Genes encoding ribonucleoside hydrolase 1 and 2 from *Corynebacterium ammoniagenes*

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Two kinds of nucleoside hydrolases (NHs) encoded by *rih1* and *rih2* were cloned from *Corynebacterium ammoniagenes* using *deoD*- and *gsk*-defective *Escherichia coli*. Sequence analysis revealed that NH 1 was a protein of 337 aa with a deduced molecular mass of 35 892 Da, whereas NH 2 consisted of 308 aa with a calculated molecular mass of 32 310 Da. Experiments with crude extracts of IPTG-induced *E. coli* CGSC 6885(pTNU23) and 6885(pTNI12) indicated that the Rih1 enzyme could catalyse the hydrolysis of uridine and cytidine and showed pyrimidine-specific ribonucleoside hydrolase activity. Rih2 was able to hydrolyse both purine and pyrimidine ribonucleosides with the following order of activity – inosine > adenosine > uridine > guanosine > xanthosine > cytidine – and was classified in the non-specific NHs family. *rih1* and *rih2* deletion mutants displayed a decrease in cell growth on minimal medium supplemented with pyrimidine and purine/pyrimidine nucleosides, respectively, compared with the wild-type strain. Growth of each mutant was substantially complemented by introducing *rih1* and *rih2*, respectively. Furthermore, disruption of both *rih1* and *rih2* led to the inability of the mutant to utilize purine and pyrimidine nucleosides as sole carbon source on minimal medium. These results indicated that *rih1* and *rih2* play major roles in the salvage pathways of nucleosides in this micro-organism.

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INTRODUCTION

The *N*-ribosidic cleaving reactions of nucleosides occur through actions of nucleoside phosphorylases (EC 2.4.2.–) and nucleoside hydrolases (NHs; EC 3.2.2.–) (West, 1988; Camici *et al.*, 1990). Nucleoside phosphorylases catalyse the phosphorolytic cleavage of nucleosides and have a reversible ribosyl transferase activity. These enzymes have been well studied and used as catalysts for the synthesis of nucleoside analogues through base-exchange reactions (Krenitsky *et al.*, 1981; Ling *et al.*, 1990; Hamamoto *et al.*, 1996). In contrast, the NHs mediating the irreversible hydrolysis of nucleosides to ribose and free nucleic bases are widely distributed in bacteria, fungi, protozoa, insects, fish, amphibians and plants, but so far not in mammals. They have been studied extensively in pathogenic protozoa as potential targets for chemotherapy, since pathogenic protozoa lack *de novo* purine biosynthetic pathways and rely on exogenous purines salvaged from the hosts (Degano *et al.*, 1996; Miles *et al.*, 1999; Shi *et al.*, 1999).

Enzymes responsible for nucleoside salvage are quite different depending on the organism. In *Escherichia coli*, exogenous ribonucleosides are predominantly metabolized by nucleoside phosphorylases encoded by *deoD*, *udp* and *xapA*, while NHs encoded by *rihA*, *rihB* and *rihC* have been reported and play a minor role (Koszalka *et al.*, 1988; Petersen & Moller, 2001). In the case of the genus *Bacillus*, the metabolic pathways mediated by only nucleoside phosphorylases have been reported and characterized (Hamamoto *et al.*, 1996; Rocchietti *et al.*, 2004), whereas in the yeast *Saccharomyces cerevisiae*, purine ribonucleosides, inosine and guanosine, and pyrimidine ribonucleosides, cytidine and uridine, were salvaged by purine nucleoside phosphorylase (encoded by *pnp1*) and uridine ribohydrolase (encoded by *urh1*), respectively (Desgranges *et al.*, 2001; Kurtz *et al.*, 2002; Mitterbauer *et al.*, 2002). In addition, NHs, but not nucleoside phosphorylases, are crucial enzymes in purine-pyrimidine salvage in protozoan parasites (Parkin *et al.*, 1991): an inosine-uridine NH and a guanosine-inosine NH from *Crithidia fasciculata* (Estupinan & Schramm, 1994; Degano *et al.*, 1996), a purine-specific inosine-adenosine-guanosine NH from *Trypanosoma brucei* subsp. *brucei* (Parkin, 1996) and a non-specific NH from *Leishmania major* (Shi *et al.*, 1999).

Corynebacterium ammoniagenes is a Gram-positive micro-organism that has been used for the industrial production of

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Abbreviation: NH, nucleoside hydrolase.

The GenBank/EMBL/DDBJ accession numbers for the sequences of *rih1* and *rih2* reported in this paper are AY603363 and AY603360, respectively.

flavour-enhancing purine nucleotides and other compounds (Chung *et al.*, 1996; Koizumi *et al.*, 2000; Noguchi *et al.*, 2003). The biosynthetic pathways leading to nucleotides/nucleosides have been reported by Chung *et al.* (1996) and Noguchi *et al.* (2003); however, very little is known about the *N*-ribosidic cleavage reactions of the salvage pathways in this organism. Although several enzymes of other coryneform bacteria were predicted to be putative NHs during genome sequencing projects, to date their functions have not been verified experimentally.

In this work, we cloned and identified two major genes involved in the salvage of ribonucleosides from *C. ammoniagenes* ATCC 6872, and designated *rih1* and *rih2*. To investigate the characteristics and physiological function of the NHs, we have determined their substrate specificity and characterized their functions using deletion mutants and gene-bearing plasmids. This is the first report of the identification and characterization of putative NHs in *Corynebacterium* species.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Wild-type *C. ammoniagenes* ATCC 6872 was used to provide the DNA template for cloning of ribonucleoside-cleaving genes. *E. coli* CGSC 6885 and DH5 α were employed for the selection of positive clones and general DNA manipulation, respectively. An *E. coli*/*Corynebacterium glutamicum* shuttle plasmid, pECCG117, was used for molecular biological work in *C. ammoniagenes* (Chung *et al.*, 1996).

Media and culture conditions. *C. ammoniagenes* was routinely grown in YPB medium (10 g yeast extract, 10 g peptone, 10 g beef extract, 2.5 g NaCl per litre water at pH 7.2) (Chung *et al.*, 1996). *E. coli* was cultured in Luria-Bertani (LB) medium (Sambrook & Russell, 2001). Nucleoside-cleaving genes were screened on MacConkey agar medium (Difco) supplemented with 2.0% inosine or uridine. Minimal (M) medium [1 g (NH₄)₂SO₄, 1 g KH₂PO₄, 1 g K₂HPO₄, 0.3 g MgSO₄·7H₂O, 10 mg CaCl₂·2H₂O, 10 mg FeSO₄·7H₂O, 1 mg ZnSO₄·7H₂O, 3.6 mg MnCl₂·4H₂O, 20 mg L-cysteine, 5 mg thiamine-HCl, 10 mg Ca-D-pantothenate, 30 μ g biotin per litre water at pH 7.2) containing 10 mM nucleoside as a carbon source was used to analyse the growth patterns of several mutants. If necessary,

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics*	Source or reference†
<i>E. coli</i>		
DH5 α	F [−] <i>endA1 hsdR17 supE44 thi-1 λ[−] recA1 gyrA96 relA1 Δ(<i>lacZYA-argF</i>)U169 F80d lacZ ΔM15</i>	Stratagene
CGSC 6885	<i>lacZ43(Fs) gsk-11 LAM[−] relA1 spoT1 thi-1 purD90 deoD80</i>	CGSC
EC101	CGSC 6885 carrying pTrc99A	This work
EC202	CGSC 6885 carrying pTNI12	This work
EC303	CGSC 6885 carrying pTNU23	This work
GM2929	<i>dam-13::Tn9 dcm-6 hsdR2 recF143 mcrA0 mcrB9999 galK2 galT22 ara-14 lacY1 xyl-5 thi-1 tonA31 rpsL136 hisG4 tsx-78 mtl-1 supE44 leuB6 rfbD fhuA13</i>	CGSC
<i>C. ammoniagenes</i>		
ATCC 6872	Wild-type strain	ATCC
CA313	ATCC 6872 Δ <i>rih1</i>	This work
CA315	ATCC 6872 Δ <i>rih2</i>	This work
CA322	ATCC 6872 Δ <i>rih1</i> Δ <i>rih2</i>	This work
<i>B. subtilis</i>		
Marburg 168	<i>trpC2</i>	Ohshima <i>et al.</i> (2002)
Plasmids		
pECCG117	<i>E. coli</i> / <i>C. glutamicum</i> shuttle vector, Km ^r	Chung <i>et al.</i> (1996)
pTrc99A	Expression vector carrying the <i>trc</i> promoter	Pharmacia
pBR322	Cloning vector, Ap ^r Tc ^r	New England BioLabs
pBRINT-Gm	Integration vector for <i>E. coli</i> , Gm ^r	Balbas <i>et al.</i> (1996)
pNI13	pECCG117 carrying 2.8 kb of <i>Sau3AI</i> fragment from <i>C. ammoniagenes</i> ATCC 6872	This work
pNU7	pECCG117 carrying 2.3 kb of <i>Sau3AI</i> fragment from <i>C. ammoniagenes</i> ATCC 6872	This work
pTNI12	pTrc99A carrying 0.9 kb of ORF1 (<i>rih2</i> gene) from pNI13	This work
pTNU23	pTrc99A carrying 1.0 kb of ORF2 (<i>rih1</i> gene) from pNU7	This work
pBG101	Integration vector for <i>C. ammoniagenes</i> , Gm ^r	This work
pBSG102	Integration vector carrying <i>sacB</i> for <i>C. ammoniagenes</i> , Gm ^r	This work
pK-Rih1	pBSG102 carrying two internal regions of <i>rih1</i> gene	This work
pK-Rih2	pBSG102 carrying two internal regions of <i>rih2</i> gene	This work

*Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance.

†ATCC, American Type Culture Collection, Manassas, VA, USA; CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, CT, USA.

kanamycin and gentamicin were used at final concentrations of 10 and 0.4 µg ml⁻¹, respectively, in *C. ammoniagenes*. For *E. coli*, 100 µg ampicillin ml⁻¹, 50 µg kanamycin ml⁻¹ or 10 µg gentamicin ml⁻¹ was added to the medium. *E. coli* was grown at 37 °C and *C. ammoniagenes* was cultured at 32 °C in a 500 ml baffled flask containing 50 ml medium with an agitation speed of 120 r.p.m.

Cloning and sequencing of nucleoside-cleaving genes

Primary screen of nucleoside cleaving genes. Genomic DNA of *C. ammoniagenes* ATCC 6872 was isolated by using a genomic DNA purification kit (Qiagen) and partially digested with *Sau3A*I. DNA fragments ranging from 2 to 7 kb were eluted from an agarose gel and cloned into the *Bam*HI site of pECCG117. These constructs were transformed into *E. coli* CGSC 6885 by electroporation (Duncan & Shivan, 1989). The *deoD*- and *gsk*-defective strain, *E. coli* CGSC 6885, unable to utilize purine nucleoside as a carbon source, did not show any colour change around the colonies on a MacConkey agar plate supplemented with 2.0% inosine or uridine, the representative purine and pyrimidine ribonucleoside (Degano *et al.*, 1996; Kurtz *et al.*, 2002). Transformants were incubated overnight at 37 °C on MacConkey agar plates containing 2.0% inosine or uridine, respectively. The positive clones showed a faint or deep red colour around colonies due to the decrease in pH caused by inosine or uridine metabolism (Peist *et al.*, 1997).

Sequencing and sequence analysis. Nucleotide sequences of clones were determined by using the dideoxy chain-termination method using double-strained DNA as a template with either universal or synthetic primers (Sambrook & Russell, 2001). The nucleotide and amino acid sequences of the putative ORFs were analysed using the BLAST server of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/). The multiple sequence alignments of related or retrieval enzymes were performed with the CLUSTAL W program (www.ebi.ac.uk/clustalw/).

Construction of expression and integration plasmids. To overexpress *rih1* and *rih2* the genes were subcloned in an *E. coli* expression vector, pTrc99A. For amplification of *rih1*, primers A (5'-CATGCCATGGATGAACGCAGATTCCACCTCACC-3') and B (5'-CCCAAGCTTTTAAAGTTGGTGTGGCC-3') were used, whereas for *rih2* primers C (5'-CATGCCATGGATGAAGATGATTTAGATCTAGA-3') and D (5'-CCCAAGCTTTTACTGGTTTGGACCA-3') were chosen. The amplified fragments were double digested with *Nco*I and *Hind*III and ligated with *Nco*I/*Hind*III-cleaved pTrc99A. The resulting plasmids were designated pTNI12 and pTNU23, respectively.

Plasmid pBSG102 harbouring the *sacB* gene was used for constructing deletion mutants based on allelic replacement. pBSG102 was constructed as follows. A vector harbouring a gentamicin marker, pBG101 (2.04 kb), was constructed by digesting pBR322 with *Ahd*I and *Nde*I (1.07 kb fragment), followed by Klenow treatment and ligation with 0.88 kb of Klenow-treated pBRINT-Gm/*Eag*I fragment, which contained gentamicin acetyltransferase (*aacC1*) and multiple cloning sites derived from pBluescript II KS+ (Balbas *et al.*, 1996). To construct a plasmid containing levansucrase, a DNA fragment of *sacB* (1.9 kb) was amplified by PCR using *Bacillus subtilis* Marburg 168 as template and primers E (5'-GATCCTTTTAAACCCATCACATAT-3') and F (5'-TCGTGATGGCAGGTTGGGCGTCGC-3') (Ohshima *et al.*, 2002). pBG101 was digested with *Sap*I, followed by filling with Klenow fragment and ligated to the *sacB* fragment, yielding plasmid pBSG102.

Construction of mutant strains. The deletion mutants were constructed by the method of allelic replacement, based on the selection of a chromosomal deletion by two recombination events (Stibitz, 1994; Ikeda & Katsumata, 1998). To construct an *rih1* deletion mutant, two regions in the *rih1* gene (0.54 and 0.58 kb), were

amplified by PCR using primer set G-H (G, 5'-CGCGGATCCATCGA-CCACGGTTTCTTTCAC-3'; H, 5'-AGCATGGCAATGGCATCGTC-3') and primer set I-J (I, 5'-GACGATGCCATTGCCATGCTTCCATCT-ATCCTGCGAACTG-3'; J, 5'-CTAGTCTAGCGACGATATCGGAA-AGACGCG-3'), and the resulting two PCR products were combined and used for another round of PCR using primers G and J. Subsequently, the fusion fragment was digested with *Bam*HI and *Xba*I, ligated with *Bam*HI/*Xba*I-digested pBSG102 vector and transformed into *E. coli* DH5 α . The transformants were selected on gentamicin plates at 37 °C. To disrupt *rih1*, *C. ammoniagenes* ATCC 6872 was transformed by electroporation with the non-replicative plasmid pK-Rih1 purified from *E. coli* GM2929. Gentamicin-resistant clones contained chromosomally integrated plasmids. Subsequently, the gentamicin marker was eliminated by negative selection with the *sacB* gene in 10% sucrose (Stibitz, 1994). *rih2* was disrupted by the same method, except that the following primer sets were used: K-L (K, 5'-TTAGATCTAGACACCGGTATCGATG-3'; L, 5'-ATCATCGCCGT-ATTTGCGCG-3') and M-N (M, 5'-CGGCGAAATACGGCGATG-ATGCTTTATGACTGCAAGTTCGC-3'; N, 5'-CGCGGATCCACGG-TCGACGTTGTTAGCAGC-3'). Primers K and N, and two PCR fragments (0.35 and 0.33 kb) were used for another round of PCR as described above. Double deletion mutant Δ *rih1* Δ *rih2* was generated starting from the parent strain, Δ *rih1*, according to the same method described above. All the deletion mutants resulting from a double chromosomal recombination event were confirmed by PCR analysis (data not shown).

Enzyme assay. After induction with 0.1 mM IPTG in LB medium, samples were taken from cultures of *E. coli* CGSC 6885(pTNI12) and 6885(pTNU23), respectively. Cells were harvested by centrifugation at 5000 g for 10 min, washed twice with 10 mM Tris/HCl (pH 7.5) and then resuspended in the same buffer. Cells were disrupted by sonication and cell debris was removed by centrifugation (10 000 g, 15 min) giving crude extracts which were used immediately or stored at -20 °C. To measure the NH enzyme activity, a 1 ml reaction mixture was used that contained 100 mM Tris/HCl (pH 7.5), 25 mM nucleoside and 1.0 mg crude extract. All of the reactions were started by adding 0.2 ml crude cell extract, incubated at 30 °C for 30 min and terminated by adding 0.1 ml 0.35% (w/v) trichloroacetic acid (Parkin *et al.*, 1991). The enzyme activity of nucleoside phosphorylase and uridine phosphorylase was determined spectrophotometrically using inosine and uridine as substrates, and 10 mM sodium arsenate was added as phosphate analogue (Krenitsky *et al.*, 1976). Inosine kinase activity was assayed by measuring the formation of IMP from inosine. The 1 ml reaction mixture consisted of 100 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) (pH 7.2), 10 mM MgSO₄, 300 mM KCl, 2 mM ATP, 5 mM inosine and 1.0 mg crude extract (Mori *et al.*, 1995). One unit (U) of enzyme activity was defined as the amount of enzyme required to convert 1 µmol substrate into the product per minute per mg protein. Each measurement was performed in triplicate.

Recombinant DNA techniques. DNA manipulations and PCR were carried out by standard procedures (Sambrook & Russell, 2001). Chromosomal DNA and PCR fragments were purified by using a Wizard genomic DNA purification kit (Promega) and a QIAquick gel extraction kit (Qiagen), respectively. Genes were amplified by PCR with *Pfu* DNA polymerase (Stratagene). All constructs involving PCR were verified by DNA sequencing according to standard methods (Sambrook & Russell, 2001).

Analyses. Cell growth was measured at 562 nm using a spectrophotometer (Beckman DU650). HPLC (Kromasil C18 column; Waters Co.) was used to separate nucleotides, nucleosides and bases, which were detected at 254 nm. A 5 µl sample was injected after equilibration with mixed solvent (solvent A:solvent B=97:3) at a flow rate of 1 ml min⁻¹. Solvent A consisted of 0.02% tetrabutylammonium

dihydrogenphosphate and 0.2% ammonium dihydrogenphosphate, adjusted to pH 2.4 with phosphoric acid, while solvent B consisted of acetonitrile. The amount of protein was determined by the dye-binding method of Bradford (Bio-Rad) with bovine serum albumin as a standard.

RESULTS

Cloning of nucleoside-cleaving genes from *C. ammoniagenes*

After ligation of partially digested genomic DNA with the cloning vector pECCG117, the genomic DNA library was transformed into the *deoD*- and *gsk*-defective *E. coli* strain CGSC 6885 on MacConkey agar supplemented with 2.0% inosine and uridine, respectively. From 20 transformants that gave rise to an apparent red colour around the colony on MacConkey agar containing inosine, genes encoding an inosine-cleaving protein were obtained. All of the plasmid DNAs isolated from the transformants displayed an approximately 1.3 kb fragment with an identical restriction enzyme pattern when digested with *NheI* and *Sall*, and had the same nucleotide sequence. Of these samples, one plasmid containing the longest DNA insert was selected and named pNI13. Genes encoding a uridine-cleaving protein were isolated from 25 clones that showed red colonies on the screening medium containing uridine as a pyrimidine nucleoside substrate. Restriction analysis of extracted plasmid DNAs from these 25 clones revealed two types of restriction pattern: one pattern contained a fragment of 0.6 kb when digested with *NcoI*; the other contained a fragment of 1.3 kb when double-digested with *NheI* and *Sall*. Sequence analysis of the 0.6 kb *NcoI* and 1.3 kb *NheI/Sall* fragments revealed that they contained different nucleotide sequences. However, the 1.3 kb *NheI/Sall* fragments in plasmids obtained from both inosine- and uridine-supplemented MacConkey agar plates had the same sequence. Therefore, one clone was selected based on the insert size and the degree of red colour around the colony from clones exhibiting a 0.6 kb fragment after digestion with *NcoI* and designated pNU7. The isolated plasmids pNI13 and pNU7 were retransformed into *E. coli* CGSC 6885 and all of the independent clones gave rise to the same red colour, as expected. Therefore, it is believed that the products of the putative genes in plasmids pNI13 and pNU7 are involved in nucleoside-cleaving reactions.

Analysis of nucleoside-cleaving genes and homology search

The nucleotide sequences of a 2.8 kb fragment of pNI13 and a 2.3 kb of pNU7 were determined. Analysis of the insert fragment of pNI13 indicated that it contained an ORF, ORF1, encoding a product of 308 aa with a calculated molecular mass of 32 310 Da. The ORF identified in pNU7, ORF2, consisted of 1011 nt, corresponding to a 337 aa product with a predicted molecular mass of 35 892 Da. To confirm the molecular mass of the putative proteins encoded by ORF1 and ORF2, crude extracts of *E. coli* EC100, EC202

Fig. 1. Expression of Rih1 and Rih2 in *E. coli* CGSC 6885. Proteins were separated by 12% SDS-PAGE. The arrows indicate the protein bands corresponding to ORF1 (NH 2, dotted arrow) and ORF2 (NH 1, solid arrow). Lanes: 1, 6885(pTrc99A); 2, 6885(pTNI12); 3, 6885(pTNU23); M, molecular size marker.

and EC303 were prepared and subjected to SDS-PAGE analysis. As shown in Fig. 1, distinct bands appeared corresponding to molecular masses of about 33 and 36 kDa, respectively, and this result correlated with the predicted molecular mass of the proteins encoded by ORF1 and ORF2.

The deduced amino acid sequences of ORF1 and ORF2 were compared to the NCBI database using the BLAST program to search for homologous proteins. The deduced amino acid sequence encoded by ORF1 displayed high sequence similarity to the sequences of the putative NHs or inosine-uridine NHs (IunH) of *Corynebacterium diphtheriae* NCTC 13129 (NP_939817.1, 53%), *Corynebacterium efficiens* YS-314 [NP_738480.1 (CE1870), 52%], *C. glutamicum* ATCC 13032 [NP_601183.1 (NCgl1902), 52%], *Pediococcus pentosaceus* ATCC 25745 (ZP_00323009.1, 48%), *Lactobacillus plantarum* WCFS1 (NP_786011.1, 46%) and *Lactobacillus reuteri* (AAX82611.1, 46%). The protein encoded by ORF2 also showed high levels of sequence similarity to putative NHs or IunHs of *Corynebacterium jeikeium* K411 (CAI38260.1, 69%), *Photobacterium luminescens* subsp. *laumondii* TTO1 (NP_931465.1, 52%), *C. efficiens* YS-314 [NP_738092.1 (CE1482), 51%], *Pasteurella multocida* subsp. *multocida* Pm70 [NP_246706.1, 51%], *C. glutamicum* ATCC 13032 [NP_600581.1 (NCgl1309), 50%], *C. diphtheriae* NCTC 13129 (NP_940091.1, 50%) and *Crithidia fasciculata* (Q27546, 49%) (see Fig. 2). All of these enzymes, except IunH of *C. fasciculata*, were predicted as putative NHs or IunHs from whole-genome sequencing projects and are still

Rih1	MNADSTSPAQKII-----LCCDPGHDAIAHLLAHGNPNLELLAYTTYAGQTLEKYVTNARAYARVAGIT	66
Rih2	M-----KHI-----LCLDTGIDCAFALAYAIHPGIDLIGYGTGYGTYTIEQGHANTQALLTLGLAA	57
RihA	HAL-----P-IL-----LCCDPGHDAIAIYVALASPELDYKAITSSAGQTPKTLRNLYRLMTLLNRT	59
RihB	HEK-----RKII-----LCCDPGHDAIAIHMAAKHPAIDLLGITTYAGQTLDTKTLINGLNYCQKLE-I	59
RihC	HRL-----P-IF-----LCLDPGIDAYATAAAIFAPELDLQLHTTYAGQYSYEKTTNRNALQLLHFWN-A	58
Urh1	HESADFFTSRNLLKQIISLICKYGEGLDSNNQRLFECKNTYSKIPWLDCCDPGHDAIAIILLGCFHPAFNLLGISTCFGAPPENDYHARSLLTAHGKA	100
IunH	M-----PKKII-----LCCDPGLDQAYAILLAHGNPEIELLAITTVYGGQTLAKYTRNAQLYADIAGIT	59
Rih1	D-IPFAAGASRPLVGPQLIPDEIHGDSGLDGPQLPEP-SYPLEEIHAYNLIADYISENEPGSYIIP#TGSLTNIALFARMYPQLVERVGGITLHGGG-HH	163
Rih2	D-VPPYAGRAIDGFVEYSEASARIHGRNGYGEYDIAA--DDAQSAAGDALEFLSEAAKYG-DOLYIYPTGCAQTTARALEKDPALRG-IRHYSHGGAL-TY	151
RihA	D-IPYAGGAYKPLHRELIIADNYHGESGLDGPALPEP-TFAPQNCATVELHAKTLRES-AEPTYIYSTGPQTNVALLNSHPELHSHKARIYIHGGA-HG	165
RihB	M-VPPYAGMPQPIHRQIYADNIHGETGLDGPYFEPL-TRQAEETHAVKYIIDTLHAS-DGIDITLVYPYGPLSNIAAYAHMQAPILPKIREIYVHGGG-YG	155
RihC	E-IPLAQGAAYPLYRAPDAASYHGESGHAGYOFVEH-NRKPLGIPAFLAIRDALHRA-PEPYTLVAIGPLTNIALLLSQCECKPYIRRLYIHGGS-AG	154
Urh1	QAIPYKGAQRPWKREPHYAPDTHGISGLDGTSLPKPTFEARTDKTYIEAIEEAALANNGEISFYSTGALTTLATVFRCKPYLKKSYKYSIHGGGLHG	200
IunH	G-YPIAAGCDKPLYRKIMTAGHIGESGHGTAYPAEFKNKYDERHAVNLIIDLYSHSEPKTITLYPTGGLTNIAHAARLEPRIVDRYKEVYVHGGG-YH	167
Rih1	TGHTPA--SEFIIADPEAAAIYFEESWPY---THYGLDYTHQVLAYPERHEQL--KAIGTDYAGFIAELYEFFGASYNKERN-YGPPH#HPLAYAAY	255
Rih2	PGYSPA--AEAIISQDPYSSNTYQLAEDH---THYGLDYTHQTLTRAEADSW--R--GTPAGDYFADHAGGYIDAYQENPNPHDGCALHPLAYAYA	242
RihA	LGNHTPA--AEFIYIYDPEAAEYFQSGIPY---YHAGLDYTHKAQIHYEDTERF--RAIGNPYSTIYAEALLDFLEYHDKWKGYGAPLHOPCTIAWL	248
RihB	TGNFTPS--AEFIIFADPEAAARYFTSGYPL---YHMGDLTNQTYCTPDYIARH--ERAGGPAGELFSDIHNF TLKTQFEN-YGLAGGPVHDATCIGYL	248
RihC	RCNCTPN--AEFIIAADPEAAACYFRSGIEI---YHCGLDYTNQAIL---TPDY--LSTLPQLNRTGKHLHALFSHYRSGSHQS--GLRHHOLCAIAWL	241
Urh1	LGCNPNLSAEFIYIWDPAANYIFRDPOYKDKCIYYPNLTHKAIATYKYNHIEYKNNKSLRELFLFLQFFAHTYKDHQGFESGPPIHOPVYALHPL	300
IunH	EGQATSY--AEFIIIIDPEAAHIYFNESWQV---THYGLDLTHQALATPPIQLRY--KEYDTNPARFMLEIHQYTYKIYQSNRY-HAAAVYH#PCAYAYY	249
Rih1	ADPSILRTYHAPIYVETQGGHARGQTIVDFRRTWSNNDPSSGLGYTDOPDFEAPNTAYAYDYDAEKFWDLLVDAL-TRIGN-TNF	337
Rih2	ADPDLVDCLILPLQVDTEGP-TIGRTIG----RWGSE-----YETRYAYGVNYDSFYPDFIDKLAQLFGRLYQNG	308
RihA	LKPELFTSYERWYGVETQGGYTQCHTYDYD-----YYLTGNK--PNATYHYDYDRQGFYDLLADRL-KEYA	311
RihB	INPDGIKTQEHYYEVDYNSGPCYGRTYCDE-----LGVLGKP--ANTKYGITIDTDFWGLYECCY-RGYIK-T--H	313
RihC	YRPDLFTLKPCFYAYETQGEFTSGTITYDI-----DGCLGKP--ANYQVALDLYKGFQQWYAEVL-ALAS	304
Urh1	LE---FYGWDPSAYGFRYKRHDISCIDYFNENSGKIIIEKEYPNDSD--YGTIIGLDLNIQYFWDQIFEALNRA-DKMSTIG	378
IunH	IDPSVHTTERYPYDIELTGKLTGMYADFNRPREH-----CHTQVAYKLDFEKFWGLYLDAL-ERIGD-P--Q	315

Fig. 2. Comparison of Rih1 and Rih2 with characterized NHs from different organisms. The alignment was performed with the CLUSTAL W program. Based on the characterized structure of IunH in *C. fasciculata*, conserved amino acid residues which occur at the same position in all sequences are indicated by a black background. The symbols #, + and @ indicate involvement of hydrogen bonds with the ribose hydroxyl groups, Ca^{2+} binding and a proton donor for the leaving group, respectively. Rih1 and Rih2, NHs of *C. ammoniagenes*; RihA, B and C, NHs of *E. coli*; Urh1, uridine ribohydrolase of *S. cerevisiae*; IunH, inosine/uridine-preferring NH of *C. fasciculata*.

uncharacterized. This comparison of protein identity suggested that the deduced polypeptides of ORF1 and ORF2 are putative NHs related to the salvage pathways of nucleoside cleavage. However, the identity (26 %) and similarity (44 %) between the two proteins were very low.

Substrate specificity of the two NHs

To characterize the function and substrate specificity of the nucleoside-cleaving enzymes, crude extracts prepared from IPTG-induced *E. coli* EC202 [6885(pTNI12)] and EC303 [6885(pTNU23)], respectively, were used to measure the

enzyme activity of NHs toward different purine and pyrimidine nucleosides. As shown in Table 2, the deduced enzyme 1 encoded by ORF1 in pTNI12 can hydrolyse both purine and pyrimidine ribonucleosides with the following activity order: inosine > adenosine > uridine > guanosine > xanthosine > cytidine. The deduced enzyme 2 encoded by ORF2 in pTNU23 had stronger activity toward uridine than cytidine, but no detectable activity toward purine ribonucleosides, except for inosine where a little activity was measured, indicating that it had an essentially pyrimidine-specific activity. No enzyme activity was detected toward deoxynucleosides in crude extracts of EC202 and EC303.

Table 2. Substrate specificity of NHs

The crude extracts prepared from IPTG-induced *E. coli* EC101 [6885(pTrc99A)], EC202 [6885(pTNI12)] and EC303 [6885(pTNU23)] were used to measure enzyme activities. –, No detectable activity. *E. coli* EC101 [6885(pTrc99A)] showed no detectable activity with any of the substrates.

Strain (<i>E. coli</i>)	$10^3 \times$ Enzyme activity (units)					
	Uridine	Cytidine	Adenosine	Guanosine	Inosine	Xanthosine
EC202	16.7	5.1	23.8	12.2	27.1	11.5
EC303	945	115.1	–	–	3.0	–

Furthermore, the enzyme activities of nucleoside phosphorylase, uridine kinase and inosine-guanosine kinase were not detected at the same level in EC202 and EC303 compared to EC100 (data not shown). Thus, according to earlier reports regarding the classification of characterized NHs based on their substrate specificity (Petersen & Moller, 2001; Versees & Steyaert, 2003), we concluded that the deduced protein 1 belongs to a class of non-specific NHs and was designated Rih2. The deduced protein 2 had NH activity for pyrimidine ribonucleosides and was named Rih1.

Effect of the *rih1* and *rih2* in *C. ammoniagenes*

To investigate the physiological role of *rih1* and *rih2* in *C. ammoniagenes*, deletion mutants CA313 (Δ *rih1*), CA315 (Δ *rih2*) and CA322 (Δ *rih1 Δ *rih2*), and *rih1*- and *rih2*-expressing strains CA313(pNU7) and CA315(pNI13) were constructed. Based on the analysis of the substrate specificity of Rih1 and Rih2 in the Table 2, the cell density of each strain was measured on M medium supplemented with different nucleosides or glucose, respectively.*

The Δ *rih1* strain, CA313, exhibited a severe decrease in cell growth on uridine and cytidine compared with the wild-type strain, but growth recovered substantially by introducing pNU7 (Table 3). As described above, since Rih1 showed only pyrimidine-specific activity, we expected that an *rih1* deletion mutant would grow well in M medium supplemented with purine nucleosides as carbon source. Contrary to our expectation, CA313 also exhibited decreased growth on purine nucleosides compared with the wild-type strain. However, unlike the growing pattern on pyrimidine medium, CA313(pNU7) harbouring *rih1* exhibited a similar level of cell growth compared to CA313, indicating that the *rih1* gene could not complement cell growth of Δ *rih1* on purine ribonucleosides. These results suggested that Rih1 in *C. ammoniagenes* has strong activity with respect to the salvage of pyrimidine nucleosides. The Δ *rih2* strain displayed very low levels of growth on M medium with purine

nucleosides, especially medium containing inosine or xanthosine. In addition, the expression of Rih2 substantially recovered growth of strain CA315. The observed growth of strain CA315 (Δ *rih2*) was also slower than that of the wild-type strain on pyrimidine nucleosides, but the introduction of Rih2 complemented its growth. Therefore, it is likely that Rih2 in *C. ammoniagenes* has a major function in the salvage of both purine and pyrimidine nucleosides. The double deletion mutant, CA322, was unable to grow on M medium with both purine and pyrimidine nucleosides. These results indicated that *rih1* and *rih2* play major roles in the salvage pathways of nucleosides in this micro-organism.

DISCUSSION

C. ammoniagenes can grow on M medium supplemented with purine or pyrimidine ribonucleosides as sole carbon source. Auling & Moss (1984) identified a uridine phosphorylase and also suggested that there may be two different pyrimidine NHs in *C. ammoniagenes*. In the present study, when we attempted to identify all of the genes responsible for the nucleoside salvaging pathway by complementation in *E. coli*; contrary to our expectations, two genes, *rih1* and *rih2*, annotated as putative NHs or IunHs, instead of uridine phosphorylase were identified.

Previously, the classification of NHs was based on substrate specificity and they were divided into four different classes (Versees & Steyaert, 2003). According to our assay of NH enzyme activities with different purine and pyrimidine nucleosides (Table 2) and the complementation test using knockout mutants and plasmids containing each gene (Table 3), we identified that the Rih1 enzyme in *C. ammoniagenes* belongs to the third class of pyrimidine-specific NHs and had a similar function to RihA and RihB in *E. coli* (Petersen & Moller, 2001) and URH1 in *S. cerevisiae* (Mitterbauer *et al.*, 2002). Rih2 was closely related to the first class of non-specific NHs and representative examples of

Table 3. Growth comparison of wild-type and deletion mutants of *rih1* and/or *rih2*

A culture grown in YPB medium overnight was washed with saline and 1% broth was inoculated into M medium supplemented with glucose or a nucleoside as sole carbon source. The final cell density shown was measured after 48 h of culture. The relative growth of mutants compared to wild-type is shown in parentheses. Each experiment was performed with three independent cultures.

Strain	6872 (wild type)	CA313 (Δ <i>rih1</i>)	CA315 (Δ <i>rih2</i>)	CA322 (Δ <i>rih1</i> Δ <i>rih2</i>)
Carbon source	–	–	With pNU7	–
Glucose	0.60	0.59	0.60	0.62
Pyrimidine nucleoside			With pNI13	
Uridine	0.24 (100)	0.08 (33)	0.20 (83)	0.12 (50)
Cytidine	0.19 (100)	0.09 (47)	0.17 (89)	0.12 (63)
Purine nucleoside				
Adenosine	0.40 (100)	0.21 (53)	0.19 (48)	0.10 (25)
Guanosine	0.37 (100)	0.28 (78)	0.24 (65)	0.09 (24)
Inosine	0.32 (100)	0.14 (44)	0.13 (41)	0.03 (9)
Xanthosine	0.26 (100)	0.15 (58)	0.13 (50)	0.04 (15)

this group include IunH of *C. fasciculata* and the RihCs of *E. coli* and *Salmonella enterica* serovar *Typhimurium* (Hansen & Dandanell, 2005). Considering the sequence homology among these enzymes, pyrimidine-specific Rih1 was much more homologous to the non-specific IunH of *C. fasciculata* (49 % sequence identity) and the pyrimidine-specific RihA of *E. coli* (43 % identity), whereas Rih2 displayed a very low level of sequence identity with the enzymes described above. Thus, there was no high correlation between function and the sequence homology of these proteins.

According to the crystal structure of the IunH protein in *C. fasciculata*, Asp10, Asp14, Asp15, Asn39, Asn160, Glu166, Asn168 and Asp242 are required for hydrogen bond formation with the ribose of the substrate, and three Asp residues (positions 10, 15, 242) and one Thr residue (position 126) are involved in the binding of a Ca^{2+} ion at the active centre (Petersen & Moller, 2001; Versees & Steyaert, 2003). The highly conserved His241 residue has been shown to be a proton donor for the leaving group in the catalytic mechanism of IunH of *C. fasciculata* (Kurtz *et al.*, 2002). In addition, the characteristic sequence of NHs is a recurring N-terminal DXDXXXDD motif (Versees & Steyaert, 2003). The sequence alignment between Rih1 and Rih2 of *C. ammoniagenes* and the functionally identified NHs demonstrated that all of these residues involved in substrate and Ca^{2+} ion binding, the proton donor and the N-terminal motif of NH activity were well conserved in Rih1 and Rih2 (Fig. 2). Even though NH 1 and 2 had the same conserved amino acid residues, they displayed different substrate specificity. These discordances might be derived from a small number of amino acid residues that restrict the access of nucleosides to the active site, as shown in a study on RihA, RihB and RihC of *E. coli* (Petersen & Moller, 2001).

Although several putative NHs have been annotated in the genomes of *Corynebacterium* species from genome sequencing projects (Ikeda & Nakagawa, 2003; Kalinowski *et al.*, 2003), none of these has been characterized. We searched for ORFs in the complete genome sequence of *Corynebacterium* species, including *C. glutamicum*, *C. efficiens*, *C. diphtheriae* and *C. jeikeium*, that would encode putative nucleoside salvage enzymes. As a result of searching for NHs using the BLAST program, three putative NHs or inosine-uridine nucleoside *N*-ribohydrolases (NP_600581.1,

NP_601183.1 and NP_602026.1) were predicted in *C. glutamicum* ATCC 13032. *C. efficiens* YS-314 had two putative NHs (NP_738092.1 and NP_738480.1) and *C. diphtheriae* NCTC 13129 contained three putative NHs (NP_939817.1, NP_940091.1 and NP_940641.1). Finally, two IunHs (CAI36385.1 and CAI38260.1) were annotated in *C. jeikeium* K411. Structurally, Rih1 exhibited more than 50 % sequence identity to NP_600581.1 of *C. glutamicum*, NP_738092.1 of *C. efficiens*, NP_940091.1 of *C. diphtheriae* and CAI38260.1 of *C. jeikeium*, whereas Rih2 was much more homologous to NP_601183.1 of *C. glutamicum*, NP_738480.1 of *C. efficiens* and NP_939817.1 of *C. diphtheriae*. Thus, based on quite high homology relationships, we suppose that enzymes of the first group in *Corynebacterium*, CAI38260.1, NP_600581.1, NP_940091.1 and NP_940641.1, may be assigned as pyrimidine-specific ribonucleoside hydrolases, whereas the proteins in the second group, NP_601183.1, NP_738480.1 and NP_939817.1, may be annotated as non-specific ribonucleoside hydrolases.

The complementation tests confirmed that the *rih1* and *rih2* deletion mutants displayed a decrease in cell growth on pyrimidine and purine/pyrimidine nucleosides, respectively, compared with the wild-type strain (Table 3). Also, growth of each mutant was substantially complemented by introducing *rih1* and *rih2*, respectively. Furthermore, surprisingly, cell growth was completely abolished in CA322 ($\Delta\text{rih1}\Delta\text{rih2}$) on M medium with purine or pyrimidine nucleosides, although Auling & Moss (1984) reported the presence of uridine phosphorylase in *C. ammoniagenes*. Thus, we suggest that *rih1* and *rih2* in *C. ammoniagenes* play a prominent role in the salvage pathways of ribonucleosides on M medium supplemented with different ribonucleosides as sole carbon source, whereas uridine phosphorylase might be induced under different physiological conditions. Considering that Rih1 acts only on pyrimidine nucleosides, growth retardation in the *rih1* deletion mutant on purine nucleosides medium was unexpected. In terms of genomic organization (Fig. 3), a putative permease of the major facilitator superfamily is preceded by *rih1* followed by a 37 nt intergenic region and an ORF encoding a putative membrane protein in the reverse direction is located 81 nt upstream of *rih1*. Therefore, we suppose that the growth inhibition of the Δrih1 strain on purine nucleosides might be related to the deletion of a region in the *rih1* ORF which

Fig. 3. Diagrammatic representation of the DNA region containing *rih1* (shaded grey). The number of amino acids for the annotated ORFs is shown within the arrows. The arrows indicate the direction of transcription. The (putative) functions of the gene products are indicated below the arrow. The numbers of intergenic base pairs are given in parentheses.

may affect the expression of the putative permease or the putative membrane protein.

We have demonstrated that two genes, *rih1* and *rih2*, play major roles in the salvage pathways of nucleosides in *C. ammoniagenes* on the basis of enzyme assays and complementation tests. Information on the salvage pathway in this organism is expected to offer more rational strategies for developing a nucleotide or nucleoside overproducing *C. ammoniagenes* strain with greater potential.

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