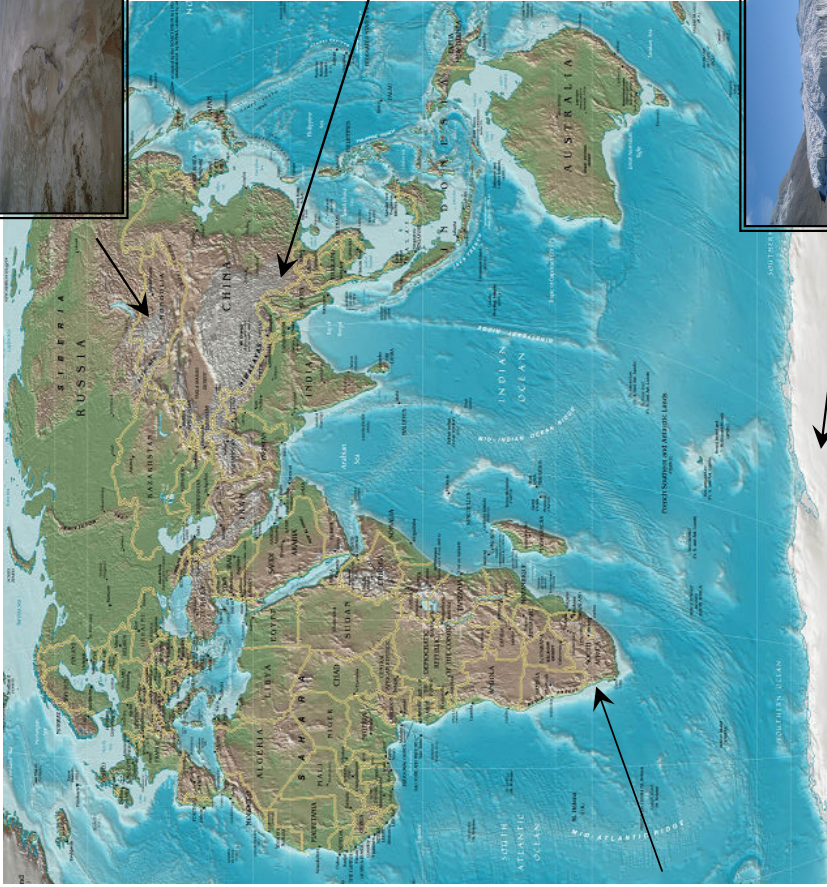


China, Long Pu, hot springs

Delftia sp. I24

Antarctica

Pseudomonas sp. N7
and G7, *Arthrobacter* sp.
E7 and F7



South Africa

See Figure 6

Figure 5: World wide distribution of bacterial isolates possessing hydantoinase activity. (Photographs are examples of the collecting areas but do not show the original place of sampling. Photographs were taken within the Chinese-EU-SA MGAtech project and Waikato University Antarctic Terrestrial Biology Program (provided by Prof. D.A. Cowan) and by the author.)

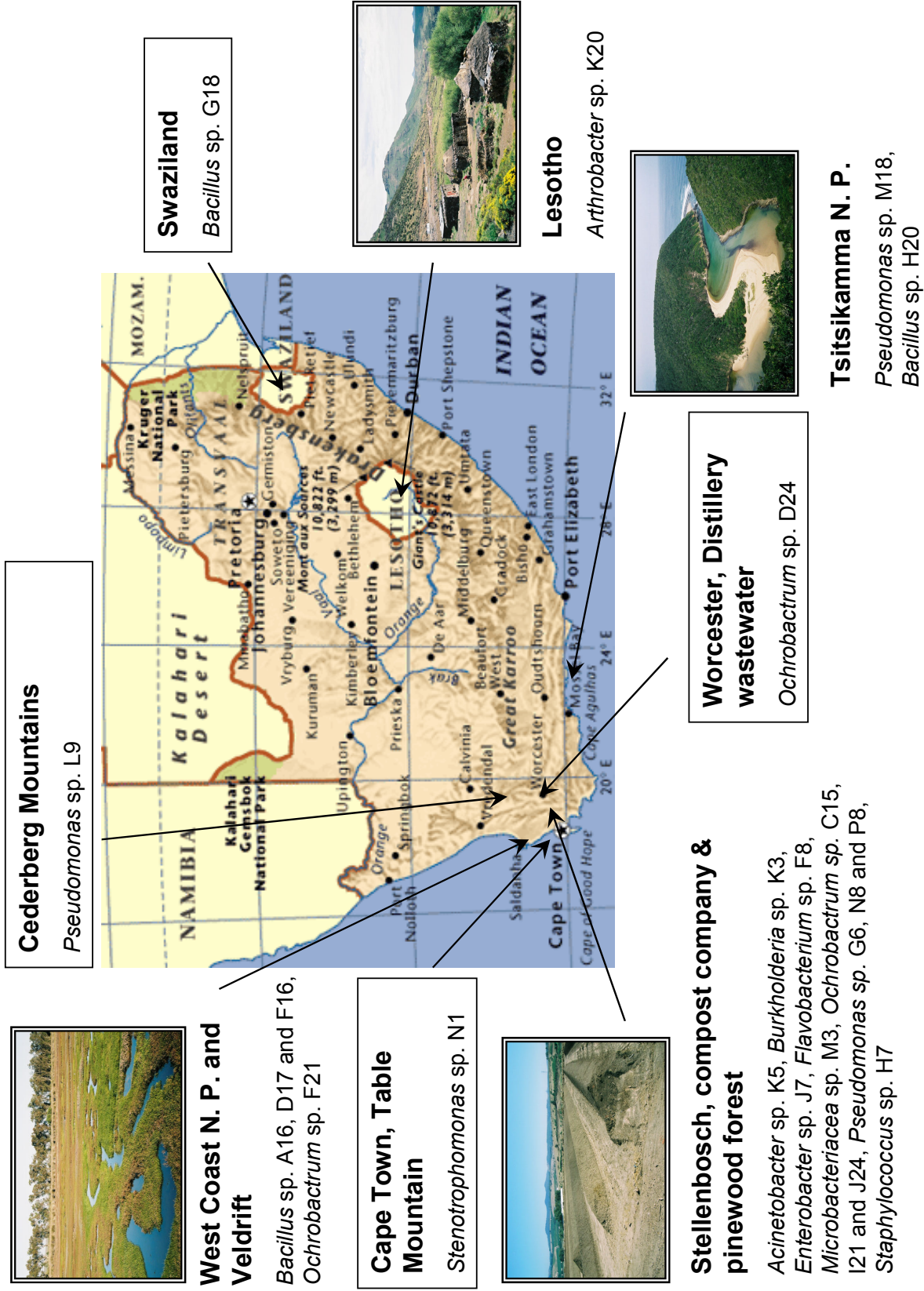


Figure 6: Distribution of bacterial isolates possessing hydrantoinase activity in South Africa, Lesotho and Swaziland. (Photographs are examples of the collecting areas but do not show the original place of sampling. Photographs were taken by the author.)

The screening and isolation for various microorganisms with hydantoinase activity is described in literature, but usually no precise data are available on the exact origin and nature of the environmental samples used in the screening experiments. In this study it is shown that microorganisms with the ability to metabolise hydantoins are widely dispersed in respect to the nature of the soil sample and to geographical origin. Nevertheless, most bacteria were found in environmental samples with a high amount of degrading material (e.g. compost soil or guano) which confirms the hypothesis that hydantoinases are involved in catabolic pathways to access amino acids as metabolic substrates. Interestingly, microorganisms with hydantoinase activity were also found in soil samples originating from extreme environments. Growth under different conditions (as temperature and media composition) showed that the two *Arthrobacter* sp. F7 and G7 and the two *Pseudomonas* sp. N7 and G7 could be designated as psychrotrophic. This is the first report of psychrotrophic microorganisms from Antarctica with hydantoinase activity. In literature only one halophilic strain with hydantoinase activity, *Pseudomonas* sp. NCIM5109 is reported. This study describes more halophilic bacteria with hydantoinase activity but from different genera: *Bacillus* sp. F18 and K18, *Ochrobactrum* sp. G21 and *Streptomyces* sp. I20. All of them were isolated from a saline environment and were designated as moderate halophilic due to their ability to grow in media supplemented with up to 10% NaCl.

Microorganisms with hydantoinase activity were predominantly found in the genera *Arthrobacter*, *Bacillus*, *Ochrobactrum* and *Pseudomonas* but we also can report hydantoinase activity in genera for which no hydantoinase activity was known before: *Stenotrophomonas*, one "Microbacteriaceae", *Staphylococcus*, *Acinetobacter*, *Delftia* and *Streptomyces*.

In summary, it was 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.
- (IV) Furthermore, the presence of hydantoinase activity was demonstrated for genera in which hydantoinase activity has not previously been reported.

4.2 Summary Chapter II: Biodiversity of Hydantoinases

Complete title:

BIODIVERSITY OF HYDANTOIN CLEAVING ENZYMES

The UNCED (1992) defined the term "biological diversity" or "biodiversity" to be the variety of genes, species and ecosystems found on our planet. It embraces all life forms – from plant and animal life to microorganisms – and the water and land in which they interact. Regarding the "biodiversity of hydantoinases" that would mean the variety of hydantoinases. It is not possible to measure this but the following questions can try to highlight the term "variety of hydantoinases":

1. It is known that hydantoinases have a broad and diverse substrate spectrum (see Introduction, Table 1). The question remains if there is a correlation between substrate spectrum and origin of hydantoinases concerning bacterial genera and/ or ecological habitat.
2. Can the hydantoinase gene sequence of different microorganisms be clustered into specific groups either related to a genus and/ or to a certain habitat?
3. Is there a correlation between the biochemical properties of hydantoinases and certain hydantoinase groups?
4. Are hydantoinase genes also present in microorganisms without any hydantoinase activity?

To answer these points several bacterial isolates from distinct environmental habitats were chosen for biochemical and genetic characterisation. The selection criteria were (i) if a strain showed high activity towards the substrates D,L-5-benzylhydantoin and 5,6-dihyrouacil (Dürr *et al.*, 2006); (ii) if a strain was isolated from an extreme or unique environmental habitat like a hot spring or a biofilm; (iii) if no hydantoinase activity had been reported in literature for that certain genus before. The investigation on these bacterial isolates led to the following conclusions:

1. The hydantoinases selected for this study showed a broad substrate spectrum but no correlation between a certain hydantoinase activity and certain bacterial genera and/ or ecological habitat could be obtained. However we propose that environmental conditions like a high organic content of the soil can evoke hydantoinase activity, since many strains were recovered from soil samples like guano and compost. The preference of most of the bacterial strains for higher conversion of 5,6-dihydrouracil than for the non-substituted hydantoin lead to the conclusion that these enzymes can be considered as dihydropyrimidinases and are part of the reductive pathway of pyrimidine degradation. Syldatk *et al.* (1999) demonstrated

that D-hydantoinases are not necessarily identical to dihydropyrimidinases and the term hydantoinase should be used for enzymes being able to hydrolyse hydantoin or 5-monosubstituted hydantoins. Since all strains were able to cleave hydantoin or 5-monosubstituted hydantoins they were declared as hydantoinases. Regarding the other substrates tested most enzymes expressed higher activity towards the 5-monosubstituted hydantoin 5-benzylhydantoin than for the dihydropyrimidine counterpart D,L-6-phenyl-5,6-dihydrouracil. The other substrates D,L-(5-*tert*-butylhydantoin, D,L-5-(3-indolylmethyl) hydantoin) were hydrolysed with lower rates.

A range of *Arthrobacter* strains was tested for the ability to cleave the above mentioned dihydropyrimidines and hydantoins. Two different groups were obtained: In whole-cell biotransformation the first group showed hydantoinase activity with a higher preference for 5,6-dihydrouracil and D,L-6-phenyl-5,6-dihydrouracil than for hydantoins. They seem to be quite different from the hydantoinases of other *Arthrobacter* strains described in literature. The other group showed no activity. No activity could be explained by the absence of hydantoin-hydrolysing enzymes or to their non-expression due to the absence of an adequate inducer.

2. Degenerate primers were developed or retrieved from a recent publication (Lin *et al.*, 2005) for the amplification of a partial hydantoinase gene sequence. The primer pairs were not specific enough for the amplification of hydantoinases of all strains tested. One reason could be the low sequence homology of hydantoinases. A hydantoinase gene fragment could be amplified from a few bacteria like *Delftia* sp. I24, *Burkholderia* sp. M3, *Bacillus* sp. G18, *Pseudomonas* sp. M18, *Arthrobacter citreus* DSM20133 and several *Ochrobactrum* isolates. The gene fragments obtained had different sizes due to the two primer pairs used. Phylogenetic analysis was conducted using hydantoinase gene fragments from the above mentioned strains and hydantoinase and dihydropyrimidine genes from the database. For a confident analysis a certain DNA-region of each hydantoinase was used namely the part of the smallest amplified fragment (app. 320 bp). In a phylogenetic tree the hydantoinase genes were predominantly found to be clustered according to their phylogenetic origin. Interestingly, *A. citreus* DSM20133, *Delftia* sp. I24 and the *Ochrobactrum* hydantoinases were not clustered together with known hydantoinases but with putative hydantoinases. These results could have been obtained because each genus showed a specific nucleotide usage (which is seen e.g. in different GC contents). However, the data obtained above is supported by a phylogenetic tree of protein sequences (Chapter IV).

In summary, we found that hydantoinases can be clustered according to their bacterial origin.

3. No correlation was obtained between the biochemical properties of hydantoinases within a certain cluster of the phylogenetic tree. One reason could be that not all nucleotide positions encoding the catalytic sites and/ or functional recognition sites are included in the gene sequence used for the phylogenetic analysis.
4. Two DIG-labelled probes were generated from hydantoinase gene fragments (app. 320 bp) amplified from genomic DNA of *Delftia* sp. I24 or *Ochrobactrum* sp. G21 (see Chapter IV). These probes were used to detect hydantoinase genes in other microorganisms. The probes were only positive for a few microorganisms since very stringent conditions were used. Positive signals of the DIG-labelled probe of *Ochrobactrum* sp. G21 were obtained for *A. ilicis* DSM20138, *A. psychrolactophilus* DSM15612 and *A. methylotrophus* DSM14008 indicating the presence of a hydantoinase gene in these microorganisms. These strains had not shown hydantoinase activity in whole-cell bio-transformation assays before, and also no amplification of a hydantoinase fragment had been achieved. Therefore, these hydantoinase genes were considered as cryptic or silent genes being not expressed during growth due to the absence of an appropriate inducer.

4.3 Summary Chapter III: Properties of Selected Hydantoinases

Complete title:

DESCRIPTION AND CHARACTERISATION OF THE D-HYDANTOINASES FROM THREE MICROORGANISMS ISOLATED FROM EXTREME ENVIRONMENTS.

In Chapter I and II it was shown that it is still possible to find microorganisms with hydantoinase activity not having been described before, and from habitats in which the pressure of survival is high due to extreme conditions. The amplification and comparison of partial hydantoinase gene sequences showed that hydantoinases are closely related to their phylogenetic origin. Some hydantoinases are clustered apart from known hydantoinases. This makes them attractive for further characterisation e.g. the hydantoinases from various *Ochrobactrum* species or the one from *Delftia* sp. I24. The questions arise if "novel" hydantoinases of microorganisms or hydantoinases of bacteria from extreme environments show unique growth- and hydantoinase properties or any adaption to the special environmental conditions. Therefore three bacterial strains were selected for:

1. further identification of the microbial strains;
2. characterisation and optimisation of growth conditions in relation to hydantoinase activity;
3. characterisation of the biochemical properties of the hydantoinases.

The selection criteria for the microorganisms used included the environment of isolation, type of bacteria, hydantoinase activity and/ or substrate range. The first strain, *Delftia* sp. I24, was selected because this strain was isolated from a Chinese hot spring at a screening temperature of 50°C (Fig. 5 & 7b), and no hydantoinase activity had been reported for this genus before. *Ochrobactrum* sp. G21 and *Bacillus* sp. F18 were selected because they both were found in soil from hypersaline lakes, Inner Mongolian Autonomous Region, China (Fig. 5 & 7a). The hydantoinase of each strain showed good activity and had a broad substrate spectrum.

Bacterial Identification

The three bacterial isolates were further classified using molecular biological and classical bacterial identification methods. They could be characterised as follows:

1. Comparison of the 16S rDNA sequence of strain *Delftia* sp. I24 to known sequences showed the highest similarity to *Delftia acidovorans* or *Delftia tsuruhatensis* at the same similarity level. Colonies of *Delftia* sp. I24 were rod shaped with a size of 4–6 µm (Fig. 8a), motile, Gram-staining negative, aerobic, catalase and oxidase positive. *Delftia* sp.



(a) Unnamed Mongolian salt lake

(b) Algal mat from site LP4

Figure 7: Environmental sites from which the bacterial strains *Ochrobactrum* sp. G21 and *Bacillus* sp. F18 (subfigure a), *Delftia* sp. I24 (subfigure b) were isolated. (Photographs are examples of the collecting areas but do not show the original place of sampling. Photographs were taken within the Chinese-EU-SA MGATech project and Waikato University Antarctic Terrestrial Biology Program and provided by Prof. D.A. Cowan.)

I24 could not be identified to the species level because the biochemical criteria did not show an exact concordance to one of the two *Delftia* species mentioned above. Therefore this strain was assigned as *Delftia* sp. I24.

2. The 16S rDNA sequence of *Ochrobactrum* sp. G21 showed highest homology to three type strains *Ochrobactrum anthropi*, *Ochrobactrum lupine* or *Ochrobactrum triciti*. *Ochrobactrum* sp. G21 cells were coccoide rods with a size of $2\ \mu\text{m}$ (Fig. 8b), colonies yellow, motile, Gram-staining negative, aerobic, catalase and oxidase positive. Concerning different characteristics, *Ochrobactrum* sp. G21 could not be assigned with the utmost probability to one of the type strains mentioned above.
3. *Bacillus* sp. F18 could be undoubtedly assigned as *Bacillus megaterium* concerning the 16S rRNA gene sequence. Therefore this strain was designated as *Bacillus megaterium* F18. The colonies of this strain were rod shaped with a size of $5\text{--}6\ \mu\text{m}$ (Fig. 8c), Gram-staining positive, facultative anaerob, catalase positive and oxidase negative.

All three strains were deposited at the German Collection of Microorganisms and Cell Cultures: *Delftia* sp. I24: DSM18833; *Ochrobactrum* sp G21: DSM18828; *Bacillus megaterium* F18: DSM18825.

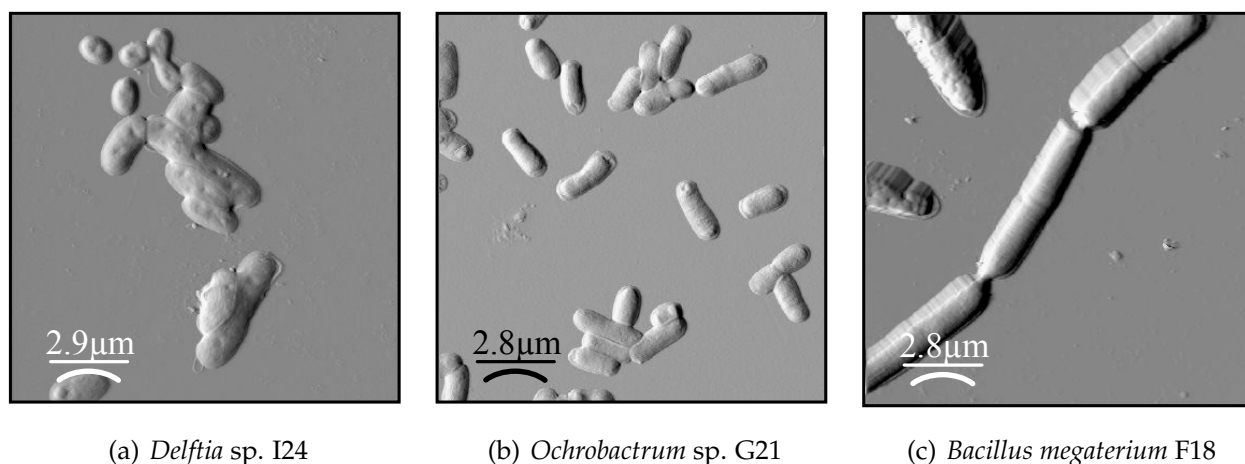


Figure 8: Atomic force microscopy (AFM) images of the three bacterial isolates being identified.

Growth Characteristics of the Three Bacterial Isolates in Relation to Hydantoinase Activity

Gross *et al.* (1990) showed that the treatment of cells of *A. aurescens* DSM3747, a Gram-staining positive bacteria, with sodium-desoxycholate led to enhanced cell permeabilisation and therefore higher hydantoinase and carbamoylase activity. To overcome cell membrane transport problems for substrates and products *Ochrobactrum* sp. G21 was chosen as a model strain and were treated with different methods. Addition of toluene (20 $\mu\text{l}/\text{mL}$) to the biotransformation solution led to highest hydantoinase activity.

Concerning the carbon source required for growth no optimisation of the media was necessary for the strains *Ochrobactrum* sp. G21 and *B. megaterium* F18 since they grew very well on glucose, whereas *Delftia* sp. I24 showed low growth on this carbon source. Addition of fructose to the media led to high growth of this strain. Concerning the origin of the strain, a Chinese hot spring, this strain should be able to grow at high temperatures which could not be confirmed. However, this strain was isolated at a screening temperature of 50°C and therefore declared as thermophilic. It is supposed that *Delftia* sp. I24 might be a contamination of this habitat, only possessing the ability to survive at high temperatures.

The influence of salt on growth and hydantoinase activity were investigated for the strains *Ochrobactrum* sp. G21 and *B. megaterium* F18, because they were isolated from hypersaline lakes and previously shown to be halophilic (Chapter I). For both strains the growth behavior was found to be opposite compared to hydantoinase activity characteristics. Whilst growth was highest at a low salt concentration of the medium, hydantoinase activity was stimulated in media with higher salt concentrations (Table 3). An attractive hypothesis for *Ochrobactrum* sp. G21 and *B. megaterium* F18 is, that the hydantoin degrading system of these bacteria is co-regulated with salt stress proteins and other metabolic pathways triggered by salt stress presumably to get access to amino acids under limited conditions. This was derived from studies on the effect of salt stress on microorganisms: Salt stress had

an important effect on the synthesis of degradative enzymes in *Bacillus subtilis* (Kunst *et al.*, 1995). Another study on *Listeria monocytogenes* showed that the synthesis of 40 proteins was repressed or induced during salt stress. This includes general stress proteins, transporters and general metabolism proteins (Duché *et al.*, 2002).

Table 3: Influence of different parameters on growth and hydantoinase activity. For each parameter the optimal conditions concerning growth or hydantoinase activity are given. (**I24:** *Delftia* sp. I24; **G21:** *Ochrobactrum* sp. G21; **F18:** *B. megaterium* F18; **GM:** growth media; **IMH:** D,L-5-(3-indolylmethyl) hydantoin; **NMH:** D,L-2-naphtylmethylhydantoin; **PheDU:** D,L-6-phenyl-5,6-dihydrouracil; **t- BH:** D,L-5-*tert*-butylhydantoin)

	I24	G21	F18
carbon source	fructose	glucose	glucose
growth medium	GM/ GM+PheDU	GM	GM+2%NaCl
NaCl conc. for hyd. activity	not tested	5%	5%
inductor	IMH/ PheDU	NMH	<i>t</i> -BH
Medium for hyd. activity	GM	GM+5%NaCl +NMH	GM+5%NaCl + <i>t</i> -BH

The addition of different inducers to the growth media had a significant effect on hydantoinase activity. The dissimilarity of each hydantoinase was shown: The hydantoinase of *Delftia* sp. I24 showed highest induction with the addition of the polycyclic aromatic hydantoin D,L-5-(3-indolylmethyl) hydantoin or the aromatic dihydropyrimidine derivative D,L-6-phenyl-5,6-dihydrouracil to the growth media (Table 3). In case of the hydantoinase of *Ochrobactrum* sp. G21 addition of the polycyclic aromatic hydantoin D,L-2-naphtylmethylhydantoin led to a dramatic increase of hydantoinase activity and the hydantoinase of *B. megaterium* F18 was stimulated highest with the aliphatic hydantoin D,L-5-*tert*-butylhydantoin (Table 3).

Hydantoinase activity was highest in the late exponential phase of growth for several *Pseudomonas* species (Morin *et al.*, 1986b, c; Gokhale *et al.*, 1996; Sudge *et al.*, 1998), *Agrobacterium* (Kim and Kim 1993) and in the exponential phase of *Arthrobacter* sp. DSM3747 (Syldatk *et al.*, 1990).

All previous results were obtained neglecting the variation of hydantoinase activity during growth since harvesting was done routinely at a certain point of time. In order to find the best media and growth phase for highest hydantoinase activity different media were compared and hydantoinase activity was determined during growth (Table 3).

Hydantoinase activity of *Delftia* sp. I24 was found to be highest in the exponential phase when grown on pure GM – or slightly lower on GM supplemented with 0.2 g/L D,L-6-phenyl-5,6-dihydrouracil. The favourable media for *B. megaterium* F18 hydantoinase

activity was GM supplemented with 5%NaCl and 0.2 g/L D,L-5-*tert*-butylhydantoin, as well with highest activity in the late exponential phase. Highest hydantoinase activity of *Ochrobactrum* sp. G21 was obtained when grown on GM supplemented with 5%NaCl and 0.2 g/L D,L-2-naphthylmethylhydantoin after 96 h but growth was very little. The results of the halophilic strains *Ochrobactrum* and *Bacillus* showed that highest hydantoinase expression could be achieved by adjustment of the salt concentration of the medium and with the choice of the appropriate inductor.

Properties of Hydantoinases

In order to determine the biochemical properties of the hydantoinases bacterial strains were grown in their favourable media as described in the previous section.

(I) Optimal temperature and pH (Table 4)

The temperature optimum of the hydantoinase of *Delftia* sp. I24 was very low with 30°C. This is in accordance to the results obtained for the growth characteristics of this strain (see previous section) and confirms the hypothesis that *Delftia* sp. I24 is probably a contamination of this habitat. Together with the D-hydantoinase from a halophilic *Pseudomonas* sp. NCIM5109 (Sudge *et al.*, 1998) this is the lowest temperature optimum of D-hydantoinases reported. The optima of temperature and pH for *Ochrobactrum* sp. G21 were 40°C and pH 8.0–8.5, respectively. The hydantoinase of the only *Ochrobactrum* strain described in literature, *Ochrobactrum anthropi*, showed a pH-optimum of pH 9.0 (Poza *et al.*, 2002). Unfortunately the influence of temperature was not tested in this study. The highest optimal temperature was obtained for the hydantoinase of *B. megaterium* F18 at 50–55°C and a pH-optimum of 7.5–8.0. The optimal temperature of this hydantoinase is quite low since hydantoinases from other *Bacilli* showed higher optimal temperatures: *Bacillus* sp. AR9, *B. stearothermophilus* SD-1 and *B. thermocatenulatus* GH-2 at 65°C (Sharma and Vohra, 1997; Lee *et al.*, 1994, 1995; Park *et al.*, 1998; Kim *et al.*, 1997a), *B. stearothermophilus* NS1122A at 60–70°C (Ishikawa *et al.*, 1994) and *B. circulans* at 75°C (Luksa *et al.*, 1997).

Table 4: Properties of the hydantoinases from *Delftia* sp. I24, *Ochrobactrum* sp. G21 and *B. megaterium* F18. The optimal value of each parameter is shown. (**I24:** *Delftia* sp. I24; **G21:** *Ochrobactrum* sp. G21; **F18:** *B. megaterium* F18)

	I24	G21	F18
T [°C]	30	40	50–55
pH	9.0	8.0–8.5	7.5–8.0
cofactor	Ni ²⁺ / Co ²⁺	no preference	Mn ²⁺ / Co ²⁺
salt conc.	no influence	0–2.5%	no influence

(II) Cofactor requirements (Table 4)

Hydantoinases are known to be dependent on cofactors, e.g. the L-hydantoinase from *Arthrobacter aurescens* DSM3745 contains about 2.5 mol Zn²⁺/mol subunit (May *et al.*, 1998a, b) and the hydantoinase of *Bacillus* sp. AR9 contains Mn²⁺ in the active centre (Rhada Kishan *et al.*, 2005). It has to be mentioned that biochemical studies can give a hint on the cofactor being present in the enzyme but for verification complex methods such as atomic absorption spectroscopy, inductive coupled plasma-atomic emission spectrometry and/ or X-ray crystallography have to be used.

Biochemical studies on the cofactor requirements of the three strains showed that all hydantoinases are cofactor dependent because addition of EDTA inhibited or completely removed hydantoinase activity. Since the hydantoinase activities of *Delftia* sp. I24 and *B. megaterium* F18 were enhanced by the addition of Ni²⁺ or Co²⁺ and Mn²⁺ or Co²⁺, respectively, it is supposed that one of these ions is present in one of the hydantoinase enzymes. In contrast, the hydantoinase from *Ochrobactrum* sp. G21 showed no enhanced activity caused by one of the ions tested. The hydantoinase from *Ochrobactrum anthropi* was described in literature to show increased activity by addition of Ca²⁺, Na⁺, Cu²⁺ or Co²⁺ (Pozo *et al.*, 2002). Unfortunately the D-hydantoinases of *Ochrobactrum* sp. G21 described in this study and of *Ochrobactrum anthropi* cannot be compared in detail because both hydantoinases were studied in other aspects except for pH and cofactor requirements. Taking into account these two aspects, or particularly the unequal cofactor requirements, we can conclude that these hydantoinases are rather different enzymes.

(III) Influence of salt concentration on hydantoinase activity (Table 4)

The hydantoinase activities of *Delftia* sp. I24 and *Bacillus megaterium* F18 remained the same at salt concentrations ranging from 2.5 to 20% NaCl, whereas the hydantoinase activity of *Ochrobactrum* sp. G21 decreased with raising NaCl-concentrations. This seem to be contrary to the growth experiments using different salt concentrations of the media in which hydantoinase activity was enhanced in media with higher salt concentrations. We have to consider that we were using growing cells on the one hand and resting cells on the other. Growing cells are able to respond to factors like salt stress for example with altered enzyme expression, whereas resting cells remain in the current-state of harvest. Consequently, in assays using resting cells only enzymes or enzyme systems can directly be affected by the salt concentration, e.g. inhibited or enhanced. In the present study only the hydantoinase of *Ochrobactrum* sp. G21 was inhibited by higher salt concentrations.

(IV) Enzyme stability

The hydantoinase enzyme of whole cells of *Delftia* sp. I24 did not loose activity at a storage temperature of 4°C and 20°C within four days, whereas activity was almost lost at 40°C after one day of storage. Hydantoinase in cells treated with toluene showed lower stability

since the activity decreased at all storage temperatures after one day. The hydantoinase activity of whole cells and toluene treated cells of *Ochrobactrum* sp. G21 decreased at all storage conditions significantly. Best storage was achieved at 4°C.

(V) Substrate range of the hydantoinases

All three strains showed a broad substrate range and were able to cleave the following substrates: 5,6-dihydrouracil, hydantoin, D,L-5-*tert*-butylhydantoin, D,L-5-benzylhydantoin, D,L-5-(3-indolylmethyl) hydantoin and D,L-6-phenyl-5,6-dihydrouracil (except of *Delftia* sp. I24 which was not able to cleave the latter). The hydantoinase from *Delftia* sp. I24 displayed for all substrates tested a significantly lower specific activity than the ones from the two other strains. The hydantoinase showed highest activity for 5,6-dihydrouracil indicating that this enzyme could be involved in the pyrimidine reductive catabolism. Interestingly, the hydantoinase of *Delftia* sp. I24 was not able to cleave the best inducer in growth experiments being D,L-6-phenyl-5,6-dihydrouracil. The highest specific activities were obtained for the hydantoinase of *Bacillus megaterium* F18. This enzyme showed preference to 5-monosubstituted hydantoins or dihydropyrimidines bearing an aryl side chain compared to the corresponding aliphatic derivatives.

In summary this section describes microorganisms with novel hydantoinase properties: a D-hydantoinase from *Delftia* sp. I24 as well as the D-hydantoinases of the two halophilic strains *Ochrobactrum* sp. G21 and *Bacillus megaterium* F18. All of the hydantoinases showed unique properties regarding salt and inductor requirements during growth for enzyme expression and they displayed different properties like variation in pH and temperature optima and cofactor requirements.

4.4 Summary Chapter IV: Gene Clusters for Hydantoin Degradation

Complete title:

GENES RESPONSIBLE FOR HYDANTOIN DEGRADATION OF A HALOPHILIC *Ochrobactrum* SP. G21 AND *Delftia* SP. I24 – NEW INSIGHT INTO RELATION OF D-HYDANTOINASES AND DIHYDROPYRIMIDINASES.

Different gene clusters responsible for the cleavage of hydantoins and dihydropyrimidines are elucidated for a range of microorganisms (Fig. 2). Nevertheless, no gene cluster responsible for hydantoin degradation has been reported for any halophilic species or for bacteria belonging to the genus *Ochrobactrum* and *Delftia*.

In the preceding chapter growth characteristics in relation to hydantoinase activity and the biochemical properties of the hydantoinases of the two moderate halophiles *Ochrobactrum* sp. G21 and *Bacillus megaterium* F18 as well as of *Delftia* sp. I24 were accurately described. The genes responsible for hydantoin hydrolysing activity were of particular interest since the wild type strains originated from extreme environments and the biochemical properties of the hydantoinases described showed unique properties. The amplification of a hydantoinase gene fragment using degenerate primers was possible for *Ochrobactrum* sp. G21 and *Delftia* sp. I24 but not for *Bacillus megaterium* F18 (see Chapter II). The hydantoinase gene clusters were identified by screening of genomic libraries with DIG-labelled probes. These probes were derived from hydantoinase gene fragments amplified by PCR. The genomic libraries were obtained by digestion of genomic DNA using selected restriction enzymes and cloning of the respective DNA fragments, bearing the hydantoinase gene into *E. coli*. Clones bearing the hydantoinase gene were obtained by probe hybridisation. The complete DNA sequences were received by sequencing of the plasmids. Since amplification of a partial hydantoinase gene sequence was crucial to prepare a DIG-labelled probe, *Ochrobactrum* sp. G21 and *Delftia* sp. I24 were chosen for characterisation of the hydantoin hydrolysing gene cluster.

The gene clusters responsible for hydantoin hydrolysis are shown in Figure 9. The cluster of *Delftia* sp. I24 consisted of four genes: A hydantoinase gene (*hyuH*), a putative dihydroprimidine dehydrogenase gene (*pydA*), a putative permease gene (*hyuP*) and an incomplete glutamate synthase gene (*gltB*). *HyuH* was first incomplete but completed by PCR amplification of the missing DNA fragment. The deduced amino acid sequence showed highest similarity to the hydantoinase of *Pseudomonas putida* DSM84. The hydantoinase was expressed in *E. coli*, and activity was shown for D,L-5-benzylhydantoin and D,L-5-(3-indolylmethyl) hydantoin. The putative dihydroprimidine dehydrogenase and the putative permease were located upstream of the hydantoinase gene. The permease was probably responsible for hydantoin or dihydropyrimidine transport. The presence of *pydA* and the preference for dihydrouracil in activity tests (see Chapter III) indicated that the gene cluster is

involved in pyrimidine degradation, and *hyuH* is rather a dihydropyrimidinase than a hydantoinase. Nevertheless it was named as D-hydantoinase according to Syldatk *et al.*, 1990, since D,L-5-benzylhydantoin was degraded D-specific. This is the first gene cluster reported for the family *Comamonadaceae* not taking into account putative hydantoin hydrolysing gene clusters of other members of the family *Comamonadaceae* obtained from the sequencing of whole bacterial genomes. A comparison of these putative gene clusters with the one from *Delftia* sp. I24 revealed a high similarity between these clusters. They probably form a unique group of hydantoin hydrolysing gene clusters with the following characteristics: an identical orientation of all genes, presence of at least a hydantoinase and a carbamoylase gene (assuming the presence of a carbamoylase for *Delftia* sp. I24) and additionally the presence of either a permease, a dihydropyrimidine dehydrogenase or a dihydroorotate dehydrogenase.

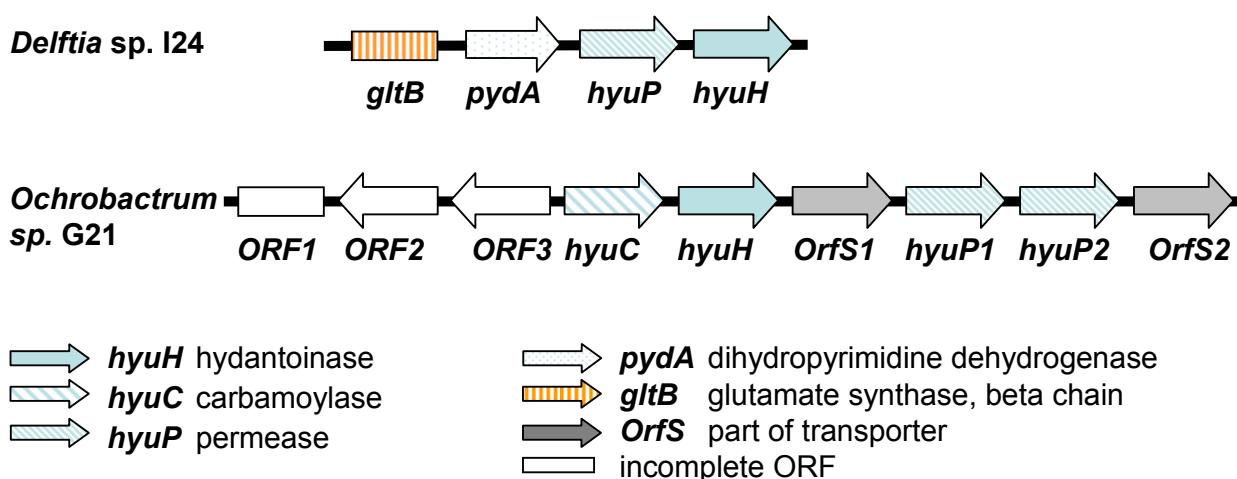


Figure 9: Reaction scheme of enzymes involved in hydantoin and dihydropyrimidine conversion.

The hydantoin hydrolysing gene cluster of *Ochrobactrum* sp. G21 consisted of six genes: The first gene, *hyuC*, probably encoding for a *N*-carbamyl amino acid amidohydrolase, also called carbamoylase and the second gene, *hyuH*, encoding for a hydantoinase followed by two transporters *OrfS1* and *OrfS1* and two permeases *hyuP1* and *hyuP2*. In detail, the deduced amino acid sequence of the hydantoinase gene showed 47% identity with the D-hydantoinase of *Bacillus* sp. AR9. The hydantoinase was analogous to the hydantoinase from *Delftia* sp. I24 expressed in *E. coli* and activity was shown for the same substrates. Taking biochemical data of the wild-type strains and of the cloned hydantoinases into account *hyuH* and *hyuC* are responsible for hydantoin conversion. The other four enzymes, especially the two permeases, are supposedly involved in hydantoin transport.

An unique pattern of *hyuC*/*hyuH* was found in the hydantoin-hydrolysing gene cluster of *Ochrobactrum* sp. G21 and in clusters from phylogenetically related bacteria.

Hydantoinases are known to belong to the protein superfamily of amidohydrolases related to ureases. Enzymes of this superfamily have evolved from a common ancestor and are a product of a divergent evolution (May *et al.*, 1998e, Holm and Sander 1997).

The question of the phylogenetic relation of hydantoinases/ dihydropyrimidinases was raised. Therefore a phylogenetic tree was built using protein sequences of hydantoinases and dihydropyrimidinases including putative sequences. It is remarkable, that as a result, predominantly hydantoinases from related bacterial families were grouped together in this tree. Taking into account functional recognition sites and the formed branches of the phylogenetic tree, six major groups were obtained and specified in families as follows:

- 1) L-hydantoinases, disjoined from the major tree;
- 2) *Rhizobiales* family (Rhizo-Fam), the most inconsistent group comprising hydantoinases from bacteria of the order *Rhizobiales* or of the same bacterial class, except of one *Pseudomonas*; *Ochrobactrum* sp. G21 is included in this family;
- 3) *Comamonadaceae* family (Com-Fam) including *Delftia* sp. I24;
- 4) *Pseudomonas* family (Pseud-Fam);
- 5) *Bacilli* family (Bac-Fam), including *Brevibacillus agri* and several *Bacillus* species;
- 6) *Agrobacterium* family (Agro-Fam), being separated from the other *Rhizobiales* and including two hydantoinases from *Agrobacterium* species and a hydantoinase from *Burkholderia pickettii*.

A deeper insight into amino acids being involved in the cleavage of hydantoins revealed that all hydantoinases used in the phylogenetic tree showed the highly conserved amino acid motif of the superfamily of amidohydrolases (GxxDxHxH). This motif is suggested to be involved in metal centre assembly (Holm and Sander, 1997). The motif known to form the active site of hydantoinases consisting of His58, His60, Lys150, His183, His239 and Asp315 (Cheon *et al.*, 2002, 2003; Rhada Kishan *et al.*, 2005; Abendroth *et al.*, 2002a, b; Xu *et al.*, 2003) is also found in all amino acid sequences.

The substrate recognition sites of several hydantoinases belonging to different bacteria are known. These known amino acid positions involved in the substrate recognition sites are investigated within the families obtained in the phylogenetic tree mentioned above. Interestingly, most families of this tree exhibited conserved amino acids residues at positions forming functional recognition sites of known hydantoinases. In detail, members of the Bac-Fam and Rhizo-Fam showed a similarity in amino acids involved in the substrate recognition sites of *Bacillus stearothermophilus* SD-1 (Met63, Leu65, Phe152, Tyr155 and Phe159; Cheon *et al.*, 2002). The Bac-Fam showed only a difference at position 65 in which Phe can be found instead of Leu. No effect on activity and substrate selectivity of this substitutions for the hydantoinase of *Bacillus stearothermophilus* SD-1 could be obtained (Cheon *et al.*, 2004). The Rhizo-Fam showed an additional substitution at position 159 (Leu instead of Phe). The same substitution for the hydantoinase of *Bacillus stearothermophilus* SD-1 led to lower activity

(Cheon *et al.*, 2004).

Completely identical were the amino acids of the exocyclic substituent recognition site of *Burkholderia pickettii* (Xu *et al.*, 2003) and the other members of the Agro-Fam being determined as Thr62, Ser64, Gln93, Phe150, Tyr153 and Asn157. We propose that the amino acid residues of the members of the Bac-, Rhizo- and Agro-Fam possibly form the substrate recognition sites as shown by comparison to known recognition sites. This assumption can be used concerning the Bac- and Rhizo-Fam since activity was not lost for substitutions at relevant amino acid positions at the hydantoinase of *B. stearrowthermophilus*. The Com- and Pseud-Fam shared the same amino acid residues at relevant amino acids residues but did not share similarity like the families mentioned above to any known substrate recognition site.

The family-/ order-specific clusters obtained in the phylogenetic study and the strong similarity of the proposed families absolutely confirms the theory of a common ancestor and that the members of the superfamily of amidohydrolases are product of a divergent evolution. Still the question of the differences of hydantoinases and dihydropyrimidinases remains. Up to now it is only possible to distinguish between them by using biochemical assays. One idea is to differentiate between them on a genetical level or at the genetical organisation of the gene clusters. No certain gene pattern or organisation of the gene clusters was observed that allows to differentiate between hydantoinases and dihydropyrimidinases. For example, a permease should only been found in connection with hydantoinases to act as transmembrane transporter to access hydantoins (as in the case of the hydantoin hydrolysing gene cluster of *A. aureescens* DSM3747). A permease would not be necessary for dihydropyrimidinases since the preferred substrate is a cellular compound. However, the hydantoin hydrolysing gene cluster of *Delftia* sp. I24 is supposed to be involved in the pyrimidine degradation pathway and the "hydantoinase" is rather a dihydropyrimidinase. But this gene cluster exhibit a permease (Figure 9). That means that the dramatically increasing number of putative hydantoinase and dihydropyrimidinase gene sequences will not solve the questions of the differentiation of these enzymes. Additionally, the question of the natural function of hydantoinases cannot be answered since exact biochemical data is missing.

5 Future Development

Further studies should focus on the novel hydantoin hydrolysing gene clusters introduced in the previous chapter. There are still some investigations to be accomplished to confirm all the results obtained in this study:

- We suppose that the hydantoin-hydrolysing gene cluster of *Delftia* sp. I24 also includes a carbamoylase downstream the hydantoinase. Experiments should be conducted to confirm its presence.
- It is remarkable that permeases were found in most of the hydantoin hydrolysing gene clusters, but in literature nothing is reported on the substrate specificity and mechanism of these enzymes. Further investigations should concentrate on these enzymes which can provide insight into the transmembrane transport (uptake and release) of hydantoins and carbamoyl amino acids. These findings would be of special interest for industrial production of enantiomerically pure amino acids by the hydantoinase process.
- To clarify the amino acids involved in functional and catalytic recognition of the proposed hydantoinase families at least the hydantoinase of *Ochrobactrum* sp. G21 and *Delftia* sp. I24 should be purified and crystallised. This would provide insight into the catalytic and functional recognition sites of the two hydantoinases. As well, this could give further information on the presence of metal ions in these enzymes.

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7 Publications and Manuscripts

7.1 Chapter I: Distribution of Hydantoinases

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, 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 Microbacteriaceae. We have shown that microorganisms with hydantoinase activity are

- distributed in various geographically distinct environmental habitats,
- distributed worldwide,
- 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.

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* DSM3745 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 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: are microorganisms with hydantoinase activity

- (i) only found in a small range of similar environmental habitats;
- (ii) are they distributed worldwide; and
- (iii) are hydantoinases limited to certain bacterial genera?

Experimental Procedures

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-(hydroxymethyl) hydantoin; **IMH:** D,L-5-(3-indolylmethyl) hydantoin; **RBC:** Rotating Biological Contactor, **SA:** South Africa; **TBE:** tris-borate buffer.

Chemicals

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

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).

Media

The following media were used: Medium I, according to [9], used for the screening experiments, contained: 10.0 g/L HMH or IMH, 0.2 g/L fructose, 3.9 g/L $(\text{NH}_4)_2\text{SO}_4$, 0.95 g/L KH_2PO_4 , 2 g/L $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.02 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 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 $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$, 40 mg/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mg/L $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 20 mg/L FeCl_3 , 10 mg/L KI, 4 mg/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. 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, 5 g/L NaCl. GM, according to [9], used as growth medium for biotransformation assays, contained: 10 g/L glucose, 6.5 g/L $(\text{NH}_2)_4\text{SO}_2$, 0.2 g/L MgSO_4 , 0.02 g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.02 g/L FeSO_4 , 0.02 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5.54 g/L KH_2PO_4 , 7.6 g/L $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.32 g/L citrate-1-hydrate, 1 g/L IMH at pH 6.8. TBE (10x), used for agarose gel electrophoresis, was prepared as follows: 108 g Tris and 55 g boric acid were dissolved in 900 mL water. 40 mL 0.5 M Na_2EDTA (pH 8.0) was added and the volume adjusted to 1 liter.

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 supplemented with either IMH or HMH. Chinese soil samples were also incubated at 40°C 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, approx. 1 g of soil sample was added to 10 mL sterile water, containing 0.0005 g nystatin (as fungicide) and 10 μ L Triton[®]X100, mixed well and 100 μ L 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.

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. 2 mL 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, 8,000 rpm and 4°C; Beckman, Avanti[™] 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 μ L 33% trifluoroacetic acid to 500 μ L of the reaction solution and centrifugation at 13,000 rpm for 7 min (Heraeus, Biofuge pico).

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% H₃PO₄) 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 derivatisation. 300 μ L of the cell free biotransformation supernatant was added to 800 μ L Ehrlich Reagent (1 g 4-dimethylaminobenzaldehyde, 5 mL H₂O, 5 mL 6 M HCl) and 900 μ L water. The quantification of the yellow product was performed photometrically at 430 nm (Unicam, Helos a).

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 μ L/100 mL) and quantified under UV-light in comparison to standard gene fragments of 10 ng and 25 ng. Polymerase chain reaction (PCR) was used for the amplification of the 16S rRNA gene and performed using a Thermo-Hybrid PCR Sprint Thermocycler under the following conditions: 0.2 mM dNTPs (Promega, USA), 5 μ L Taq-Polymerase, 10x-reaction buffer (Promega, USA), 0.5 mM forward primer, 0.5 mM reverse primer, 1.5 mM MgCl₂ (Promega, USA), 1 μ L genomic DNA, and PCR water combined to a total volume of 50 μ L. 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 thirty 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 GFXTM 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/>).

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 characterised using 16S rRNA gene amplification, sequencing and database comparisons. The isolates were assigned according to the closest 16S rDNA match (Table 1). Further characterisation via classical bacterial identification is in progress.

In total, thirty-two bacterial strains were isolated from terrestrial soil samples of different environmental sources (Table 1). *Pseudomonas putida* strains and an *Agrobacterium tumefaciens* strain have previously been isolated from South Africa and characterised [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, 17, 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).

Table 1: Hydantoinase positive bacterial isolates. (%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))

description of soil sample	isolate no.	genus	%homology /bp	DU-biotr		BnH-biotr	
				C-Ala	L-C-P	D-C-P	D-C-P
SA, Table Mountain, 700 m a.s.l., soil	N1	<i>Stenotrophomonas</i> sp.	99%/472	+	-	-	++
SA, Stellenbosch, pinewood forest, soil	K3	Und. <i>Microbacteriaceae</i>	100%/445	++	+	+	+
	M3	<i>Burkholderia</i> sp.	98%/513	+	+	+	-
SA, Worcester, Distell Brandy Distillery, biofilm of a RBC for wine distillery wastewater	D24	<i>Ochrobactrum</i> sp.	98%/749	+++	-	-	+
SA, Stellenbosch, Reliance Compost Company, compost of wine yards	G6	<i>Pseudomonas</i> sp.	98%/692	++	-	-	+
	C15	<i>Ochrobactrum</i> sp.	99%/530	+++	-	-	++
	H7	<i>Staphylococcus</i> sp.	99%/592	++	-	-	+
SA, Stellenbosch, Reliance Compost Company, compost of fiber material	F8	<i>Flavobacterium</i> sp.	98%/563	++	-	-	+
SA, Stellenbosch, Reliance Compost Company, shredder material (five weeks old)	K5	<i>Acinetobacter</i> sp.	98%/356	++	-	-	+
	I21	<i>Ochrobactrum</i> sp.	99%/585	+++	-	-	+
	J24	<i>Ochrobactrum</i> sp.	100%/586	++	-	-	+
SA, Stellenbosch, Reliance Compost Company, shredder material (twelve weeks old)	J7	<i>Enterobacter</i> sp.	97%/384	+	-	-	+
	N8	<i>Pseudomonas</i> sp.	99%/492	+++	-	-	+
	P8	<i>Pseudomonas</i> sp.	99%/754	++	-	-	+
SA, West Coast N. P., guano from a small pond at Langebaan Lagoon	A16	<i>Bacillus</i> sp.	99%/727	+++	-	-	++
SA, Veldrift, "guano" from a salt lake with bird dung	F16	<i>Bacillus</i> sp.	100%/520	++	-	-	++
SA, West Coast N. P., algal material of	D17	<i>Bacillus</i> sp.	100%/688	++	-	-	+

description of soil sample	isolate no.	genus	%homology /bp	DU-biotr		BnH-biotr	
				C-Ala	L-C-P	L-C-P	D-C-P
Langebaan Lagoon	F21	<i>Ochrobactrum</i> sp.	100%/735	+++	-	-	+
SA, Cederberg Mountains, 1100 m a.s.l., dry soil	L9	<i>Pseudomonas</i> sp.	99%/584	++	-	-	+
SA, Tsitsikamma N.P., soil of an Afromontane Forest	M18 H20	<i>Pseudomonas</i> sp. <i>Bacillus</i> sp.	99%/445 100%/565	++ ++	-	-	+
Swaziland, Ezulweni Valley, red soil	G18	<i>Bacillus</i> sp.	100%/501	++	-	-	++
Lesotho, Blue Mountain Pass, 2500 m a.s.l., soil	K20	<i>Arthrobacter</i> sp.	100%/587	+	-	-	+
Inner Mongolia, IHX	F18	<i>Bacillus</i> sp.	99%/736	++	-	-	++
Inner Mongolia, BJ1	K18	<i>Bacillus</i> sp.	99%/633	++	-	-	++
Inner Mongolia, GHI, hypersaline lake	I20	<i>Streptomyces</i>	-	+	-	-	+
Antarctic, Dry Valley, soil	G21	<i>Ochrobactrum</i> sp.	95%/351	+++	-	-	+
Antarctic, Dry Valley, soil	N7	<i>Pseudomonas</i> sp.	98%/524	+++	-	-	+
Antarctic, Bratina Island, sediment of a saline pond	E7 F7 G7	<i>Arthrobacter</i> sp. <i>Arthrobacter</i> sp. <i>Pseudomonas</i> sp.	99%/764 98%/593 99%/711	++ ++ +++	-	-	+
Long Pu, Kunming, SW China, algal mat, 60 – 65°C (screening at 50°C)	I24	<i>Delftia</i> sp.	99%/588	+++	-	-	+

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 thirty-two 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°C 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. 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 (Figure 1), whereas two *Bacillus* strains (isolates F18 and K18) were 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, 23, 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-ethylthioethyl) 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 & Figure 1) and from a biofilm of a 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 activity. 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 *Arthrobacter aurescens* strains DSM3745 and 3747 have been well-described [28] and both produce α -amino acids from 5-monosubstituted hydantoins [28]. The hydantoinase

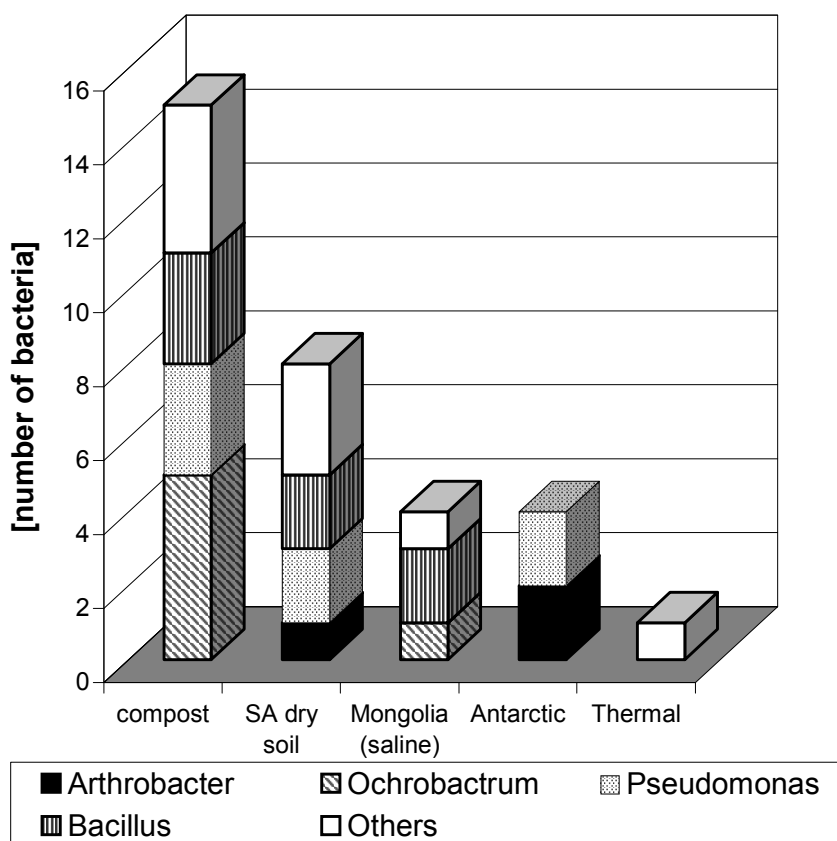


Figure 1: Distribution of isolated bacterial genera in relation to environmental type. (Soil samples (described in Table 1) are grouped by the dominant property: **compost:** derived from compost or from habitats with a high amount of degrading material; **SA dry soil:** samples from South African desert 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).)

from *A. aurescens* DSM3745 belongs to the amidohydrolase superfamily [5] and has been shown to be a Zn²⁺-metalloenzyme [29, 30] with L-selectivity in the conversion of D,L-5-(3-indolylmethyl) hydantoin. The hydantoinase and carbamoylase genes from *Arthrobacter aurescens* DSM3747 and DSM3745 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, 36, 37, 38, 39]. Psychrotrophic bacteria isolated from cyanobacterial mats in the McMurdo Dry Valleys, Antarctica, have been recently characterised 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 ($T_{\text{opt}} = 80^{\circ}\text{C}$) 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 *Bacillus stearothermophilus* NS1122A, with optimal temperatures of between 60°C and 70°C [24].

D-hydantoinase activity has been reported in *Burkholderia pickettii* [44], towards D,L-5-mercaptopethyl-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, *Microbacteriaceae* sp. K3, *Staphylococcus* sp. H7, *Acinetobacter* sp. K5, *Delftia* sp. I24 and an unidentified isolate I20 (possibly *Streptomyces*; Dr. Eberspächer pers. 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.

Conclusions

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*).
- (iii) 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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7.2 Chapter II: Biodiversity of Hydantoin Cleaving Enzymes

BIODIVERSITY OF HYDANTOIN CLEAVING ENZYMES

Introduction

"Biogeography" is the "science that attempts to document and understand spatial patterns of biodiversity, distribution of organisms, both past and present, and of related patterns of variation over the earth in the numbers and kinds of living things" (Brown and Lomolino, 1998). Baas-Becking (1934) postulated that microorganisms are highly dispersed resulting in a cosmopolitan microbial world ("everything is everywhere") and a lack of biogeographical patterns for free living bacteria. This theory is still under strong debate within the microbiologist community. Recent investigations demonstrated biogeographical patterns for some bacterial taxa. Whitaker *et al.* for example (2003) found a correlation between the geographical and genetic distance of hot spring archaean *Sulfolobus* isolates from geographically distinct regions.

The United Nations Conference on Environment and Development, UNCED (1992) defined the term "biological diversity" or "biodiversity" to be the variety of genes, species, and ecosystems found on our planet. It embraces all life forms – from plant and animal life to microorganisms – and the water and land in which they interact. An introduction on hydantoinases as well as the distribution and occurrence of hydantoinase possessing microorganisms was shown by Dürr *et al.* (2006). In summary, hydantoinase-positive bacterial isolates are very widely dispersed, both geographically and with respect to environmental conditions. Among culturable aerobes hydantoinase-positive bacteria are predominantly found in certain genera: *Pseudomonas*, *Ochrobactrum*, *Bacillus* and *Arthrobacter*.

This chapter will focus on the biodiversity of hydantoin cleaving enzymes meaning the variety of hydantoinases hereby referring to the UNCED definition. Therefore a detailed investigation of the following topics was conducted for the hydantoinases previously described (Dürr *et al.*, 2006):

1. It is known that hydantoinases have a broad and diverse substrate spectrum (compare Introduction, Table 1). The remaining question is if there is a correlation between substrate spectrum and origin of hydantoinases concerning bacterial genera and/ or ecological habitat?
2. Can the hydantoinase gene sequence of different microorganisms be clustered into specific groups either related to a genus and/ or to a certain habitat?
3. Is there a correlation between the biochemical properties of hydantoinases and certain groups of hydantoinase gene sequences?
4. Are hydantoinase genes as well present in microorganisms for which hydantoinase activity could not be shown, yet?

The screening for hydantoinases is normally done by classical ways using enrichment techniques which enable the isolation of microorganisms with hydantoinase activity.

Additionally, molecular and immunological attempts are described to get access to new enzymes being able to cleave dihydropyrimidines/ hydantoins.

Hydantoinase gene sequences were found by searching for hydantoinase positive clones in a genomic library either by activity assays (LaPointe *et al.*, 1994; Kim *et al.*, 1997) or growth on hydantoin as the sole nitrogen source (Mukohara *et al.*, 1994; Chien *et al.*, 1998). Polyclonal antibodies were successfully developed for the detection of the L-hydantoinase from *Arthrobacter aurescens* but they were not specific for the detection of the D-hydantoinase from *Agrobacterium* sp. (Siemann *et al.*, 1993 a, b). LaPointe *et al.* (1994) developed a 1.5 kb DIG-labelled DNA probe derived from a recombinant hydantoinase gene from *Pseudomonas putida* DSM84. The probe only hybridised with total DNA from *Pseudomonas* strains of rRNA group I showing hydantoinase activity. No hybridisation occurred with DNA from bacteria outside rRNA group I indicating the divergence of genes responsible for hydantoin conversion. The authors used a 122 bp DNA probe obtained from the same organism mentioned above to detect D-hydantoinase genes in other bacterial genera by DNA and by colony hybridisation. The probe was specific, allowing 32% mismatch, to detect known D-hydantoinase activity in microorganisms belonging to the genera *Pseudomonadaceae*, *Agrobacterium*, *Serratia*, *Corynebacterium* and *Arthrobacter*. In an experiment under environmental conditions also bacteria belonging to the genera *Pseudomonas*, *Klebsiella*, *Enterobacter* and *Enterococcus* showed a positive signal towards the probe. Hydantoinase activity was shown for the mentioned bacteria (LaPointe *et al.*, 1995).

Recently, degenerate primers were reported for the amplification of a 330 bp dihydropyrimidinase gene fragment of *Bacillus* sp. TS-23 (Lin *et al.*, 2005). They were designed from conserved motifs obtained from the comparison of the amino acid sequence of hydantoinases/ dihydropyrimidinases from *Bacillus stearothermophilus*, *Pseudomonas putida*, *Agrobacterium tumefaciens* and *Streptomyces coelicor*. The amplified 330 bp DNA fragment was used as a probe to identify the dihydropyrimidinase gene within a genomic library. The isolated dihydropyrimidinase gene consisted of 1422 bp and was deduced to contain 472 amino acids sharing 40–87% sequence identity with *Arthrobacter aurescens*, *Thermus* sp., *Burkholderia pickettii* and *Bacillus stearothermophilus*.

Materials and Methods

Abbreviations

BnH: 5-benzylhydantoin; **CA:** Corynebacterium medium; **CA-plates:** Corynebacterium agar plates; **C-Ala:** *N*-carbamoyl- β -alanine; **C-Gly:** *N*-carbamoyl-glycine; **CH3-IMH:** D,L-5-(3-indolylmethyl)-3-*N*-methylhydantoin; **D-/L-C-Phe:** *N*-carbamoyl-D/ L-phenylalanin; **D-/L-C-*t*-Leu:** *N*-carbamoyl-D/L-*tert*-leucine; **D-/L-C-Try:** *N*-carbamoyl- D-/L-tryptophane; **N-C- β Phe:** *N*-carbamoyl- β -phenylalanine; **DIG-G21:** random primed DNA probe labeled with digoxigenin-dUTP, DNA derived from hydantoinase amplification of genomic DNA from *Ochrobactrum* sp. G21; **DIG-I24:** see DIG-G21, genomic DNA from *Delftia* sp. I24; **DU:** 5,6-dihydrouracil; **GM:** growth medium; **Hyd:** hydantoin; **IMH:** D,L-5-(3-indolylmethyl) hydantoin; **SA:** South Africa; ***t*-BH:** D,L-5-*tert*-butylhydantoin; **PheDU:** D,L-6-phenyl-5,6-dihydrouracil.

Chemicals

All chemicals were obtained from commercial sources and were of reagent grade except for hydantoins, dihydropyrimidines and their derivatives. They were kindly supplied by Degussa AG, Germany. If not otherwise stated enzymes, chemicals and kits for genetical experiments were obtained from Fermentas, Germany. PheDu and N-C- β Phe were synthesised using an optimised method (modified following instructions of Dakin and Dudley, 1914): **N-C- β Phe:** 200 mg 3-amino-3-phenylpropionic acid, 120 mg KNCO and 10 mL water were poured in a 25 mL round-bottomed flask and allowed to react at 80°C with a condenser connected to the flask. As the reaction proceeded the initial white suspension became a clear solution. After one hour the flask was cooled down to room temperature and the pH was adjusted to about 3 with 4N HCl. The precipitate was left 30 min to settle down. Then it was filtered through a Buchner funnel, washed with 5 mL of water and dried. **PheDu:** 150 mg N-C-Phe were poured in a 25 mL round-bottomed flask, 4 mL of water were added and 4 mL of HCl 32% were slowly dropped into the milky mixture. The reaction was carried out for approximately 2 hours at 80°C with a condenser connected to the flask. The flask was cooled to 4°C; after 30 min the precipitate was collected by filtration, washed with 5 mL of cold water and dried. Both reactions were followed by HPLC.

Bacterial Strains

Isolation and characterisation of the bacterial isolates used was described elsewhere (Dürr *et al.*, 2006).

Media and General Growth Conditions

The media used in this study were: **CA**, used for the starter cultures and growth medium (Dürr *et al.*, 2006); **GM** according to Sylđatk *et al.* (1990), used as growth medium for biotransformation assays either with 0.2–1 g/L IMH or CH3-IMH as inducer. Bacterial strains were routinely maintained at CA-plates at 30°C and stored at 4°C (Dürr *et al.*, 2006). For long term storage bacterial isolates were kept on 20% glycerol at –80°C.

Cell Growth and Biotransformations

Cell growth and biotransformations were performed as described elsewhere (Dürr *et al.*, 2006) but with the following modifications: 200 mM K-phosphate buffer (pH 8.0) was used instead of 100 mM; the total volume of the biotransformation reaction was 1 mL and the substrates were dissolved by heating if necessary (40–50°C). Biotransformations were stopped by addition of 100 μ L of 50% H₃PO₄. The final concentrations of the substrates were as follows: 25 mM DU, 20 mM Hyd, 8 mM t-BH, 5 mM BnH, 2 mM IMH, 4 mM PheDU.

Determination of Dry Weight

A volume of 0.5 mL–1 mL of the cell suspension used for biotransformation assays was centrifuged (10 min, 13,000 rpm), the supernatant discarded and the pellet dried over night at 100°C. The biomass dry weight was determined by weighing of the dried pellet. The average of three measurements was used for the determination of the dry weight.

Analysis

HPLC

HPLC-analysis (High Performance Liquid Chromatography) was the general detection and quantification method for hydantoins and their corresponding derivatives except for DU and Hyd (see below). HPLC-analysis was routinely carried out on an Agilent 1100 series HPLC System (Agilent Technologies, Germany). In most cases substrates and products were first analysed using non-chiral separation. For selected samples chiral analysis of the carbamoyl amino acid followed. HPLC analysis was conducted as follows:

1a) Non-chiral analysis of D,L-5-*tert*-butylhydantoin (*t*-BH) and N-carbamoyl-D/L-*tert*-leucin (C-*t*-Leu)

column	Thermo Hypersil-Keystone BDS C18 (250 x 4.6 mm, 5 μ m)
mobile phase	20% MeOH/ 80% (0.1% H ₃ PO ₄)
flow rate	1 mL/min
detection wavelength	210 nm
retention times	<i>t</i> -BH: 15.7 min C- <i>t</i> -Leu: 13.5 min

1b) Chiral analysis of N-carbamoyl-D/L-*tert*-leucin (D- or L-C-*t*-Leu)

column	Macherey-Nagel, Nucleodex β -PM (150 x 4 mm, 5 μ m)
mobile phase	5% MeOH/ 95% (0.1% H ₃ PO ₄), pH 3.0
flow rate	0.5 mL/min
detection wavelength	200 nm
retention times	L-C- <i>t</i> -Leu: 9.2 min D-C- <i>t</i> -Leu: 10.2 min

2a) Non-chiral analysis of D,L-5-benzylhydantoin (BnH) and N-carbamoyl-D/L-phenylalanin (C-Phe)

column	Thermo Hypersil-Keystone BDS C18 (250 x 4.6 mm, 5 μ m)
mobile phase	20% MeOH/ 80% (0.1% H ₃ PO ₄)
flow rate	1 mL/min
detection wavelength	210 nm
retention times	BnH: 22.8 min C-Phe: 25.5 min

2b) Chiral analysis of N-carbamoyl-D/L-phenylalanin (D- or L-C-Phe)

column	Macherey-Nagel, Nucleodex β -PM (150 x 4 mm, 5 μ m)
mobile phase	10% MeOH/ 90% (0.1% H ₃ PO ₄), pH 3.0
flow rate	0.5 mL/min
detection wavelength	210 nm
retention times	L-C-Phe: 18.0 min D-C-Phe: 14.6 min

3a) Non-chiral analysis of D,L-5-(3-indolylmethyl) hydantoin (IMH) and N-carbamoyl-D-/L-tryptophan (C-Try)

column	Thermo Hypersil-Keystone BDS C18 (250 x 4.6 mm, 5 μ m)
mobile phase	20% MeOH/ 80% (0.1% H ₃ PO ₄)
flow rate	1 mL/min
detection wavelength	210 nm
retention times	IMH: 37.0 min C-Try: 29.0 min

3b) Chiral analysis of N-carbamoyl-D-/L-tryptophan (D- or L-C-Try)

column	Chiral Technology Europe, Chiralpak QN-AX (150 x 4 mm, 5 μ m)
mobile phase	50% MeOH/ 50% (0.2M AcOH); pH 6.0 (25% NH ₃)
flow rate	0.8 mL/min
detection wavelength	280 nm
retention times	D-C-Try: 32.8 min L-C-Try: 52.9 min

4) Non-chiral analysis of D,L-6-phenyl-5,6-dihydrouacil (PheDU) and N-carbamoyl- β -phenylalanine (C- β Phe)

column	Thermo Hypersil-Keystone BDS C18 (250 x 4.6 mm, 5 μ m)
mobile phase	20% MeOH/ 80% (0.1% H ₃ PO ₄)
flow rate	1 mL/min
detection wavelength	210 nm
retention times	PheDU: 19.0 min C- β Phe: 16.0 min

Ehrlich Test

The Ehrlich Test (described by Dürr *et al.*, 2006) was applied for detection and quantitative determination of C-Ala and C-Gly, the products of DU- and Hyd-biotransformations, respectively.

DNA Manipulation Methods

General recombinant DNA techniques were carried out by standard procedures (Sambrock *et al.*, 1989). Chromosomal bacterial DNA was extracted using the Qiagen DNeasy Tissue kit (Qiagen, Germany) following the manufacturer's protocol. DNA fragments were purified from standard agarose gels by a DNA extraction kit. Pure plasmids were obtained by a GeneJETTM Plasmid Mini Prep Kit. Sequencing was done commercially (JenaGen GmbH, Germany) and DNA-analysis was carried out using BlastN (Altschul *et al.*, 1997) and ClustalW (Chenna *et al.*, 2003). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar *et al.*, 2004).

Amplification of a Hydantoinase DNA-Fragment

Polymerase chain reaction (PCR) was applied for the amplification of a hydantoinase gene fragment using genomic bacterial DNA as template. Amplification was carried out on one of the following thermocyclers: Eppendorf Mastercycler gradient (Germany) or MJ Research PTC-200 (USA). The conditions were: 10–100 ng template DNA, 1x-Taq buffer with (NH₄)₂SO₄, 1.5 U Taq DNA Polymerase, 50 pmol upstream primer and downstream primer each, 0.2 mM dNTP's, and PCR-water, combined to a volume of 20 μL. The following degenerate oligonucleotide primers were used synthesised by MWG-Biotech, Germany:

dhpf: 5'-GCSGCVTTYGGNGGNACNAC-3',

dhpr: 5'-TCNCCRTTYTCNGCRTGNAC-3' (Lin *et al.*, 2005) or

Hyd-f: 5'-GCCGCAGCATGCGGNGGNACNAC-3',

Hyd-r: 5'-GAYGCNGAYHTAGTCATATGGGACCCTAATGGTG-3' (provided by Dr. A. Puñal, University of Karlsruhe).

PCR-amplification was initiated with DNA denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 40–60°C for 2 min and primer extension at 72°C for 1 min. The cycles were finalised by 7 min extension at 72°C. After amplification PCR-products of 320–330 bp or approximately 950 bp were purified. Purified PCR-products were either sequenced directly or cloned.

Cloning of PCR-Products

To obtain an adequate amount of DNA for sequencing the purified PCR-products were cloned using the Strata CloneTM PCR Cloning kit (Stratagene, USA). The inserts of the purified plasmids were sequenced.

Dot Blot Analysis

Dot blot analysis was carried out using the DIG DNA Labeling and Detection Kit (Roche, Germany) using standard procedures. A volume of 1 μ L of pure genomic DNA was used for dot blot analysis. The probes used for DIG-labelling and detection were DIG-I24 and DIG-G21 (see Chapter 4).

Results and Discussion

The discovery of various microorganisms with hydantoinase activity from different geographical origins was described previously (Dürr *et al.*, 2006). Several bacterial isolates from this screening program were selected for further biochemical and genetical characterisation. The selection criteria were:

- (i) high activity of the hydantoinase of a strain towards the substrates BnH and DU;
- (ii) isolation of the strain from an extreme or unique environmental habitat like hot springs or biofilms;
- (iii) no report in literature on hydantoinase activity of the chosen bacterial genus.

Substrate Spectrum of Hydantoinases

Different hydantoins and dihydropyrimidines were used as a substrate to determine the substrate spectrum of the hydantoinases from the chosen bacterial strains. The chemical structures of the six substrates tested are shown in Figure 1.

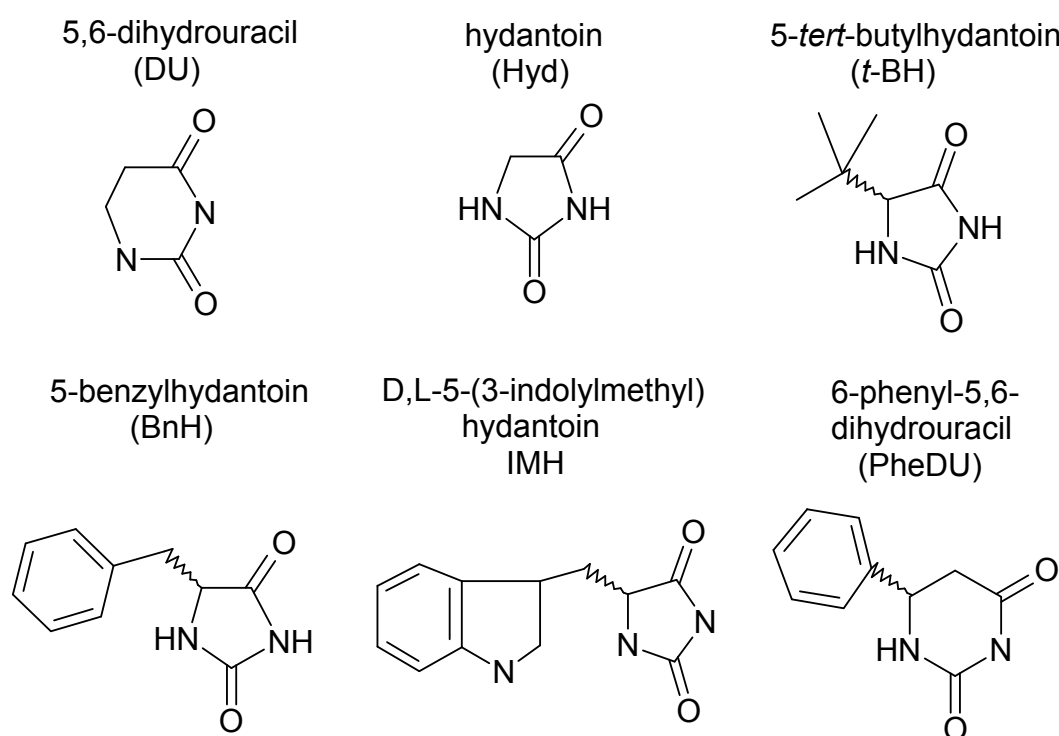


Figure 1: Chemical structure of hydantoins and dihydropyrimidines used as substrates in this study.

Looking at the wild-type strains isolated from different geographical regions (South Africa, China and Antarctica) it is noticeable that nearly all strains are able to cleave the following substrates (Table 1): the non-substituted hydantoin Hyd (with the exception of *Microbacteriaceae* sp. K3) and the dihydropyrimidine derivative DU, as well as BnH being a compound bearing an aryl-side chain. The hydantoinase activities were higher for the non-substituted

substrate DU than for Hyd for almost all strains tested for a similar substrate concentration. The opposite result is obtained for *Bacillus* sp. A16 and F18. The preference towards DU for most of the bacteria tested in this study leads to the assumption that the enzymes being responsible for hydantoin and dihydropyrimidine conversion can be considered as dihydropyrimidinases and are part of the reductive pathway of pyrimidine degradation. Syldatk *et al.* (1999) demonstrated that D-hydantoinases are not necessarily identical to dihydropyrimidinases and the term "hydantoinase" should be used for enzymes being able to hydrolyse hydantoin or 5-monosubstituted hydantoins. Consequently, enzymes being responsible for hydantoin and dihydropyrimidine conversion are named as hydantoinases in this study.

Table 1: Substrate spectrum of hydantoinases in whole-cell biotransformations. The specific hydantoinase activity for each substrate is shown as the concentration of the formed carbamoyl amino acid in relation to the biomass used and time [mM/(h*g)]. Different substrate concentrations were used because of different aqueous solubility of hydantoin and dihydropyrimidines. If determined the configuration of the formed product enantiomer is given in brackets. (* growth in CA medium supplemented with 0.2 g/L CH3-IMH; SA: South Africa)

Isolate	substrate		DU (25mM)	Hyd (20mM)	t-BH (8mM)	BnH (5mM)	IMH (2mM)	PheDU (4mM)
	origin	product						
<i>Delftia</i> sp. I24	China, hot springs		54.54	133.45*	2.80* (D)	0.97 (D)	5.37*	0
<i>Microbacteriaceae</i> sp. K3	SA pinewood forest		60.02	0	0	0.43 (D,L)	0	0.90
<i>Burkholderia</i> sp. M3	pinewood forest SA		57.83	10.76	0.33 (D)	0.13 (L)	0.35	0.10
<i>Flavobacterium</i> sp. F8	SA, compost		68.04	11.27	0.33 (D)	1.58 (D)	0.56	0.62
<i>Streptomyces</i> sp. I20	Mongolia, saline lake		90.55	5.06	0	0.32 (D)	0	0
<i>Bacillus</i> sp. A16	SA, guano of a pond		30.57	45.6	5.69 (D)	0.76 (D)	2.70 (D)	1.49
<i>Bacillus</i> sp. F18	Mongolia, saline lake		61.77	85.91	2.53 (D)	0.72 (D)	1.47	0.30
<i>Bacillus</i> sp. G18	Swaziland, red soil		0.81	96.48	0.12	4.26 (D)	0.54 (D)	1.70
<i>Bacillus</i> sp. H20	SA forest soil		37.48	2.22	0	-	0	0
<i>Pseudomonas</i> sp. G7	Antarctica		51.48	4.63	0.85 (D)	0.93 (D)	0	0.33
<i>Pseudomonas</i> sp. M18	SA, forest soil		40.18	50.83	0.72	1.69 (D)	2.1 (D)	0.05
<i>Pseudomonas</i> sp. L9	SA, mountain soil		11.23	4.73	0	0	0	0
<i>Ochrobactrum</i> sp. C15	SA, compost		69.48	7.48	0	3.11 (D)	0.44	0.51
<i>Ochrobactrum</i> sp. G21	Mongolia, saline lake		63.91	34.99	0	2.33 (D)	0.48 (D)	0.82
<i>Ochrobactrum</i> sp. D24	SA, biofilm		41.93	58.99	0.13	1.21 (D)	0.44 (D)	0
<i>Ochrobactrum</i> sp. F21	SA, algal material		37.71	38.43	0.18	3.12 (D)	0.23 (D)	0.30
<i>Ochrobactrum</i> sp. I21	SA, compost		21.85	26.95	0.42	2.18 (D)	0.38 (D)	0.40
<i>Arthrobacter</i> sp. E7	Antarctica		24.25	1.44	0	-	0	0.51
<i>Arthrobacter</i> sp. K20	Lesotho, mountain soil		18.74	1.31	1.05 (D)	0.09	0.238	
<i>A. polychromogenes</i> DSM20136	DSMZ		32.77	0.68	0	0	0	4.86
<i>A. polychromogenes</i> DSM342	DSMZ		38.29	0.30	0	0	0	5.73

Isolate	origin	substrate		DU (25mM) C-Ala	Hyd (20mM) C-Gly	<i>t</i> -BH (8mM) C- <i>t</i> -Leu	BnH (5mM) C-Phe	IMH (2mM) C-Try	PheDU (4mM) C-βPhe
		product	product						
<i>A. aureescens</i> DSM20116	DSMZ			50.75	1.40	0	0	0	0
<i>A. nicotinovorans</i> DSM420	DSMZ			0.52	2.86	1.04 (D)	0.15	0	2.46
<i>A. citreus</i> DSM20133	DSMZ			24.96	2.69	0	0.17	0	1.35
<i>A. sulfureus</i> DSM20167	DSMZ			20.76	96.62	0	0	0	0

Comparing the conversion of the substituted hydantoin BnH and the substituted dihydropyrimidine PheDu (both bearing an aryl-side chain and consisting either of a 5- or 6-membered ring, Fig. 1) with DU and Hyd the opposite behaviour could be observed for most of the strains tested: BnH was converted by more strains and with a higher hydantoinase activity compared to PheDU conversion. This shows that substrate binding and turnover are not only dependent on interactions between enzyme and substrate ring, but also on interactions between enzyme and substrate side chain (additionally see Chapter IV). The substrates *t*-BH and IMH in comparison to the substrates mentioned above were cleaved by less bacteria.

In the case of IMH the latter result attracts attention: All strains should be able to convert the 5-monosubstituted hydantoin IMH since they were isolated on media containing IMH as C- and N-source. It has to be mentioned that fructose and ammonia were as well present in the screening media (see Dürr *et al.*, 2006) but nevertheless strains possessing an active hydantoinase were screened.

A second observation is that almost all bacteria screened are D-specific documented by the formation of either D-C-*t*-Leu, D-C-Phe and/ or D-C-Try (Table 1). L-specific hydantoinases would be expected since other microorganisms were isolated on IMH like *Flavobacterium* sp. I-3 (Nishida *et al.*, 1987), *Flavobacterium* sp. AJ-3912 (Sano *et al.*, 1977; Yokozeki *et al.*, 1987a, b) and *Arthrobacter aurescens* 3747 (Gross *et al.*, 1987) and were reported to form L-tryptophan from IMH. However, stereoselectivity can vary depending on the substrate: The L-hydantoinase from *A. aurescens* DSM3745 showed to be L-selective for D,L-BnH whilst conversion of D,L-methylthioethylhydantoin was D-specific (May, 1998). Similar results were obtained for *Flavobacterium* sp. AJ-3912. D-specificity was only obtained for benzyloxymethylhydantoin whereas the other substrates tested were cleaved L-selectively (Yokozeki *et al.*, 1987b). In this study a change of selectivity for different substrates was obtained for *Burkholderia* sp. M3 (Table 1).

The enzymes involved in hydantoin degradation of *Arthrobacter aurescens* DSM3747, *Arthrobacter aurescens* DSM3745 and *Arthrobacter crystallopoietes* DSM20117 are well described (e.g. May, 1998; Waniek, 2000; Werner, 2001 and Wiese, 2000). The question arises as to whether more members of the genus *Arthrobacter* possess hydantoinase activity? Since only three *Arthrobacter* species were isolated in the previously reported screening program (Dürr *et al.*, 2006) more *Arthrobacter* species were included for hydantoinase screening. Therefore various *Arthrobacter* strains were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and were tested for the ability to cleave various hydantoins and dihydropyrimidines (Table 1). The ability to cleave BnH was formerly reported for *A. citreus* DSM20133/ ATCC11624 (LaPointe *et al.*, 1995) which could be confirmed within this study (Table 1).

It is remarkable that almost all *Arthrobacter* strains from the DSMZ with hydantoinase

activity preferred DU and PheDu as substrates and were able to cleave non-substituted hydantoin and in some cases 5-monosubstituted hydantoin derivatives with very low activity. This indicates that the enzymes from these *Arthrobacter* species can be considered as dihydropyrimidinases and are quite different from the known hydantoin hydrolysing enzymes like *Arthrobacter aurescens* DSM3747, DSM3745 and *Arthrobacter crystallopoietes* DSM20117 since these enzymes were able to cleave various 5-monosubstituted hydantoins. None of the following *Arthrobacter* strains obtained from DSMZ showed conversion of the hydantoins and dihydropyrimidines tested: *A. agilis* DSM20550, *A. atrocyaneus* DSM20127, *A. chlorophenicus* DSM12829, *A. ilicis* DSM20138, *A. methylotrophus* DSM14008, *A. nasiphocae* DSM13988, *A. nicotianae* DSM20123, *A. oxydans* DSM20119, *A. pascens* DSM20545, *A. protomorphiae* DSM20644, *A. psychrolactophilus* DSM15612, *A. roseus* DSM14508, *A. sulvonivorans* DSM14002, *A. uratoxydans* DSM20647 and *A. ureafaciens* DSM20126. No conversion of hydantoins/ dihydropyrimidines by these strains could be observed because either they all do not possess a hydantoinase and/or a carbamoylase, or hydantoinase/ carbamoylase genes are present but they were not induced with the appropriate inducer. For all biotransformation assays IMH was added to the growth medium as inducer. Various inducers were reported in literature for hydantoin-hydrolysing activity in bacteria for example: uracil for *Agrobacterium* sp. IP-671 (Runser *et al.*, 1990; Runser and Meyer, 1993), D,L-5-(3-indolylmethyl) methylhydantoin and D,L-5-(3-indolylmethyl)-3-N-hydantoin for *Arthrobacter aurescens* DSM3747 and *Arthrobacter aurescens* DSM3745 (Syldatk *et al.*, 1990; Syldatk and Pietsch, 1995), hydantoin and isopropylhydantoin for *Pseudomonas fluorescens* DSM84 (Morin *et al.*, 1986), hydantoin for *Pseudomonas* sp. RUKM1 and hydantoin, dihydro-uracil and thiouracil for *Pseudomonas* sp. RUKM₃ (Burton *et al.*, 1998). Nevertheless, no inducer was needed for hydantoin-hydrolysing activity in *Agrobacterium radiobacter* NRRLB11291 (Oliveri *et al.*, 1981; Deepa *et al.*, 1993). In addition, the activity of the hydantoinase of a halophilic *Pseudomonas* sp. NCIM5109 was not increased by the addition of different hydantoins or dihydropyrimidines to the media (Sudge *et al.*, 1998).

The question still remains whether there is a correlation between the origin of the strain and "good" hydantoinase activity represented by activity and a broad substrate spectrum. This question cannot be easily answered since strains with good activity originated from various different environmental habitats (Table 1). For example: *Bacillus* sp. F18 from a saline lake, *Pseudomonas* sp. M18 from forest soil and *Ochrobactrum* sp. D24 from a biofilm. The author reported (Dürr *et al.*, 2006) that the bacteria with hydantoinase activity mentioned above were predominantly recovered from soil or samples with a high organic load. Probably such soil conditions promote "good" hydantoinase activity since many of the respective microorganisms originated from compost soil, guano or wastewater biofilm.

Amplification of Hydantoinase Gene Fragments

Lin *et al.* (2005) reported degenerate primers to amplify a gene fragment encoding for dihydropyrimidinase. In this study the same degenerate primers called dhpf/dhpr were used in order to amplify hydantoinase gene fragments from the genomic DNA of various environmental isolates with and without hydantoinase activity (Table 2).

It was not feasible to amplify all potential hydantoinase genes by this primer pair. Hence, degenerate primers (Hyd-f/Hyd-r) which were designed for the amplification of D-hydantoinase gene fragments were used subsequently (provided by Dr. A. Puñal). The primer pairs tested were not able to amplify a hydantoinase gene fragment from all strains tested. No amplification was obtained for the following environmental isolates: *Microbacteriaceae* sp. K3, *Flavobacterium* sp. F8, *Streptomyces* sp. I20, *Bacillus* sp. F18, *Bacillus* sp. H20, *Pseudomonas* sp. L9, *Arthrobacter* sp. E7 and *Arthrobacter* sp. K20; and for the following *Arthrobacter* strains obtained from the DSMZ: *A. polychromogenes* DSM20136, *A. polychromogenes* DSM342, *A. aurescens* DSM20116, *A. nicotinovorans* DSM420, *A. sulfureus* DSM20167, *A. nasiphocae* DSM13988, *A. chlorophenicus* DSM12829, *A. sulfonivorans* DSM14002, *A. roseus* DSM14508, *A. oxydans* DSM20119, *A. nicotianae* DSM20123, *A. ureafaciens* DSM20126, *A. atrocyaneus* DSM20127, *A. pascens* DSM20545, *A. agilis* DSM20550, *A. protophormiae* DSM20644 and *A. uratoxydans* DSM20647.

The primer pair dhpf/dhpr was primarily designed for the amplification of the dihydropyrimidinase of *Bacillus* sp. TS-23 (Lin *et al.*, 2005). In this study only one hydantoinase gene fragment of a member of the genus *Bacillus*, namely *Bacillus* sp. G18, could be amplified. The amplified gene fragment showed 80% homology to the hydantoinase of *Bacillus* sp. KNK245 (Table 2). No hydantoinase gene of any other *Bacillus* could be amplified. Similar results were obtained for the genus *Pseudomonas*. A gene fragment of the only one hydantoinase, namely from *Pseudomonas* sp. M18, was amplified. More successful was the amplification of several hydantoinase gene fragments from *Ochrobactrum* species. Four hydantoinase gene sequences were obtained using the primer dhpf/dhpr and one with Hyd-f/Hyd-r. They all showed highest homology to a putative hydantoinase from *Brucella suis* 1330. A partial hydantoinase homolog gene sequence could be obtained for *Delftia* sp. I24 and *Burkholderia* sp. M3. It is notable that in literature neither gene nor protein sequences involved in hydantoin degradation have been reported for the genus *Delftia* or *Ochrobactrum*.

Table 2: Amplification of hydantoinase gene fragments and Dot-Blot analysis (Labelling see next page).

isolate	PCR- primer pair	identity %/ bp (bp aligned)	highest match: organism / enzyme	accession number	DIG -I24	DIG -G21
<i>Delftia</i> sp. I24	dhpf/dhpr ¹	82%/ 485 (1140)	<i>Pseudomonas putida</i> / D-Hyd	L24157	+	-
<i>Burkholderia</i> sp. M3	Hyd-f/Hyd-r	88%/ 914 (957)	<i>Burkholderia xenovorans</i> LB400/ put DHP	CP000270.1	-	-
<i>Bacillus</i> sp. G18	dhpf/dhpr	80%/ 248 (323)	<i>Bacillus</i> sp. KNK245/ Hyd	AB222979	-	-
<i>Pseudomonas</i> sp. M18	dhpf/dhpr	83%/ 284 (320)	<i>Pseudomonas putida</i> / D-Hyd	U84197	-	-
<i>P. putida</i> RU-KM1	-	-	-	-	+	+
<i>Ochrobactrum</i> sp. C15	dhpf/dhpr	86%/ 263 (317)	<i>Brucella suis</i> 1330/ put D-Hyd	AE014291.4	-	+
<i>Ochrobactrum</i> sp. G21	dhpf/dhpr ¹	86%/ 1347 (1455)	<i>Brucella suis</i> 1330/ put D-Hyd	AE014291.4	-	+
<i>Ochrobactrum</i> sp. D24	dhpf/dhpr	93%/ 293 (317)	<i>Brucella suis</i> 1330/ put D-Hyd	AE014291.4	+	+
<i>Ochrobactrum</i> sp. F21	dhpf/dhpr	92%/ 293 (318)	<i>Brucella suis</i> 1330/ put D-hyd	AE014291.4	+	+
<i>Ochrobactrum</i> sp. I21	Hyd-f/Hyd-r	90%/ 884 (958)	<i>Brucella suis</i> 1330/ put D-hyd	AE014291.4	-	+
<i>A. aureus</i> DSM3745	-	88%/ 409 (958)	<i>Pseudomonas</i> sp. KNK003A/ Hyd	AB232933	-	+
<i>A. crystallopoietes</i> DSM20117	dhpf/dhpr	-	-	-	-	-
<i>A. citreus</i> DSM20133	Hyd-f/Hyd-r	84%/ 269 (967) ²	<i>Frankia alni</i> / put D-Hyd, DHP	-	-	+
<i>A. ilicis</i> DSM20138	-	-	-	-	-	+
<i>A. psychrolactophilus</i> DSM15612	-	-	-	-	-	+
<i>A. methylotrophus</i> DSM14008	-	-	-	-	-	+

Additional description of Table 2: Each hydantoinase gene fragment is shown in relation to its organism, to the primer pair used for PCR and the size of the obtained gene fragment (bp aligned). Further, consistency of the hydantoinase gene fragment is given as the highest match obtained by comparison to enzymes from the database. The last two rows show the detection of a possible hydantoinase using two DIG-labelled probes derived from the putative hydantoinase gene of *Delftia* sp. I24 (**DIG-I24**) and *Ochrobactrum* sp. G21 (**DIG-G21**), respectively (for comparison see Chapter IV). ⁽¹⁾ Sequences obtained as described in Chapter IV; ⁽²⁾ Partial deduced amino acid sequence (322 amino acids) of the putative hydantoinase of *A. citreus* DSM20133 showed 47% sequence similarity to the hydantoinase of *Bacillus* sp. TS-23 by 321 amino acids aligned and 44% sequence similarity to the D-hydantoinase of *A. crystallopoietes* DSM20117.

Unexpected results were obtained for strains from the genus *Arthrobacter*. No amplification was obtained for the L-hydantoinase of *A. aurescens* DSM3745 with all primer pairs whereas the D-hydantoinase of *A. crystallopoietes* DSM20117 could be amplified by dhpf/dhpr. A gene fragment was obtained with the primer pair Hyd-f/Hyd-r for *A. citreus* DSM20133 with highest sequence similarity to a putative hydantoinase of *Frankia alni* with 84% identity at 269 bp of a total of 967 bp aligned (Table 2). Nevertheless, the deduced amino acid sequence of this gene fragment revealed 47% identity to the hydantoinase of *Bacillus* sp. TS-23 and 44% identity to the D-hydantoinase of *A. crystallopoietes* DSM20117. This indicates that the amplified DNA-fragment encodes for a hydantoinase of *A. citreus* DSM20133.

It is remarkable that not all hydantoinases could be amplified by using the primer pair dhpf/dhpr. This primer pair was designed based on the amino acid motifs AAAFGG and VHAENG obtained by the alignment of the amino acid sequences of dihydropyrimidinases/ hydantoinases from *Bacillus stearothermophilus* NS1122A, *Burkholderia pickettii*, *Pseudomonas putida*, *Agrobacterium tumefaciens* and *Streptomyces coelicolor* (Lin *et al.*, 2005). Comparing the amino acid sequences of other hydantoinases and dihydropyrimidinases at the positions mentioned above we found that the amino acids were not identical (data not shown, amino acid sequences used are from microorganisms shown in Fig. 3, Chapter IV). Moreover, comparing the nucleotides sequence of a greater number of hydantoinases/ dihydropyrimidinases and the corresponding gene sequence of the degenerate primer at the appropriate position the sequences are nearly identical for a small majority of sequences but not for all sequences. This explains why not all hydantoinases used in this study could be amplified. The major problem in hydantoinase primer design is the low sequence homology of hydantoinases and the lack of conserved regions not allowing successful design of degenerate primer.

Phylogenetic Relation of D-Hydantoinases

In literature the evolutionary relationship of hydantoinases and related enzymes was investigated based on amino acid sequence and structure homology data. Hence, the L-hydantoinase of *A. aurescens* DSM3745, dihydropyrimidinase, allantoinase, dihydroorotase, urease and others such as adenine deaminase, adenosine aminoacylase, AMP deaminase, arylphosphatase, cytosine deaminase, chlorohydrolase, formylmethyldehydrogenase, imidazolonepropionase and phosphotriesterase are shown to belong to a superfamily of cyclic amidases (for review see Sylđatk *et al.*, 1999). All these enzymes share a low sequence similarity. They have evolved from a common ancestor by divergent evolution and are members of a very old protein family. This is in agreement with the speculation that in the primitive hydrosphere the formation of hydantoins and N-carbamoyl amino acids was higher than that of α -amino acids (Talliades *et al.*, 1998). Thus, first primitive microorganisms on earth had to develop an enzyme system to use hydantoins and N-carbamoyl amino acids as C- and/ or N-source.

To our knowledge no study has focussed on the comparison of a larger number of hydantoinases and dihydropyrimidinases and on the phylogenetic and evolutionary relation of these enzymes. Therefore, a phylogenetic evolutionary analysis was performed (Fig. 2) in this study using hydantoinase DNA sequences obtained beforehand (Table 2), described hydantoinase sequences and putative hydantoinase sequences. For the analysis the distance based method Minimum Evolution was used since it provides a fast analysis and a model for sequence evolution (Knoop and Müller, 2006). In total, 36 gene fragments were aligned and used for phylogenetic analysis with the aim to classify the gene sequences obtained in this study (Fig. 2).

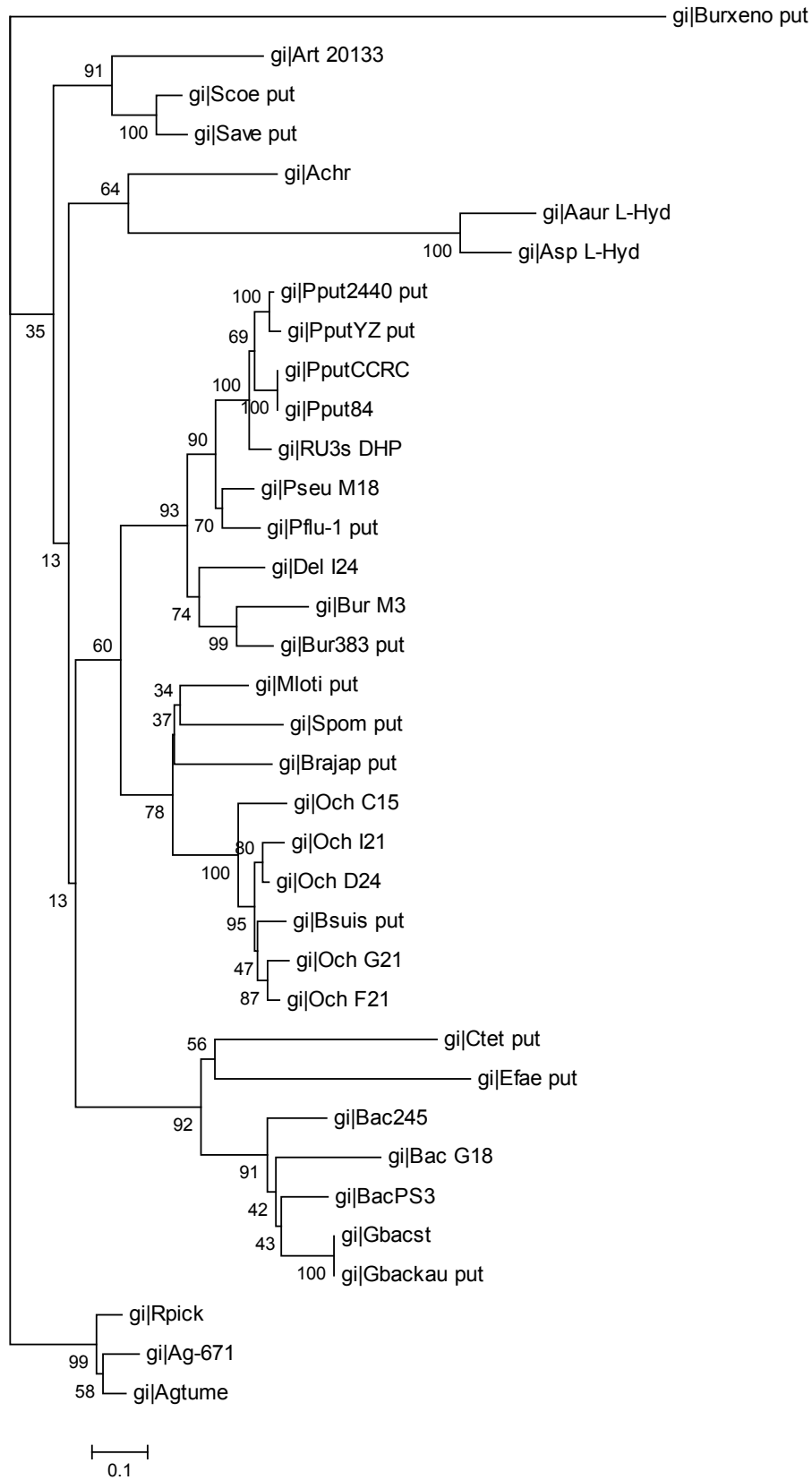


Figure 2: Phylogenetic tree of partial gene fragments of hydantoinases and dihydropyrimidinases

Additional description of Figure 2: Phylogenetic tree of partial gene fragments of hydantoinases and dihydropyrimidinases (called nucleotide phylogenetic tree). The gene sequences used were amplified from wild type strains (Table 2) or were from hydantoinases obtained from databases. Since most of the obtained gene sequences had a different length a gene fragment for each hydantoinase was determined spanning the region between the primer pair dhpf/dhpr, primers included. The tree was constructed using MEGA with the minimum evolution method (Kumar *et al.*, 2004). Bootstrap values represent 1000 replicates. (**put**: putative enzyme; **Ac**: accession numbers for the nucleotide sequence; the size of the obtained hydantoinase gene fragment is shown in brackets)

The hydantoinases sequences used were: **Art 20133**: *A. citreus* DSM20133 (320 bp); **Bac G18**: *Bacillus* sp. G18 (323 bp); **Bur M3**: *Burkholderia* sp. M3 (321 bp); **Del I24**: *Delftia* sp. I24 (320 bp); **Och G21**: *Ochrobactrum* sp. G21 (317 bp); **Och D24**: *Ochrobactrum* sp. D24 (318 bp); **Och F21**: *Ochrobactrum* sp. F21 (318 bp); **Och I21**: *Ochrobactrum* sp. I21 (320 bp); **Och C15**: *Ochrobactrum* sp. C15 (317 bp); **Pseu M18**: *Pseudomonas* sp. M18 (320 bp); **Asp L-Hyd**: L-Hyd from *Arthrobacter* sp. BT801, Ac: AY069990 (320 bp); **Achr**: D-Hyd from *Arthrobacter crystallopoietes* DSM20117, Ac: AY185303 (318 bp); **Aaur L-Hyd**: L-Hyd from *Arthrobacter aurescens* DSM3747, Ac: AF146701 (320 bp); **Ag-671**: D-Hyd from *Agrobacterium* sp. IP I-671, Ac: AF335479 (320 bp); **Agtume**: D-Hyd from *Agrobacterium tumefaciens*, Ac: X91070 (320 bp); **Bac245**: Hyd from *Bacillus* sp. KNK245, Ac: AB222979 (323 bp); **BacPS3**: D-Hyd from *Bacillus* sp. PS3, Ac: E01160 (323 bp); **Brajap put**: putative DHP from *Bradyrhizobium japonicum* USDA110; Ac: NC004463 (323 bp); **Bsuis put**: putative Hyd from *Brucella suis* 1330, Ac: NP697312 (317 bp); **Burxeno put**: put DHP from *Burkholderia xenovorans* LB400, Ac: CP000270 (323 bp); **Bur383 put**: putative Hyd from *Burkholderia* sp. 383, Ac : CP000150 (320 bp); **Ctet put**: putative DHP from *Clostridium tetani* E88, Ac: NP782372 (317 bp); **Efae put**: putative Hyd from *Enterococcus faecalis* V583, Ac: AE016830 (317 bp); **Gbacst**: Hyd from *Geobacillus stearothermophilus* NS1122A, Ac: S73773 (323 bp); **Gbackau put**: putative Hyd from *Geobacillus kaustophilus* HTA426, Ac: BA000043 (323 bp); **Mloti put**: put DHP from *Mesorhizobium loti* MAFF303099, Ac : NC 002678 (320 bp); **PputCCRC**: D-Hyd from *Pseudomonas putida* isolate CCRC 12857, Ac: U84197 (320 bp); **Pput84**: D-Hyd from *Pseudomonas putida* DSM84, Ac: L24157 (322 bp); **Pput2440 put**: putative Hyd from *Pseudomonas putida* KT2440, Ac: NC 002947 (320 bp); **PputYZ put**: putative D-Hyd from *Pseudomonas putida* strain YZ-II6, Ac: AY387829 (320 bp); **Pflu-1 put**: put Hyd from *Pseudomonas fluorescens* PfO-1, Ac: CP000094 (320 bp); **Rpick**: D-Hyd from *Ralstonia/ Burkholderia pickettii*, Ac: AF320814 (320 bp); **RU3s L-DHP**: L-DHP from *Pseudomonas putida* RU-KM3s, (sequence not in database, personal communication) (320 bp), **Save put**: putative Hyd from *Streptomyces avermitilis* MA-4680, Ac: NC 003155 (320 bp); **Scoe put**: putative Hyd from *Streptomyces coelicolor* A3(2),

Ac: AL939127 (320 bp); **Spom put:** putative Hyd from *Silicibacter pomeroyi* DSS-3, Ac: CP000031 (320 bp); **Stok put:** put DHP from *Sulfolobus tokodaii* str. 7, Ac: NC 003106 (311 bp).

In the resulting tree (Fig. 2, in this context called nucleotide phylogenetic tree) it is noteworthy that hydantoinases were in most cases grouped according to their phylogenetic origin. That means that hydantoinases from bacteria belonging either to the same genus or to the same order/ family were grouped together. A genus specific grouping was obtained for hydantoinases from *Bacillus* species and *Pseudomonas* species. Order or family specific grouping was obtained for the *Burkholderiales* *Delftia* sp. I24 and the two *Burkholderias*, *Burkholderia* sp. 383 and *Burkholderia* M3 as well as for the *Brucellaceae* including five *Ochrobactrum*s species and *Brucella suis* 1330. These results were obtained probably due to the problem that each genus showed a specific nucleotide usage which is seen e.g. in different GC contents. The results obtained in the nucleotide phylogenetic tree are supported by a phylogenetic tree of protein sequences of various hydantoinases and dihydropyrimidinases (see Fig. 3, chapter IV, in this context called protein phylogenetic tree). Consequently, applying the mentioned theory of a common ancestor of the protein superfamily of cyclic amidases on hydantoinases the observed behaviour would be expected. Thus it is assumed that all D-hydantoinases evolved from one ancestral hydantoinase/ dihydropyrimidinase by divergent evolution excluding L-hydantoinases. The L-hydantoinases from *Arthrobacter* sp. BT801 and *Arthrobacter aurescens* DSM3747 were shown to be closely related to the D-hydantoinase from *A. crystallopoietes* DSM20117 (Fig. 2). This is in contrast to the results obtained in the protein phylogenetic tree for hydantoinases and dihydropyrimidinases (Fig. 3, chapter IV). The location of the L-hydantoinases in a separate branch of the protein phylogenetic tree is definitely correct since the information used comprises the complete amino acid sequence of the enzymes. This includes all amino acids being involved in the active site and as well in the specific exocyclic recognition of hydantoins. Precisely because this information is not included in the sequences used for the nucleotide phylogenetic tree an incorrect classification of the different *Arthrobacter* hydantoinases may be obtained.

All hydantoinase gene sequences from the wild-type strains obtained in this study can be related to putative and certain hydantoinase/ dihydropyrimidinase gene or protein sequences from closely related bacteria. One exception is the hydantoinase/ dihydropyrimidinase from *Arthrobacter citreus* DSM20133 being related to two putative hydantoinases from *Streptomyces avermilitis* and *S. coelicolor*. We assume that this enzyme is a novel type of *Arthrobacter* hydantoinase due to its substrate spectrum with preference to dihydropyrimidines (Table 1) and its location in the phylogenetic tree (Fig. 2).

Detection of Hydantoinase Genes Using Dot Blot Analysis

Two different DIG-labelled DNA probes were used for the detection of hydantoinase genes. One probe was derived from a 320 bp-hydantoinase gene fragment amplified from the genomic DNA of *Delftia* sp. I24, so called DIG-I24, and one from *Ochrobactrum* sp. G21, so called DIG-G21 (for details see Chapter IV). The probe DIG-I24 showed only a positive hybridisation signal for *P. putida* RU-KM1 and for two *Ochrobactrum* species. *P. putida* RU-KM1 exhibited hydantoinase activity towards hydantoin, methylhydantoin and hydroxyphenylhydantoin (Burton *et al.*, 1998) and was used in this study as a control strain for *Pseudomonads*. The other *Pseudomonas* strains tested such as *Pseudomonas* sp. G7 or M18 showed no positive signal.

More interesting were the hybridisation signals obtained for DIG-G21 which was positive for all *Ochrobactrum* species. This was expected since all hydantoinase gene fragments of this genus are closely related and share high homology as shown in Table 2 and Fig. 2. Further, the L-specific hydantoinase gene of *Arthrobacter aurescens* DSM3745 was detected but not the D-specific hydantoinase gene of *A. crystallopoietes* DSM20117. By using the DIG-labelled probe DIG-G21 the four *Arthrobacter* species *A. citreus* DSM20133, *A. ilicis* DSM20138, *A. psychrolactophilus* DSM15612 and *A. methylotrophus* DSM14008 showed positive hybridisation signals. Except for *A. citreus* DSM20133 none of these four strains showed hydantoinase activity. For repetition, *A. citreus* DSM20133 showed hydantoinase activity (see Table 1) and a positive hydantoinase gene fragment amplification by PCR. This was confirmed by literature. A 120 bp DNA probe derived from *P. putida* DSM84 was specific for *A. citreus* DSM20133 in hybridisation experiments (LaPointe *et al.*, 1995).

Hall *et al.* (1987) defined the term cryptic genes as "phenotypically silent DNA sequences not normally expressed during the life cycle of an individual but capable of activation as a rare event in a few members of a large population by mutation recombination, insertion elements, or other genetic mechanisms". In many cases cryptic genes are silent at experimental conditions and their expression is induced in the natural environment (Tamburine and Mastromei, 2000). Cryptic genes can be classed in two groups. One group encodes for a known function not being expressed and the other is expressed but their function is unknown or the protein product is not active for example because of misfolding (Innes *et al.*, 2001). One example for cryptic genes are nitrile hydratase genes which were considered to be cryptic genes because they were amplified from eight *R. erythropolis* strains but the strains were not able to grow on acetonitrile and benzonitrile (Brandão *et al.*, 2003). Hence, the possible presence of hydantoinase genes for the three strains *A. ilicis* DSM20138, *A. psychrolactophilus* DSM15612 and *A. methylotrophus* DSM14008 is shown by positive hybridisation signals with the DNA-probe DIG-G21 (Table 2). The hydantoinase genes were considered as cryptic genes because no activity for the substrates tested could be obtained (Table 1). We assume that the hydantoinase genes are not expressed due to the absence of

an adequate inducer during growth.

Genomic DNA of strains with hydantoinase activity did not show hybridisation for one of the digoxigenin labelled probes (Table 2). One reason could be that the conditions used for hybridisation were too stringent to allow pairing with distantly related sequences. This is supported by the fact that hybridisation was predominantly found for closely related species as for the *Delftia* sp. I24 probe with *P. putida* RU-KM1 which is a member of the closely related *Pseudomonas* group (see Fig. 1). Another example is the hybridisation of the *Ochrobactrum* sp. G21 probe to *Ochrobactrum* species. In general, lowering of the very high hybridisation temperature (68°C) would tolerate mismatches and would allow the detection of more hydantoinases. However, hybridisation conditions need to be adjusted adequately since not desired sequences can lead to undesired signals. LaPointe *et al.* (1995) showed that the adequate adjustment of hybridisation conditions for a 120 bp DNA-probe from *P. putida* DSM84 was necessary. Since the hybridisation conditions of the digoxigenin probes were very stringent they were only specific to similar hydantoinase gene sequences and did not show hybridisation for all hydantoinase positive strains. To use them as universal probes to detect hydantoinase genes in microorganisms or even in environmental samples the hybridisation conditions need to be optimised.

Conclusion

After some decades of research on hydantoinases, novel enzymes can still be discovered which differ from already known enzymes. These enzymes can be considered as novel when differences in biochemical and genetical characterisation are obtained. For example several bacteria with hydantoinase activity recovered in this study showed a broad substrate spectrum and are phylogenetically distantly related to known enzymes. Examples are *Delftia* sp. I24 and the *Ochrobactrum* isolates. This makes them potential candidates for industrial use e.g. for the production of β -amino acids. The main issue of this chapter was the biodiversity of hydantoin cleaving enzymes resulting in the following conclusions:

1. Hydantoinases obtained in this study showed a broad substrate spectrum. No correlation between hydantoinase properties and a certain bacterial genera and/ or origin of an ecological habitat could be observed.
2. Hydantoinases can be clustered on the DNA-level according to their bacterial lineage.
3. No pattern for the biochemical properties of hydantoinases within a certain cluster (point II) was found. One reason could be that not all nucleotide positions encoding for active site and/ or functional recognition are included in the 320 bp DNA fragment used for clustering.

4. Cryptic hydantoinase genes are assumed for *A. ilicis* DSM20138, *A. psychrolactophilus* DSM15612 and *A. methylotrophus* DSM14008. They can be considered as silent since the hydantoinase is not expressed during growth possibly due to the absence of an appropriate inducer.

One remaining question about hydantoinases is that the physiological function is still unknown. We showed in this chapter that the enzymes used cleave dihydropyrimidines and hydantoins, highlighting that these enzymes probably are involved in pyrimidine degradation. Besides these findings, their diverse biochemical properties as indicated by different substrate specificities show that they also have another unknown metabolic function. Nevertheless, the biochemical experiments conducted have not answered all the questions of this problem.

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7.3 Chapter III: Properties of Selected Hydantoinases

DESCRIPTION AND CHARACTERISATION OF THE D-HYDANTOINASES FROM THREE MICROORGANISMS ISOLATED FROM EXTREME ENVIRONMENTS

submitted to
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Abstract

Three microorganisms with hydantoinase activity were previously isolated from extreme environments (saline lakes and a hot spring). In this study, growth requirements for high hydantoinase activity and the biochemical properties of the hydantoinases are described. Based on 16S rDNA and classical identification methods the bacterial isolates were identified as *Delftia* sp. strain I24, as *Ochrobactrum* sp. strain G21 and as *Bacillus megaterium* strain F18, respectively. This is the first report on a hydantoinase from a bacterium of the genus *Delftia* and on a hydantoinase from a moderately halophilic *Ochrobactrum* sp. and a moderately halophilic *Bacillus megaterium* species. The influence of bacterial growth on hydantoinase activity was investigated. In the case of the two halophilic strains the addition of NaCl turned out to be favourable to achieve an efficient conversion of the model substrate D,L-5-benzylhydantoin. We propose an activation of degradative enzymes and also the hydantoinase system by salt stress. The hydantoinase of *Ochrobactrum* sp. G21 showed maximal induction by D,L-2-naphthylmethylhydantoin and the enzyme of *Bacillus megaterium* F18 by D,L-*tert*-butylhydantoin. Growth of *Delftia* sp. I24 was improved by replacing glucose with fructose, and the highest enzyme activity levels were found during the end of the exponential growth phase. The same results were obtained for *Bacillus megaterium* F18. Temperature and pH optima of the hydantoinases were 30°C and pH 9.0 for *Delftia* sp. I24, 40°C and pH 8.0–8.5 for *Ochrobactrum* sp. G21 and 45–50°C and pH 7.5–8.0 for *Bacillus megaterium* F18, respectively. All three hydantoinases are found to be cofactor dependent with preference to Ni²⁺ and Co²⁺ for *Delftia* sp. I24, Mn²⁺ and Co²⁺ for *Bacillus megaterium* F18. No preference to a cofactor was obtained for the hydantoinase of *Ochrobactrum* sp. G21. All hydantoinases had a broad substrate spectrum being able to cleave various hydantoins and dihydropyrimidines.

Introduction

The biotechnological production of amino acids and derivatives is of economical interest. In the list of important fermentation products demanded by the global market amino acids follow immediately behind ethanol and antibiotics. Amino acids can be produced by extraction, chemical synthesis, fermentation and enzymatic catalysis. Among others, the following enzyme systems are examples for enzymatic production of amino acids by the chemical industry: (i) Aspartase from *E. coli* and aspartate β -decarboxylase from *Pseudomonas decunhae* for the production of L-aspartate and L-alanine; (ii) Leucine dehydrogenase with cofactor recycling using formate dehydrogenase for the production of L-*tert*-leucine from trimethyl pyruvate. Of considerable interest is the use of the hydantoinase/ carbamoylase system. The targets are D-serine, L-methionine and L-phosphinocitrine; of special significance are D-phenylglycine and p-hydroxy-D-phenylglycine as building blocks for the production of the semisynthetic antibiotics ampicillin and amoxicillin (16).

Hydantoins are converted to the corresponding amino acids in a two step reaction: Hydrolysis of hydantoins via a hydantoinase which forms the corresponding *N*-carbamoyl amino acid, followed by the action of a *N*-carbamoyl amino acid amidohydrolase (so-called carbamoylase), leading to the respective amino acid. Hydantoinases belong to the group of cyclic amidases (E.C. 3.5.2) including dihydropyrimidinases, dihydroorotases and allantoinases (38). Hydantoinase activity has been found for a wide spectrum of microorganisms belonging to, amongst others, the genera *Arthrobacter*, *Pseudomonas*, *Bacillus* and *Flavobacterium* (34, 38).

In the present work we describe the identification of bacteria with hydantoinase activity, the influence of growth parameters on hydantoinase activity and the biochemical characterisation of D-hydantoinase from three different microorganisms isolated from geographically distinct environmental habitats. The isolates are the two moderately halophilic species *Ochrobactrum* sp. G21 and *Bacillus* sp. F18 and a *Delftia* sp. I24 (6).

Hydantoinase activity has been reported for an *Ochrobactrum anthropi* strain (25) isolated from soil. The strain was able to cleave D,L-(2-methylthioethyl)-hydantoin to methionine and was induced by this hydantoin compound. The enzyme had a pH optimum of 9.0 and activity was significantly increased by the addition of Ca^{2+} , Na^{2+} , Cu^{2+} , Co^{2+} , Mg^{2+} , Zn^{2+} and Fe^{3+} .

In literature several *Bacillus* strains with the ability to cleave hydantoins have been reported, most of them possessing thermostable hydantoinases: *Bacillus stearothermophilus* SD-1, *Bacillus* sp. AR9, *Bacillus stearothermophilus* NS1122A (15, 29, 10). No halophilic *Bacillus* strain with hydantoinase activity has been reported. The only halophilic strain with hydantoinase activity reported so far was *Pseudomonas* sp. NCIM5109 isolated from sea water (32). The best medium for growth and enzyme production was nutrient broth with 2% NaCl. The D-hydantoinase was not inducible by addition of different hydantoins, dihydrouracil, uracil or β -alanine to the growth medium. The D-hydantoinase had a broad substrate spectrum and

was able to convert dihydrouracil, hydantoin, allantoin and phenylhydantoin. The optimal pH and temperature were 9.0–9.5 and 30°C, respectively.

Delftia sp. I24, isolated by our group (6), is the only species known of this genus to be able to convert hydantoins. The gram-negative genus *Delftia* belongs to the *Comamonadaceae* lineage in the β -subclass of *Proteobacteria* together with other genera including *Acidovorax*, *Brachymonas*, *Hydrogenophaga*, *Polaromonas*, *Rhodoferax*, *Variovorax*, *Xenophilus* (41). In literature some type strains of *Delftia* are described: *Delftia tsuruhatensis* sp. nov. with the ability to assimilate terephthalate was isolated from activated sludge of a domestic wastewater treatment plant in Japan (30), *Delftia tsuruhatensis* AD 9 isolated from soil surrounding a textile dyeing plant and having the ability of aniline degradation (17) and *Delftia acidovorans* bearing a stereospecific D-amino acid amidase exhibiting high enzyme activity against D-phenylalanine amide, D-tryptophan amide and D-tyrosine amide (9).

Materials and Methods

Abbreviations

AFM: atomic force microscopy; **BnH:** D,L-5-benzylhydantoin; **t-BH:** D,L-tert-butylhydantoin; **CA:** Corynebacterium medium; **CA-plates:** Corynebacterium agar plates; **CH3-IMH:** D,L-5-(indolylmethyl)-3-N-methylhydantoin; **C-Ala:** N-carbamoyl- β -alanine; **C-Gly:** N-carbamoyl-glycine; **D-/L-C-Phe:** N-carbamoyl-D-/L-phenylalanin; **N-C- β Phe:** N-carbamoyl- β -phenylalanine; **DU:** 5,6-dihydrouracil; **GM:** growth medium; **GMF:** growth medium with fructose; **Hyd:** hydantoin; **HMH:** D,L-5-(hydroxymethyl) hydantoin; **IMH:** D,L-5-(3-indolylmethyl) hydantoin; **Methyd:** D,L-5-(2-methylthioethyl) hydantoin; **NMH:** D,L-2-naphthylmethylhydantoin; **PheDU:** D,L-6-phenyl-5,6-dihydrouracil; **PN:** productivity number.

Chemicals

All chemicals used were obtained from commercial sources and were of reagent grade except for hydantoins, dihydropyrimidines and their derivatives. They were kindly supplied by Degussa AG, Germany. PheDu and N-C- β Phe were synthesised using a optimised method (modified after (4)) as follows:

N-C- β Phe: 200 mg 3-amino-3-phenylpropionic acid, 120 mg KNCO and 10 mL water were poured in a 25 ml round-bottomed flask and allowed to react at 80°C with a condenser connected to the flask. As the reaction proceeded the initial white suspension became a clear solution. After one hour the flask was cooled down to room temperature, and the pH was adjusted to about 3 with 4N HCl. The precipitate was left 30 min to settle down. Then it was filtered through a Buchner funnel, washed with 5 ml of water and dried.

PheDu: 150 mg N-C- β Phe were poured in a 25 ml round-bottomed flask, 4 ml of water were added and 4 ml of HCl 32% were slowly dropped into the milky mixture. The reaction was carried out for approximately 2 hours at 80°C with a condenser connected to the flask. The flask was cooled to 4°C, after 30 min the precipitate collected by filtration, washed with 5 ml of cold water and dried. Both reactions were followed by HPLC.

Bacterial Strains

The bacterial isolates used in this study were the two moderately halophilic strains *Ochrobactrum* sp G21, *Bacillus* sp. F18 and a "thermophilic" strain *Delftia* sp. I24. The bacterial strains were deposited at DSMZ (German Collection of Microorganisms and Cell Cultures) under the following accession numbers: *Delftia* sp. I24: DSM18833; *Ochrobactrum* sp G21: DSM18828; *Bacillus* sp. F18: DSM18825. The isolates *Ochrobactrum* sp. G21 and *Bacillus* sp. F18 were isolated from hypersaline lakes, Inner Mongolian Autonomous Region, China and the isolate *Delftia* sp. I24 from an algal mat of a hot spring (60–65°C), Long Pu, Yunnan Province, China (6). Since this strain was obtained at a screening temperature of 50°C it was assumed to be thermophilic. The bacterial strains were routinely maintained

at CA-plates at 30°C (6).

Media

The media used in this study were: **CA** for the starter cultures (6); **GM** according to (35) as growth medium for *Ochrobactrum* sp. G21 and *Bacillus* sp. F18. GM contained: 10 g/L glucose, 6.5 g/L (NH₂)₄SO₂, 0.2 g/L MgSO₄, 0.02 g/L MnCl₁₂·4H₂O, 0.02 g/L FeSO₄, 0.02 g/L CaCl₂·2H₂O, 5.54 g/L KH₂PO₄, 7.6 g/L K₂HPO₄, 0.32 g/L citric acid, 0.2–1 g/L IMH or another hydantoin compound as inductor. The pH of the medium was adjusted to pH 6.8; **GMF**, used as growth medium for *Delftia* sp. I24, contained the same ingredients as GM but 9 g/L fructose instead of glucose.

Phylogenetic Description

16S rDNA amplification, sequence determination and database analysis were conducted as described by (6). Classical bacterial identification was carried out as described by (33).

Cell Growth and Harvesting

An overnight culture of cells in CA-medium was added to 100 mL of growth medium in an Erlenmeyer flask (300 mL). *Delftia* sp. I24 was first cultured in GM, later routinely in GMF, *Ochrobactrum* sp. G21 and *Bacillus* sp. F18 in GM at 30°C and 140 rpm. For growth experiments, GM and GMF were supplemented with different concentrations of NaCl and/or with different hydantoins or dihydropyrimidines (0.2 g/L). Whole cells were harvested by centrifugation (10,000 rpm, 10 min, 4°C; AvantiTM J-30 I, Beckmann Coulter). Resting cells were obtained by washing twice with 0.2 M K-phosphate buffer (pH 8.0) followed by centrifugation and resuspension in the same buffer.

Biotransformation

The substrates DU, Hyd, *t*-BH, BnH, IMH and PheDU were dissolved in 0.2 M potassium-phosphate buffer (pH 8.0) assisted, if necessary, by heating to 40–50°C. For biotransformation assays 500 μL (200 μL) of resting cell suspension were added to 500 μL (800 μL) of the substrate solution (pre-incubated at 40°C) and usually allowed to react over 20 to 24 hours at 40°C and 1,100 rpm (Thermomixer compact, Eppendorf). BnH was used as a model substrate. Toluene (20 μL/mL) was added to the biotransformations in order to enhance cell permeability. The reaction was stopped by addition of 100 μL of 50% H₃PO₄ followed by centrifugation at 13,000 rpm for 7 min. For pH-assays the pH was adjusted using the following buffer solutions: pH 6.0 to 8.0, 0.2 M potassium-phosphate buffer; pH 7.5 to 9.5, 0.2 M Tris-HCl-buffer; pH 10, 0.2 M carbonate buffer. For detection of the temperature optimum the biotransformations were carried out between 20°C and 60°C. For testing the influence of metal-ions 10 mM EDTA and the following compounds (final concentration 1 mM each) were added to the biotransformation solutions in form of their sulphates or

chlorides: Ca^{2+} , Mn^{2+} , Ni^{+} , Zn^{2+} , Na^{2+} , Co^{2+} , Cu^{2+} , Fe^{3+} , Mg^{2+} .

Analysis

The analysis of most of the substrates was performed using High Performance Liquid Chromatography (HPLC) on an Agilent 1100 series instrument (Agilent, Germany). The concentrations of BnH, IMH, PheDU, *t*-BH and their corresponding carbamoyl amino acids were determined using a Hypersil-Keystone BDS C18 column (Thermo, Germany). The mobile phase contained 20% MeOH/ 80% (0.1% H_3PO_4) solution. The flow rate was 1 mL/min. Detection was carried out at 210 nm or 280 nm, respectively. The products of DU- and Hyd-conversion C-Ala and C-Gly were analysed using the Ehrlich Test (6).

Calculations

It is not possible to define the catalytic activity of enzymes involved in a reaction for whole cell systems. However, the efficiency of the conversion of substances using microorganisms can be characterised by the so-called productivity number (PN) modified after (31):

$$PN = \frac{\text{amount of product [mM]}}{\text{DWB [g/mL]} * \text{time [min]}} \quad (1)$$

DWB representing the dry weight of biocatalyst per reaction volume in this equation [g/mL]. The PN resembles the specific activity as defined for pure enzymes but instead of referring to the turnover of one single enzyme in a certain time it implies the dry weight of the utilised cells and also includes other factors such as inhibition and transport phenomena. Hence, hydantoinase activity is given as the amount of carbamoyl amino acid produced in relation to the dry weight of the catalyst used and time. If not stated otherwise, PN is referred to the amount of D-/L-C-Phe produced, since BnH was used as a model substrate.

Results and Discussion

Phylogenetic Characterisation

16S rDNA Amplification

Phylogenetic characterisation using 16S rDNA amplification to the genus level was described previously for all three strains (6). More detailed 16S rDNA comparison revealed that *Delftia* sp. I24 could be either classified as *Delftia tsuruhatensis* or *Delftia acidovorans*, *Ochrobactrum* sp. G21 as *Ochrobactrum anthropi*, *O. lupine* or *O. tritici* and *Bacillus* sp. F18 as *Bacillus megaterium* (Table 1).

Table 1: 16S rDNA amplification with the primer pair U151OR and E9F of the previously isolated bacterial strains.

bacterial isolate	closest 16S rDNA match	% identity (identical bp/aligned bp)
<i>Delftia</i> sp. I24	<i>Delftia tsuruhatensis</i>	99.4% (1393/1402)
	<i>Comamonas (Delftia)</i> cf. <i>acidovorans</i>	99.1%(1393/1405)
	<i>Ochrobactrum</i> sp. G21	
<i>Ochrobactrum</i> sp. G21	<i>Ochrobactrum anthropi</i>	99.4% (1081/1087)
	<i>Ochrobactrum tritici</i>	99.4% (1081/1087)
	<i>Ochrobactrum lupine</i>	99.4% (1081/1087)
<i>Bacillus</i> sp F18	<i>Bacillus megaterium</i>	99.9% (1315 /1316)

Classical Bacterial Identification

Colonies of the strain *Delftia* sp. I24 were rod shaped with a size of 4–6 μm (see Fig. 1a), motile, Gram-staining negative, aerob, catalase and oxidase positive. Classical identification showed that the strain *Delftia* sp. I24 could not be assigned undoubtedly to *Delftia acidovorans* or *tsuruhatensis*, since not all criteria tested accorded to one of these strains (data not shown, references were taken from (40)). *Ochrobactrum* sp. G21 cells were coccoide rods with a size of 2 μm (Fig. 1b), colonies yellow, motile, Gram-staining negative, aerob, catalase and oxidase positive. Concerning different characteristics *Ochrobactrum* sp. G21 could not be assigned with assurance to one of the known *Ochrobactrum* species (data not shown, references from (2)). For exact classification of the strains *Delftia* sp. I24 and *Ochrobactrum* sp. G21 at least DNA-DNA hybridisation or further classification methods should be used. *Bacillus* sp. F18 were rod shaped with a size of 5–6 μm (see Fig. 1c), Gram-staining positive, facultative anaerob, catalase positive and oxidase negative. 16S rRNA gene amplification and classical bacterial identification showed that this strain could undoubtedly be assigned as *Bacillus megaterium*.

Resuming the phylogenetic characterisation, the strains were designated as *Delftia* sp. I24, *Ochrobactrum* sp. G21 and *Bacillus megaterium* F18.

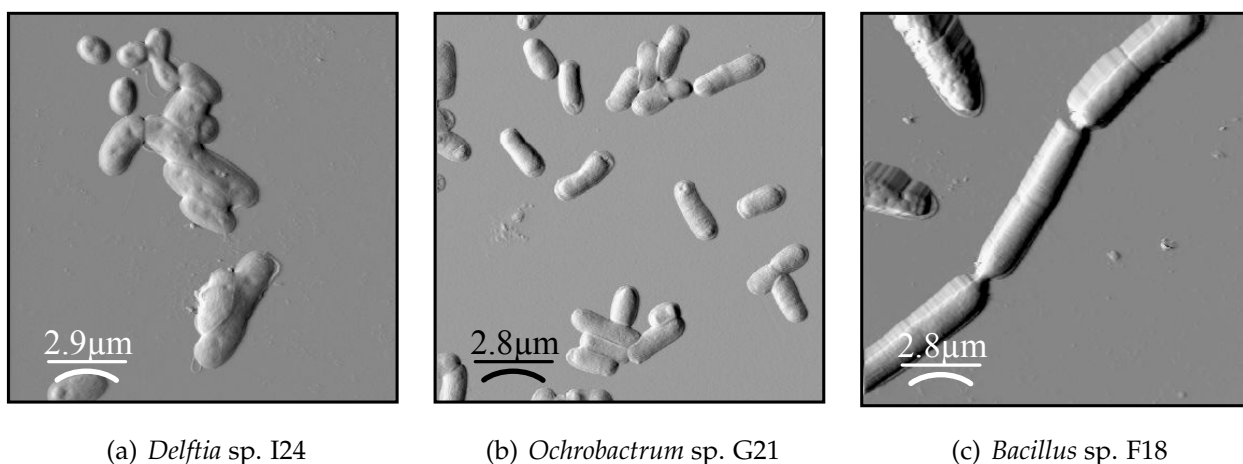


Figure 1: Atomic force microscopy (AFM) pictures of each bacterial strain: **a:** *Delftia* sp. I24, **b:** *Ochrobactrum* sp. G21, **c:** *Bacillus* sp. F18.

Growth Conditions Influencing Hydantoinase Activity

a) Cell permeability

Gross *et al.* (8) showed that the treatment of cells of *A. aureescens* DSM3747 with sodium--desoxycholate leads to enhanced cell permeability and higher hydantoinase and carbamoylase activity. To overcome cell membrane transport problems for substrates and products, and consequently increase hydantoinase activity, *Ochrobactrum* sp. G21 cells were used as a model strain and treated by different methods in biotransformation assays. Highest enzyme activity was obtained by the addition of toluene (20 $\mu\text{L}/\text{mL}$) to the biotransformation solution, followed by Triton X-100 addition. No effect or low activity was obtained by sodium-desoxycholate addition or cell disruption. Cell disruption probably led to instability of the enzymes and therefore to decreased enzyme activity.

b) Carbon source

Delftia sp. I24 showed poor growth in GM. Shigematsu *et al.* (30) reported that *Delftia tsuruhatensis* sp. nov. was not able to use D-glucose, lactose, L-serine, starch, sucrose, L-valine and D(+)-xylose as carbon and energy source but was able to utilize citrate, D-fructose, glycerol, isobutyrate, propionate, terephthalate and D- and L-tryptophan. Therefore other carbon sources were investigated in order to enhance growth of *Delftia* sp. I24. It turned out that growth was highest in GM supplemented with fructose instead of glucose. Slower growth was obtained with glycerol and tryptophan, no growth with citrate and sucrose. Additionally, no growth was obtained at a growth temperature of 40°C using GM but at 30°C growth was satisfactory. Growth at 40°C was obtained in CA-medium (data not shown).

The results described above were routinely applied for the improvement of growth and enzyme activity: Cell permeability was enhanced by the addition of toluene; good growth

was achieved for *Delftia* sp. I24 in GM supplemented with fructose instead of glucose.

c) Salt concentrations during growth

Ochrobactrum sp. G21 and *Bacillus megaterium* F18 were able to grow at NaCl concentrations of 5% and 7%, respectively, but with lower growth rates compared to NaCl free conditions. Hydantoinase activity, however, was stimulated by higher salt concentrations (Table 2).

Table 2: Influence of the NaCl content of the medium on hydantoinase activity. (Cells of *Ochrobactrum* sp. G21 and *Bacillus megaterium* sp. F18 were grown in GM supplemented with 0.2 g/L IMH and at different salt concentrations. Biotransformations were conducted at standard conditions.)

strain	NaCl content [%]	PN [nM/(min * g)]
F18	0	39.8
F18	2	39.2
F18	5	36.7
F18	7	46.0
G21	0	7.35
G21	2	9.59
G21	5	40.92

It has been shown that salt stress has an important effect on the synthesis of degradative enzymes in *Bacillus subtilis* (14). The synthesis of 40 proteins was found to be repressed or induced during salt stress in *Listeria monocytogenes* cells which can tolerate certain environmental stress. The 40 proteins include general stress proteins, transporters and proteins involved in general metabolism (5). An attractive hypothesis for *Ochrobactrum* sp. G21 and *Bacillus megaterium* F18 is a co-regulation of the hydantoin degrading system and other metabolic pathways together with salt stress proteins, triggered by salt stress. The function of the co-regulation of the hydantoinase system could be the supply of α - and/ or β -amino acids for the metabolism during salt stress. This could be verified by investigating the enzyme expression using 2-D-gel electrophoresis or gene expression using DNA-array-techniques of cells grown under normal and salt stress conditions.

d) Effect of inducers on hydantoinase activity

Various hydantoin- and dihydropyrimidine derivatives were added as potential inducers to the growth medium (Fig. 2).

All three strains tested showed hydantoinase activity in the absence of any inducer, nevertheless the hydantoinase activity could be increased by the presence of inducers. The hydantoinase of *Delftia* sp. I24 showed highest activity in the presence of the aromatic hy-

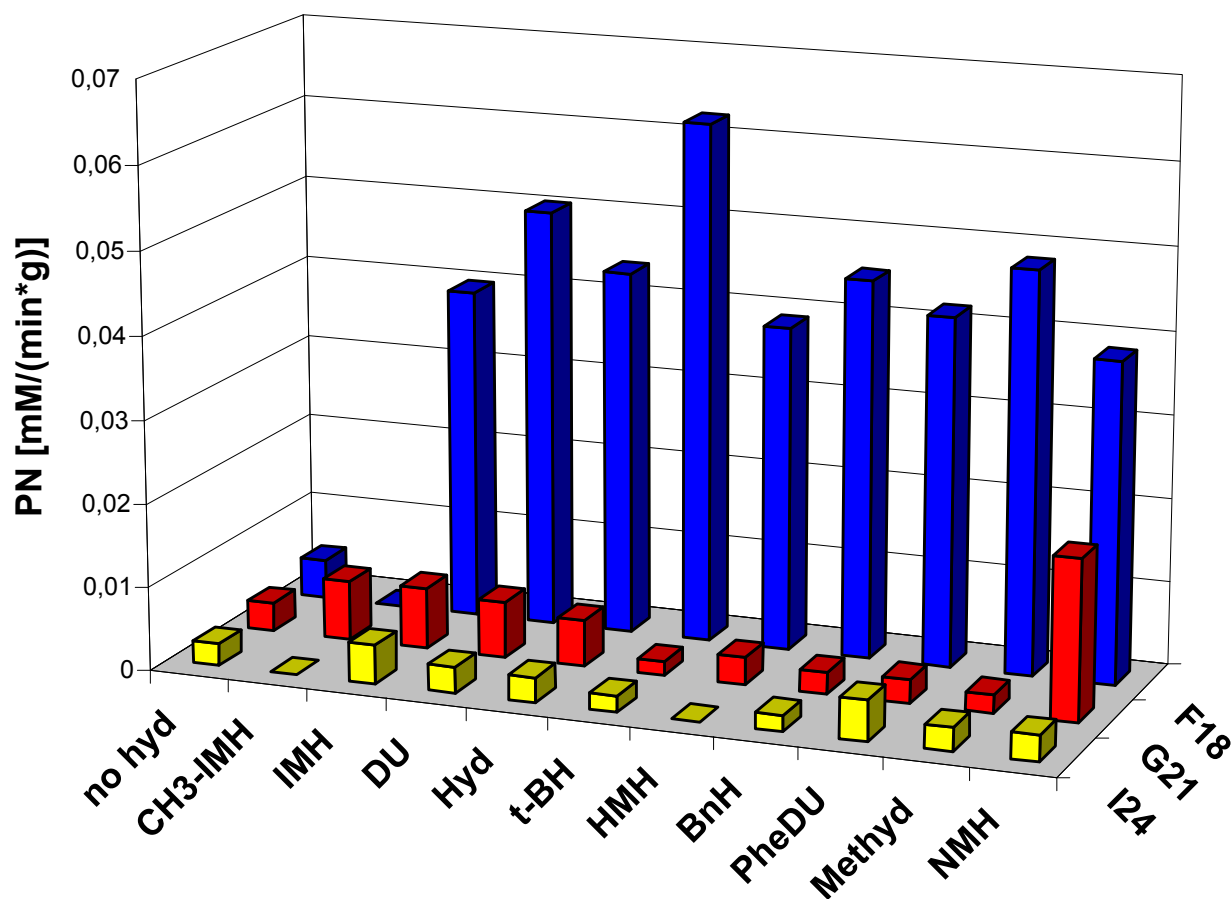


Figure 2: Effect of inducers on hydantoinase activity. (Cells were grown in the presence of different inducers. Biotransformations were conducted with 2.5 mM BnH as substrate for *Ochrobactrum* sp. G21 and *Delftia* sp. I24 and 5 mM BnH for *Bacillus megaterium* F18. **no hyd:** no inducer added to the growth medium; for abbreviations of the inducers see Materials and Methods.)

dantoin IMH and the aromatic dihydropyrimidine PheDU. A dramatic increase in activity was obtained by addition of NMH for *Ochrobactrum* sp. G21 (Fig. 2). At least a double increase in activity was obtained by addition of CH3-IMH and IMH, followed by DU and Hyd. In contrast, the hydantoinase of *Bacillus megaterium* F18 showed highest activity in the presence of *t*-BH, a hydantoin with a sterically demanding side chain, followed by DU and Methyd.

These results are not surprising since a positive effect of various inducers for hydantoin cleaving activity has repeatedly been reported in literature. An optimal induction can vary widely between species and strains: Induction by 2-thiouracil was found for *Agrobacterium* sp. IP-671 (27, 28), by D,L-5-(3-indolylmethyl)-3-*N*-methylhydantoin and D,L-5-(3-indolylmethyl)-3-*N*-hydantoin for *Arthrobacter aureescens* DSM3747 and 3745 (35, 36), by hydantoin and isopropylhydantoin for *Pseudomonas fluorescens* DSM84 (21), by hydantoin for *Pseudomonas* sp. RUKM1 and by hydantoin, dihydrouracil and thiouracil for *Pseudo-*

monas sp. RUKM3_S (3). The activity of the hydantoinase of a halophilic *Pseudomonas* sp. NCIM5109 (32) was not increased by addition of different hydantoins or dihydropyrimidines to the media. In contrast, the three strains used in this study did not show the maximum induction with one of the hydantoins or dihydropyrimidines reported above showing that induction could be strain or species specific and indicating that the three described hydantoinases could have novel unique properties.

e) Enzyme activity during growth

Hydantoinase activity was found to be highest in the late exponential phase of growth for different *Pseudomonas* species (22, 7, 32), for *Agrobacterium* (11) and in the exponential phase for *Arthrobacter* sp. (DSM3745) (35). In the present study we investigated the hydantoinase activity during growth for different growth media (data not shown): *Delftia* sp. I24 grew equally well in GMF and GMF + PheDU. Enzyme activity was highest at the mid exponential phase when grown in GMF. *Ochrobactrum* sp. G21 was grown in GM, GM + 5% NaCl and GM + 5% NaCl + NMH. Growth was very good in GM, as seen before, lower in GM + 5% NaCl and very slow in GM + 5% NaCl + NMH. Interestingly, at a growth time of 94 h hydantoinase activity was highest in GM+ 5% NaCl + NMH. This is probably an effect of hydantoinase induction by NMH and the assumed co-regulation of the hydantoinase system with salt stress proteins due to salt stress, resulting in increased levels of hydantoinases as potential amino acid suppliers. Similar results were obtained for *Bacillus megaterium* F18 showing highest enzyme activity during the late exponential phase with the preferred medium for highest enzyme production being GM + 5% NaCl or GM + 5% NaCl + *t*-BH.

Summary of growth conditions

The growth and hydantoin enzyme synthesis of all three isolates studied varied considerably depending on composition of the growth medium and cultivation time. The adjustment of different parameters such as media composition and cultivation time can significantly influence hydantoinase activity. The two halophilic strains *Ochrobactrum* sp. G21 and *Bacillus megaterium* F18 were able to grow in media with higher salt concentrations but high growth rates are obtained for media with a low NaCl content. High levels of enzyme activity can be achieved by the addition of NaCl and/ or hydantoins to the growth media.

Properties of Hydantoinases

The hydantoinase enzymes of the three bacterial isolates were characterised in biotransformation assays using resting cells. Cells were grown under optimised conditions, as described above. *Delftia* sp. I24 was grown to mid exponential phase in GMF, *Bacillus megaterium* F18 in GM supplemented with 5% NaCl and 0.2 g/L *t*-BH and *Ochrobactrum* sp. G21 in GM supplemented with 5% NaCl; For *Ochrobactrum* sp. G21 GM with 5% NaCl

was used because cell growth was very slow in the best medium for high activity GM + 5% NaCl + NMH. Toluene ($0.2 \mu\text{l}/\text{mL}$) was added to all biotransformation assays to achieve higher cell permeability for substrates and products.

a) Temperature and pH optima

The hydantoinase of *Delftia* sp. I24 exhibited a pH optimum of 9.0 and a temperature optimum of 30°C (Fig. 3 and Fig. 4). Above 45°C no hydantoinase activity could be detected. This was surprising since this strain had been isolated from a Chinese hot spring, and, taking in account the temperature growth characteristics (see: Growth conditions influencing hydantoinase activity, b) carbon sources), we conclude that this strain might be a contamination of this habitat with the ability to only survive at high temperatures.

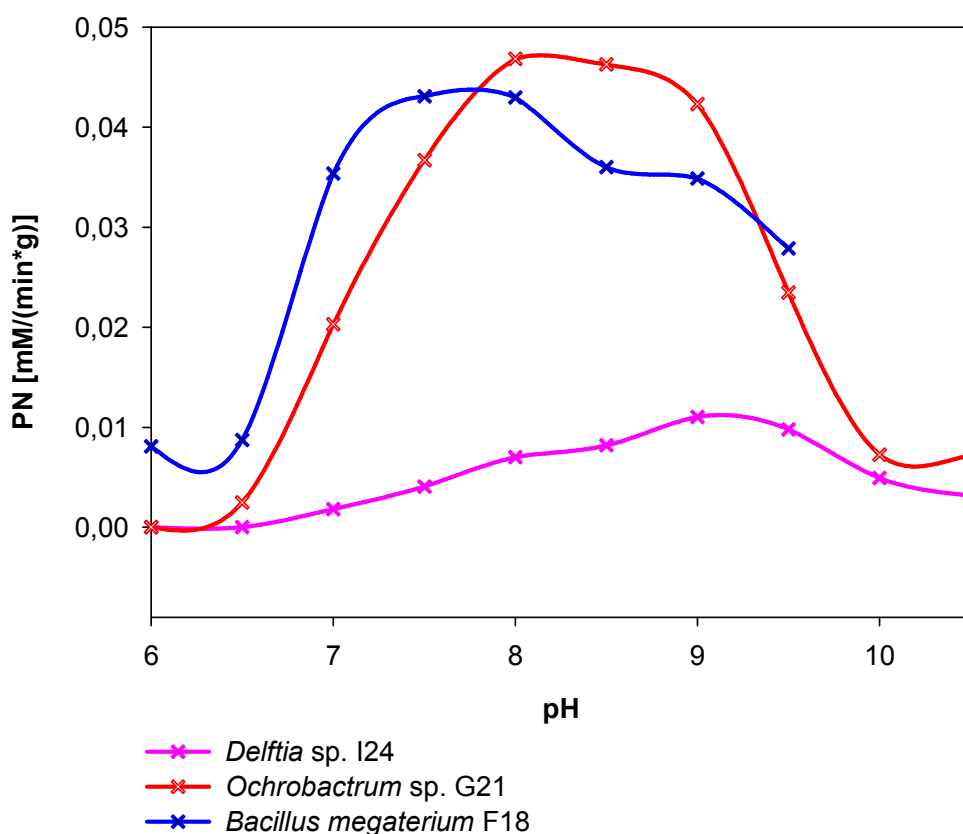


Figure 3: Effect of pH on hydantoinase activity. (Biotransformations were conducted with 10 mM BnH as substrate. Different buffers were used for the adjustment of the pH: potassium-phosphate buffer for pH 6.0–8.0; Tris-HCl-buffer for pH 7.5–9.5; carbonate-buffer for pH 10.0.)

The temperature optimum of the hydantoinase of *Delftia* sp. I24 is remarkably low, since the lowest temperature optima of D-hydantoinases reported in literature are at $45\text{--}50^\circ\text{C}$ for different *Pseudomonas* strains (37, 22, 23) and a phenylhydantoinase of *E. coli* K-12 (13). An exception is the D-hydantoinase of a halophilic *Pseudomonas* sp. NCIM5109 with a 30°C optimum (32).

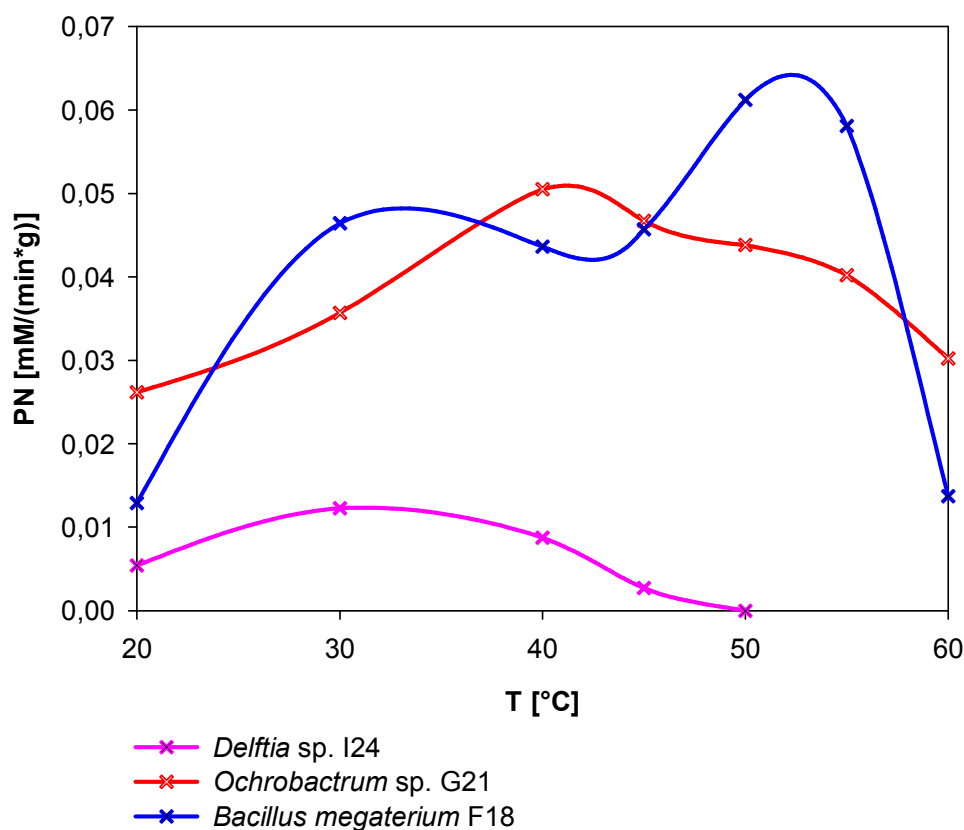


Figure 4: Effect of temperature on hydantoinase activity. (Biotransformations were conducted with 5 mM BnH as substrate.)

The hydantoinase of *Ochrobactrum* sp. G21 had a pH and temperature optimum of 8.0–8.5 and 40°C, respectively (Fig. 3 and Fig. 4). The hydantoinase of the sole *Ochrobactrum* strain described in literature, *Ochrobactrum anthropi*, showed a pH optimum of 9.0 (25). Unfortunately the influence of temperature was not tested in this study. Of all three strains tested in this study, the hydantoinase from *Bacillus megaterium* F18 showed the highest optimal temperature at 50–55°C and a pH optimum at 7.5–8.0 (Fig. 3 and Fig. 4). The optimal temperature of this strain is relatively low since other *Bacilli* show higher optimal temperatures: *Bacillus* sp. AR9, *Bacillus stearothermophilus* SD-1 and *Bacillus thermocatenuatus* GH-2 at 65°C (29, 15, 24, 12), *Bacillus stearothermophilus* NS1122A at 60–70°C (10) and *Bacillus circulans* at 75°C (18).

b) Cofactor requirements

Hydantoinases were described to be cofactor-dependent; e.g. the L-hydantoinase of *Arthrobacter aurescens* DSM3745 contains about 2.5 mol Zn²⁺ /mol subunit as shown by atom absorption spectroscopy and inductive coupled plasma-atomic emission spectrometry. The zinc ions have a catalytic and a structural function (19, 20). This was proven in crystallising the hydantoinase and by a homology model (1). However, first reactivation experiments with the *Arthrobacter* enzyme showed best activation with Mn²⁺- or Co²⁺-ions,

indicating that activity studies can not be regarded as proof for cofactor requirement. On the other hand the thermostable hydantoinase from *Bacillus* sp. AR9 was activated, amongst other ions, by MnCl_2 , and the presence of Mn^{2+} in the active centre was shown by atom absorption spectroscopy (26). A positive effect of Mn-ions was also reported for *Bacillus stearothermophilus* SD-1 (15) and *Bacillus circulans* (18).

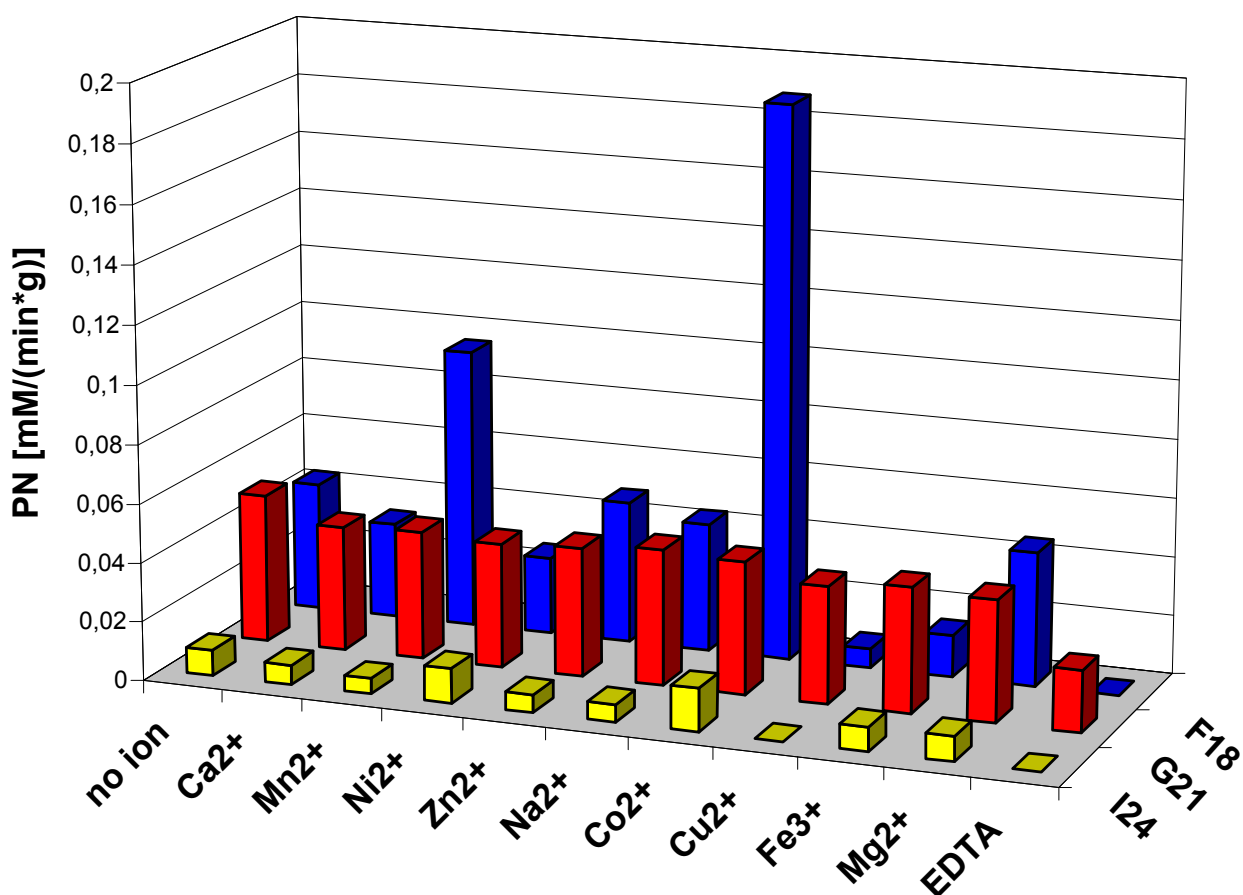


Figure 5: Effect of metal ions on hydantoinase activity. (Biotransformations were conducted with 5 mM BnH as substrate, 1 mM of the respective metal ion, or 10 mM EDTA. **no ion:** no metal ion added to the biotransformation solution.)

All three strains of this study seemed to be cofactor dependent, since the addition of the chelator EDTA resulted in a significant loss of activity or in complete inactivation of the hydantoinase (Fig. 5). The hydantoinase from *Delftia* sp. I24 was activated with Ni^{2+} and Co^{2+} , whereas Cu^{2+} led to a complete inactivation. Like other *Bacillus* hydantoinases, the activity of the hydantoinase from *Bacillus megaterium* F18 was dramatically increased by the addition of Mn^{2+} and Co^{2+} . In contrast to the strains mentioned above and the sole hydantoinase described from *Ochrobactrum* which was activated by Ca^{2+} , Na^+ , Cu^{2+} or Co^{2+} (25), the hydantoinase from *Ochrobactrum* sp. G21 showed no preference to one of the ions tested.

c) Enzyme stability

The stability of the three hydantoinases was investigated at different storage temperatures (Fig. 6): In the initial activity test no difference was observed between whole cells and toluene treated cells of *Delftia* sp. I24 which was in contrast to the results obtained for *Ochrobactrum* sp. G21. Hydantoinase in whole cells of *Delftia* sp. I24 did not lose activity at 4°C and 20°C during a four days storage, quite contrary to toluene treated cells. Toluene treated cells showed a significant loss in activity during storage at 4°C and 20°C either due to the negative effect of toluene on the hydantoinase at long term storage times or because the hydantoinase was no longer in the appropriate environment due to the permeabilised cell membrane. The influence of toluene to cell membrane permeabilisation was higher for *Ochrobactrum* sp. G21, indicated by higher initial activity in toluene treated cells as for untreated cells. Enzyme activity decreased under all storage conditions and almost all of the activity was lost at 40°C storage in both strains. Hydantoinase enzyme stability was also tested for *Bacillus megaterium* F18 (data not shown). For this strain activity increased during a four days storage. One explanation could be that the cells still had enough nutrients to grow because it was not possible to wash the cells properly due to the slimy and dense character of the cells.

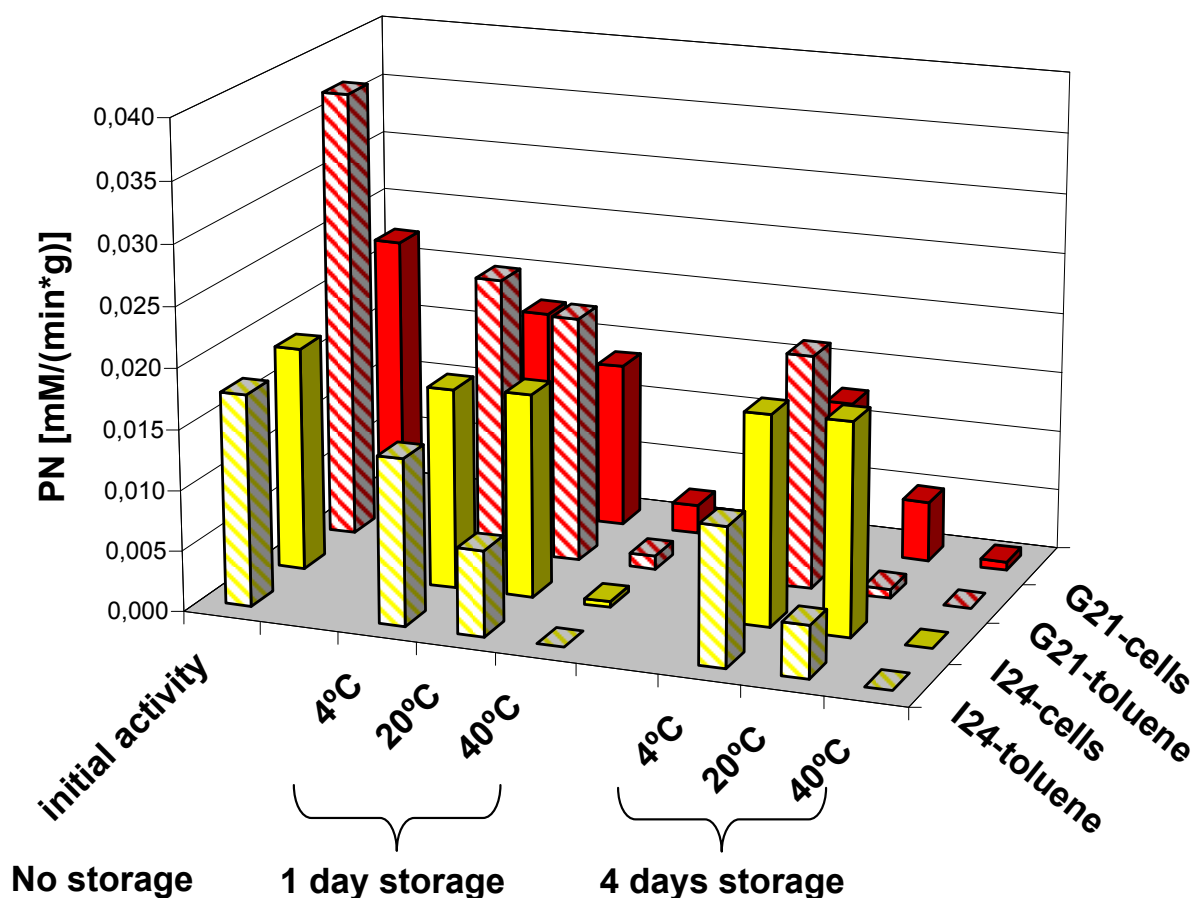


Figure 6: Effect of storage on hydantoinase activity. (Biotransformations were conducted with 5 mM BnH as substrate. Cells were stored at 4°C, 20°C and 40°C with or without 20 μ L/mL toluene for 1 to 4 days. **initial activity (no storage):** Biotransformations were directly conducted after harvesting of cells.)

d) Influence of NaCl on hydantoinase activity

The effect of NaCl on hydantoinase activity in biotransformations was tested in the concentration range from zero to 20% NaCl (data not shown). No influence or only a very slight decrease of hydantoinase activity for high NaCl concentrations on the hydantoinases from *Delftia* sp. I24 and *Bacillus megaterium* F18 was observed. The hydantoinase from *Ochrobactrum* sp. G21 was approximately twice as active when tested in 2.5% NaCl than in 20% NaCl. These results seem to be contradictory to the observations of growing cells. Obviously growing cells respond to salt stress resulting in activation of certain enzyme systems, e.g. the hydantoinase system; whereas resting cells do not show response to higher salt concentrations and only the enzymes can be affected.

e) Substrate spectrum

An *Ochrobactrum anthropi* isolate is reported to hydrolyse D,L-(2-methylthioethyl) hydantoin to methionine (25). Unfortunately no other hydantoinins were tested. The hydantoinase from

Ochrobactrum sp. G21 described within this study showed a broad substrate spectrum and was able to cleave all substrates tested in this study (Figure 7 and Table 3).

Table 3: Substrate range of the D-hydantoinases from *Delftia* sp. I24 (**I24**), *Ochrobactrum* sp. G21 (**G21**) and *Bacillus megaterium* F18 (**F18**). (Cells were grown as follows: *Delftia* sp. I24 in GMF for 62 h or in CA supplemented with 0.2 g/L CH3-IMH as inducer ⁽¹⁾, *Ochrobactrum* sp. G21 in GM supplemented with 2.5% NaCl for 48 h and *Bacillus megaterium* F18 in GM supplemented with 5% NaCl and 0.2 g/L *t*-BH for 14 h at standard conditions. Biotransformations were carried out at optimised conditions: *Delftia* sp. I24 at pH 9.0, 30°C and 1 mM Co²⁺, *Ochrobactrum* sp. G21 at pH 8.0 and 40°C and *Bacillus megaterium* F18 at pH 8.0, 50°C and 1 mM Co²⁺; all assays were supplemented with 20 µL/mL toluene, except for ⁽¹⁾. Biotransformations were conducted at initial conditions. The chemical structures of the substrates used are shown in Table 7.)

substrate	conc. [mM]	PN [mM/(min*g)]		
		I24	G21	F18
DU	25	1.8	2.52	1.26
Hyd	20	0.45	2.18	3.90
<i>t</i> -BH	8	0.05 ⁽¹⁾	0.11	0.26
IMH	2	0.09 ⁽¹⁾	0.09	0.17
BnH	5	0.01	0.25	0.44
PheDU	4	0	0.08	0.39

The hydantoinase from *Bacillus megaterium* F18 had a similar substrate range as the one from *Ochrobactrum* sp. G21 but showed higher enzyme activity than the other two strains tested. The higher enzyme activity for BnH and PheDU in comparison to *t*-BH indicates that 5-monosubstituted hydantoin or dihydropyrimidines with an aromatic side chains are favoured over aliphatic ones. As well, hydantoin was hydrolysed at higher rates than DU. The same result was reported for the purified hydantoinase enzyme from *Bacillus stearothermophilus* SD-1 (15) and *Bacillus* sp. AR9 (26). The hydantoinase of *Delftia* sp. I24 showed the highest activity for DU, indicating that this enzyme might be involved in pyrimidine reductive catabolism. Other substrates were cleaved but at much lower reaction rates compared to *Ochrobactrum* sp. G21 and *Bacillus megaterium* F18.

Comparing enzyme induction (Fig. 2) and enzyme activities towards different substrates (Table 3) it is surprising that high induction by a certain inducer is not correlated with high enzyme activity towards this compound.

Many different microorganisms with the ability to cleave hydantoin and/or dihydropyrimidines were isolated and characterised during the last decades. In conclusion we

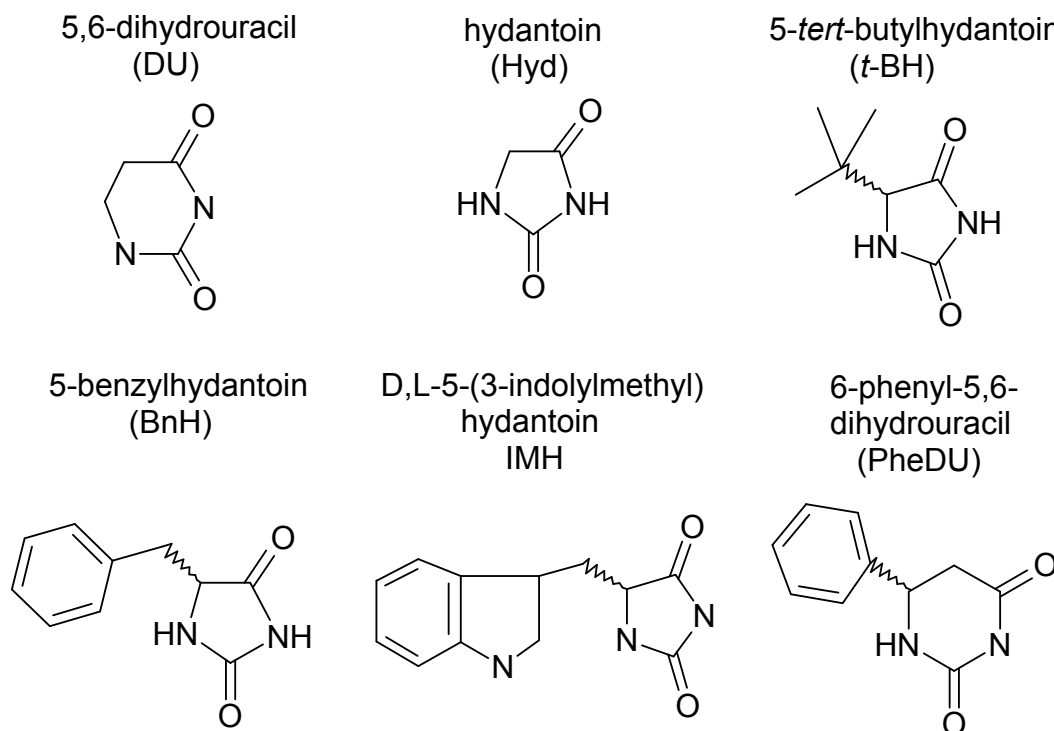


Figure 7: Chemical structures of hydantoins and dihydropyrimidines used for determination of the substrate range.

showed that it is still possible to find and describe new microorganisms with hydantoinase activity and as well with novel properties. In our particular case we described a novel D-hydantoinase from *Delftia* sp. I24, isolated from a Chinese hot spring, as well as the D-hydantoinases of two halophilic strains, *Ochrobactrum* sp. G21 and *Bacillus megaterium* F18, respectively. All of the hydantoinases showed unique properties regarding salt and inductor requirements during growth for enzyme expression and they displayed different properties like variation in pH and temperature optima and co-factor requirements. For a comparison with known hydantoinases it is still necessary to obtain more information on the amino acid or gene sequence of these enzymes.

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7.4 Chapter IV: Gene Clusters for Hydantoin Degradation

GENES RESPONSIBLE FOR HYDANTOIN DEGRADATION OF A HALOPHILIC *Ochrobactrum* SP. G21 AND *Delftia* SP. I24 – NEW INSIGHT INTO RELATION OF D-HYDANTOINASES AND DIHYDROPYRIMIDINASES

submitted to

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Abstract

Delftia sp. I24 and a moderately halophilic *Ochrobactrum* sp. G21 are able to hydrolyse dihydropyrimidines and hydantoins D-specific. The genes being with the utmost probability involved in dihydropyrimidine and hydantoin degradation of these two microorganisms were cloned into an appropriate vector and transformed into *E. coli*. The putative gene cluster of *Delftia* sp. I24 included four genes: an incomplete NADPH-dependent glutamate synthase gene (*gltB*), dihydropyrimidine dehydrogenase gene (*pydA*), permease gene (*hyuP*) and an incomplete D-hydantoinase gene (*hyuH*). The hydantoinase gene sequence was completed by PCR amplification. The putative gene cluster of *Ochrobactrum* sp. G21 comprised nine ORFs, six being potentially involved in hydantoin-hydrolysis: carbamoylase (*hyuC*), D-hydantoinase (*hyuH*), two transporters (*OrfS1* and *OrfS2*) and two permeases (*hyuP1* and *hyuP2*). Expression of the D-hydantoinases from *Delftia* sp. I24 and from *Ochrobactrum* sp. G21 in *E. coli*, followed by biotransformation assays confirmed hydantoinase activity. This is the first report of the genetical organisation of hydantoin-degradation within the genera *Delftia* and *Ochrobactrum*. Phylogenetic analysis of the two "novel" hydantoinases and known hydantoinases and dihydropyrimidinases, including putative protein sequences, revealed that they can be classed with some exceptions in the following groups: L-Hydantoinases (L-Hyd), *Rhizobiales* family (Rhizo-Fam), *Comamonadaceae* family (Com-Fam), *Pseudomonas* family (Pseud-Fam), *Bacilli* family (Bac-Fam) and *Agrobacterium* family (Agro-Fam). The highly conserved "histidine motif" for the superfamily of amidohydrolases could be found for all hydantoinases of this study but differences were found in the substrate recognition sites, whereas some of the above mentioned groups showed to possess the same recognition sites as known hydantoinases.

Introduction

Hydantoinases can be found, amongst other organisms, in microorganisms and are classed as cyclic amidases (EC 3.5.2). Hydantoinases are able to hydrolyse the five-membered ring of hydantoins or/and 5-monosubstituted hydantoin derivatives forming *N*-carbamoyl α -amino acids. A carbamoylase can transform this intermediate product to the corresponding amino acid. The natural function of hydantoinases is still unknown. In comparison, dihydropyrimidinasases are involved in the reductive pathway of pyrimidine degradation and therefore are able to cleave 5,6-dihydrouracil to the corresponding *N*-carbamoyl β -amino acid. Various enzymes with the ability to catalyse the cleavage of dihydropyrimidines and hydantoins are known: e.g. *Arthrobacter crystallopoietes* DSM20117 [1], *Bacillus* sp. AR9 [2], *Bacillus stearothermophilus* SD-1 [3,4], *Pseudomonas* sp. NCIM5109 [5]. It is notable that in the EC-nomenclature the name hydantoinase is used as a synonym for dihydropyrimidinase. Contrary, the hydantoinases of *Agrobacterium* IP-671 and *Arthrobacter aurescens* DSM3745 are able to cleave the five-membered ring of hydantoin but not dihydrouracil [6, 7]. Due to this fact, Syldatk *et al.* [8] proposed that the name hydantoinase should be used for all enzymes that hydrolyse hydantoin or/and 5-monosubstituted derivatives and not as synonym for dihydropyrimidinasases. However, in this study we will not strictly distinguish between these two enzymes because only a few real hydantoinases are known and except for biochemical data no further differentiation is known. Therefore the focus of this work will be on hydantoinases and dihydropyrimidinasases.

The hydantoinase process is widely used in industry for production of D- and L-amino acids, e.g. for the production of the aromatic amino acids D-phenylglycine and p-hydroxyl-D-phenylglycine as building blocks for semisynthetic antibiotics (ampicillin and amoxicillin). Economic targets are as well D-serine, L-methionine, L-phosphinotricine [9].

During recent years the gene clusters of different microorganisms, encoding for the enzymes being involved in hydantoin and/ or dihydropyrimidine conversion, have been elucidated (Fig. 1). The genes of hydantoinase/ dihydropyrimidinase and carbamoylase have been found to form part of a unique operon, whereas other genes coding for e.g. racemase and permease are not commonly present. However, the genetic organisation within the cluster has been found to be very diverse in positioning and transcriptional orientation. Nevertheless, the only pattern observed is a similar transcriptional orientation in bacteria from the same genus or suborder (Fig. 1): All *Agrobacterium* species show an opposite orientation of the hydantoinase and carbamoylase genes. A similar orientation of all genes involved in hydantoin conversion was obtained for the two *Arthrobacter* species and *Microbacterium liquefaciens*. In spite of this, the two *Pseudomonas* gene clusters show a complete different organisation.

Different techniques have been used to identify these clusters. A genomic phage library of *Agrobacterium* sp. IP-I671 was screened by plaque hybridisation using a DIG-labelled *hyuC*-

DNA fragment. The *hyuC*-DNA was obtained by amplification of the carbamoylase gene by degenerate primers. These primers were derived by consensus analysis using known carbamoylase sequences from *Agrobacterium* strains [12]. Other clusters were obtained in a similar manner using information derived from the amino acid sequence of purified proteins [10, 14, 17]. The hydantoinase gene cluster from *A. crystallopoietes* DSM20117 was identified by cloning the hydantoinase and expanding the flanking DNA regions by inverse PCR. Thus, the complete hydantoinase, D-N-carbamoylase and a putative L-N-carbamoylase gene could be found [11]. Recently, the dihydropyrimidinase and β -ureidopropionase gene of *Pseudomonas putida* RU-KM3_s were identified by transposon mutagenesis and selection of altered growth phenotypes [16].

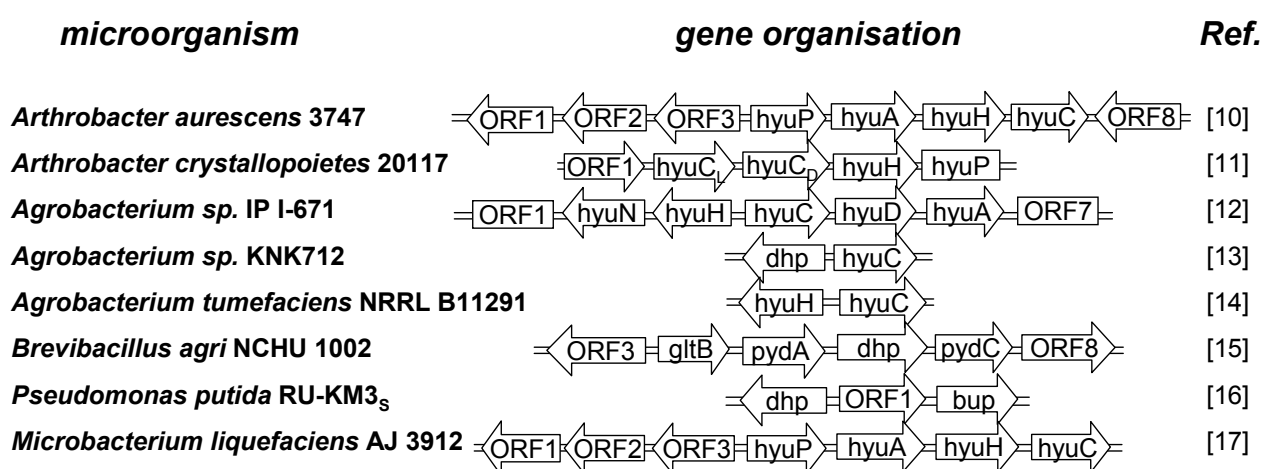


Figure 1: Genetic organisation of genes encoding for the enzymes involved in hydantoin- and/ or dihydropyrimidine hydrolysis from different microbial sources. (**hyuP**: permease; **hyuA**: racemase; **hyuH**: hydantoinase; **hyuC**: carbamoylase; **hyuN**: NADP-flavin oxidoreductase; **hyuD**: amino acid dehydrogenase; **dhp**: dihydropyrimidinase; **gltB**: glutamate synthase; **pydA**: dihydropyrimidine dehydrogenase; **pydC**: β -alanine synthase; **bup**: β -ureidopropionase. Incomplete ORFs are shown in rectangles.)

In the present study we describe the detection and characterisation of the genes involved in hydantoin cleavage of *Ochrobactrum* sp. G21 and *Delftia* sp. I24. This was achieved by screening a genomic library of each strain with the aid of a DIG-labelled hydantoinase DNA-fragment. The genomic library was obtained by digestion of the genomic DNA of each strain and cloning and transformation of the resulting DNA-fragments into an appropriate vector and competent *E. coli* cells.

Ochrobactrum sp. G21 was isolated from a hypersaline lake, Inner Mongolian Autonomous Region, China and *Delftia* sp. I24 from an algal mat of hot springs (60–65°C) Long Pu, Yunnan Province, China [18]. Growth conditions and hydantoinase activity of both strains have been studied intensively (manuscript in preparation). In literature only one *Ochrobactrum anthropii* with D,L- hydantoinase activity has been reported, isolated from a soil sample in

Spain [19]. No hydantoinase activity from the family *Delftia* was reported before. As well, none is known about the genetical organisation of hydantoin cleaving enzymes in these two families.

Materials and Methods

Abbreviations

Agro-Fam: *Agrobacterium* family; **Bac-Fam:** *Bacilli* family; **BnH:** D,L-5-benzylhydantoin; **Com-Fam:** *Comamonadaceae* family; **DIG-G21:** random primed DNA probe labeled with digoxigenin-dUTP, DNA derived from hydantoinase amplification of genomic DNA from *Ochrobactrum* sp. G21; **DIG-I24:** see DIG-G21, genomic DNA from *Delftia* sp. I24; **dhp:** dihydropyrimidinase; **DIG:** digoxigenin; **gltB:** glutamate synthase; **D-Hyd:** D-Hydantoinase; **L-Hyd:** L-Hydantoinase; **hyu:** hydantoin utilization; **hyuH:** hydantoinase; **hyuP:** permease; **IMH:** D,L-5-(3-indolylmethyl) hydantoin; **LB:** Luria-Bertani medium; **LB-Amp:** Luria-Bertani medium supplemented with ampicillin; **ORF:** open reading frame; **pydA:** dihydroprimidine dehydrogenase; **pydO:** dihydroorotate dehydrogenase; **Pseud-Fam:** *Pseudomonas* family; **Rhizo-Fam:** Rhizobiales family; **SGLs:** stereochemistry gate loops.

Chemicals

All chemicals used were obtained from commercial sources and were of reagent grade. If not stated otherwise, enzymes, chemicals for molecular work and molecular kits were obtained from Fermentas, Germany.

Bacterial Strains and Media

The bacterial isolates used within this study were a halophilic *Ochrobactrum* sp. G21 (DSM18828) and a *Delftia* sp. I24 (DSM18833) described by Dürr *et al.* [18]. The media and growth conditions of *Ochrobactrum* sp. G21 and *Delftia* sp. I24 have been described previously [18]. For cloning *E. coli* XL-1 blue and *E. coli* JM109 was used routinely, being cultivated in LB medium at 37°C. Ampicillin (100 µg/mL) was added (LB-Amp) if *E. coli* cells were cultured carrying recombinant plasmids.

DNA Manipulation Methods

General recombinant DNA techniques were carried out by standard procedures [20]. Chromosomal bacterial DNA was extracted using the Qiagen DNeasy Tissue kit (Qiagen, Germany) following the manufacturer's protocol for gram-negative bacteria. DNA fragments were purified from standard agarose gels by a DNA extraction kit. Pure plasmids were obtained by a GeneJETTM Plasmid Mini Prep Kit. Sequencing was done commercially (JenaGen GmbH, Germany) and DNA and protein sequence analysis were carried out using BlastN and BlastP [21], ORF finder (at the National Centre for Biotechnology Information website), ClustalW [22], BPRM (available at www.softberry.com) and NEBcutter [23]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 [24].

Preparation of a 320 bp DNA Fragment

Two homologous probes for hydantoinases were generated by polymerase chain reaction (PCR) using genomic DNA from *Ochrobactrum* sp. G21 or *Delftia* sp. I24 as template. Amplifications were carried out either on a Eppendorf Mastercycler gradient (Germany) or MJ Research PTC-200 (USA) at the following conditions: 50–150 ng template DNA, 1 x Taq buffer with (NH₄)₂SO₄, 1.5 U Taq DNA Polymerase, 50 pmol upstream primer and downstream primer each, 0.2 mM dNTP's, and PCR-water, combined to volume of 20 μL. The following degenerate oligonucleotide primers were used [25], synthesised by MWG-Biotech, Germany: 5'-GCSGCVTTYGGNGGNACNAC-3' and 5'-TCNCCRTTYTCNGCRTGNAC-3'. Amplification was initiated with DNA denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 2 min and primer extension at 72°C for 1 min. The PCR reaction was completed at 72°C for 7 min. After PCR amplification a 320 bp DNA fragment was purified for each strain. In order to verify hydantoinase amplification purified PCR products were sequenced and compared to known hydantoinase sequences. To obtain an adequate amount of DNA for DIG-labelling the purified PCR products were cloned using the Strata CloneTM PCR Cloning kit (Stratagene, USA). The obtained plasmid inserts were excised by different restriction enzymes and purified.

Preparation of DIG-Labelled Probes

The DIG DNA Labeling and Detection Kit (Roche, Germany) was used for DIG-random primed DNA labelling of the 320 bp DNA fragments following the manufacturer's instructions. This results in two probes: DIG-I24 obtained from hydantoinase DNA amplification of *Delftia* sp. I24 and DIG-G21 of *Ochrobactrum* sp. G21.

DNA Digest and Southern Blot

Chromosomal DNA was digested using different restriction enzymes (*Eco*RI, *Hind*III) and separated on standard agarose gels. The DNA was blotted on a nylon membrane (Roti[®] Nylon plus, Roth, Germany) using standard methods. The nylon membrane was hybridised with the DIG-labelled probe by 68°C followed by immunological detection applying the DIG DNA Labeling and Detection Kit (Roche, Germany). NBT/BCIP was used for the colour reaction. By this reaction DNA fragments possessing the hydantoinase genes could be located. The DNA fragments were afterwards purified.

Plasmid Preparation, Cloning and Screening

Plasmid DNA (pUC19) was digested using either *Eco*RI or *Hind*III. Subsequent dephosphorylation was conducted by addition of shrimp alkaline phosphatase. Ligation was carried out under the following conditions: 50 ng vector DNA (pUC19/*Eco*RI or pUC19/*Hind*III), 2 x molar excess of the purified DNA fragment, 1 x ligation buffer, T4-DNA-ligase and water (nuclease free), combined to a volume of 20 μL. The ligated DNA was transformed into *E.*

E. coli XL-1 [26], plated on LB-Amp supplemented with 38.4 $\mu\text{g/ml}$ IPTG and 40 $\mu\text{g/ml}$ X-Gal (in DMSO) and incubated at 37°C. *E. coli* XL-1 cells bearing recombinant plasmids were obtained by blue/white screening. Positive clones were picked and transferred on a master plate and on a nylon membrane on LB-Amp plates. Cells were grown overnight at 37°C. DNA transfer from *E. coli* colonies to the nylon membrane were conducted as followed (modified after [27]): the membrane was transferred on 3MM Whatman paper saturated with denaturation solution (0.5 M NaOH, 1.5 M NaCl, 0.1% SDS) and incubated for 15 min followed by neutralisation on 3MM Whatman paper saturated with neutralisation solution (1.5 M NaCl, 1 M TRIS/ HCl, pH7.5) for 5 min. The nylon membrane was transferred on 3MM Whatman paper saturated with 2x SSC for 15 min. Cell debris were washed out with 2x SSC. DNA fixation on the nylon membrane was carried out for 2 h at 80°C. Hybridisation with the DIG-labelled probe and immunological detection was conducted as described before. DIG-labelled clones bearing a plasmid containing an insert with the hydantoinase gene were re-plated on LB-Amp. Plasmid DNA was isolated. For verification of the correct insert and to estimate the location of the hydantoinase gene in the DNA fragment PCR reactions were carried out as described above using the plasmid DNA as template. In addition, plasmid DNA was digested by the same restriction enzymes as used for cloning in combination with restriction enzymes cutting in the hydantoinase fragment. These restriction enzymes were *PauI* for *Ochrobactrum* sp. G21, *Eco72I* and *Eco52I* for *Delftia* sp. I24. The digested plasmids were run on an agarose gel, blotted on a nylon membrane, hybridised and the immunological detection performed as described above. To obtain high concentration of plasmid DNA, plasmid DNA was isolated by the method of [23] or by a QIAfilter Plasmid Maxi Kit (Qiagen, Germany). The obtained plasmids were sequenced and analysed (see above).

Completion of the Hydantoinase Gene from *Delftia* sp. I24 and Heterologous Expression of the Hydantoinases in *E. coli*

The hydantoinase gene sequence from *Delftia* sp. I24 was completed by PCR amplification using chromosomal DNA from *Delftia* sp. I24. The complete hydantoinase gene was amplified with the primers s4740 (5'-AAAAAACATATGGGTCAATCACAGGGTTCGGTAT-3') and s4741 (5'-AAAAAGCTTAGCGCGCCACGGCCGTGGGCT-3'), the resulting gene fragment cloned into the vector pJOE4786.1 which was cut by *Sma*I. The resulting plasmid was cut by *Bam*H1 and *Nde*I and the hydantoinase gene inserted into the expression vector pJOE5697.2 resulting in the plasmid pJOE5704.1. The hydantoinase gene from *Ochrobactrum* sp. G21 was amplified by PCR using chromosomal *Ochrobactrum* sp. G21 DNA and the primers s4738 (5'-AAAAACATATGGCAAAGGTCATCAAAGGCGGA-3') and 4739 (5'-AAAAAAGCTTAGACCCCTATCGGCATGTGTTTCGGCGCT-3'). The fragment was cut by *Hind*III and *Nde*I and inserted into the expression vector pJOE5427.5 resulting in the vector pJOE5702.1. Both plasmids pJOE5704.1 and pJOE5702.1 were transformed into *E. coli* JM109 using standard procedures.

Biotransformation Experiments

E. coli cells for biotransformation experiments were grown in LB-AMP with 0.2% rhamnose as inducer for 12 h at 30°C. Cell harvesting and biotransformations were performed as described by [18]. 3 mM BnH and IMH were used as substrates. The analysis of the substrates and products was performed using High Performance Liquid Chromatography (HPLC) on an Agilent 1100 series instrument (Agilent, Germany). The concentrations of BnH and IMH and their corresponding carbamoyl amino acids were determined using a Hypersil-Keystone BDS C18 column (Thermo, Germany). The mobile phase contained 20% MeOH/ 80% (0.1% H₃PO₄) solution. The flow rate was 1 mL/min. Detection was carried out at 210 nm or 280 nm, respectively.

Results and Discussion

Amplification of a 320 bp Hydantoinase Fragment

A 320 bp DNA fragment was amplified for both strains, *Ochrobactrum* sp. G21 and *Delftia* sp. I24, using degenerate primers. The DNA sequence obtained for *Delftia* sp. I24 showed the highest homology to a putative D-hydantoinase of *Burkholderia* sp. 383 (85% similarity of 167 bp), and the DNA sequence for *Ochrobactrum* sp. G21 showed the highest homology to a putative D-hydantoinase of *Brucella suis* 1330 (93% similarity of 248 bp). This indicated the amplification of a hydantoinase DNA fragment for both strains. The DNA fragments were used for preparation of a DIG-labelled probe.

Cloning and Screening of a DNA Fragment Containing the Gene Cluster Responsible for Hydantoin Degradation

The DIG-labelled probes were used to screen a genomic library of each of the two strains. These genomic libraries were derived from digested genomic DNA followed by cloning and transformation into pUC19 and *E. coli* XL-1. Four plasmids, two for each strain, carrying the genetic information likely to code for enzymes involved in hydantoin conversion were obtained. Restriction enzyme and DNA sequence analysis revealed that only a partial putative hydantoinase gene sequence of *Delftia* sp. I24 was cloned into each of the two plasmids. Therefore approximately 4 kb of a 15 kb plasmid, designated pUC19-M50, were sequenced. In the case of *Ochrobactrum* sp. G21 two plasmids with a size of approximately 6.6 kb (pUC19-H1-10) and 11.1 kb (pUC19-L1-61) containing the hydantoinase homolog gene were obtained. DNA analysis showed that both plasmids contained the same DNA information, except for pUC19-L1-61 more in downstream direction. Hence, the plasmid pUC19-L1-61 was completely sequenced.

Sequence Analysis of pUC19-M50 (*Delftia* sp. I24)

The sequencing and analysis of 4469 bp of the plasmid pUC-M50, containing the hydantoinase homolog gene sequence of *Delftia* sp. I24, revealed two complete open reading frames (ORFs) and two incomplete ORFs (Table 1). Since the hydantoin encoding sequence was first incomplete it was completed by PCR amplification resulting in known 4790 bp of the gene cluster. Primers were designed from the putative dihydropyrimidinase sequence of *Delftia acidovorans* SPH-1. All ORFs are transcribed in the same direction and the overall GC-content of the obtained DNA fragment was 67%. This is in concordance to other species of this genus since a GC content of 66–69 mol% is reported for other *Delftia* species [29, 30]. Analogously to other hydantoin gene clusters the genes involved in hydantoin degradation were abbreviated as *hyu* "hydantoin utilization" [31]. The ORFs can be attributed to the following proteins (Table 1 and for overview Figure 2):

Table 1: Overview of ORFs obtained from plasmid pUC19-M50 (*Delftia* sp. I24). (The putative Shine Dalgarno sequence is underlined and italics. The start codons of the ORFs are bold. * ORF not complete.)

gene	start	end	length [bp]	GC [%]	putative Shine Dalgarno sequence	assumed function
<i>gltB</i> *	-	319	-	-	-	glutamate synthase
<i>pydA</i>	354	1673	1320	67	GAGA <u><i>A</i>GGAGCTTCGCAATG</u>	dihydro- pyrimidine dehydrogenase
<i>hyuP</i>	1806	3299	1494	65	AGA <u><i>A</i>GGAGCTTCGCAATG</u>	permease
<i>hyuH</i>	3330	4790	1461	68	<u><i>C</i>AGGAGGCATGACGCTATG</u>	hydantoinase

***gltB*:** The deduced amino acid sequence of this ORF could be assigned to the family of NADPH-dependent glutamate synthases (beta chain) and related oxidoreductases by comparison to a conserved domain database [32].

***pydA*:** This gene was named *pydA*, since the deduced protein sequence showed the highest homology to the dihydropyrimidine dehydrogenase (*pydA*) of *Brevibacillus agri* [15] with 59% identity. This group of enzyme is known to catalyse the reduction of uracil and thymine to the corresponding 5,6-dihydropyrimidines.

***hyuP*:** The highest homology of the obtained amino acid sequence of this ORF to a "non-putative" protein was to the allantoin permease *Dal4* from *Saccharomyces cerevisiae* with 28% identity. A comparison to a conserved database showed that this ORF can be associated to the family of cytosine/ uracil/ thiamine/ allantoin permeases [32]. Additionally, a Kyte-Doolittle hydrophathy plot [33] revealed a hydrophobic character of this protein (data not shown) indicating a transmembrane protein. Resuming all the data mentioned above, we assume that this ORF encodes for a permease with its function in hydantoin transport and therefore named as *hyuP*.

***hyuH*:** The highest identity (66%) of the deduced amino acid sequence of this ORF to a "non-putative" enzyme was obtained to the D-hydantoinase of *Pseudomonas putida* DSM84 [34, 35]. Interestingly, the highest homologies (compare Fig. 2) of the deduced protein sequence was found with 98% to a putative dihydropyrimidinase of *Delftia acidovorans* SPH-1 (accession ZP01581256), with 84% to a putative D-hydantoinase of *Acidovorax avenae* subsp. *citrulli* AAC00-1 (accession ZP01403855), with 81% to a putative dihydropyrimidinase of *Verminephrobacter eiseniae* EF01-2 (accession EAT75980) and with 78% to a putative D-hydantoinase of *Polaromonas naphthalenivorans* CJ2 (accession ZP01019452). All these organisms, including *Delftia* sp. I24, belong to the lineage of proteobacteria, burkholderiales, family of comamonadaceae. The expression of this gene in *E. coli* followed by biotransformations showed the conversion of the substrates BnH and IMH (Table 2). This proves the

assumed function as hydantoinase and this ORF was therefore named as *hyuH*. The complete hydantoin-hydrolysing gene cluster of *Delftia* sp. I24 is accessible at the following gene bank entry at NCBJ: 878499.

Table 2: Activity tests of the *E. coli* clones JM109 (pJOE5702.1) and JM109 (pJOE5704.1) using the substrates BnH and IMH (+: formation of the corresponding carbamoyl amino acid).

clone	insert	BnH	IMH
JM109 (pJOE5702.1)	<i>hyuH</i> of <i>Ochrobactrum</i> sp. G21	+	+
JM109 (pJOE5704.1)	<i>hyuH</i> of <i>Delftia</i> sp. I24	+	+

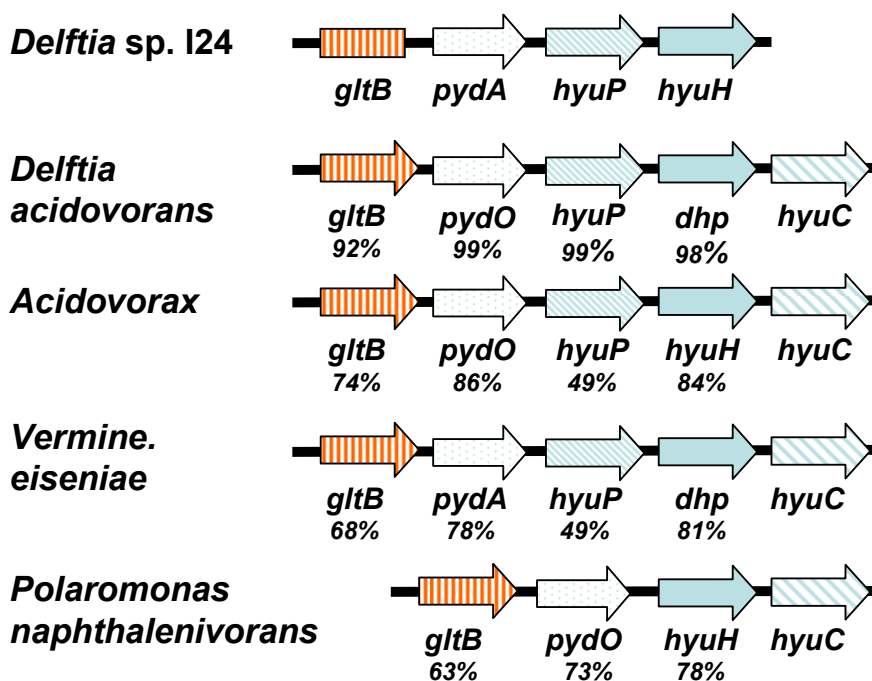


Figure 2: Overview of different gene clusters probably coding for the conversion of hydantoins, including *Delftia* sp. I24. (The other putative gene clusters were obtained from the database and originated from completely sequenced genomes: *Delftia acidovorans* SPH-1 (AAVD01000005), *Verminephrobacter eiseniae* EF01-2 (AASQ01000001); *Polaromonas naphthalenivorans* CJ2 (NZ AANM01000002); *Acidovorax avenae* subsp. *citrulli* AAC00-1 (NZ AASX01000002). Incomplete ORFs are shown in rectangles. Legend see Fig. 3.)

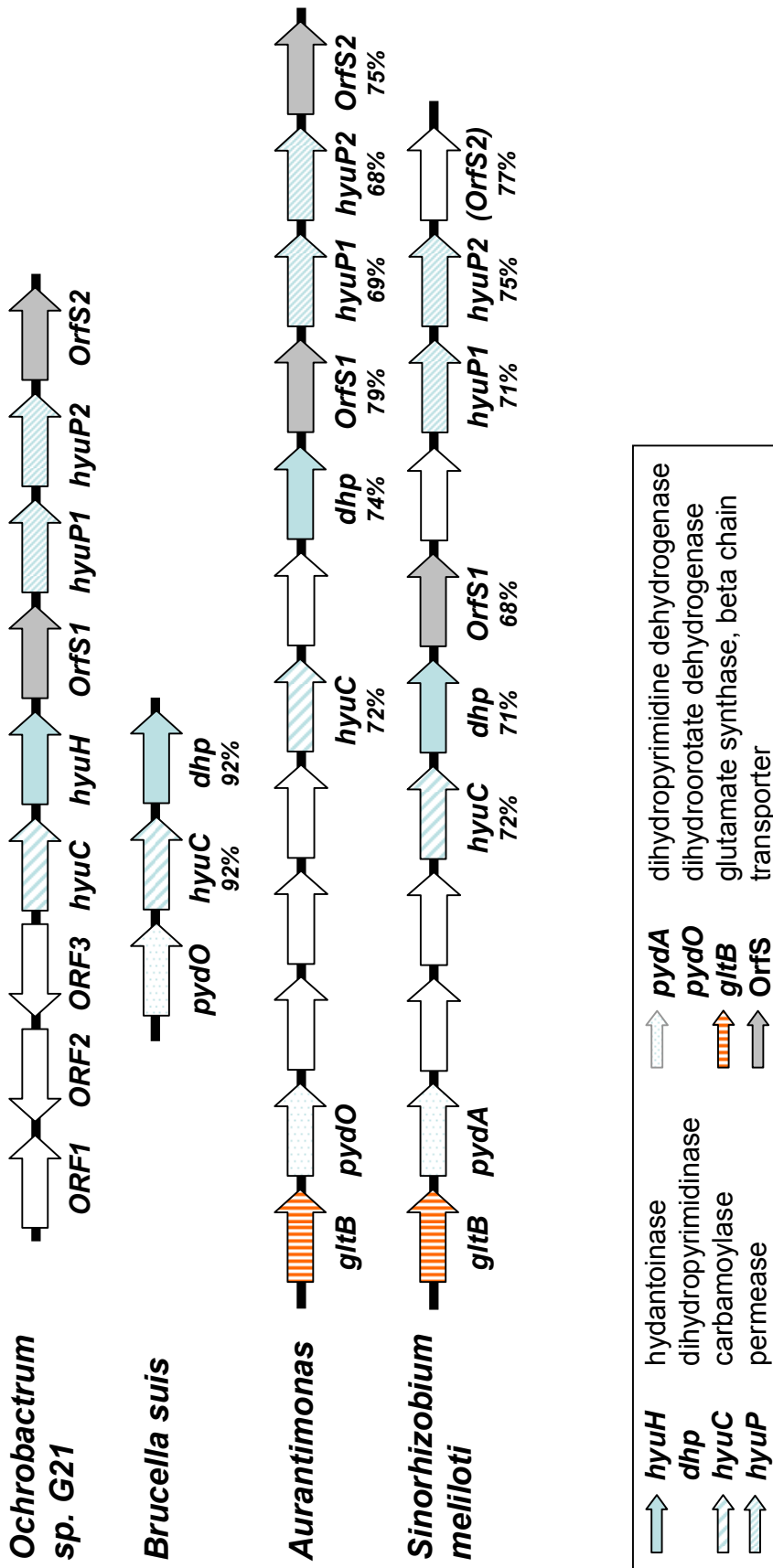


Figure 3: Different gene clusters probably coding for the conversion of hydantoin, including *Ochrobactrum* sp. G21. (The other putative gene clusters were: *Brucella suis* 1330 (AE014291); *Aurantimonas* sp. SI85-9A1 (NZ AAPJ01000001); *Sinorhizobium meliloti* 1021 (SME591790). The identity of homologous genes from other species to the obtained genes from *Delftia* sp. I24 and *Ochrobactrum* sp. G21 are shown below the assigned gene name. White arrows represent ORFs not involved in hydantoin conversion.)

Sequence Analysis of pUC19-L1-61 (*Ochrobactrum* sp. G21)

The cloned chromosomal DNA in the plasmid pUC19-L1-61 comprised 8453 bp with an overall GC content of 56%. In total eight complete ORFs and one incomplete ORFs were obtained coding for the proteins listed (Table 3 and Fig 3). Except for *hyuC* and *hyuH* all other deduced protein sequences are classified by comparison to a Conserved Domain Database [32]:

ORF1: The first ORF, incomplete, probably belongs to the family of ABC-type sugar transport systems, ATPase components.

ORF2: The second ORF, oppositely directed to ORF1, shows identity to the MutT/ Nudix family of proteins.

ORF3: This second, oppositely directed ORF could be assigned as a bacterial regulatory protein belonging to the TetR-family or as transcriptional regulator AcrR. With high probability all first three ORFs mentioned are not involved in hydantoin conversion.

***hyuC*:** Highest similarity of the deduced amino acid sequence was obtained to the *N*-carbamoyl-L-amino acid amidohydrolase, also called L-carbamoylase, of *Bacillus stearothermophilus* strain NS1122A with 34% identity, followed by the L-carbamoylase of *Arthrobacter* BT801 (32%). We assume that this ORF is responsible for carbamoyl amino acid degradation since *Ochrobactrum* sp. G21 was able to degrade carbamoyl phenylalanin to phenylalanine (data not shown) and therefore named as carbamoylase (*hyuC*).

***hyuH*:** The deduced amino sequence consists of 484 amino acids and showed highest homology to a putative hydantoinase of *Brucella suis* 1330 and to a putative dihydropyrimidinase of *Aurantimonas* sp. SI85-9A1 with 92% and 74% identity, respectively. Both strains, like *Ochrobactrum* belong to the order of rhizobiales. An identity of 47% was obtained for the D-hydantoinase of *Bacillus* sp. AR9 as first "non-putative" enzyme. This ORF is responsible for hydantoin conversion and was named *hyuH* since the expressed gene in *E.coli* showed conversion of BnH and IMH (Table 2).

***OrfS1*:** This 867 bp long gene fragment shows to encode for an ATP-binding subunit of the bacterial ABC-type nitrate and sulfonate transport system, respectively. These enzymes are involved in the transport of different compounds like sugars, ions, peptides and more complex organic molecules.

***hyuP1* and *hyuP2*:** Both ORFs can be associated to a family of permease ABC transporter proteins. Homologies were only obtained to putative enzymes (Fig. 3). Probably they are acting as permeases being involved in the transport of hydantoins and therefore named as *hyuP*.

***OrfS2*:** The last ORF can be assigned to the family of TauA, ABC-type nitrate/ sulfonate/ bicarbonate transport systems.

Table 3: Overview of ORFs obtained from plasmid pUC19-L1-61 (*Ochrobactrum* sp. G21). (The putative Shine Dalgarno sequence is underlined and italics. The start codons of the ORFs are bold. * ORF not complete.)

gene	start	end	length [bp]	GC [%]	putative Shine Dalgarno sequence	assumed function
<i>ORF1*</i>	-	207	-	-	-	glucose transporter
<i>ORF2</i>	602	204	399	56	CGAGAATTCGTCATCATG	Nudix-protein
<i>ORF3</i>	1297	599	699	53	AAAGGTGTCAAATG	regulator
<i>hyuC</i>	1517	2752	1239	58	GCGGAGGCAGAGCGCATG	carbamoylase
<i>hyuH</i>	2827	4281	1455	57	AAGGGGAACGACGAACAATG	hydantoinase
<i>OrfS1</i>	4285	5151	867	55	ATAGGGGTCTGAGACATG	part of transporter
<i>hyuP1</i>	5188	6081	894	57	GCTGGAGATCGTCCATG	permease
<i>hyuP2</i>	6078	6938	861	54	AAAGGGCAGCCACATG	permease
<i>OrfS2</i>	7052	8050	999	56	AGAGGAGAACTGAAATG	part of transporter

The complete gene cluster which is responsible for hydantoin-hydrolysis of *Ochrobactrum* sp. G21 is accessible at the following gene bank entry at NCBJ: 883016.

Promoter regions (-10 and -35 respectively) were found in front of the hydantoinase and carbamoylase genes. This leads to the suggestion that *hyuC* and *hyuH* are regulated independently. The control of the expression of dihydropyrimidinase (*dhp*) and β -ureidopropionase (*bup*) with two promoters is as well found for *P. putida* RU-KM3_s [16], but the *dhp* and *bup* genes are orientated oppositely.

In conclusion, this is the first report on genes responsible for hydantoin and dihydropyrimidine degradation of members of the genera *Delftia* and *Ochrobactrum*.

Comparison to Other Hydantoin Cleaving Gene Clusters

The highest homology of *hyuH* in *Delftia* sp. I24 was obtained to putative hydantoinases/dihydropyrimidinases of other members of the family *Comamonadaceae* (see previous chapter). Therefore the obtained gene cluster of *Delftia* sp. I24 was primarily compared to gene clusters from other members of the family *Comamonadaceae*, obtained by complete sequencing of the genomes, and to known hydantoin-hydrolysing gene clusters (see Fig. 1 and Fig. 2). Interestingly, the *Comamonadaceae* gene clusters showed a very high conservancy of enzymes involved in hydantoin degradation:

- All ORFs were shown to be orientated in the same direction.
- The identity of the deduced amino acid sequences of *pydA*, *hyuP* and *hyuH* from *Delftia* sp. I24 in comparison to the other enzymes was quite high (Fig. 2).
- In all clusters hydantoinase and carbamoylase homologous genes were present, all of

them in the same orientation. To date, the presence of a carbamoylase in *Delftia* sp. I24 has not been shown but it is very likely that a carbamoylase gene is present downstream the hydantoinase.

(d) The general structure of the gene clusters is *pydA/pydO-hydH-hyuC*". This is supported by the observation that the identity of these enzymes is quite high (Fig. 2). The presence of *pydA* together with *hyuH* and *hyuC* and the possibility of *Delftia* sp. I24 to transform dihydrouracil [18] indicate that the hydantoin metabolising enzymes of *Delftia* sp. I24 could be part of the pyrimidine reductive catabolism.

(e) The presence of *gltB* in most of the gene clusters shown in Fig. 2 and in the gene cluster of *Brevibacillus agri* (Fig. 1) raises the question of whether there is any association of hydantoinase-cleaving enzymes with glutamate metabolism and/or nitrogen metabolism. Comparing the above-mentioned gene clusters (Fig. 2) to other clusters shown in Fig. 1, no conserved pattern could be observed for this group, excepting *Brevibacillus agri*. Taking into account all of these observations we can assume that gene clusters of these members of the family *comamonadaceae* and *Brevibacillus agri* form their own class of hydantoinase gene clusters with a highly conserved organisation.

Completely different gene organisation could be observed for the gene cluster of *Ochrobactrum* sp. G21 and putative gene clusters of related microorganisms (Fig. 3). Very obviously, and unique for all these gene clusters is that the carbamoylase gene is located upstream in front of the hydantoinase gene, forming a *hyuC/hyuH* motif. This organisation is only found in the known hydantoinase gene cluster of *Arthrobacter crystallopoietes* DSM20117. Other analogies could not be obtained for all of these gene clusters. The only concordance could be demonstrated for the gene clusters of *Ochrobactrum* sp. G21, *Aurantimonas* and of *Sinorhizobium meliloti* in which hypothetical transport proteins and permeases could be observed downstream of *hyuC/hyuH*. Notably, *gltB* genes could be observed in the upstream direction of *hyuC/hyuH* for all bacteria, except for *Ochrobactrum* sp. G21.

Phylogenetic and Structural Comparison of Hydantoinases

The non-ATP dependent L-hydantoinase from *A. aurescens* DSM3745 belongs, together with dihydropyrimidinase/ D-hydantoinase and allantoinase, to the superfamily of amidohydrolases related to ureases [36, 37]. N-methylhydantoinase or 5-oxoprolinase are not associated to this family. The authors suggested that the enzymes of this superfamily have evolved from a common ancestor and are the product of a divergent evolution. The amino acid sequence of members of this superfamily show a low sequence identity (always less 30%) but the tertiary structure always consists of the same TIM (triose phosphate isomerase) barrel fold [38, 39, 40, 41, 42, 43, 44, 45] indicating a very similar central (α/β)₄-barrel with strong variation in the β -sheet. They all belong to a metalloenzyme family with a significant conserved binuclear metal centre. The active site of known hydantoinases is located at one side of the barrel distant from the β -rich domain at the end of a hydrophobic cleft. In all

of the enzymes of this superfamily the structure responsible for the activation of the hydrolytic reaction is highly conserved: A water molecule is situated between the two metal ions resulting in a reduction of the pK_a of this water molecule. The ligands of this metal ion (either zinc or nickel) are two histidines each, one aspartate and one carboxylated lysine (summarised from [38]).

As described above hydantoinases are members of a superfamily of amidohydrolases related to ureases but one remaining question concerns the phylogenetic relationship of hydantoinases/ dihydropyrimidinases themselves. In order to investigate this a phylogenetic tree was built in this study using different D- and L-hydantoinases/ dihydropyrimidinases from various bacterial genera, including putative D-hydantoinases (Figure 4). Clearly, the L-hydantoinases, including the L-hydantoinase from *Arthrobacter aurescens* DSM3745, were disjoined from the other D-hydantoinases. The major tree consists of five branches each branch accommodating hydantoinases of phylogenetically related bacteria disregarding several exceptions. Six major groups can be distinguished by this phylogenetic tree (Fig. 3) and an alignment of all hydantoinases used (data not shown):

The *Rhizobiales* family (Rhizo-Fam) is the most inconsistent group in which most bacteria belong to the order *Rhizobiales* or to the same bacterial class with exception of one *Pseudomonas*. This *Pseudomonas*, *Pseudomonas* sp. KNK003A, is more likely to be an *Agrobacterium* species (personal communication). The hydantoinase of *Ochrobactrum* sp. G21 can be located in this group. More unique and limited are the other groups in which only hydantoinases from bacteria of a certain class or family can be found: *Comamonadaceae* family (Com-Fam) including *Delftia* sp. I24, *Pseudomonas* family (Pseud-Fam), *Bacilli* family (Bac-Fam), including *Brevibacillus agri* and several *Bacillus* species. Interestingly, two hydantoinases from *Agrobacterium* species were separated from the other *Rhizobiales*, together with one hydantoinase from *Burkholderia* and due to the higher number of hydantoinases from the genus *Agrobacterium* designated as *Agrobacterium* family (Agro-Fam). One reason could be that these three hydantoinases are plasmid-encoded of the respective wild-type strains and the other hydantoinases from the Rhizo-Fam are found in the chromosomal DNA. The other strains remaining can not be classed into a unique group. In summary, we have shown that hydantoinases can be clustered according to their phylogenetic origin and form unique families. This is not surprising since members of the superfamily of amidohydrolases are a product of a divergent evolution [36]. We assume, since hydantoinases are members of this family they are expected to show an evolution similar to the superfamily because divergent evolution can lead to a close relationship of phylogenetically related species. In this study we confirmed the theory of the common ancestor for this superfamily, since the same was observed for hydantoinases.

In literature several crystal structures and the respective catalytic and functional sites of hydantoinases/ dihydropyrimidinases are reported: *Bacillus stearothermophilus* SD1 [42], *Bacillus* sp. AR9 [39], *Arthrobacter aurescens* DSM3745 [38], *Burkholderia pickettii* [47] and *Thermus* sp.

[39]. A highly conserved motif for the superfamily of amidohydrolases is the motif GxxDx-HxH, suggested to be involved in metal centre assembly [37]. The histidines of this motif are known to be ligands for metal binding of ureases and dihydroorotate synthases [48, 49]. The active site of hydantoinases consists of His58, His60, Lys150 (in most cases carboxylated), His183, His239 and Asp315 [38, 39, 42, 46, 47]. It was shown by Cheon *et al.* [42] that the catalytic structure of hydantoinases is highly conserved, whereas the substrate recognition is not. The hydantoinase from *Bacillus stearothermophilus* SD-1 has been well studied [42, 50, 51] and it was shown that the recognition of the exocyclic site chain of 5-D-monosubstituted hydantoin takes place in a completely buried hydrophobic substrate binding pocket. This pocket is formed by the hydrophobic and bulky residues of three loops, so called stereochemistry gate loops (SGLs; see Figure 5), whereas the side chains of the residues Met63, Leu65, Phe152, Tyr155 and Phe159 play the mayor role. In particular, the hydrophobic and bulky residues of amino acids in SGL-3, Phe152, Tyr155 and Phe159 are responsible for interaction with the chiral exocyclic substituent of the substrate. Mutagenesis studies of residues involved in the hydrophobic substrate binding pocket showed that only a small effect on hydantoinase activity or substrate specificity was observed when Leu65 was changed to Phe65. A predominant role has been proposed for Tyr155 for substrate binding and for the transition state [39, 42]. Phe159 is also part of the hydrophobic lid and interacts strongly with the exocyclic substituent of the substrate. Mutagenesis studies showed that this residue can affect the size of the binding pocket and modulate the substrate specificity [51].

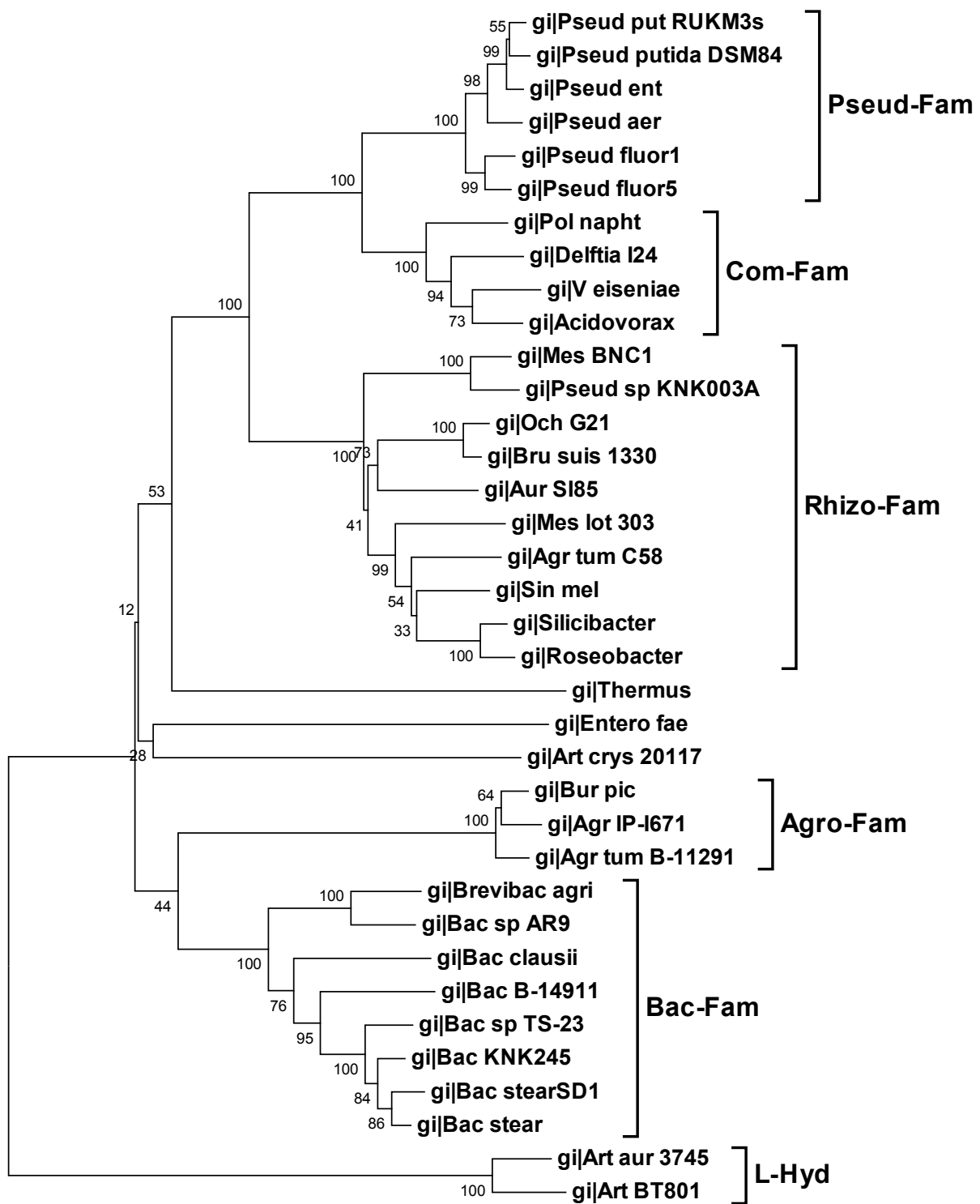


Figure 4: Phylogenetic tree of hydantoinases and dihydropyrimidinasases.

Additional description of Figure 4: The sequences were aligned using MEGA, version 3.1 based on ClustalX (Gonnet-matrix). The tree was constructed using the same program selecting the minimum evolution method [24]. Bootstrap analysis was performed with 1000 replicates. The hydantoinases described in this study are highlighted in boxes. Sources of amino acid sequences were: **Acidovorax**: putative D-Hyd, *Acidovorax avenae* subsp. *citrulli* AAC00-1 (EAT96435.1); **Agr IP-I671**: D-Hyd, *Agrobacterium* sp. IP I-671 (AF335479 3); **Agr tum B-11291**: D-Hyd, *Agrobacterium tumefaciens* NRRL B-11291 (Q44184); **Agr tum C58**: putative DHP; *Agrobacterium tumefaciens* str. C58 (NP 533058.1); **Art BT801**: L-Hyd, *Arthrobacter* sp. BT801 (AAL55412.1); **Art aur 3745**: L-Hyd, *Arthrobacter aurescens* DSM3745 (P81006); **Art crys 20117**: D-Hyd, *Arthrobacter crystallopoietes* DSM20117 (AAO24771.1); **Aur SI85**: putative DHP, *Aurantimonas* sp. SI85-9A1 (ZP 01226320.1); **Bac sp AR9**: D-Hyd, *Bacillus* sp. AR9 (AAV65953.1); **Bac B-14911**: putative DHP, *Bacillus* sp. NRRL B-14911 (ZP 01170481.1); **Bac KNK245**: putative DHP, *Bacillus* sp. KNK245 (BAE16757.1); **Bac sp TS-23**: DHP, *Bacillus* sp. TS-23 (AAY18594.1); **Bac clausii**: putative DHP, *Bacillus clausii* KSM-K16 (YP 177275.1); **Bac stear**: D-Hyd, *Bacillus stearothermophilus* NS1122A (AAC60487.1); **Bac stearSD1**: D-Hyd *Bacillus stearothermophilus* SD-1 (1K1D E); **Brevibac agri**: DHP, *Brevibacillus agri* (AAO66292.1); **Bru suis 1330**: putative D-Hyd, *Brucella suis* 1330 (AAN29227.1); **Bur pic**: D-Hyd, *Burkholderia/ Ralstonia pickettii* (1NFG D); **Delftia I24**: D-Hyd, *Delftia* sp. I24 (this study); **Entero fae**: putative DHP, *Enterococcus faecalis* V583 (NP 816221.1); **Mes BNC1**: putative DHP, *Mesorhizobium* sp. BNC1 (YP 675206.1); **Mes lot 303**: putative DHP, *Mesorhizobium loti* MAFF303099 (BAB48959.1); **Och G21**: D-Hyd, *Ochrobactrum* sp. G21 (this study); **Pol naph**: D-Hyd, *Polaromonas naphthalenivorans* CJ2 (ZP 01019452.1); **Pseud sp KNK003A**: Hyd, *Pseudomonas* sp. KNK003A (BAE20330.1); **Pseud aer**: putative DHP, *Pseudomonas aeruginosa* PAO1 (NP 249132.1); **Pseud ent**: putative DHP, *Pseudomonas entomophila* L48 (YP 608802.1); **Pseud fluor1**: putative Hyd, *Pseudomonas fluorescens* PfO-1 (YP 349170.1); **Pseud fluor5**: putative Hyd, *Pseudomonas fluorescens* Pf-5 (YP 259655.1); **Pseud putida DSM84**: D-Hyd, *Pseudomonas putida* DSM84 (Q59699); **Pseud put RUKM3s**: L-Hyd, *Pseudomonas putida* RUKM3s(sequence not in database, personal communication); **Roseobacter**: putative DHP, *Roseobacter* sp. MED193 (ZP 01055868.1); **Silicibacter**: D-Hyd, *Silicibacter* sp. TM1040 (YP 613492.1); **Sin mel**: putative DHP, *Sinorhizobium meliloti* (ABG76935.1); **Thermus**: D-Hyd, *Thermus* Sp. (1GKP); **V eiseniae**: putative DHP, *Verminephrobacter eiseniae* EF01-2 (EAT75980.1).

In this study a more detailed consideration of the hydantoinase amino acid sequences of the newly defined families revealed homologies concerning the amino acids involved in the catalytic site and substrate recognition, as described above. Therefore we used the amino acid sequence information of the hydantoinases, also used in the phylogenetic tree, for a ClustalW alignment. With exception of the one from *Bacillus stearothermophilus* SD-1, hydantoinases from each hydantoinase group showed conserved amino acid residue at amino acid positions being involved in the active site and substrate recognition. For a simplified display of the aligned sequences one bacterial member of each family was chosen as representative (Figure 5). The conserved motif of the metal centre assembly (GxxDxHxH) and the conserved amino acids of the active site, were found for all hydantoinases compared in this study (Fig. 5).

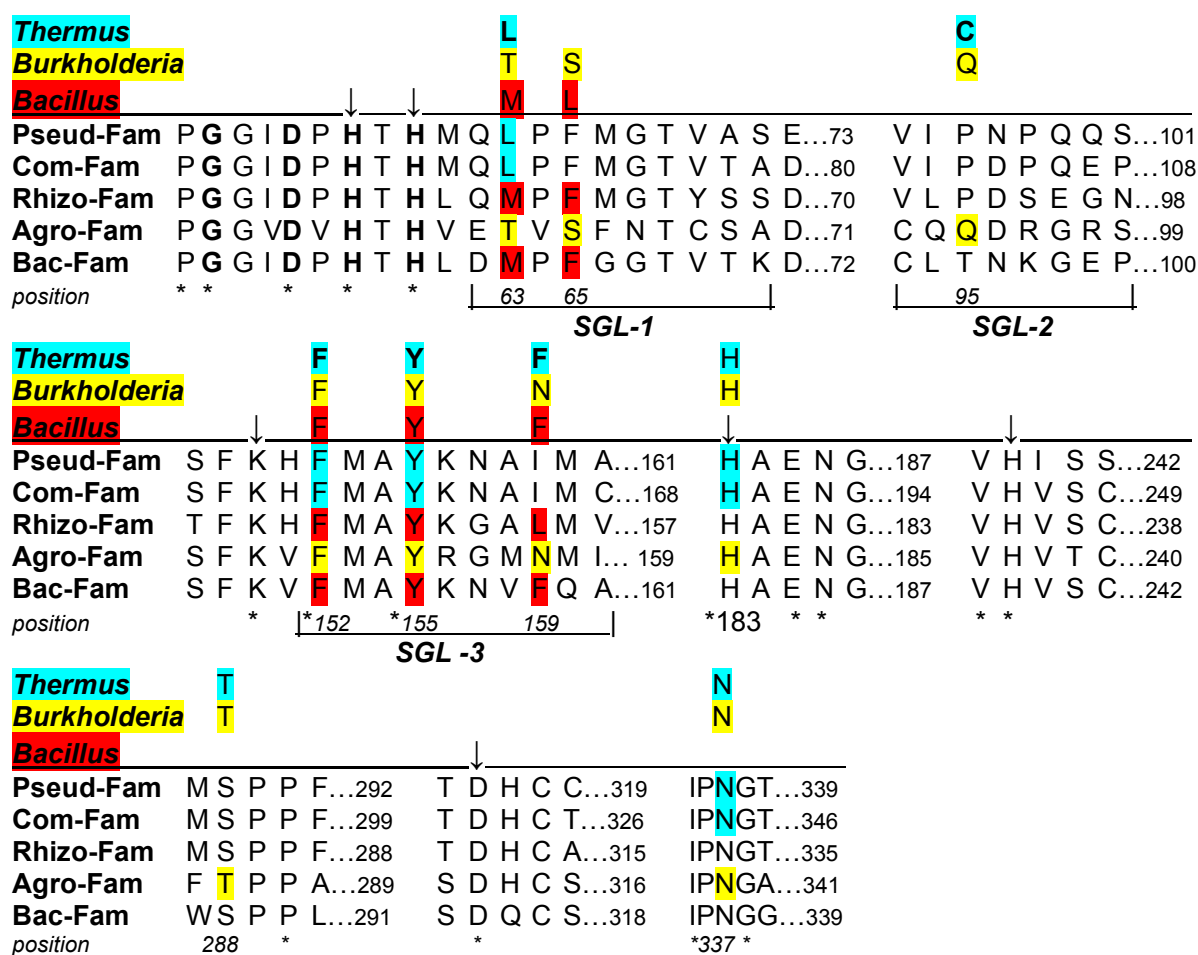


Figure 5: Alignment of selected amino acid regions significant for D-hydantoinase conversion. One representative strain was used for each hydantoinase family: **Pseud-Fam:** *Pseudomonas putida* RUKM3s; **Com-Fam:** *Delftia* sp. I24; **Rhizo-Fam:** *Ochrobactrum* sp. G21; **Agro-Fam:** *Agrobacterium* sp. IP I-671; **Bac-Fam:** *Bacillus* sp. TS-23. *Bacillus* sp. TS-23 was used as a basis for numbering of important amino acids.

Additional description of Figure 5: Identical amino acids of all 36 D-hydantoinases aligned are indicated by asterisks (*). The GxxDxHxH-motif is bold. Amino acids associated with the catalytic centre are highlighted by an arrow from the top. Amino acids involved in recognition sites of hydantoinases described by crystal structure determination and structural analysis are shown on top of the alignment. *Bacillus*: *Bacillus stearothermophilus* SD1 [42, 51]; *Burkholderia*: *Burkholderia pickettii* [47], *Thermus*: *Thermus* sp. [39]. The stereochemistry gate loops (SGL) obtained for the hydantoinase of *Bacillus stearothermophilus* SD1 are shown below the sequence at the appropriate positions. Amino acids being part of the functional recognition sites of hydantoinases with determined crystal structures and shown to be identical within the proposed families are highlighted by different colours.

Hydantoinases of the Bac-Fam and Rhizo-Fam showed high similarity with respect to the amino acids involved in the functional recognition sites of *Bacillus stearothermophilus* SD-1, as described above. The Bac-Fam showed only one difference in position 65, in which Phe can be found instead of Leu (Fig. 5). Only a minor effect on activity and substrate selectivity of this substitution for the hydantoinase of *Bacillus stearothermophilus* SD-1 was observed [51]. These authors also reported the influence of a mutation at position 159 of the hydantoinase of *Bacillus stearothermophilus* SD-1 from Phe to Leu. This mutation led to lower activity for all substrates tested but no loss in activity [51]. As seen in Figure 5 the same substitutions were obtained for all members of the Rhizo-Fam.

Quite prominent is the identity of all amino acids involved in the recognition sites of *Burkholderia pickettii* and the other members of the Agro-Fam (Fig. 3). The crystal structure of the D-hydantoinase of *Burkholderia pickettii* has been solved and the overall structure and catalytic site was found to be similar to other hydantoinases [47]. A significant feature was found in the functional recognition site of the D-hydantoinase of *Burkholderia pickettii*: Tyr153, His 181, Thr286 and Asn335 are responsible for recognition of the amide group of hydantoin by formation of hydrogen bonds. It is notable that the substitution of Thr286 by Ser, as found in the hydantoinase of *Thermus* sp., does not affect the interaction with the substrate. The exocyclic substituent recognition sites were determined as Thr62, Ser64, Gln93, Phe150, Tyr153 and Asn157. Comparing amino acids involved in the substrate recognition of the hydantoinase from *B. pickettii* and the other Agro-Fam members, absolutely identical amino acids were obtained (Fig. 5).

In literature the structures of the dihydropyrimidinase of *Thermus* sp. and the L-hydantoinase of *Arthrobacter aurescens* DSM3745 were described, too [38, 39]. In both studies the importance of the hydrophobic pocket, consisting of Leu64, Cys95, Phe152, Tyr155 and Phe159, of the D-hydantoinase of *Thermus* sp. on substrate specificity was shown. A remarkable role was shown for Tyr155 which is part of this hydrophobic pocket and stabilises the transition state with its hydroxyl group. In this transition state additionally Ser288, His183 and Asn336 were involved.

Comparing all these amino acid positions to the equivalent amino acid residues of the remaining two families, the Com-Fam and Pseud-Fam, no significant homology could be obtained. In fact, there are conserved amino acids but no high concordance, as seen for the other hydantoinase families described above. Nevertheless, the amino acids of hydantoinases of the Com-Fam and Pseud-Fam showed 100% identity in positions shown to be involved in recognition sites of the hydantoinases mentioned above.

Summarising the comparison of structurally important amino acids we can conclude that we have found a very high similarity in "functional" amino acids of the Bac-Fam and Rhizo-Fam to the ones of *Bacillus stearothermophilus* SD-1 as well as identical amino acids of the Agro-Fam and *Burkholderia pickettii*. An attractive hypothesis for these three groups would be that the conserved amino acids described form the substrate recognition sites of all members of each family. However, this has to be proved at least by modelling of the hydantoinases. Taking into account the two other families, we are at least able to state that each of the proposed families is highly conserved as a result of parallel evolution of the families.

Conclusions

We have elucidated two gene clusters responsible for the hydantoin degradation in *Delftia* sp. I24 and a moderate halophilic *Ochrobactrum* sp. G21. No information on the genetic organisation of hydantoin degradation of members of these genera has been described in literature previously.

We have shown a high similarity of these gene clusters to putative gene clusters from related bacteria. Phylogenetic comparison of different hydantoinases and grouping of them into specific families with identical sites for the recognition of hydantoins showed that in most cases hydantoinases from related bacteria share the highest homology. This supports the theory that hydantoinases evolved from a common ancestor and their evolution takes place parallel to phylogenetic evolution for each genus.

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Various bacteria with hydantoinase activity were recovered from terrestrial soil samples of different geographic origins (Antarctica, South Africa, China). Based on these findings it is shown that microorganisms with hydantoinase activity are (i) distributed in various geographically distinct environmental habitats (ii) distributed worldwide (iii) found in certain bacterial genera.

Three microorganisms were selected for further characterization: a halophilic *Bacillus megaterium* F18, a halophilic *Ochrobactrum* sp. G21 and *Delftia* sp. I24. The hydantoinases of these three strains showed unique enzyme properties.

The gene clusters responsible for hydantoin degradation of *Ochrobactrum* sp. G21 and *Delftia* sp. I24 were elucidated. Furthermore, in phylogenetic studies we have shown that hydantoinases can be classed according to their origin in different families.