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ORIGINAL PAPER

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Cloning and characterization of genes from *Agrobacterium* sp. IP I-671 involved in hydantoin degradation

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Abstract Cloning and sequencing of a 7.1 kb DNA fragment from *Agrobacterium* sp IP I-671 revealed seven open reading frames (ORFs) encoding D-hydantoinase, D-carbamoylase and putative hydantoin racemase, D-amino acid oxidase and NAD(P)H-flavin oxidoreductase. Two incomplete ORFs flanking the hydantoin utilization genes showed similarities to genes involved in transposition. Expression of the D-hydantoinase and D-carbamoylase gene in *Escherichia coli* gave mainly inactive protein concentrated in inclusion bodies, whereas homologous expression on an RSF1010 derivative increased hydantoinase and D-carbamoylase activity 2.5-fold and 10-fold, respectively, in this strain. Inactivation of the D-carbamoylase gene in *Agrobacterium* sp IP I-671 led to a complete loss of detectable carbamoylase activity whereas the low hydantoinase activity remaining after inactivation of the D-hydantoinase gene indicated the presence of a second hydantoinase-encoding gene. Two plasmids of 80 kb and 190 kb in size were identified by pulsed-field gel electrophoresis and the cloned hydantoin utilization genes were found to be localized on the 190 kb plasmid.

Introduction

D-Amino acids like D-4-hydroxyphenylglycine and D-phenylglycine are important precursors of semisynthetic β-lactam antibiotics. A convenient way to produce such enantiomerically pure D-amino acids is the hydrolysis of the corresponding 5-monosubstituted hydantoins via enzymatic reactions employing D-hydantoinase and D-carbamoylase. Chemical or enzymatic racemization allows a complete bioconversion of racemic hydantoins to D-amino acids. Several gram-positive and gram-negative bacteria that are capable of hydrolysing hydantoins to D-amino acids have been described, including *Agrobacterium*, *Arthrobacter*, *Pseudomonas*, *Comomonas* and *Blastobacter* (for review, see Syldatk et al. 1999). From some of them, the relevant genes have been cloned and sequenced and/or the enzymes isolated and characterized. However, less is known about additional genes involved in hydantoin utilization, the genetic organization and genomic localization of the genes, their regulation, and the natural substrates of hydantoinases and carbamoylases. In order to get more information about these biotechnologically important enzymes, we isolated a 7.1 kb DNA fragment from a genomic library of *Agrobacterium* sp. IP I-671 (Runser et al. 1990) encoding D-hydantoinase and D-carbamoylase, sequenced the two genes and the flanking regions, characterized the gene products and finally located the genes on a large plasmid.

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Materials and methods

Bacterial strains

Agrobacterium sp. IP I-671 was described by Runser et al. (1990). *Agrobacterium* sp. RifR3 is a spontaneous rifampicin-resistant mutant and was obtained by plating 3×10^9 cells of *Agrobacterium* sp. IP I-671 on rifampicin-containing agar plates. *Escherichia coli* JM109 (Yanisch-Perron et al. 1985) was used for cloning experiments. For mobilization of plasmids from *E. coli* to *Agrobacterium* sp. RifR3 via conjugation, *E. coli* S17-1 (Simon et al. 1983) or, in the case of R6K-derived plasmids, *E. coli* S17-1/λpir (de Lorenzo and Timmis 1994) was used. The λRES phages were propagated in *E. coli* TAP90 (Patterson and Dean 1987) and converted into plasmids in HB101 F′*lac*[::Tn*1739tnpR*] (Altenbuchner 1993).

Culture conditions

E. coli cells were grown in LB liquid broth and on LB agar plates (Luria et al. 1960) at 37°C, and *Agrobacterium* strains in AM com-

plex medium at 30°C (Runser and Ohleyer 1990). Antibiotics were added in the following concentrations: ampicillin (100 μg ml^{-1}), tetracycline (15 μg ml^{-1}), nalidixic acid (40 μg ml^{-1}), rifampicin (50 μg ml^{-1}). For conjugation experiments, the two bacterial strains were harvested in the exponential phase of growth (OD_{600}=0.6) and mixed (1 ml of each). After centrifugation, cells were resuspended in 100 μl LB, transferred to a NC20 nitrocellulose filter (Schleicher & Schuell, Dassel), and then the filter was placed on a LB agar plate. After overnight incubation at 30°C, the cells were washed off the filters and plated on LB agar plates with antibiotics. In order to induce the L-rhamnose inducible expression system, *E. coli* was grown in LB at 30°C. At OD_{600}=0.3–0.5, L-rhamnose was added to a final concentration of 0.2% and the cells incubated for a further 4 h. Phages were propagated on LB agar plates overlaid with LB soft agar containing 0.2% maltose and 10 mM $MgCl_2$.

Construction of plasmids

For amplification of *hyuC*, the primers S783 (5′-CAT ATG ACA CGT CAG ATG TAC-3′) and S784 (5′-TCA GA(G/A) TTC CGC GAT CAG A-3′) were used. For amplification of *hyuH*, the primers S1003 (5′-AAA AAA GCT TCC GGA TTT ATT GCT TGT ATC-3′) and S1005 (5′-AAA ACA TAT GGA TAT CAT CAT CAA AAA C-3′) were used. The fragments were inserted as *Nde*I/*Bam*HI fragments between the *Nde*I/*Bam*HI sites of the vector pJOE2702 (Volff et al. 1996).

The suicide vector pMHS119 was constructed from the R6K-derived plasmid pUT-mini-Tn*5*Cm (deLorenzo and Timmis 1994) and the tetracycline resistance (*tet*) genes of Tn*1721* (Allmeier et al. 1992). The *tet* genes were amplified from pJOE105 with the primers Tc1 (5′-GAG CTC TCA GCG ATC GGC TCG TTG-3′) and Tc2 (5′-GAG CTC TCA ATC GTC ACC CTT TCT-3′); the resulting PCR fragment was cleaved with *Sac*I and inserted between the *Sac*I sites of pIC20H (pANK19). From here it was isolated again as a *Hin*dIII fragment and inserted into the *Hin*dIII site of pIC20R (Marsh et al. 1984) to give pMHS18. The chloramphenicol resistance gene was deleted from pUT-mini-Tn*5*Cm by *Eco*RI/*Sca*I and replaced by an *Eco*RI/*Sca*I fragment of pIC20R which was first deleted by *Nar*I/*Sma*I. Between the *Eco*RI/*Sph*I site of this new pUT derivative, pSB310, the tetracycline resistance genes from Tn*1721* were inserted as an *Eco*RI/*Sph*I fragment obtained from pMHS18 to give pMHS77. The *hyuC* gene and flanking regions were inserted into pIC20R as an *Eco*RV/*Kpn*I fragment (pMHS101), the new plasmid cleaved by *Sal*I and the *Sal*I site filled in by Klenow polymerase. Finally, this mutated fragment was isolated again as an *Eco*RI fragment and inserted into the *Eco*RI site of pMHS77 to give pMHS119.

To construct the suicide vector pMHS325, the *oriT* of RP4, amplified with the primers S1594 (5′-TTA TAG AGC TCC CGG CCA GCC TCG CAG AGC A-3′) and S1595 (5′-TAT AGG ATC CTG TAG ACTT TCC TTG GTG T-3′) from RP4 (Pansegrau et al. 1994) was first inserted between the *Sac*I/*Bam*HI sites of pUC18. The *tet* genes of pMHS18 were obtained on a *Bam*HI/*Xba*I fragment and inserted into this new plasmid cut with the same enzymes to give pMHS298. A *Nru*I fragment of pMHS242 with the *hyuH* gene was inserted into pIC20R and a 71 bp *Eco*NI fragment deleted. The *Eco*NI ends were filled in by Klenow polymerase, ligated and the modified *Eco*RI fragment inserted into the *Eco*RI site of pMHS298 to give pMHS325.

For expression of *hyuC* and *hyuH*, respectively, the vector pMHS93 was constructed by fusion of a *Hin*dII fragment of RSF1010 encoding the rep- and mob-functions with an *Ssp*I/*Xmn*I fragment from pMHS18 carrying the *tet* genes, followed by a deletion of a small *Eco*RI fragment. The *hyuC* gene was obtained from pMHS101 (see above) as an *Eco*RI fragment and inserted into the *Eco*RI site of pMHS93 to give pMHS108. The plasmid pMHS296 with the *hyuH* gene was constructed in the same way. First, an *Nru*I fragment from pMHS242 was inserted into the *Sma*I site of pIC20R (pMHS276) and from here inserted into the *Eco*RI site of pMHS93.

DNA manipulations

Plasmid DNA for sequence analysis was isolated using the Qiawell 8 Plasmid-Kit from Qiagen (Hilden, Germany). Genomic DNA of *Agrobacterium* ssp. strains was isolated using the Nucleospin C+T DNA Isolation kit from Macherey & Nagel (Düren, Germany). To construct a genomic library of *Agrobacterium* sp. IP I-671, chromosomal DNA was partially digested with *Xho*II. Fragments of 10–14 kb were cloned in *Bam*HI/*Sal*I-cleaved phage λ RESIII and in vitro packaged as described (Altenbuchner 1993). Standard DNA manipulation techniques were performed according to Sambrook et al. (1989).

DNA sequencing

Templates for DNA sequencing were prepared by subcloning DNA fragments in pIC vectors (Marsh et al. 1984). Double-stranded DNA was sequenced using the ALFexpress sequencer and the ALFexpress AutoRead Sequencing Kit from Pharmacia Biosystems (Freiburg, Germany) as described (Volff et al. 1996). Database searches were done with the programs BlastP, BlastN and BlastX (Altschul et al. 1990).

Pulsed-field gel electrophoresis

Conditions for embedding *Agrobacterium* cells in agarose, purification of the DNA, S1 nuclease treatment and separation of the DNA by pulsed-field gel electrophoresis (PFGE) were essentially as described by Barton et al. (1995). Cells of an overnight culture (corresponding to an OD_{600} of 2.5) were washed, embedded in Incert agarose (BioRad) and treated with lysozyme and protease. The protease was inactivated with PMSF and 3-mm-long agarose strips were treated with 1 U S1 nuclease for 15 min at 37°C. The DNA was separated by PFGE using a CHEF-MAPPER, Model 1000 Mini Chiller, Chef Electrophoresis Cell from Bio-Rad (Munich, Germany).

Southern blotting

DNA labeling and hybridization was done with the DIG DNA random-primed labeling and detection kit from Roche Diagnostics on positive-charged nylon membranes. Hybridization and washing was performed at 65°C.

Enzyme assays

Cells were harvested by centrifugation and washed with lysis buffer (50 mM Tris pH 8, 10 mM β-mercaptoethanol). Cell suspension (1 ml; OD_{600}=10) in lysis buffer was disrupted by sonification. Cell debris was separated by centrifugation. The cleared crude extract was used for enzymatic assays. D-Carbamoylase activity was measured at 37°C by adding 100 μl of crude extract to 800 μl *N*-carbamyl-D-4-hydroxyphenylglycine (2 g l^{-1}) in 50 mM Tris, pH 8. The reaction was stopped with 500 μl 10% phosphoric acid followed by centrifugation. Detection of the amino acid in the supernatant was done at 210 nm after HPLC separation (Sykam, Fürstenfeldbruck) at 20°C. The mobile phase used was methanol:0.3% phosphoric acid (1:9), the solid phase was a Lichrospher 100 RP-18 5 μm column (Grom, Herrenberg) and the flow rate was 0.8 ml min^{-1}. D-Hydantoinase activity was measured in the same manner, using 1 g l^{-1} 4-hydroxyphenylhydantoin as a substrate. If not stated otherwise, *E. coli* cells were harvested 4 h after L-rhamnose addition, and *Agrobacterium* ssp. cells 24 h after the culture was started.

Protein analysis by SDS-PAGE

Crude extract of the soluble fraction, and the resuspended insoluble fraction obtained after cell disruption and centrifugation, was

applied to 12% polyacrylamide gels (Minigel-Twin, Biometra). Electrophoresis and staining with Coomassie blue was performed according to Sambrook et al. (1989). Protein concentrations were determined according to Bradford (1976) using bovine serum albumin as a standard.

Nucleotide sequence accession number

The sequence of the 7125 bp fragment of *Agrobacterium* sp. IP I-671 has been submitted to GenBank; accession number AF335479.

Results

Amplification and cloning of *hyuC* from *Agrobacterium* sp. IP I-671

D-Carbamoylase genes have been cloned and sequenced from *Agrobacterium radiobacter* NRRL B11291, renamed *A. tumefaciens* NRRL B11291 (Buson et al. 1996; Grifantini et al. 1998), *Agrobacterium* sp. 80/44-2A (Neal et al. 1994) and *A. radiobacter* KNK712 (Nanba et al. 1998). The DNA sequences were aligned and used to design oligonucleotides S783 and S784 for PCR amplification of the D-carbamoylase gene from *Agrobacterium* sp. IP I-671 (Runser and Meyer 1993). The amplified DNA fragment was cloned in *E. coli* and sequenced. The deduced protein sequence of the newly isolated D-carbamoylase gene (*hyuC*) showed about 92% identity in amino acid sequence with the three already known D-carbamoylase enzymes of *Agrobacterium* sp., and 59% identity with this enzyme in *Pseudomonas* sp. KNK003.

Isolation and sequence analysis of a larger, *hyuC*-containing genomic fragment from *Agrobacterium* sp. IP I-671

In order to find more genes involved in hydantoin utilization, a genomic phage library of *Agrobacterium* sp. IP I-671 was constructed and screened by plaque hybridization with the DIG-labeled *hyuC* DNA. The *hyuC* gene was carried by 5 out of 7,000 phages tested. The inserts in the phage were converted into plasmids as described by Altenbuchner (1993) and the presence of the *hyuC* gene on the plasmids confirmed by Southern hybridization. Restriction enzyme analysis revealed that most of the cloned genomic fragments ended very near the *hyuC* gene (<2 kb) and only in plasmid pMHS242 was *hyuC* flanked on both sides by more than 2 kb of *Agrobacterium* sp. IP I-671 genomic DNA (data not shown).

From pMHS242, overlapping restriction fragments were isolated and analyzed by DNA sequencing until database searches revealed the beginning of transposon-derived genes. In the sequenced 7125 bp fragment, seven open reading frames (ORFs) with similarities to genes in databases could be identified using the BLAST programs. The results are summarized in Fig. 1 and Table 1. At the C-terminal end of *hyuC*, and in the same orientation, two ORFs were found encoding putative proteins similar to the small subunit of a D-amino acid dehydrogenase (*hyuD*) and a hydantoin racemase (*hyuA*). The non-coding regions between these genes were 15 and 18 bp, respectively. At a distance of 470 bp upstream of *hyuC*, and in opposite orientation, two further ORFs were found encoding a putative D-hydantoinase (*hyuH*) and a NAD(P)H-dependent flavin oxidoreductase (*hyuN*). This latter gene might start at an ATG codon 24 bp behind the *hyuH* stop codon although similarities to other enzymes, as well as the presence of a putative ribosomal binding site (Table 1), indicate a GTG start codon 117 bp further downstream. ORFI and ORFIV were found at distances of 130 and 773 bp, respectively, to the neighboring *hyu* genes and the corresponding proteins showed similarities to transposon-borne enzymes.

Expression of *hyuC* and *hyuH* in *E. coli*

For heterologous expression in *E. coli*, the genes *hyuC* and *hyuH* were amplified by PCR and inserted in the L-rhamnose-inducible expression vector pJOE2702 (Volff

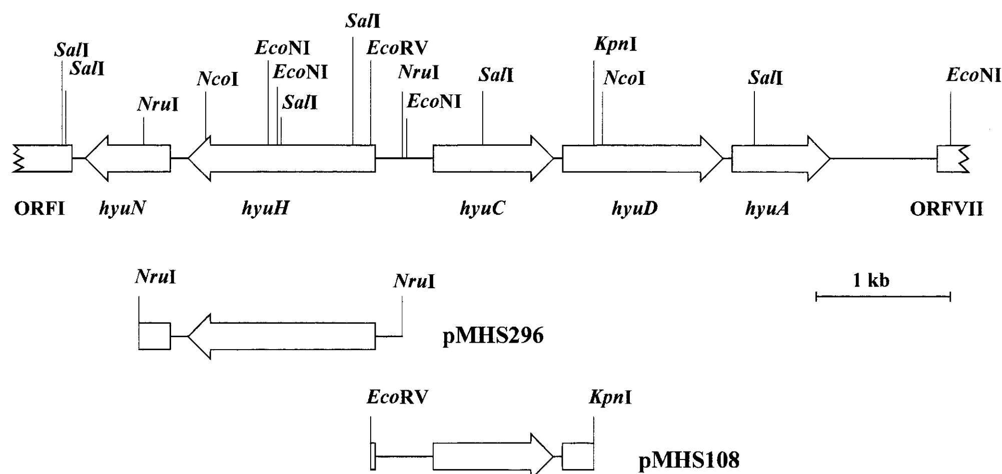

Fig. 1 Restriction map of the 7125 bp fragment from *Agrobacterium* sp. IP I-671 encoding the hydantoin utilization genes. The fragments used to construct the plasmids pMHS101, *pMHS108*, pMHS119, *pMHS296* and pMHS325, are shown below

Table 1 Analysis of the ORFs and the presumed ribosomal binding sites (*RBS*) of the sequenced *Agrobacterium* sp. IP I-671 fragment. Start-codons are bold and in italics, the presumed RBS bold and underlined. In the case of *hyuN*, both possible start-sites are listed

Name	Length [bp]	Calculated size of the deduced protein [kDa]	Presumed RBS	Protein with highest amino acid identity	Host	Database accession number	Identical amino acids [%]	Reference
ORF I	–	–	GGAT**GGAG**TCGGCA**ATG**	Transposase from Tn*21*	*Escherichia coli*	sp|P006694	37	Diver et al. (1983)
hyuN	711 597	25 21	TTTGT**GAG**AACCACT**ATG** TCCGCC**GACG**AACTG**GTG**	NADPH flavin oxido reductase	*Vibrio harveyi*	sp|Q56691	38	Lei et al. (1994)
hyuH	1374	50	CCGTA**GAG**CTGCATC**ATG**	D-hydantoinase	*Agrobacterium tumefaciens*	sp|Q44184	83	Grifantini et al. (1998)
hyuC	915	34	TTGA**AGGAG**CATAGGTTC**ATG**	D-carbamylase	*A. tumefaciens*	emb|CAA62550	93	Grifantini et al. (1998)
hyuD	1254	45	GGCC**GGGG**TGAAACGC**ATG**	D-Amino acid dehydrogenase (small subunit)	*E. coli*	sp|P29011	40	Lobocka et al. (1994)
hyuA	744	26	TAGAA**AGGA**CGCCTT**ATG**	Hydantoin racemase	*Pseudomonas* sp.	sp|Q00924	46	Watabe et al. (1992)
ORF VII	–	–	TT**GAGG**TTTGTCGTGC**ATG**	resolvase from Tn*5501*	*Pseudomonas pseudoalcaligenes*	gb|AAB94125	72	Davis et al. (2000)
E. coli consensus sequence:			NNN**AGGAGG**NNNNN**ATG**					

et al. 1996). The *hyuC* and *hyuH* expression plasmids were called pJOE2743 and pJOE2929, respectively. The recombinant cells were induced with 0.2% L-rhamnose for 4 h and enzyme activity determined in crude extract. In *E.coli* cells carrying pJOE2743 (*hyuC*), a specific D-carbamoylase activity of 21 mU mg^{-1} was measured. No D-hydantoinase activity was found in *E. coli* pJOE2929. For comparison, the D-carbamoylase activity determined in crude extract of *Agrobacterium* sp. IP I-671 was 70 mU mg^{-1} and that of D-hydantoinase was 46 mU mg^{-1} (see below). SDS-PAGE revealed the presence of two inducible proteins with the expected molecular mass of hydantoinase and carbamoylase in the insoluble fraction of the L-rhamnose-induced cells, which explains the lack, or low amount, of enzyme activity in *E. coli* cells (data not shown).

Overexpression of *hyuC* and *hyuH* in *Agrobacterium* sp. IP I-671

The heterologous expression of *hyuC* and *hyuH* in *E. coli* did not produce enough active enzyme for further biochemical studies. Therefore, attempts were made to overexpress the genes under control of their own promoters on plasmids in *Agrobacterium*. A *Kpn*I-*Eco*RV fragment (Fig. 1) containing *hyuC* and its 5′-non-coding promoter region was cloned into the vector pMHS93 resulting in plasmid pMHS108 (the vector pMHS93 is an RSF1010 derivative with the tetracycline resistance genes *tetA* and *tetR* of transposon Tn*1721*). The plasmid was introduced into *E. coli* S17-1 (Simon et al. 1983) for conjugation into *Agrobacterium*. For selection of transconjugants, a spontaneous rifampicin-resistant mutant of *Agrobacterium* sp. IP I-671 was isolated (*Agrobacterium* sp. RifR3). A tetracycline- and rifampicin-resistant transconjugant was grown in AM medium with tetracycline at 30°C and carbamoylase activity was determined between 10 h and 24 h of growth. A strain without the plasmid, grown in AM medium without antibiotic was examined in the same way. After about 22 h of growth the plasmid-free strain showed a maximum activity of 73 mU mg^{-1}, while the recombinant strain reached an activity of 765 mU mg^{-1} in this time.

Overexpression of *hyuH* in *Agrobacterium* sp. RifR3 was achieved in the same way. An *Nru*I fragment from pMHS242 with the *hyuH* gene and the intercistronic region between *hyuC* and *hyuH* containing the putative *hyuH* promoter (Fig. 1) was inserted into pMHS93 and the new plasmid, pMHS296, introduced into *Agrobacterium* sp. RifR3 via conjugation. Whereas the hydantoinase activity measured in the plasmid-free strain was 46 mU mg^{-1}, the strain containing pMHS296 expressed 113 mU mg^{-1}. SDS-PAGE analysis of the cell extract revealed that a protein of the molecular size of the D-hydantoinase was present both in the soluble and in the insoluble fraction of the cell extract, which indicated inclusion body formation (data not shown).

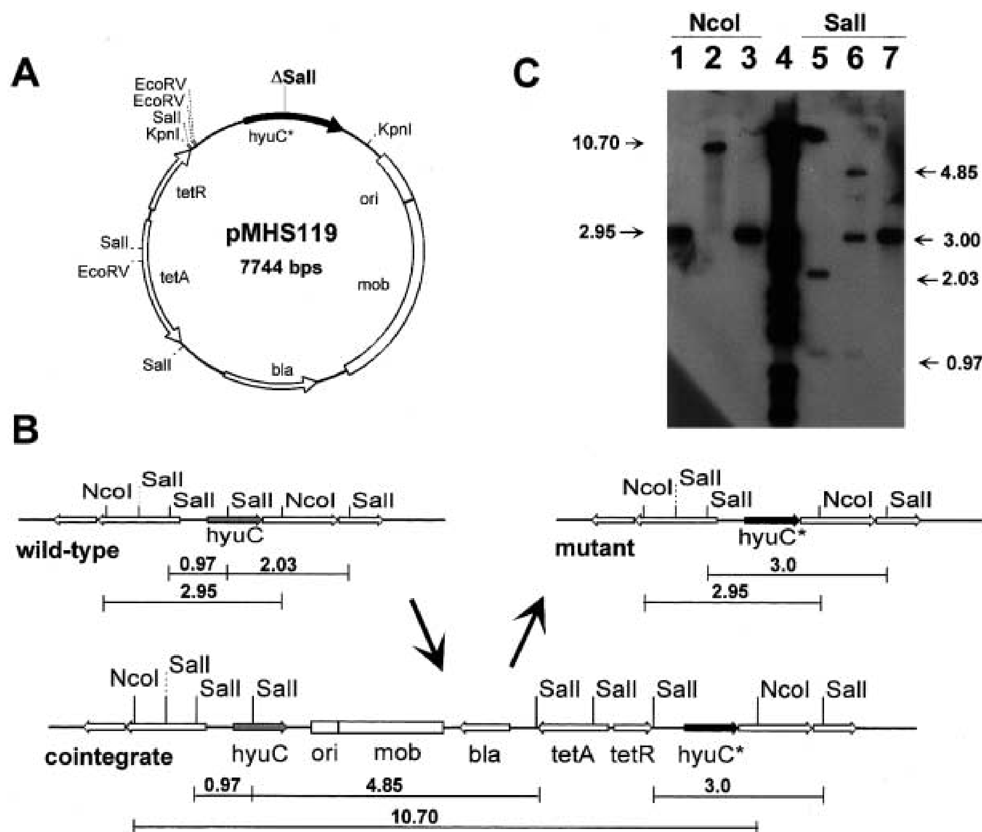

Fig. 2A–C Disruption of the *hyuC* gene in *Agrobacterium* sp. RifR3. **A** Restriction map of the suicide plasmid *pMHS119*. **B** Restriction map of the *hyu* gene cluster of wild-type *Agrobacterium* sp. RifR3, with *pMHS119* integrated (cointegrate) and of the mutant disrupted in *hyuC* (*hyuC**). **C** Southern blot of genomic DNA of *Agrobacterium* sp. RifR3 (lanes *1*, *5*), M4.1 (cointegrate; lanes *2*, *6*) and the mutant M4.1.1 (lanes *3*, *7*). Lane *4*: λ *Bgl*I size standard

Gene disruption of *hyuC* in *Agrobacterium* sp. IP I-671 and complementation

To further prove that the cloned *hyuC* was the only gene responsible for D-carbamoylase activity in *Agrobacterium* sp. IP I-671, a mutant strain with an inactivated *hyuC* gene was isolated. The suicide plasmid pMHS119 (Fig. 2) was constructed from the R6K-derived plasmid pUT-mini-Tn*5*Cm, and contains the tetracycline resistance genes of Tn*1721* and the *hyuC* gene carrying a frame-shift mutation created by filling in a *Sal*I site in the gene. The plasmid is efficiently mobilized and can only replicate in strains providing the *pir* gene. Using strain *E. coli* S17-1/λpir as a donor, pMHS119 was transfered by conjugation into the rifampicin-resistant mutant *Agrobacterium* sp. RifR3. Integration of pMHS119 into the *Agrobacterium* sp. IP I-671 chromosome was selected by growth on rifampicin- and tetracycline-containing medium. One of the colonies (*Agrobacterium* sp. M4.1) obtained on this medium was cultivated for about 100 generations without antibiotics in liquid culture and plated on medium without tetracycline. From 2,500 colonies, 21 tetracycline-sensitive colonies were identified by replica plating. Five of six strains tested showed no D-carbamoylase activity. One of the D-carbamoylase-negative strains (*Agrobacterium* sp. M4.1.1) was further investigated. The two *hyuC* alleles can be distinguished by the lack of the *Sal*I site in the mutant copy. Total DNA of the mutant and parental strains *Agrobacterium* sp. M4.1 and RifR3was digested with the restriction enzymes *Nco*I and, separately, *Sal*I, blotted on filters and hybridized with DIG-labeled *hyuC*-DNA. Figure 2, lanes 1, 2 and 3 show the 2.95 kb *Nco*I fragment of the wild-type strain RifR3, the 10.7 kb *Nco*I fragment of strain M4.1, which is due to the integration of pMHS119, and again a 2.95 kb *Nco*I fragment in strain M4.1.1, which indicates that the integrated plasmid was excised again. Lanes 5, 6 and 7 demonstrate the replacement of the wild-type allele in strain M4.1.1. Two *Sal*I fragments were found in Rif3R (lane 5), three in M4.1 due to the integration of pMHS119, and one in strain M4.1.1 caused by the replacement of the wild-type allele with the *hyuC* allele lacking the *Sal*I site.

Finally, D-carbamoylase activity in mutant M4.1.1 was restored with the plasmid pMHS108 carrying *hyuC*. The mutant M4.1.1 showed no D-carbamoylase activity as expected, the wild-type *Agrobacterium* sp. RifR3 had an activity of 70 mU mg^{-1} and in the complemented strain, *Agrobacterium* sp. M4.1.1/pMHS108, a specific D-carbamoylase activity of 723 mU mg^{-1} was measured. This showed that *hyuC* seems to be the only gene in *Agrobacterium* sp. IP I-671 encoding carbamoylase activity.

Gene disruption of *hyuH* in *Agrobacterium* sp. IP I-671

The D-hydantoinase gene was inactivated in *Agrobacterium* sp. IP I-671 in a similar way. Instead of the R6K-derived suicide vector, a pUC18-derived plasmid was con-

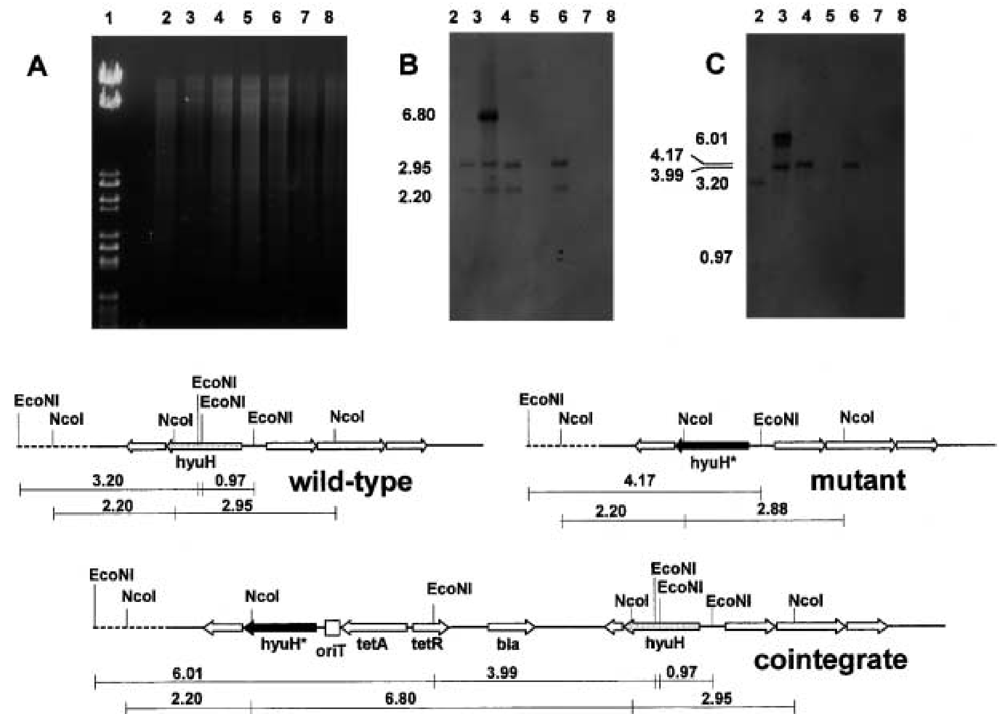

Fig. 3A–C Disruption of the *hyuH* gene in *Agrobacterium* sp RifR3. **A** Agarose gel with the genomic DNA of wild-type and mutants digested with *Nco*I. **B, C** Southern blot hybridization with the labeled fragment encoding *hyuH* to *Nco*I- (**B**) and *Eco*NI-digested (**C**) genomic DNA. Lanes: *1* λ *Bgl*I size standard, *2 Agrobacterium* sp RifR3 (wild-type), *3* M7.1 (pMHS325 integrated in the *hyu* gene cluster of *Agrobacterium* sp RifR3), *4–8* M7.1.1–M7.1.5 (tetracycline-sensitive derivatives of M7.1). The restriction maps of the *hyu* gene cluster of wild-type, M7.1 with pMHS325 integrated (cointegrate), and the mutant M7.1.1 disrupted in *hyuH* (*hyuH**), are shown below

structed which is also incapable of autonomous replication in *Agrobacterium*. This vector, pMHS298, contains the *oriT* of RP4 for mobilization and the tetracycline resistance genes from Tn*1721*. A copy of the *hyu*H gene with a deletion of a 71 bp *Eco*NI fragment and the *Eco*NI site was inserted into the suicide vector pMHS298. The resulting plasmid, pMHS325, was introduced into *Agrobacterium* RifR3 via mobilization from *E. coli* S17-1. One of the tetracycline- and rifampicin-resistant clonies (M7.1) was grown in liquid culture without tetracycline for about 100 generations and, after replica plating, seven colonies were finally obtained which were sensitive to tetracycline again. Testing for hydantoinase activity of the seven strains showed that two still had hydantoinase activity at the wild-type level. The other five strains (M7.1.1–M7.1.5) showed hydantoinase activity too, however at a significantly lower level – about 15% of wild-type activity. These five strains were chosen for further analysis. Southern blotting and hybridization with DIG-labeled *hyuH* of *Eco*NI- and *Nco*I-digested genomic DNA after agarose gel electrophoresis (Fig. 3) showed an additional *Nco*I-band in strain M7.1 compared to the wild-type. This is due to the integration of the suicide plasmid pMHS325. Of the tetracycline-sensitive M7.1 derivatives, only two strains showed again two *Nco*I fragments similar to the wild-type; three strains did not hybridize at all with *hyuH*, indicating a deletion. With *Eco*NI, M7.1 showed the loss of the 3.2 kb *Eco*NI fragment due to the integration of pMHS325, and two new fragments as would be expected if integration took place upstream of the *Eco*NI sites. Additionally, there is a fourth signal at a size corresponding to the vector, which may be explained by double integration of the suicide plasmid. Of the tetracycline-sensitive derivatives, again only two showed hybridization. The new *Eco*NI fragment of 4.17 kb in these two mutants, instead of the two *Eco*NI fragments of 3.2 kb and 0.97 kb in the wild-type, indicates that the *hyuH* wild-type gene was replaced by the mutant allele.

Localization of the *hyu* genes

To find out whether the cloned and sequenced *hyu* gene cluster is plasmid-borne or chromosomal, PFGE was performed to separate chromosomal DNA from plasmids. Prior to electrophoresis, the cells are embedded in agarose plugs, lysed and the DNA purified by protease treatment. Finally the agarose plugs are incubated with S1 nuclease to linearize circular plasmids. According to the PFGE results, *Agrobacterium* sp. IP I-671 harbors two plasmids (about 80 kb and 190 kb in size, Fig. 4A). Southern blot and subsequent hybridization with DIG-labeled *hyuC* DNA revealed that the *hyuC* gene is located on the 190 kb plasmid (Fig. 4B). The same experiment was carried out with the mutant *Agrobacterium* M7.1.2, a strain which did not hybridize with the *hyuH* gene probe (see above). M7.1.2 harbors the same 80 kb plasmid. Instead of the 190 kb plasmid, a plasmid of about 180 kb in size was found. No hybridization was found with the *hyuC* gene probe indicating a deletion of the complete *hyu* gene cluster. In addition, *A. tumefaciens* NRRL B 11291, another strain well known for exhibiting hydantoinase and carbamoylase activity, was analyzed by PFGE. Here again, two plasmids were found (160 kb and 240 kb in size) and the D-carbamoylase gene was located on the 160 kb plasmid (data not shown).

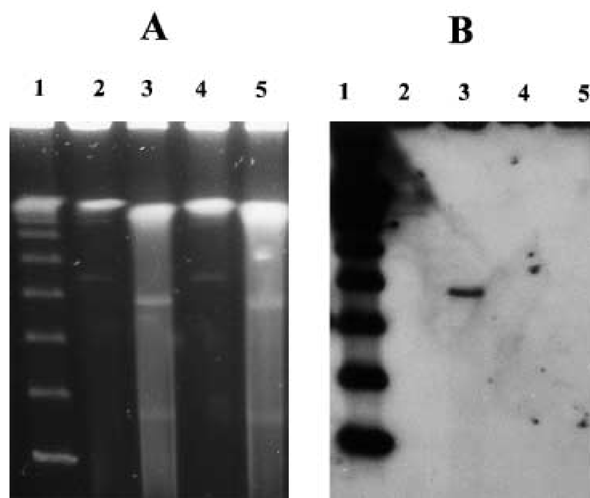

Fig. 4 **A** Pulsed-field gel electrophoresis (PFGE) of *Agrobacterium* sp. IP I-671 and *Agrobacterium* M7.1.2, and **B** Southern blot hybridization with the labeled *hyuC* gene probe. Lanes: *1* λ ladder (50, 100, 150 kb etc.), *2*, *3 Agrobacterium* sp. IP I-671, *4*, *5 Agrobacterium* M7.1.2. In lanes *3* and *5* the DNA was treated with S1 nuclease

Discussion

For the first time, it could be shown by PFGE and hybridization that the *hyu* genes in *Agrobacterium* sp. IP I-671 and NRRL B 11291, and maybe also in other *Agrobacteria*, are located on large plasmids of sizes similar to the Ti plasmids. Sequence analysis of a 7,125 bp DNA fragment from *Agrobacterium* sp. IP I-671 revealed seven ORFs. The genes *hyuCDA* and ORFVII were in the same orientation on the fragment; *hyuHN* and ORFI in opposite orientation. The C-terminal truncated ORFI and ORFVII showed similarity to transposases and resolvases, respectively. They obviously do not belong to the *hyu* gene cluster. However, they might be part of a transposon carrying the *hyu* genes.

From other *Agrobacteria*, only *hyuC* or *hyuC* and *hyuH* have been sequenced. *HyuH* and *hyuC* are oriented divergently in all strains, but the non-coding intergenic sequence varies, being 470 bp in *Agrobacterium* sp. IP I-671, 465 bp in *Agrobacterium* sp. 80/44–2A and 214 bp in *A. tumefaciens* NRRL B 11291. From *A. radiobacter* KNK712, a region 643 bp downstream of *hyuC* was sequenced. The high identity in nucleotide sequence of the *hyuC* genes stops immediately after the ends of the genes. A significant level of identity was found 200 bp further downstream of the *hyuC* gene from KNK712, and in the non-coding sequence between *hyuA* and ORFVII of *Agrobacterium* sp. IP I-671 (70% identity in 400 bp) indicating a deletion of *hyuD* and *hyuA* in *A. radiobacter* KNK712. In *Pseudomonas* sp. KNK003A, there is an additional ORF upstream of *hyuC* and in the same orientation which is 54% identical at the amino acids level to a spermidine/putrescine transport ATP-binding protein *potA* (Furuchi et al. 1991), which might be involved in the uptake of the substrate. In *Agrobacterium* ssp., no genes for the transport of hydantoins have been reported.

The functionality of *hyuC* as a D-carbamoylase gene was proven by gene inactivation, complementation and by expression in *E. coli* and *Agrobacterium* sp. IP I-671. HyuC shows high identity to D-carbamoylases of other bacteria. Further similarities were found with amidases, nitrilases, β-alanine synthetases or ureidosuccinases in BLAST searches. An multialignment shows a common cysteine residue for all these enzymes at position 172 that is essential for catalytic activity of HyuC (Grifantini et al. 1996). A reaction mechanism for D-carbamoylases involving this cysteine residue has been suggested by Nakai et al. (2000) based on the 3D-structure of the *Agrobacterium* sp. KNK712 enzyme.

The expression of *hyuC* seems to be correlated to the growth phase of the bacterial culture. The D-carbamoylase activity reached a maximum value when the culture had already entered stationary phase. Runser and Meyer (1993) measured an increase of D-carbamoylase activity after addition of 2,4-thiouracil to the culture broth. In our experiments we could not reproduce this induction (data not shown). A difference between the two experiments was that Runser and Meyer (1993) used whole cells whereas we used crude cell extracts for measuring the hydantoinase activity. Therefore the data reported by Runser and Meyer (1993) might rather be due to an increased permeability of the cell membranes for the substrates.

HyuH is a member of the subfamily of dihydropyrimdinases and collapsin response mediator proteins that belong to the superfamily of the amidohydrolases (Holm and Sander 1997). Inactivation of the *hyuH* gene in *Agrobacterium* sp. IP I-671 resulted in strains with 15% residual D-hydantoinase activity. Even when the whole gene was deleted (strain *Agrobacterium* sp. M7.1.2, M7.1.4, M7.1.5), this activity could be measured. Runser and Meyer (1993) purified a D-hydantoinase without dihydropyrimidinase activity from *Agrobacterium* sp. IP I-671. The N-terminal sequence of this purified hydantoinase does not share any similarity with HyuH. Apparently *Agrobacterium* sp. IP I-671 contains at least two different enzymes with hydantoinase activity. Indeed, the presence of a second hydantoinase with dihydropyriminidase activity in *Agrobacterium* sp. IP I-671 was also observed by Runser and Meyer (1993).

HyuA shares 46% identity in amino acid sequence with the hydantoin racemase of *Pseudomonas* sp. NS671. No hydantoin racemase activity has been observed in *Agrobacterium* ssp. so far. This might be due to the high rate of spontaneous chemical racemization of D,L-phenylhydantoins and D,L-*p*-hydroxyphenylhydantoins (the racemization half-lives for these substrates are about 20–40 min depending on pH and reaction temperatures; Lee and Fan 1999). On the other hand, the comparison of the KNK712 and IP I-671 sequences indicates that the racemase gene is deleted in KNK712. A racemase should be useful in strains with high hydantoinase

and carbamoylase activity because in such strains the racemization of the hydantoin will be the bottleneck in amino acid production.

HyuD is homologous to the small subunit of D-amino acid dehydrogenases of *E. coli* and *Klebsiella aerogenes* responsible for the catabolism of alanine into pyruvate and ammonium. D-Amino acid dehydrogenase is a heterodimer and contains a flavin adenine dinucleotide and nonheme iron in its active center (Lobocka et al. 1994; Janes and Bender 1998). HyuD might be responsible for oxidative deamination of the D-amino acids released by HyuC.

Finally, HyuN is homologous to the NAD(P)H-flavin oxidoreductase found in luminous bacteria. The enzyme catalyzes the reduction of flavin by NAD(P)H and is believed to provide the reduced form of FMN for luciferase (Lei et al. 1994). HyuN might have a similar function in reducing the flavin of the D-amino acid dehydrogenase.

According to the functions identified in the *hyu* gene cluster of *Agrobacterium* sp. IP I-671, one can speculate that the D-enantiomer of a 5-monosubstituted hydantoin racemate is enantioselectively converted into a D-amino acid via the N-carbamyl amino acid, and the amino acid then deaminated to an α-keto acid. The HyuN keeps the flavin of the D-amino acid dehydrogenase in a reduced state and the continuous racemization by HyuA ensures that the hydantion is completely converted into the keto acid. The natural substrate of the hydantoin-metabolizing enzymes remains unknown. Since *Agrobacteria* interact intimately with plants, the natural substrates might be secondary plant metabolites similar to agropinic acid, which is converted to mannopinic acid by a *N*-methylhydantoinase-homologous gene product encoded on a Ti plasmid (Lyi et al. 1999).

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