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Genes responsible for hydantoin degradation of a halophilic *Ochrobactrum* sp. G21 and *Delftia* sp. I24 — New insight into relation of D-hydantoinases and dihydropyrimidinases

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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 (*gltB*), dihydropyrimidine dehydrogenase (*pydA*), permease (*hyuP*) and an incomplete D-hydantoinase (*hyuH*). The hydantoinase gene sequence was completed by PCR amplification. The putative gene cluster of O*chrobactrum* sp. G21 comprised nine ORFs, six being potentially involved in hydantoin-hydrolysation: 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 organization 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), Comamonadacae 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 posses the same recognition sites as known hydantoinases.
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1. 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, dihydropyrimidinases 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.

Abbreviations: Agro-Fam, Agrobacterium family; Bac-Fam, Bacilli family; BnH, 5-benzylhydantoin; Com-Fam, Comamonadacae 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 familiy; Rhizo-Fam, Rhizobiales family; SGLs, stereochemistry gate loops.

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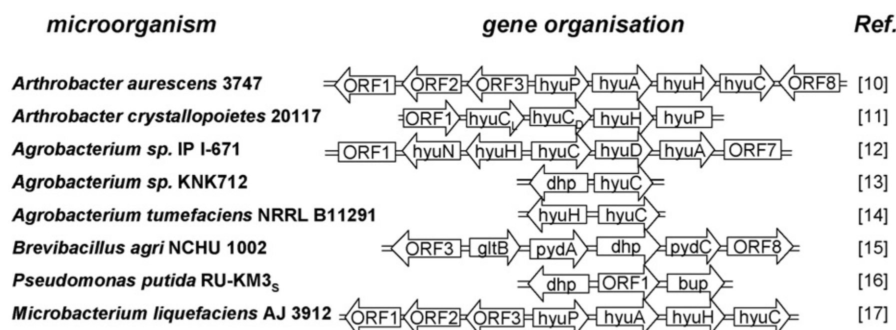

Fig. 1. Genetic organization of genes encoding for the enzymes involved in hydantoin and/or dihydropyrimidine hydrolysis from different microbial sources (hyuP: permease; hyuA: racemase; hyuH: hydantoinase; hyuC: carbamoylase; hyuN: NADP-flavin oxidoreductase; hyuD: amino acid dehydrogenase; dhp: dihydropyrimidinase;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

Arthrobacter crystallopoietes DSM20117 [1], *Bacillus* sp. AR9 [2], *Bacillus stearothermophilus* SD-1 [3,4], *Pseudomonas* sp. NCIM 5109 [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 dihydropyrimidinases. 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 dihydropyrimidinases.

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-methinonine, 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 an unique operon, whereas other genes coding for e.g. racemase and permease are not commonly present. However, the genetic organization 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 organization.

Different approaches have been used to identify these clusters. A genomic phage library of *Agrobacterium* sp. IP-I671 was screened by plaque hybridisation using a DIG-labeled *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 dihyropyrimidinase and β-ureidopropionase gene of *Pseudomonas putida* RU-KM3s were identified by transposon mutagenesis and selection of altered growth phenotypes [16].

In the present study we describe the isolation and characterization 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 aid of a DIG-labeled 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 organization of hydantoin cleaving enzymes in these two families.

2. Materials and methods

2.1. 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.

2.2. 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 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.

2.3. 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 GeneJET™ 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], BPROM (available at www.softberry.com) and NEBcutter [23]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 [24].

2.4. Preparation of a 320 bp DNA fragment

Two homologous probes for hydantoinase 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× Taq buffer with $(NH_4)_2SO_4$, 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 Clone™ PCR Cloning kit (Stratagene, USA). The obtained plasmid inserts were excited by different restriction enzymes and purified.

2.5. Preparation of DIG-labeled 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.

2.6. DNA digest and southern blot

Chromosomal DNA was digested using different restriction enzymes (EcoRI, HindIII) 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.

2.7. Plasmid preparation, cloning and screening

Plasmid DNA (pUC19) was digested using either EcoRI or HindIII. Subsequent dephosphorylation was conducted by addition of shrimp alkaline phosphatase. Ligation was carried out under the following conditions: 50 ng Vector DNA (pUC19/EcoRI or pUC19/HindIII), 2× molar excess of the purified DNA fragment, 1× ligation buffer, T4-DNA-ligase and water (nuclease free), combined to a volume of 20 μL. The ligated DNA was transformed into *E. coli* XL-1 [26], plated on LB-Amp supplemented with 38.4 μg/mL IPTG and 40 μg/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 2× SSC for 15 min. Cell debris were washed out with 2× 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 the restriction enzymes used for

cutting 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, bloted 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 [28] or by a QIAfilter Plasmid Maxi Kit (Qiagen, Germany). The obtained plasmids were sequenced and analysed (see above).

2.8. 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′-AAAAAGCTT AGCGCGCCACGGCCGTGGGCT-3′), the resulting gene fragment cloned into the vector pJOE4786.1 which was cut by SmaI. The resulting plasmid was cut by BamHI and NdeI 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′-AAAAAAAGCTTAGACCCCTATCGGCATGTGTTCGGCGCT-3′). The fragment was cut by HindIII and NdeI and inserted into the expression vector pJOE5427.5 resulting in the vector pJOE5702.1. The expression vectors pJOE5697.2 and pJOE5427.5 are both derived from the L-rhamnose-inducible vector pWA21 [29] by deletion of two small NaeI fragments. The plasmid pJOE5697.2 has in addition a polylinker sequence downstream of the eGFP gene. In both plasmids the eGFP gene is replaced during cloning of the hydantoinase genes. The hydantoinase plasmids pJOE5704.1 and pJOE5702.1 were brought into *E. coli* JM109 by transformation using standard procedures.

2.9. 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% H_3PO_4) solution. The flow rate was 1 mL/min. Detection was carried out at 210 nm or 280 nm, respectively.

3. Results and discussion

3.1. 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. These DNA fragments were used for preparation of a DIG-labelled probe.

3.2. 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 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 contain the same DNA information, except pUC19-L1-61 more in downstream direction. Hence, the insert of plasmid pUC19-L1-61 was completely sequenced.

3.3. Sequence analysis of the insert in the plasmid 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 4790 known 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 [30,31]. Analogously to other hydantoin gene clusters the genes involved in hydantoin degradation were abbreviated as *hyu* "hydantoin utilization" [32]. The ORFs can be attributed to the following proteins (Table 1 and for overview Fig. 2a):

Table 1
Overview of ORFs obtained from plasmid pUC19-M50 (*Delftia* sp. I24)

Gene	Start	End	Length (bp)	GC (%)	Putative Shine Dalgarno sequence	Assumed function
gltB[a]	–	319	–	–	–	Glutamate synthase
pydA	354	1673	1320	67	GAGA*AGGAGC*TTCGCA**ATG**	Dihydropyrimidine dehydrogenase
hyuP	1806	3299	1494	65	AGA*AGGAGC*TTCGCA**ATG**	Permease
hyuH	3330	4790	1461	68	C*AGGAGG*CATGACGCT**ATG**	Hydantoinase

The putative Shine Dalgarno sequence is underlined and italics. The start codons of the ORFs are bold.
[a] ORF not complete.

gltB: The deduced incomplete amino acid sequence of this first ORF could be assigned to the family of NADPH-dependent glutamate synthases (beta chain) and related oxidoreductases by comparison to a conserved domain database [33].

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 [33]. Additionally, a Kyte–Doolittle hydropathy plot [34] 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 *P. putida* DSM84 [35,36]. Interestingly, the highest homologies (compare Fig. 2a) of the deduced protein sequence was found with 98% to a putative dihydropy-

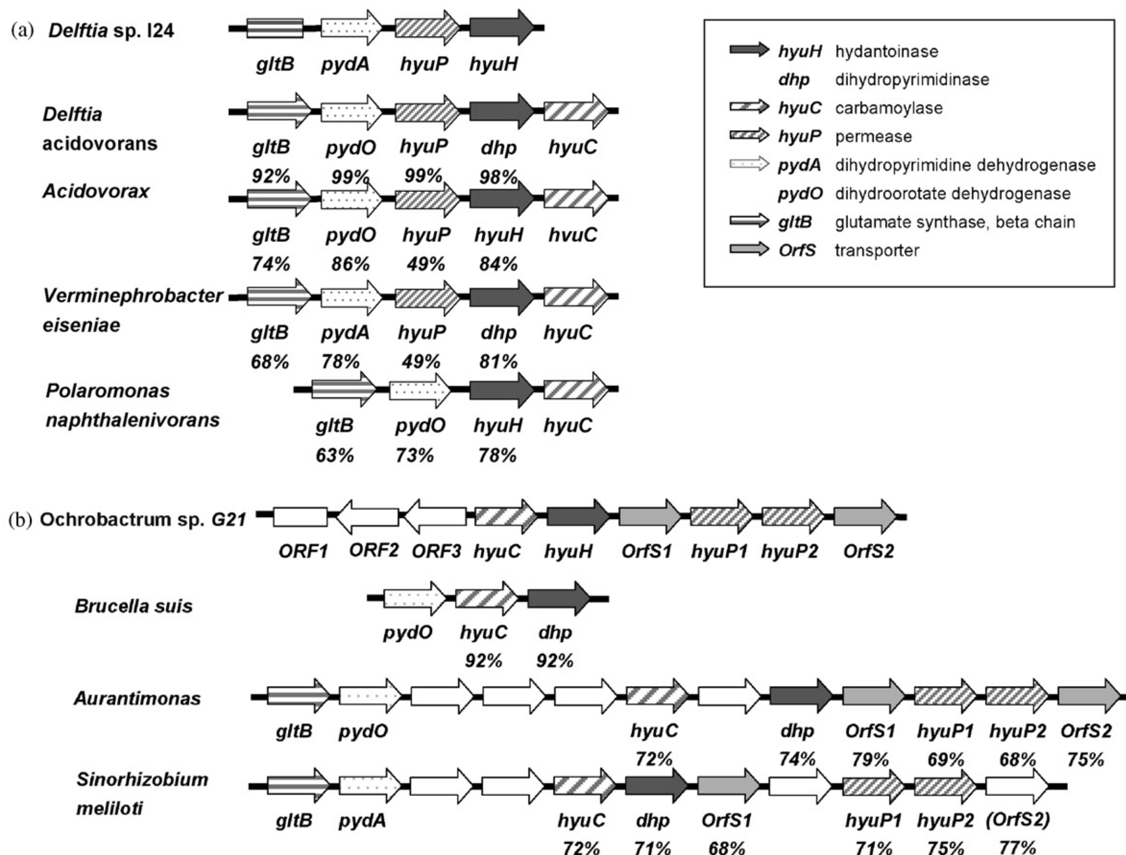

Fig. 2. (a and b) Overview over different gene clusters probably coding for the conversion of hydantoins (the clusters of *Delftia* sp. I24 and *Ochrobactrum* sp. G21 were obtained within this study. The identity of homologous genes from other species to the obtained genes from *Delftia* sp. I24 and *Ochrobactrum* sp. G21 are shown below the assigned gene name. 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 (CP000542); *Polaromonas naphthalenivorans* CJ2 (NZ_AANM01000002); *Acidovorax avenae* subsp. *citrulli* AAC00-1 (NZ_AASX01000002); *Brucella suis* 1330 (AE014291); *Aurantimonas* sp. SI85-9A1 (NZ_AAPJ01000001); *Sinorhizobium meliloti* 1021 (SME591790). Incomplete ORFs are shown in rectangles. White arrows represent ORFs not involved in hydantoin conversion).

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)	hyuH from *Ochrobactrum* sp. G21	+	+
JM109 (pJOE5704.1)	hyuH from *Delftia* sp. I24	+	+

rimidinase of *D. acidovorans* SPH-1 (accession ZP_01581256), with 84% to a putative D-hydantoinase of *Acidovorax avenae* subsp. *citrulli* AAC00-1 (accession ABM31567), with 81% to a putative dihyropyrimidinase of *Verminephrobacter eiseniae* EF01-2 (accession EAT75980) and with 78% to a putative D-hydantoinase of *Polaromonas naphthalenivorans* CJ2 (accession YP_984234). All these organisms, including *Delftia* sp. I24, belong to the lineage of proteobacteria, burkholderiales, family of comamonadacaea. The expression of this gene in *E. coli* followed by biotransformations showed the conversion of the substrates BnH and IMH (Table 2). This proofs the assumed function as hydantoinase and this ORF was therefore named as hyuH.

The complete gene cluster likely to be responsible for hydantoin-hydrolysis of *Delftia* sp. I24 is accessible at the following gene bank entry at NCBJ: EF407881.

3.4. Sequence analysis of the Insert in the plasmid 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 (see as well Table 3 and Fig. 2b). Except for hyuC and hyuH all other deduced protein sequences are classified by comparison to a Conserved Domain Database [33]:

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 *B. 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 *B. 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. 2b). 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.

The complete gene cluster, which is probably responsible for hydantoin-hydrolysis of *Ochrobactrum* sp. G21 is accessible at the following gene bank entry at NCBJ: EF407882.

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

Table 3
Overview of ORFs obtained from plasmid pUC19-L1-61 (*Ochrobactrum* sp. G21)

Gene	Start	End	Length (bp)	GC (%)	Putative Shine Dalgarno sequence	Assumed function
ORF1[a]	–	207	–	–	–	Glucose transporter
ORF2	602	204	399	56	CGAGAATTCGTCATC**ATG**	Nudix-protein
ORF3	1297	599	699	53	AA*AGG*T*G*TCAA**ATG**	Regulator
hyuC	1517	2752	1239	58	GC*GGAGG*CAGAGCGC**ATG**	Carbamoylase
hyuH	2827	4281	1455	57	A*AGGGG*AACGACGAACA**ATG**	Hydantoinase
OrfS1	4285	5151	867	55	AT*AGG*G*G*TCTGAGAC**ATG**	Part of transporter
hyuP1	5188	6081	894	57	GCT*GGAG*ATCGTCC**ATG**	Permease
hyuP2	6078	6938	861	54	AAAGGGCAGCCAC**ATG**	Permease
OrfS2	7052	8050	999	56	AG*AGGAG*AACTGAA**ATG**	Part of transporter

The putative Shine Dalgarno sequence is underlined and italics. The start codons of the ORFs are in bold.
[a] ORF not complete.

for *P. putida* RU-KM$_{3S}$ [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*.

3.5. 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 Figs. 1 and 2a). Interestingly, the *Comamonadaceae* gene clusters showed a very high conservancy of enzymes involved in hydantoin degradation: (a) All ORFs were shown to be orientated in the same direction. (b) 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. 2a). (c) In all clusters hydantoinase and carbamoylase homolog genes were present, all ot 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. 2a). 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 *B. 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. 2a) to other clusters shown in Fig. 1, no conserved pattern could be observed for this group, excepting *B. agri*. Taking into account all these observations, we can assume that gene clusters of these members of the family Comamonadacae and *B. agri* form their own class of hydantoinase gene clusters with a highly conserved organization.

Completely different gene organization could be observed for the gene cluster of *Ochrobactrum* sp. G21 and putative gene clusters of related microorganisms (Fig. 2b). 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 organization is only found in the known hydantoinase gene cluster of *A. 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.

3.6. Phylogenetic and structural comparison of hydantoinases

The non-ATP dependent L-hydantoinase from *A. aurescens* DSM 3745 belongs, together with dihydropyrimidinase/D-hydantoinase and allantoinase, to the superfamily of amidohydrolases related to ureases [37,38]. 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 [39–46]. 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 [39]).

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 using different D- and L-hydantoinases/dihydropyrimidinases from various bacterial genera, including putative D-hydantoinases (Fig. 3). Clearly, the L-hydantoinases, including the L-hydantoinase from *A. aurescens* DSM3745, were disjoined from the other D-hydantoinases. The major tree consists of five branches each branch accommodating hydantoinases of phylogenetically related bacteria disregarded several exceptions. Six major groups can be distinguished from 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 of bacteria from a certain class or family can be found: Comamonadacae family (Com-Fam) including *Delftia* sp. I24, Pseudomonas family (Pseud-Fam), Bacilli family (Bac-Fam), including *B. 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-

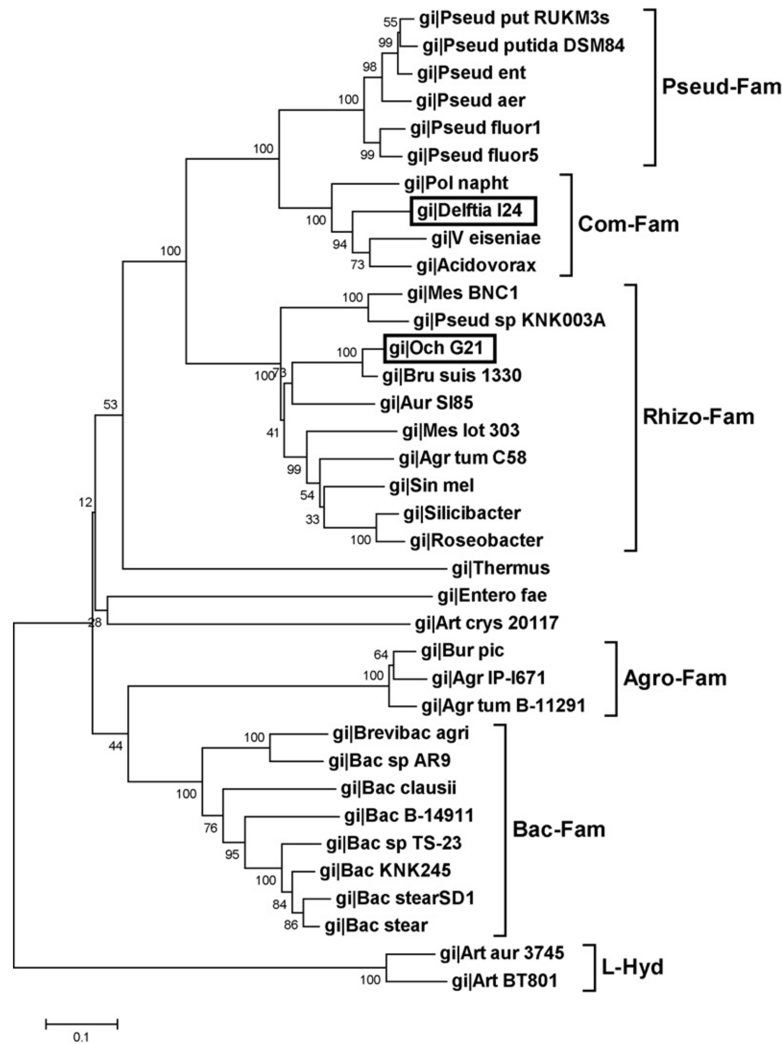

Fig. 3. Phylogenetic tree of hydantoinases and dihydropyrimidinases. The sequences were aligned using MEGA, version 3.1 based on ClustalX (Gonnet-matrix). The tree was constructed using the same program selecting the minimum evolution method [24]. Bootstrap analysis was performed with 1000 replicates. The hydantoinases described in this study are highlighted in boxes. Sources of amino acid sequences were: Acidovorax: putative D-Hyd, *A. 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* DSM 3745 (P81006); Art_crys_20117: D-Hyd, *Arthrobacter crystallopoietes* DSM 20117 (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 *B. stearothermophilus* SD-1 (1K1D_E); Brevibac_agri: DHP, *Brevibacillus agri* (AAO66292.1); Bru_suis_1330: putative D-Hyd, *B. 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_napht: D-Hyd, *P. 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* DSM 84 (Q59699); Pseud_put_RUKM3s: L-Hyd, *P. putida* $RUKM_{3s}$ (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, *S. meliloti* (ABG76935.1); *Thermus* sp. D-Hyd, *Thermus* sp. (1GKP); V_eiseniae: putative DHP, *V. eiseniae* EF01-2 (EAT75980.1).

Fam are found in the chromosomal DNA. The other strains remaining cannot 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 [37]. 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 phylogenetic related species. In this study we confirmed the theory of the common ancestor for this superfamily, since the same was observed for hydantoinases.

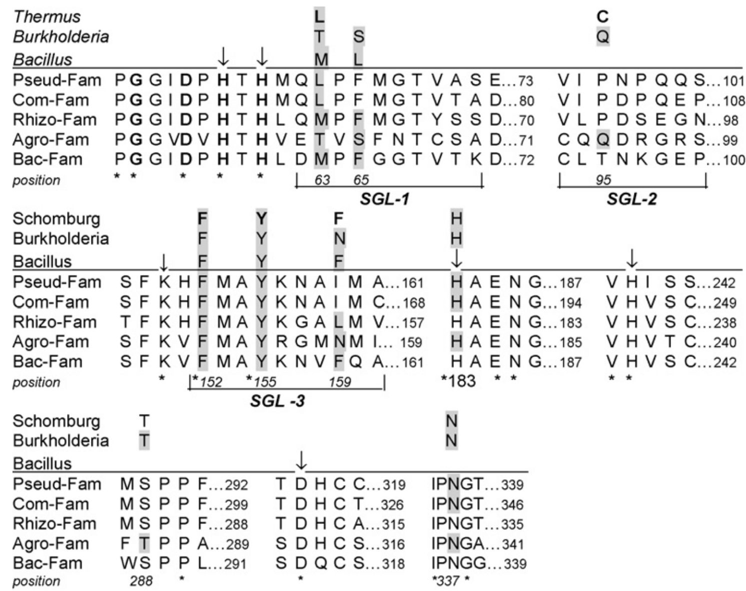

Fig. 4. Alignment of selected amino acid regions significant for D-hydantoinase conversion. One representative strain was used for each hydantoinase family: Pseud-Fam: *P. putida* RUKM$_{3s}$; Com-Fam: *Delftia* sp. I24; Rhizo-Fam: *Ochrobactrum* sp. G21; Agro-Fam: *Agrobacterium* sp. IP I-671; Bac-Fam: *Bacillus* sp. TS-23. *Bacillus* sp. TS-23 was used as a basis for numbering of important amino acids. Identical amino acids of all 36 D-hydantoinases aligned are indicated by asterisks (*). The GxxDxHxH-motiv 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 steraothermophillus* SD1 [43,52]; *Burkholderia*: *Burkholderia pickettii* [48], *Thermus*: *Thermus* sp. [40]. The stereochemistry gate loops (SGL) obtained for *Bacillus steraothermophillus* 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 grey shades.

In literature several crystal structures and the respective catalytic and functional sites of hydantoinases/dihydropyrimidinases are reported: *B. stearothermophilus* SD1 [43], *Bacillus* sp. AR9 [40], *A. aurescens* DSM 3745 [39], *Burkholderia pickettii* [48] and *Thermus* sp. [40]. A highly conserved motif for the superfamily of amidohydrolases is the motif GxxDxHxH, suggested to be involved in metal centre assembly [38]. The histidines of this motif are known to be ligands for metal binding of ureases and dihydroorotate synthases [49,50]. The active site of hydantoinases consists of His58, His60, Lys150 (in most cases carboxylated), His183, His239 and Asp315 [39,40,43,47,48]. It was shown by Cheon et al. [43] that the catalytic structure of hydantoinases is highly conserved, whereas the substrate recognition is not. The hydantoinase from *B. stearothermophilus* SD-1 has been well studied [43,51,52] and it was shown that the recognition of the exocyclic site chain of 5-D-monosubstituted hydantoins 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 Fig. 4), 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. Phe159 is also part of the hydrophobic lid and interacts strongly with the exocyclic subsitutent of the substrate. Mutagenesis studies showed that this residue can affect the size of the binding pocket and modulate the substrate specificity [52].

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 is given, 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 *B. 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 (Fig. 4). The conserved motifs 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. 4).

Hydantoinases of the Bac-Fam and Rhizo-Fam showed high similarity with respect to the amino acids involved in the functional recognition sites of *B. 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. 4). Only a minor effect on activity and substrate selectivity of this substitution for the hydantoinase of *B. stearothermophilus* SD-1 was observed [52]. These authors also reported the influence of a mutation at position 159 of the hydantoinase of *B. stearothermophilus* SD-1 from Phe to Leu. This mutation led to lower activity for all substrates tested but no loss in activity [52]. As seen in Fig. 4 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 *B. pickettii* and the other members of the Agro-Fam (Fig. 3). The crystal structure of the D-hydantoinase of *B. pickettii* has been solved and the overall structure and catalytic site was found to be similar to other hydantoinases [48]. A significant feature was found in the functional recognition sites of the D-hydantoinase from *B. pickettii*: Tyr153, His 181, Thr286 and Asn335 are responsible for recognition of the amide group of hydantoins 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. 4).

In literature the structures of the dihydropyrimidinase of *Thermus* sp. and the L-hydantoinase of *A. aurescens* DSM 3745 were described, too [39,40]. 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 Ser288, additionally 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 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 *B. stearothermophilus* SD-1, as well as identical amino acids of the Agro-Fam and *B. 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.

4. 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 organization of hydantoin degradation of members of these genera has been described in literature previously.

We have shown a high identity 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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