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Purification, Cloning, and Primary Structure of a New Enantiomer-Selective Amidase from a *Rhodococcus* Strain: Structural Evidence for a Conserved Genetic Coupling with Nitrile Hydratase

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A new enantiomer-selective amidase active on several 2-aryl propionamides was identified and purified from a newly isolated *Rhodococcus* strain. The characterized amidase is an apparent homodimer, each molecule of which has an M_r of 48,554; it has a specific activity of 16.5 μmol of *S*(+)-2-phenylpropionic acid formed per min per mg of enzyme from the racemic amide under our conditions. An oligonucleotide probe was deduced from limited peptide information and was used to clone the corresponding gene, named *amdA*. As expected, significant homologies were found between the amino acid sequences of the enantiomer-selective amidase of *Rhodococcus* sp., the corresponding enzyme from *Brevibacterium* sp. strain R312, and several known amidases, thus confirming the existence of a structural class of amidase enzymes. Genes probably coding for the two subunits of a nitrile hydratase, albeit in an inverse order, were found 39 bp downstream of *amdA*, suggesting that such a genetic organization might be conserved in different microorganisms. Although we failed to express an active *Rhodococcus* amidase in *Escherichia coli*, even in conditions allowing the expression of an active R312 enzyme, the high-level expression of the active recombinant enzyme could be demonstrated in *Brevibacterium lactofermentum* by using a pSR1-derived shuttle vector.

After a systematic search for soil microorganisms able to carry out the enantiomer-selective hydrolysis of racemic nitriles or amides, especially 2-aryl propionamides, we recently reported the purification and cloning of such an amidolytic activity from *Brevibacterium* sp. strain R312 (15). We found that the gene coding for the enantiomer-selective amidase, *amdA*, was closely linked to the genes coding for the two subunits, α and β, of nitrile hydratase, the enzyme responsible for the hydration of various nitriles to the corresponding amides (10). The structure of this region led to the hypothesis that the two genes could be translated from a polycistronic mRNA, thus supporting the view that there is a nitrile utilization operon, at least in this bacterium. These results have been confirmed by another group (9). Moreover, it was found that the identified amidase displays a striking homology to other known amidases, such as the acetamidase from *Aspergillus nidulans* and the indoleacetamide hydrolases from *Pseudomonas syringae* (*savastanoi*) and *Agrobacterium tumefaciens*. Interestingly, the homology was not scattered through the whole amino acid sequence but was mainly focused in a portion of the sequence around amino acids 150 to 220 of the R312 sequence (47 to 65% strict identity), strongly suggesting that this conserved sequence could be part of the active site of this class of enzymes.

We now report the identification of a new *Rhodococcus* strain, obtained during a microbiological screening of soil samples, which is also characterized by efficient stereospecific amidase activity on 2-aryl propionamides. The enzyme responsible for this activity was purified, cloned, and sequenced. It is significantly homologous to the R312 amidase, with the same homodimeric quaternary structure; furthermore, the gene coding for this enzyme is also found immediately upstream from genes probably coding for the two subunits of a nitrile hydratase, thus extending the validity of our previous observations. Finally, our results suggest that this recombinant protein can be expressed as a completely active enzyme in coryneform species such as *Brevibacterium lactofermentum*, but not in *Escherichia coli*.

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MATERIALS AND METHODS

Abbreviations. HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; DTT, dithiothreitol; P*trp*, *E. coli* tryptophan operon promoter; p_R, early right promoter from bacteriophage lambda; PPAmide: racemic 2-phenylpropionamide; HPPAmide, racemic 2-(4-hydroxyphenoxy) propionamide; TFA, trifluoroacetic acid; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; LB, Luria-Bertani; ORF, open reading frame; PCR, polymerase chain reaction.

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are described in Table 1. When enzymatic activities were tested, the *Rhodococcus* strain was cultivated in a semiminimal medium buffered at pH 7.2, containing (per liter) 5 g of glycerol, 1 g of yeast extract (Difco), 1 g of beef extract (Difco), 5 g of isobutyronitrile, 2 g of K_2HPO_4, 0.5 g of $MgSO_4$, 20 mg of $FeSO_4$, 20 mg of $MnSO_4$, 10 mg of NaCl, 2 mg of $CaCl_2$, 0.15 mg of Na_2MoO_4, 0.04 mg of $ZnSO_4$, 4 μg of $CuSO_4$, 4 μg of $CoCl_2$, 0.2 mg of H_3BO_3, and 0.1 mg of KI. For other purposes, the *Rhodococcus* strain and recombinant *B. lactofermentum* or *E. coli* strains were routinely grown on LB plates or in liquid LB medium at 30 or 37°C, respectively. Ampicillin at 100 μg/ml and kanamycin at 20 μg/ml were used to select for and

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
Rhodococcus	Natural isolate	This study
B. lactofermentum		ATCC 21086
B. lactofermentum RP2	Spontaneous Rif^r derivative of ATCC 21036	27a
E. coli DH5α	F$^-$ *endA1 hasR17* ($r_K^-m_K^-$) *supE44 thi-1* λ$^-$*recA1 gyrA96 relA1* φ80 dlacZΔM15	Clontech Laboratory, Palo Alto, Calif.
E. coli B	Wild-type strain	15a
E. coli E103S	*lon met*	D. L. Simon, Waksman Institute of Microbiology, Piscataway, N.J.
Plasmids and bacteriophages		
pIC20H	Multicloning site	14
pUC8, −19	Multicloning site	27
pXL534	Ap^r, P*trp* promoter vector expressing human serum albumin	12
pXL694	Ap^r, P*trp* promoter vector expressing human angiogenin	7
pXL1029	Ap^r, p_RcIts promoter vector expressing interleukin 1β	11
pXL1835	Ap^r, pUC derivative containing 3.2-kbp *amdA* insert	This study
pXL1836	Ap^r, same as pXL1835; reverse orientation of insert	This study
pXL1891	Ap^r, pIC derivative containing P*trp*, *c*II RBS[a] and the first 39 codons of amidase	This study
pXL1892	Ap^r, pIC derivative containing p_R *c*Its, *c*II RBS, and the first 39 codons of amidase	This study
pXL1893	Ap^r, *amdA* under control of *c*II RBS and P*trp*	This study
pXL1894	Ap^r, *amdA* under control of *c*II RBS and p_R *c*Its	This study
pSR1	Endogenous cryptic plasmid from *C. glutamicum*	28
pSV73	Ap^r, shuttle vector *E. coli*-corynebacteria, pUC and pSR1 derivative	This study
pYG822	Ap^r, Km^r, *amdA* under control of *E. coli* P*trp*, on pSV73	This study

[a] RBS, ribosome-binding site.

maintain *E. coli* and *B. lactofermentum* plasmids, respectively. Conditions for the expression of heterologous expression in *E. coli* have been described previously (7, 11). *B. lactofermentum* RP2 is a spontaneous rifampin-resistant isolate of *B. lactofermentum* ATCC 21086, the endogenous plasmids of which were cured after extensive protoplastisation and regeneration of the cell wall (27a). Strain RP2 was transformed by electroporation as described previously (8), using the Bio-Rad gene pulser and pulse controller unit; 10^5 transformants per μg of DNA were routinely obtained. Conditions used for the expression of recombinant amidase in *B. lactofermentum* were as follows. Five milliliters of LB medium containing kanamycin was inoculated with a fresh colony of recombinant RP2 and grown at 30°C for 24 h. This inoculum culture was then diluted 1/100 in fresh LB medium containing kanamycin, and cells were collected for protein analysis after 24 h at 30°C.

Materials and general methods. Materials and standard procedures used in molecular biology have been described previously (13). DNA sequencing was partly carried out with the 370A automated DNA sequencer and the M13 (−21) fluorescent-dye primers from Applied Biosystems (5).

Enzyme assay. A sample of source enzyme (about 0.017 to 0.034 U; 1 U is defined as 1 μmol of *S*(+)-2-phenyl propionic acid formed per min under the conditions described) was incubated at 30°C for 30 min in 500 μl of 0.1 M Tris-HCl, pH 7.5, containing 5 mM DTT and 18 mM PPAmide (prepared from racemic phenyl-2-propionitrile [Aldrich]). After incubation, 2 ml of a solution of acetonitrile and 1 N HCl (90:10) and then 2 ml of the HPLC mobile phase (50 mM H_3PO_4 and CH_3CN [75:25]) were added to the reaction mixture. After a 10-min centrifugation at 5,000 × *g*, a 10-μl aliquot of the supernatant was analyzed by HPLC on a Nucleosil 5-C18 column (4.6 by 250 mm; Macherey-Nagel). The elution was carried out at a rate of 1 ml/min; eluted compounds were detected at 215 nm.

Enantiomer selectivity assay. The enantiomer excess of reaction products was checked as follows. The incubation mixture was diluted with 10 volumes of CH_3CN–1 N HCl (90:10) and centrifuged. The organic phase was then separated by adding NaCl and dried by evaporation. The residue was solubilized in the elution buffer at the proper concentration. *S*(+) and *R*(−) enantiomers of 2-phenylpropionic acid (S and R, respectively) were assayed by HPLC on a chiral column (5-μm pore size, 100 by 4 mm; Chromtech Chiral AGP) with 10 mM sodium phosphate (pH 4.0) used as the elution buffer. If ee (%) is the enantiomer excess $[(S - R)/(S + R)] \times 100$, the enantiomeric ratio *E* can be defined as $\ln\{1 - C[1 + ee(P)]\}/\ln\{1 - C[1 - ee(P)]\}$, where *C* represents the extent of conversion and ee(P) is the enantiomeric excess of the product fraction (4). In the case of HPPAmide, when the *R* acid is obtained, the same definition holds with $ee = [(R - S)/(S + R)] \times 100$.

Purification of enantiomer-selective amidase activity. Unless otherwise mentioned, all steps were performed at room temperature and the pH of all buffers was adjusted to 7.5.

(i) Step 1: preparation of a crude extract. A cell extract of *Rhodococcus* sp. was prepared at 4°C by ultrasonic treatment of 7.0 g of wet cells in 15 ml of 0.1 M Tris-HCl–5 mM DTT and then centrifugation at 50,000 × *g* for 1 h.

(ii) Step 2: first ion-exchange chromatography. One volume of 25 mM Tris-HCl–5 mM DTT (buffer A) was added to the clear supernatant, and the solution was loaded onto a Mono Q HR 10/10 column (Pharmacia) equilibrated with buffer A. Proteins were eluted at a rate of 3 ml/min with a 180-ml linear 0 to 1.0 M gradient of KCl in buffer A. Fractions containing

amidase activity (eluted at around 0.3 M KCl) were pooled and concentrated to 2 ml with a Centriprep 10 concentrator (Amicon).

(iii) Step 3: second ion-exchange chromatography. The protein solution was mixed with 3 volumes of buffer A and applied to a Mono Q HR 5/5 column (Pharmacia) equilibrated with buffer A. Proteins were eluted at a rate of 1 ml/min with a 25-ml linear 0 to 0.5 M gradient of KCl in buffer A. Active fractions were pooled, adjusted to 15% (wt/vol) glycerol, and concentrated to 1 ml.

(iv) Step 4: hydrophobic chromatography. A 1-ml volume of 0.1 M Tris-HCl–0.5 mM DTT–1.7 M ammonium sulfate (buffer B) was added to the protein fraction from step 3, and the solution was loaded at a flow rate of 0.25 ml/min onto a Phenyl-Superose HR 5/5 column (Pharmacia) equilibrated with 0.1 M Tris-HCl–0.5 mM DTT–0.85 M ammonium sulfate. Proteins were eluted at a rate of 0.5 ml/min with a linear decreasing gradient of ammonium sulfate (0.85 to 0 M). The fraction containing activity was adjusted to 15% glycerol and mixed with 1 volume of buffer A.

(v) Step 5: hydroxyapatite chromatography. The solution was injected onto a Bio-Gel HPHT column (Bio-Rad) equilibrated with 85 mM Tris-HCl–0.5 mM DTT–10 μM $CaCl_2$–15% glycerol (buffer C). Amidase was eluted at a rate of 0.5 ml/min with a linear gradient of 0 to 100% 0.35 M potassium phosphate–0.5 mM DTT–10 μM $CaCl_2$–15% glycerol in buffer C.

Protein sequencing. An aliquot of the purified protein was applied on a TSK-G3000 SW (Tosoh Co. Ltd.) gel permeation column and eluted at a rate of 0.5 ml/min with 20 mM NaH_2PO_4–50 mM Na_2SO_4 (pH 6.8). The apparent M_r of the enzyme was estimated by using GPC6/7000 software (Perkin-Elmer). The protein was then recovered and kept frozen. One nanomole was directly subjected to N-terminal sequencing by using the Applied Biosystems model 470A apparatus; no significant signal was found, probably indicating a blocked N terminus. Another nanomole was completely digested by trypsin (5 μg), and the resulting hydrolysate was loaded on a VYDAC C18 narrow-bore column (length, 22 cm) equilibrated in 0.07% aqueous TFA. Peptides were eluted at a rate of 0.2 ml/min with the following linear gradient of acetonitrile containing 0.07% TFA: 0%, 0 to 10 min; 0 to 35%, 10 to 150 min; 35 to 50%, 150 to 170 min; 50 to 100%, 170 to 190 min. Three fractions gave clear, reliable sequences: fraction 123, NH_2-ADPATVDVPVPDYAAAL TGDVR; fraction 124, NH_2-TFLEAGELVPATDYIK; and fraction 162, NH_2-ELFQDIDVLIAPTVSSPALP.

Oligonucleotide probe, Southern blots, and colony hybridization. The 32-mer degenerated oligonucleotide probe 5′-GCIACIGTIGA(T,C)GTICCIGTICCIGA(T,C)TA(T,C)GC-3′, containing seven inosines, was deduced from the peptide sequence ATVDVPVPDYA of sequence 123 and synthesized as described previously (7). The preparation of genomic DNA, Southern blots, and colony hybridization procedures were carried out as described previously (15). Hybridization was at 55°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt solution–0.1% SDS–50 mM sodium phosphate (pH 6.5)–250 μg of single-stranded DNA per ml. Filters were washed several times in 6× SSC at room temperature and then in 2× SSC–0.1% SDS at 50°C for 5 min.

Plasmid constructions. (i) *E. coli* expression plasmids. An *Nde*I site containing the presumed ATG initiation codon of the amidase structural gene was created by using PCR, by amplifying a DNA fragment of plasmid pXL1835 between the following primers: 5′-CGCGGTGGGAGTGCATATG GGCTTGCATG-3′ and 5′-AGATCTTCGGTTCGATCTCC-3′. The 114-bp *Nde*I-*Bgl*II PCR fragment obtained after digestion with these enzymes was then ligated to either of the two *Eco*RI-*Nde*I fragments carrying a promoter and the lambda *c*II-derived ribosome-binding site, between the *Eco*RI and the *Bgl*II sites of plasmid pIC20H (14). pXL1891 was derived in this way with the 121-bp *Eco*RI-*Nde*I fragment of pXL534 (12) containing the P*trp* promoter, whereas pXL1892 contains the 1,228-bp *Eco*RI-*Nde*I fragment of pXL1029 (11) carrying both the lambda p_R promoter and *c*Its. Expression plasmids pXL1893 and pXL1894 were obtained by ligating the *Eco*RI-*Bgl*II fragment of pXL1891 and pXL1892 (containing the promoter, the ribosome-binding site, and the first 39 codons of amidase), respectively, to the 1,358-bp *Bgl*II-*Sal*I fragment (containing the rest of the amidase gene) of pXL1835 and to the 3.1-kbp *Eco*RI-*Sal*I vector fragment of pXL694 (7).

(ii) *B. lactofermentum* expression plasmid. Plasmid pSV73 (see Fig. 6) is an *E. coli*/corynebacteria shuttle vector derived from the endogenous cryptic plasmid pSR1 (3 kbp) isolated from *Corynebacterium glutamicum* ATCC 19223 (28) as follows. Plasmid pRS1 was first linearized at its *Bcl*I site and cloned into the *Bam*HI site of plasmid pUC8. The *Pst*I fragment of plasmid pUC4K (Pharmacia) containing the aminoglycoside 3′-phosphotransferase gene from transposon Tn*903* was then incorporated into the *Pst*I site of the pSRI-pUC8 hybrid plasmid to yield pSV73. Expression plasmid pYG822 (Fig. 6) was then obtained by ligating the 6.9-kbp *Sal*I-*Bgl*II vector fragment of pSV73 to the 1,696-bp *Hin*dIII-*Sal*I fragment of pXL1835 containing the whole amidase gene and to the 96-bp *Bam*HI-*Hin*dIII fragment of pXL1891 containing the *E. coli* P*trp* promoter.

Nucleotide sequence accession number. The sequence of the 3.2-kbp *Pst*I fragment of *Rhodococcus* sp. has been assigned the GenBank accession number M 74531.

RESULTS

Identification and purification from a *Rhodococcus* strain of an enantiomer-selective amidase active on 2-aryl propionamides. A new strain able to use isobutyronitrile as the sole nitrogen source was isolated during a microbiological screening of soil samples. According to current taxonomic classification criteria, this gram-positive, nonsporing strain was identified as a member of the genus *Rhodococcus* (data not shown) and will thus be referred to as *Rhodococcus* sp. in this report. Further analysis of this strain, following the experimental protocols described in Materials and Methods, indicated that it contains amidase activity able to catalyze the stereospecific hydrolysis of 2-aryl propionamides such as PPAmide or 2-aryloxy propionamides such as HPPAmide into the corresponding *S* or *R* acid, respectively. A comparison of the amidolytic activities of intact cells of *Rhodococcus* sp. and of the previously identified *Brevibacterium* sp. strain R312 (15) is presented in Table 2. We found the new strain to be at least 15- to 20-fold more active on PPAmide, but 6-fold less active on HPPAmide, than R312. The enantiomer excess of the acid product was found to be above 95% on several substrates, demonstrating the enantiomer selectivity of the amidolytic reaction. By using PPAmide as a substrate, a specific activity of about 0.4 U/mg of bacterial proteins was found in the soluble cell extract. This enzymatic activity was subsequently purified 40-fold, to a purity of at least 95%, through the different steps detailed in Materials and Methods (Table 3). The apparent M_r of the purified native enzyme was estimated at 118,000 by HPLC

TABLE 2. Comparison of amidase specific activities of *Brevibacterium* sp. strain R312 and *Rhodococcus* intact cells on PPAmide and HPPAmide

Substrate	Activity[a]		Ratio
	Brevibacterium sp. strain R312	*Rhodococcus* sp.	
PPAmide	0.016 (68)	0.333 (311)	21
HPPAmide	0.242 (92)	0.040 (ND)	0.17

[a] Values are in standard units (see Materials and Methods). The *E* value is also given (in parentheses) as an indication of the enantiomer selectivity of the reaction. Activities were normalized per milligram of total soluble proteins in the cell sample. ND, not determined.

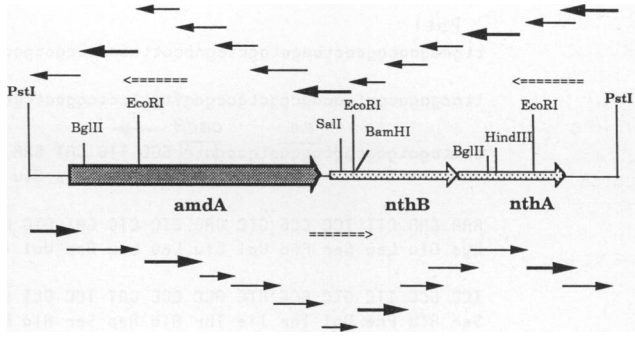

FIG. 1. Restriction map of the 3,221-bp *Pst*I fragment containing the *amdA* gene from the *Rhodococcus* genome. This fragment was cloned in both orientations in the pUC19 polylinker to yield pXL1835 and pXL1836. The positions of the genes are indicated by the large arrows. *nthA* and *nthB* are the proposed genes for the two subunits of nitrile hydratase. The sequencing strategy for both strands is indicated by the short arrows above and under the fragment. Thick and thin arrows represent automated and manual sequencing data on single-stranded templates, respectively, whereas dashed arrows indicate double-stranded sequencing data.

gel filtration (data not shown). However, a unique band of apparent M_r 53,000 ± 2,000 was detected by silver staining an SDS-polyacrylamide gradient gel (see also Fig. 5, lane H), strongly suggesting a homodimeric quaternary structure for the active enzyme, as already found in the case of the enantiomer-selective amidase recently isolated from *Brevibacterium* sp. strain R312 (15). The presence of glycerol (15%) and a reducing agent such as DTT was found to be necessary to maintain the enzymatic activity throughout the purification procedure, but the purified enzyme could be kept frozen at −20°C in the elution buffer of the last hydroxyapatite step for several weeks without noticeable loss of activity. The purified enzyme thereby obtained displayed a specific enantiomer-selective (ee > 95%) activity of about 16.5 U/mg.

Cloning of the *Rhodococcus* amidase. After a supplementary purification step on TSK-G3000 SW, the enzyme was subjected to sequencing. Since the N-terminal extremity was inaccessible to Edman-type chemistry, a total trypsin hydrolysis was carried out and three HPLC fractions of the hydrolysate—123, 124, and 162—provided peptides that allowed an unambiguous sequence to be obtained (Materials and Methods). From the sequence obtained from fraction 123, a 32-mer nucleotide probe was synthesized, corresponding to a mixture of eight oligonucleotides and containing seven inosines in positions degenerated at least three times (see Materials and Methods). This probe, labeled at the 5′ end with ^{32}P, was hybridized to a Southern transfer of genomic DNA from the *Rhodococcus* strain previously digested by one of several restriction enzymes. Conditions under which the probe gave strong, unambiguous signals (see Materials and Methods) were found; in particular, with *Bam*HI, *Kpn*I, *Sph*I, *Sst*I, *Sma*I, *Sal*I, and *Pst*I digestions, a single genomic band strongly hybridizing to the probe was found. The hybridizing band corresponding to a *Pst*I fragment of approximately 3.2 kb was chosen for further cloning. The ≈3- to ≈4-kb *Pst*I digestion fragments of genomic DNA were thus purified by preparative electrophoresis through agarose followed by electroelution, and they were then ligated to plasmid pUC19 that had been cut by *Pst*I. After the transformation of *E. coli* DH5α and selection on LB ampicillin X-gal plates, 600 white clones were repicked individually and probed by colony hybridization under stringency conditions similar to those of the Southern blot hybridization. The nine clones with particularly strong hybridization signals were then analyzed by restriction of plasmid DNA. Of six clones that each had a single copy of the same 3.2-kb *Pst*I fragment clearly inserted in the two possible orientations, two clones representing each orientation (Fig. 1, pXL1835 and pXL1836) were analyzed in more detail (detailed mapping, Southern analysis), thereby confirming that the desired fragment had been cloned.

TABLE 3. Purification of the enantiomer-selective amidase

Purification step[a]	Vol (ml)	Amt of protein (mg)	Sp act (U/mg)	Recovery (%)	Purification (fold)
Crude extract	17	264	0.4		
Mono Q 10/10	6.0	27.6	2.5	64	6.2
Mono Q 5/5	3.0	8.9	5.3	44	13
Phenyl-Superose	1.0	4.77	9.8	43	24
Bio-Gel HPHT	1.5	2.69	16.5	41	40

[a] Steps are detailed in Materials and Methods.

Nucleotide sequence of the *Pst*I fragment from *Rhodococcus* sp.: evidence that the gene coding for the enantiomer-selective amidase is coupled to a gene coding for a nitrile hydratase. The complete nucleotide sequence of both strands of the 3.2-kbp *Pst*I fragment was determined, as indicated in Fig. 1. The 3,227-bp sequence is shown in Fig. 2. The overall G+C composition of this fragment is 62.4%. Analysis of the sequence first revealed an ORF of 1,386 nucleotides (positions 210 to 1595), coding for a polypeptide of 462 amino acids (M_r, 48,554) that contains the three peptides previously obtained by sequencing the trypsic fragments. This ORF thus corresponds to the structural gene, *amdA*, of the enantiomer-selective amidase from *Rhodococcus* sp. Since an N-terminal sequence of the purified natural enzyme could not be determined, the exact position of the initiation codon remained uncertain at that stage (see below). However, the indicated codon (Fig. 2) is clearly the only possible ATG codon compatible with the apparent M_r of the protein. A putative ribosome-binding site, GGAG, is found six nucleotides upstream. In this coding sequence, the mean G+C composition of positions 1, 2, and 3 of the codons is 68.6, 50.6, and 74.5%, respectively, which is typical of organisms with high G+C compositions (1). Since additional ORFs are found 3′ to *amdA* on the nucleotide sequence of the 3.2-kbp *Pst*I fragment and because such a genetic organization had

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                Pst I
ctgcagaacggaactaagatggctcgaaccttcaccaaagacggacttgaacacagcctcgcacttgcgcgtttggagctcccggacgagcg    92

ttacgagacggtgacagcggctgccgagttggtcctcggactcgctgaggctctggatgctgtcccgctggccgagactccgatggcagccg   184

                s.D.      amdA ──►
ccttcgatgcgcggtgggagtgacgatg GGC TTG CAT GAA CTG ACG CTC GCG CAA GTC GCT GCG AAG ATC GAG AAC   260
                             Gly Leu His Glu Leu Thr Leu Ala Gln Val Ala Ala Lys Ile Glu Asn    16

AAA GAA CTT TCC CCG GTC GAG CTC CTC GAT GTG ATC CTG GCG CGC GTC GCG GAG ATC GAA CCG AAG ATC   329
Lys Glu Leu Ser Pro Val Glu Leu Leu Asp Val Ile Leu Ala Arg Val Ala Glu Ile Glu Pro Lys Ile    39

TCC GCC TTC GTC ACG ATC ACC GCC GAT TCC GCT CGG AAG GCG GCC CGG CTC GCA GCC GAC GAG ATC GCA   398
Ser Ala Phe Val Thr Ile Thr Ala Asp Ser Ala Arg Lys Ala Ala Arg Leu Ala Ala Asp Glu Ile Ala    62

GGT GGG CAC TAT CGC GGT CCG CTG CAC GGA GTT CCG ATT GGC CTC AAG GAT CTG TTC GAA GTG GCA GGC   467
Gly Gly His Tyr Arg Gly Pro Leu His Gly Val Pro Ile Gly Leu Lys Asp Leu Phe Glu Val Ala Gly    85

GTC CCG AAT ACC GCG AGT TCG CGG GTC CGA GCT GAC TAC ATC CCC TCA TCG GAT GGG GCC GCG GTC GAG   536
Val Pro Asn Thr Ala Ser Ser Arg Val Arg Ala Asp Tyr Ile Pro Ser Ser Asp Gly Ala Ala Val Glu   108

AAG CTC ACC GCC GGT GGA GCG GTC ATG ATC GGC AAG ACG CAC ACT CAC GAA TTC GCC TAC GGT GCG ATC   605
Lys Leu Thr Ala Gly Gly Ala Val Met Ile Gly Lys Thr His Thr His Glu Phe Ala Tyr Gly Ala Ile   131

ACA CCG ACC ACC CGT AAT CCA TGG GAC CCC ACC CGG ACA CCC GGC GGT TCC AGC GGT GGG ACG GCA GCA   674
Thr Pro Thr Thr Arg Asn Pro Trp Asp Pro Thr Arg Thr Pro Gly Gly Ser Ser Gly Gly Thr Ala Ala   154

GCT CTC GCG GCA GGC CTC ATC TTC GCC GGT ATG GGT ACC GAT ACC GGG GGG TCC ATT CGG ATA CCA GCC   743
Ala Leu Ala Ala Gly Leu Ile Phe Ala Gly Met Gly Thr Asp Thr Gly Gly Ser Ile Arg Ile Pro Ala   177

GCC GTC TGC GGG ACG GTA GGT CTC AAA CCC ACA TAT GGT CGC GTT TCG CGT CGT GGA GTG ACC TCC TTG   812
Ala Val Cys Gly Thr Val Gly Leu Lys Pro Thr Tyr Gly Arg Val Ser Arg Arg Gly Val Thr Ser Leu   200

TCA TGG TCT CTG GAC CAC GCG GGA CCG CTG GCC CGG ACC GTG GAA GAC GCT GCC ATC ATG CTG AAC CAG   881
Ser Trp Ser Leu Asp His Ala Gly Pro Leu Ala Arg Thr Val Glu Asp Ala Ala Ile Met Leu Asn Gln   223

ATC GCT GGC TAT GAC CGG GCT GAT CCT GCG ACG GTA GAT GTG CCC GTT CCC GAC TAC GCG GCG GCG CTG   950
Ile Ala Gly Tyr Asp Arg Ala Asp Pro Ala Thr Val Asp Val Pro Val Pro Asp Tyr Ala Ala Ala Leu   246

ACC GGA GAC GTC CGA GGG CTG CGG ATT GGT GTG CCG ACC AAT TTC TAC ACC GAC AAC GTC CAT CCC GAG  1019
Thr Gly Asp Val Arg Gly Leu Arg Ile Gly Val Pro Thr Asn Phe Tyr Thr Asp Asn Val His Pro Glu   269

GTT GCC GCA GCG GCC GAC GCT GCG GTG GCG CAA CTG GCC CAT TTG GGT GCG GTG GTC CGC GAA GTG AAG  1088
Val Ala Ala Ala Ala Asp Ala Ala Val Ala Gln Leu Ala His Leu Gly Ala Val Val Arg Glu Val Lys   292

ATC CCG ATG GCA GAG GTC ATC GTG CCC ACC GAG TGG AGC TTG CTC GTC CCG GAG GCG TCG GCC TAC CAC  1157
Ile Pro Met Ala Glu Val Ile Val Pro Thr Glu Trp Ser Leu Leu Val Pro Glu Ala Ser Ala Tyr His   315

CAG CAG ATG CTG CGC GAG CGC GCA GAT CAC TAC ACC GAC GAG ACG AGA ACC TTC CTG GAA GCC GGC GAA  1226
Gln Gln Met Leu Arg Glu Arg Ala Asp His Tyr Thr Asp Glu Thr Arg Thr Phe Leu Glu Ala Gly Glu   338

CTC GTT CCG GCG ACC GAC TAC ATC AAG GCG CTG CGG GTG CGC ACC CTC ATC CAG GCA GCC TTC CGG GAA  1295
Leu Val Pro Ala Thr Asp Tyr Ile Lys Ala Leu Arg Val Arg Thr Leu Ile Gln Ala Ala Phe Arg Glu   361

CTG TTC CAG GAC ATC GAT GTC CTG ATC GCA CCC ACG GTC AGC TCT CCG GCT CTG CCG CTC GAT GAC CTG  1364
Leu Phe Gln Asp Ile Asp Val Leu Ile Ala Pro Thr Val Ser Ser Pro Ala Leu Pro Leu Asp Asp Leu   384

GAA GTC ACT TGG CCC GAT GGC ACA TCC GAA GGC GGC ACC ATC ACC TAT GTC CGT CTC AGC GCC CCC GGC  1433
Glu Val Thr Trp Pro Asp Gly Thr Ser Glu Gly Gly Thr Ile Thr Tyr Val Arg Leu Ser Ala Pro Gly   407

AAC GTC ACC GGA CTT CCA GCG CTG TCG GTC CCC TCC GGC TTC ACC GAG CAA GGC CTT CCC ACC GGT ATC  1502
Asn Val Thr Gly Leu Pro Ala Leu Ser Val Pro Ser Gly Phe Thr Glu Gln Gly Leu Pro Thr Gly Ile   430

CAG ATC ATC GGC CGT CCC TTC GAC GAG GAG ACC GTC CTC AAC GTC GGT CAC GCC TAC GAA GGC TGC ACG  1571
Gln Ile Ile Gly Arg Pro Phe Asp Glu Glu Thr Val Leu Asn Val Gly His Ala Tyr Glu Gly Cys Thr   453

                                                    s.D.      presumed nthB ──►
GAC TGG CCG CGA CTG GCG CCG CTT  tgaactactgaccccccattggagaaaaccgaaggagagaacgatgAAT GGA GTG  1649
Asp Trp Pro Arg Leu Ala Pro Leu  ***                                  Asn Gly Val    464

TTC GAT TTG GGT GGG ACC GAC GGC ATC GGC CCG GTC GAC CCT CCC GCT GAA GAA CCG GTG TTC CGC GCG  1718
Phe Asp Leu Gly Gly Thr Asp Gly Ile Gly Pro Val Asp Pro Pro Ala Glu Glu Pro Val Phe Arg Ala   487
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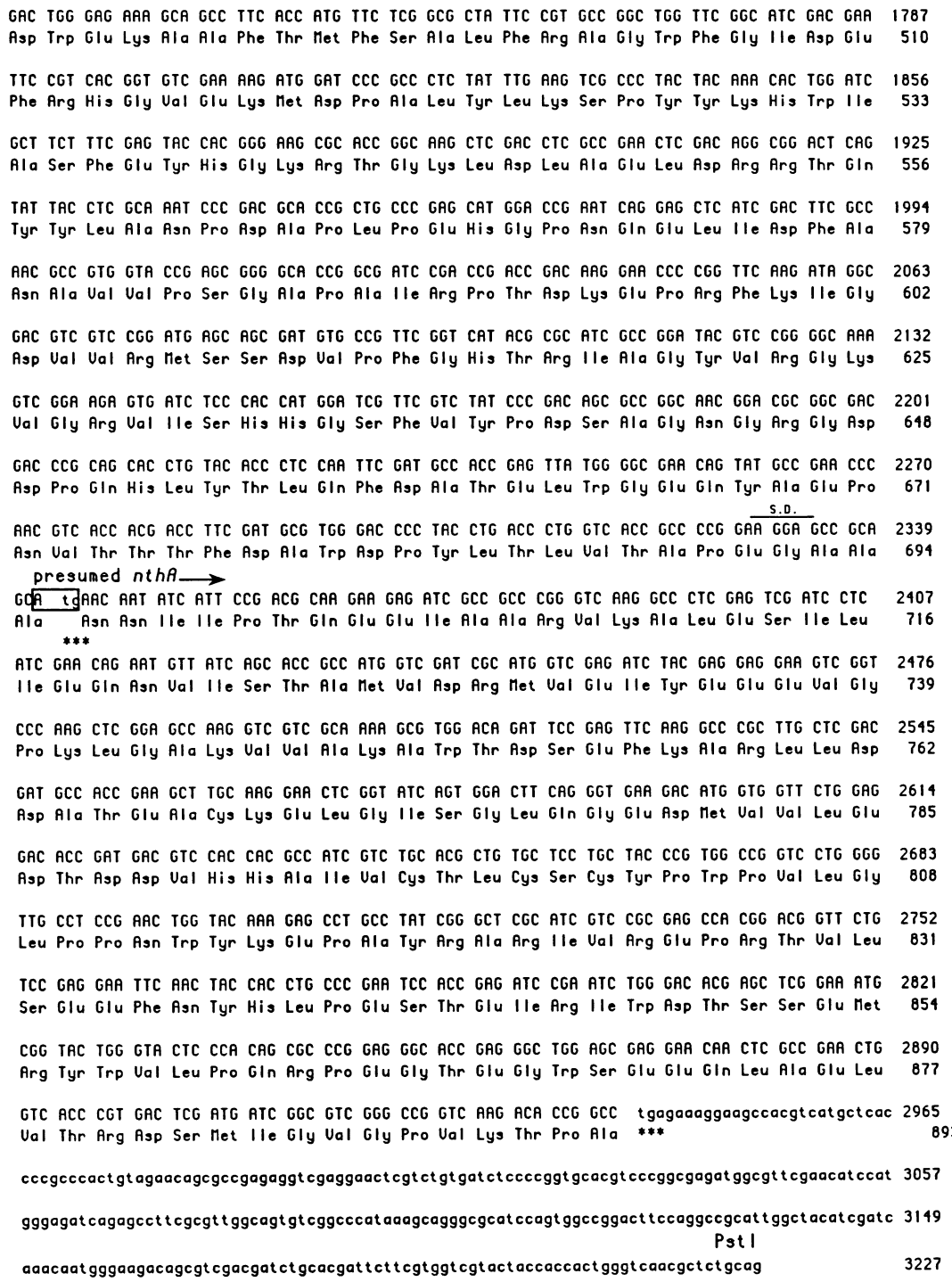

FIG. 2. Nucleotide and amino acid sequences of the *Pst*I fragment shown in Fig. 1. The presumed initiation codons are boxed. S.D., potential ribosome-binding sites. The sequenced peptides from the purified *Rhodococcus* enantiomer-selective amidase are underlined.

already been demonstrated for *Brevibacterium* sp. strain R312, we investigated whether the deduced peptide sequences bore some resemblance to the sequences of the subunits of nitrile hydratase from *Brevibacterium* sp. strain R312 (or *Rhodococcus* sp. strain N-774) (10, 15). This proved indeed to be the case: the ORF which immediately follows *amdA* (positions 1638 to 2342) encodes a protein having a significant degree of homology to the sequence of the β subunit of nitrile hydratase (31% strict identity; Fig. 3, right panel). That the homology starts from the N terminus indicates that the proposed ATG is probably the initiation codon for this ORF, which could code for a protein with an M_r of 26,000 (26K). This is further suggested by the presence of the Shine-Dalgarno sequence AGGAG six nucleotides

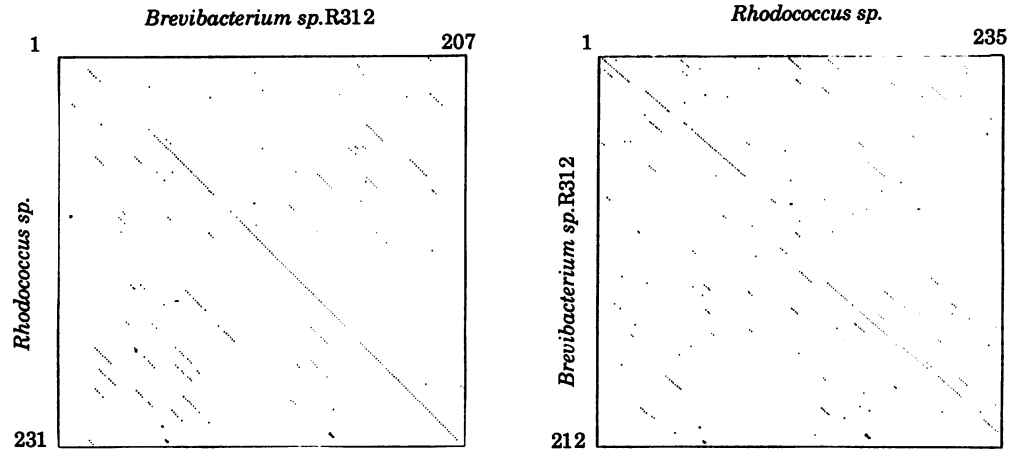

FIG. 3. Dot matrix analysis (22) of a comparison of the amino acid sequences of *Brevibacterium* sp. strain R312 nitrile hydratase and the putative nitrile hydratase from *Rhodococcus* sp. Left panel, α subunit (*nthA*); right panel, β subunit (*nthB*). Numbers in corners of panels are residue numbers.

upstream from this codon. A second ORF, probably initiated by an ATG overlapping the TGA stop codon of the previous coding sequence (positions 2,342 to 2,938; this ATG codon is also preceded by the possible Shine-Dalgarno sequence AGGAG), displays an even more important homology (49% strict identity; Fig. 3, left panel) to the α subunit of nitrile hydratase and could code for a 22.5K protein. The DNA sequence was also analyzed by the program of Staden and McLachlan (21, 23), which uses codon preference to identify the coding sequences. A codon preference table was established from the codon usage in the sequenced genes of a close gram-positive microorganism of similar G+C content, *B. lactofermentum*. According to the results of this analysis (not shown), only the above-mentioned ORFs are compatible with sequences coding for proteins in this region of the sequenced fragment.

Sequence homology with known amidases. The peptide sequences of the amidases from *Brevibacterium* sp. strain R312 (15) and *Rhodococcus* sp. have been compared, as shown in Fig. 4A. The two proteins display a highly significant level of homology, especially in the second third of the sequences, between residues 150 and 280 of the R312 sequence. The highest homology is found in the region of residues 158 to 215 of R312 (137 to 193 in the *Rhodococcus* sequence; 67% strict identity), a portion of the sequence which had been proposed to be part of the active site on the basis of its conservation in other amidases (15). Not surprisingly, a systematic search in the GENPRO protein data base of proteins displaying good local homologies to the *Rhodococcus* amidase gave proteins previously identified for their homology to the R312 amidase (15), namely, the acetamidase of *A. nidulans* (6) and the indoleacetamide hydrolases of *P. syringae* (26), *A. tumefaciens* (19), and *Bradyrhizobium japonicum* (20). Moreover, two new amidases have been identified by this procedure: the 6-aminohexanoate-cyclic-dimer hydrolases (ACDH-I) from *Flavobacterium* sp. strain K172 and *Pseudomonas* sp. strain NK87 (25). The homology between the *Rhodococcus* amidase and the *Flavobacterium* ACDH-I is important: 43% strict identity is found between positions 100 and 290 and up to 61% is found between positions 137 and 195, which is also the portion that has maximum homology with the other amidases. As shown in Fig. 4B, peptides 137 to 195 of the *Rhodococcus* enzyme are highly conserved in the different amidases, further supporting the previous hypothesis that this conserved sequence might be involved in the active sites of these enzymes. Finally, a putative amidase sequence recently identified in the yeast *Saccharomyces cerevisiae* (3) was also shown to display a significant homology to *Rhodococcus* amidase, mainly localized in the same region (59% strict identity with residues 147 to 193 of *Rhodococcus* sequence; Fig. 4B).

Expression of recombinant *Rhodococcus* amidase in *E. coli*. In order to confirm the identification of the ORF coding for the enantioselective amidase, an *Nde*I site was created by PCR at the presumed ATG initiation codon (position 210 in Fig. 2). The fragment between this site and the *Sal*I position at position 1683, containing only the region coding for the amidase, was placed under the control of strong signals functional in *E. coli* for transcription (promoters P*trp* or p_R) and translation (ribosome-binding site of the lambda *cII* gene) initiation. The vectors thereby obtained, pXL1893 (P*trp*) and pXL1894 (p_R), are similar to vectors pXL1751 and pXL1752 previously described (15) for *E. coli* expression of R312 amidase. Expression from these plasmids was studied in *E. coli* B and *E. coli* K-12 E103S, respectively. As shown in Fig. 5, in the case of pXL1894, a protein comigrating with the purified *Rhodococcus* amidase was produced specifically upon derepression of the promoter. However, this protein was always found to be produced in an insoluble, inactive form, even when growth conditions were used that were previously shown to yield a soluble and active R312 amidase (15). Other *E. coli* expression systems have been tried (results not shown); although some of them do lead to enhanced levels of expression of the protein, no soluble or active protein could ever be detected.

Expression in *B. lactofermentum*. Because *E. coli* did not seem to be a suitable host for the expression of an active *Rhodococcus* amidase, we turned to a gram-positive coryneform microorganism, *B. lactofermentum*. Plasmid pSV73 was used in these experiments as a shuttle vector between *E. coli* and *B. lactofermentum*. This plasmid is derived from plasmid pSR1 of *C. glutamicum* (28) by the insertion of plasmid pUC8 and the kanamycin resistance gene from transposon Tn*903*. As detailed in Materials and Methods, the expression plasmid pYG822 (Fig. 6) was derived from pSV73 by inserting a cassette containing the amidase coding se-

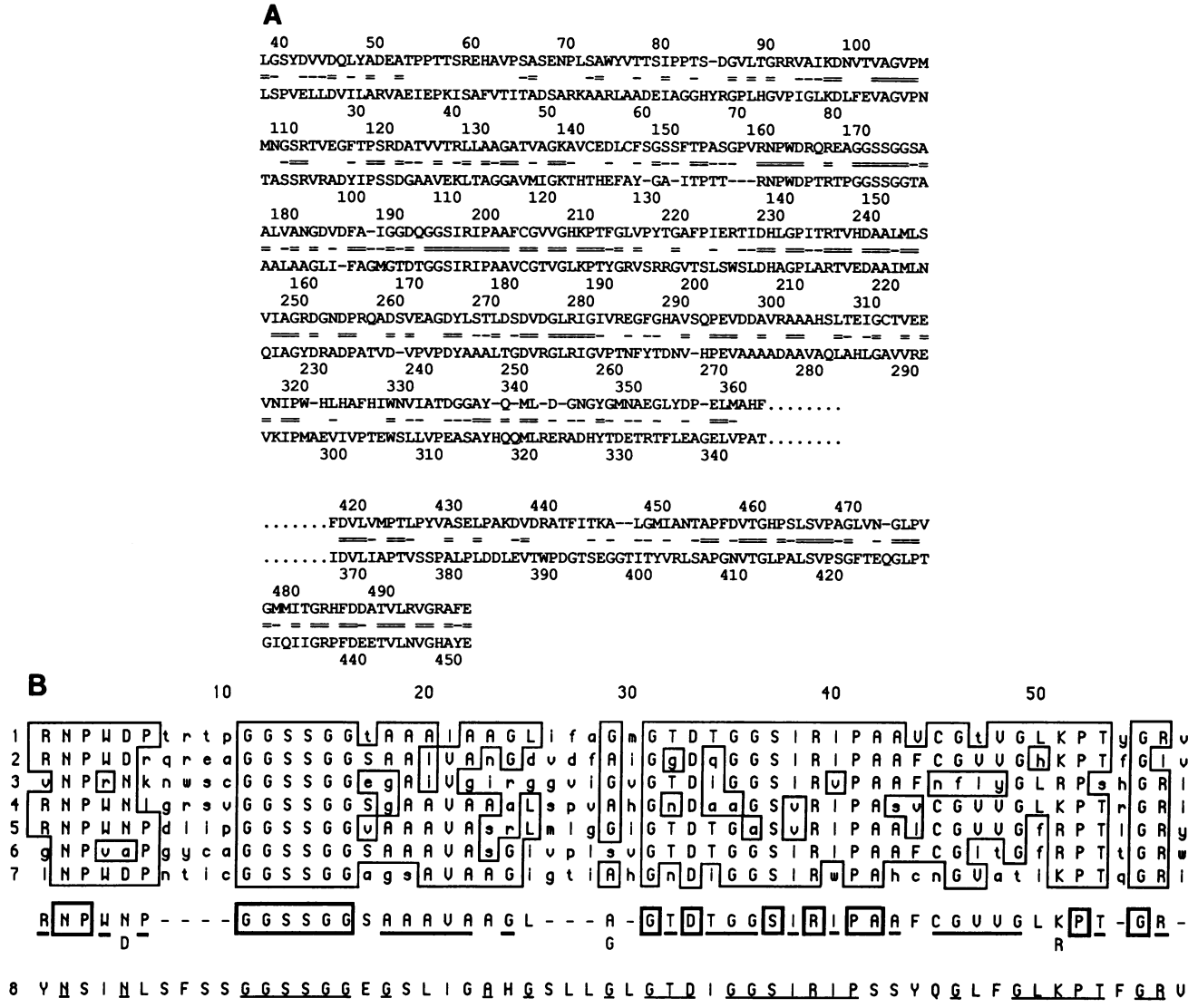

FIG. 4. Comparison of amino acid sequences of different amidases. (A) Alignment of amino acid sequences of *Brevibacterium* sp. strain R312 (upper line) and *Rhodococcus* sp. (lower line) enantioselective amidases. Similarities between the sequences are indicated as follows: =, same amino acid; −, amino acids belonging to the same group (hydroxyl/small aliphatic: A, G, S, T; acid and acid amide: N, D, E, Q; basic: H, R, K; aliphatic: M, I, L, V; or aromatic: F, Y, W). Dashes within the sequence represent gaps in the alignment. (B) Alignment of different amidases. 1, *Rhodococcus* amidase (residues 137 to 193); 2, *Brevibacterium* sp. strain R312 amidase (residues 159 to 215); 3, *A. nidulans* acetamidase (residues 192 to 248); 4, *Flavobacterium* aminohexanoate-cyclic-dimer hydrolase (residues 137 to 193); 5, *A. tumefaciens* indole acetamide hydrolase (IAH) (residues 137 to 193); 6, *P. syringae* IAH (residues 134 to 190); 7, *B. japonicum* IAH (residues 137 to 193); 8, *S. cerevisiae* putative amidase (residues 197 to 253). Consensus residues in block letters are boxed on lines 1 to 7. The next line (between 7 and 8) represents the consensus sequence based on the seven sequences of known amidases; sequences that are boxed, underlined, presented in normal letters, and indicated by hyphens represent positions that are conserved in seven of seven, six to four of seven, and three of seven sequences and nonconserved positions, respectively. Consensus residues in line 8 (putative amidase) are simply underlined.

quence (positions 1 to 1683) with its own translation initiation region under the control of the *E. coli* P*trp* promoter. This promoter was previously shown to be active in corynebacteria (16). We found that, when grown in a nonoptimized LB medium containing kanamycin, a crude protein extract of *B. lactofermentum* harboring pYG822 is characterized by a significant stereospecific hydrolysis activity towards PPAmide (0.105 U/mg), comparable (≈25%) to the activity of an extract from the original *Rhodococcus* strain grown in the indicated medium (0.408 U/mg; see Materials and Methods), whereas the control pSV73-containing *B. lactofermentum* strain is completely inactive on this substrate (<0.002 U/mg). Similar constructions using other promoters gave even much higher expression levels and activities (not shown). In all cases, the enzyme was expressed as a soluble protein. This result demonstrates that the expression of the *amdA* gene is indeed sufficient to account for the hydrolytic properties of the original strain. The recombinant enzyme was purified from the best producer and was shown to be indistinguishable from the natural enzyme on the basis of several criteria, including specific activity (not shown). Moreover, the N-terminal sequence could now be deter-

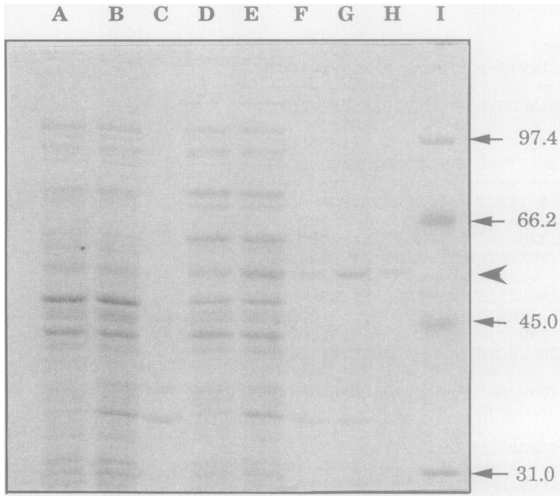

FIG. 5. Expression of *Rhodococcus* amidase from plasmid pXL1894 in *E. coli* E103S: SDS-PAGE on an 8.5% gel (Coomassie blue staining). Expression was carried out at 30°C (lanes A, B, and C) or at 42°C (lanes D, E, and F). Total proteins (lanes B and E), soluble proteins (lanes A and D), and insoluble proteins (lanes C and F) are shown. Purified *Rhodococcus* amidase was applied on lane H (the arrowhead points to the position of the protein [48.5K]). A mixture of the proteins applied in lanes F and H was applied in lane G in order to demonstrate that the purified protein from *Rhodococcus* sp. and the *E. coli*-expressed protein comigrate. Lane I, molecular size standards.

mined and was shown to correspond to the N terminus of Fig. 2. Finally, an Electro-Spray mass spectrometry study of the purified recombinant enzyme gave an absolute value for the total mass of the enzyme (≈48,500 Da) that is in very close agreement with the value expected from the sequence.

DISCUSSION

Results presented in this paper strengthen and extend previous conclusions drawn from the study of the enantiomer-selective amidase from *Brevibacterium* sp. strain R312 (15). A class of enzymes, all characterized by amidase activity, can be defined by the consensus sequence shown in Fig. 4B, derived from a comparison of seven different proteins. In this sequence of 56 residues, 15 amino acids are strictly conserved, with an overall level of homology of 53%. In particular, the GGSSGG sequence, which is the longest block of strictly conserved residues, appears as a salient feature of this consensus (hereafter referred to as the amidase consensus). It should be noted that the spacing between the conserved residues is also strictly maintained. We conclude from this consensus sequence that the recently identified putative amidase gene from *S. cerevisiae* (3) clearly belongs to this family of enzymes and thus should very probably code for an amidase (Fig. 4B). However, not all amidases belong to this class of enzymes, since the aliphatic amidase from *Pseudomonas aeruginosa* (2) does not exhibit any significant sequence homology to the amidase consensus or any other portion of the enantiomer-selective amidases (results not shown). It can thus be concluded that there are at least two different classes of amidases. Whether different amidases expressed by the same microorganism can be of the same type or belong to different classes is still an open question. We are now cloning and studying the so-called wide-spectrum amidase, another amidase from *Brevibacterium* sp. strain R312 (24), to address this question.

It is likely that such a sequence conservation reflects the involvement of this portion of the sequence in the catalytic function of these enzymes. Recently, Tsuchiya et al. (25) have shown that the only region of homology between the EI and EII ACDH enzymes from *Flavobacterium* sp. strain K172, products of the *nylA* and *nylB* genes, respectively, is precisely the sequence from amino acids 173 to 201 of ACDH-I, which is part of the amidase consensus. Moreover, their biochemical data on the EII and EI enzymes suggest that Ser-174 of EI, a strictly conserved position in the consensus, could be essential for the enzymatic activity; in addition, *p*-chloromercuribenzoate is an inhibitor of the EI enzyme, which suggests that a cysteine residue is also essential, at least in the case of ACDH-I activity (25).

The correlation, if any, between substrate specificity and the amidase consensus is not yet clear. Most amidases in this class can accommodate different substrates with various efficiencies, and only a thorough comparison of the substrate specificities and other enzymatic properties of several members of the two classes would perhaps allow an understanding of the role of the consensus sequence and the influence of given residues within the consensus sequence. In particular, although we are not yet able to predict from its sequence whether a particular amidase will be able to carry out efficiently the enantiomer-selective hydrolysis of 2-aryl pro-

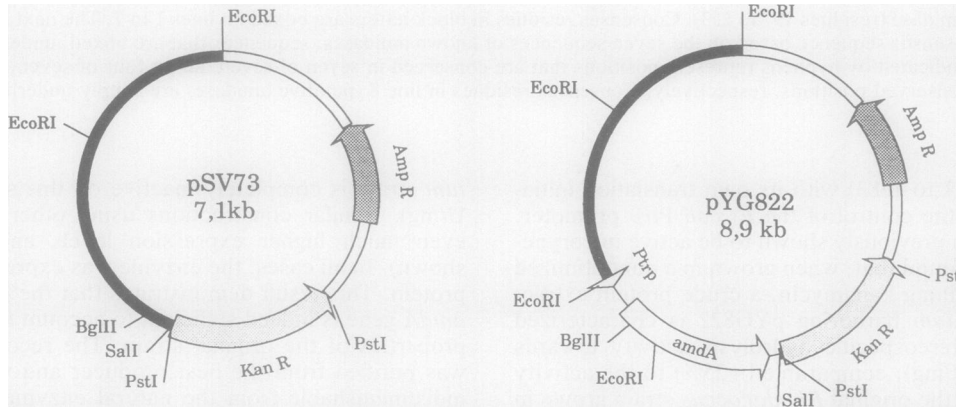

FIG. 6. Schematic representation of shuttle *E. coli*/coryneform plasmids pSV73 and pYG822. The black and white portions represent the pSR1 and pUC8 sequences, respectively.

pionamides, the activity monitored to purify the *Brevibacterium* sp. strain R312 and *Rhodococcus* amidases, it is likely that such an enzyme will exhibit the amidase consensus sequence.

We also propose that, as with the *Brevibacterium* enzyme, the gene coding for the *Rhodococcus* amidase is followed by a gene coding for a nitrile hydratase. Although this is not formally proven by our data, two pieces of evidence strongly argue in favor of this assumption. First, we have found that the *Rhodococcus* strain is able to hydrate several nitriles to the corresponding amides (not shown), which demonstrates the presence of a nitrile hydratase activity. Second, striking homologies are found with the corresponding nitrile hydratase subunits of *Brevibacterium* sp. strain R312 and *Rhodococcus* sp. strain N-774 (10, 15). If the downstream genes really code for the α and β subunits of the *Rhodococcus* nitrile hydratase (*nthA* and *nthB*, respectively), the cistrons encoding the two subunits are in the reverse order compared with the situation found in *Brevibacterium* sp. strain R312 (*amdA-nthB-nthA* instead of *amdA-nthA-nthB*). This is not a unique situation since, for instance, the order of the *thr* genes in *B. subtilis* is *hom-thrC-thrB* whereas it is *thrA-thrB-thrC* in *E. coli* (17, 18); however, this is to our knowledge the first instance of such an inversion in relatively closely related gram-positive bacteria. On another hand, it will be interesting to determine whether, in other microorganisms, the gene for nitrile hydratase is also linked to a gene coding for an amidase of this class.

Finally, we have shown that we could obtain the intracellular expression of an active recombinant amidase in *B. lactofermentum*, whereas the enzyme was always found to be insoluble and completely inactive in *E. coli*, contrary to what was found with the previously described *Brevibacterium* enzyme. This suggests either that an important cofactor for enzyme refolding and activity can be found in *B. lactofermentum*, but not in *E. coli*, or that the intracellular physicochemical conditions are significantly different in the two types of bacterial cells. In any case, this observation further suggests that different expression systems have to be tested and that all bacterial hosts may not be equivalent, even for the cytoplasmic expression of intracellular bacterial enzymes.

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