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 Mr 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., 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 amidoactive system 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 indoleacetamidase 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.

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; Ptrp, E. coli tryptophan operon promoter; pR, early right promoter from bacteriophage lambda; PPAmide: racemic 2-phenylpropionamide; HPPAmide, racemic 2-(4-hydroxyphenyl)propionic acid; 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 K2HPO4, 0.5 g of MgSO4, 20 mg of FeSO4, 20 mg of MnSO4, 10 mg of NaCl, 2 mg of CaCl2, 0.15 mg of Na2MoO4, 0.04 mg of ZnSO4, 4 μg of CuSO4, 4 μg of CoCl2, 0.2 mg of H2BO3, 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

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TABLE 1. Bacterial strains and plasmids used

<table>
<thead>
<tr>
<th>Strains</th>
<th>Plasmids and bacteriophages</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodococcus</td>
<td></td>
<td>Natural isolate</td>
<td>This study</td>
</tr>
<tr>
<td>B. lactofermentum</td>
<td></td>
<td>Spontaneous Rif’ derivative of ATCC 21036</td>
<td>ATCC 21086</td>
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<tr>
<td>B. lactofermentum RP2</td>
<td></td>
<td>F’ endA hasR17 (rK{m-}) supE44 thi-1 recA1 tyrA96 relA Δ80 lacZgam15</td>
<td>27a</td>
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<td>E. coli DH5α</td>
<td></td>
<td></td>
<td>Cientech Laboratory, Palo Alto, Calif.</td>
</tr>
<tr>
<td>E. coli B</td>
<td></td>
<td>Wild-type strain</td>
<td>ion met</td>
</tr>
<tr>
<td>E. coli E103S</td>
<td></td>
<td></td>
<td>D. L. Simon, Waksman Institute of Microbiology, Piscataway, N.J.</td>
</tr>
</tbody>
</table>

Plasmids and bacteriophages

- pUC20H: Multicloning site
- pUC8.−19: Multicloning site
- pXLE34: Ap’, Prp promoter vector expressing human serum albumin
- pXLE94: Prp promoter vector expressing human angiogenin
- pXLE1029: PpCts promoter vector expressing interleukin 1β
- pXLE935: pUC derivative containing 3.2-kbp amdA insert
- pXLE936: Same as pXLE835; reverse orientation of insert
- pXLE891: pIC derivative containing Prr, cII RBS* and the first 39 codons of amide
- pXLE982: pIC derivative containing Prk, cII RBS, and the first 39 codons of amide
- pXLE983: amdA under control of cII RBS and Prp
- pXLE984: amdA under control of cII RBS and Prk cts
- pSR1: Endogenous cryptic plasmid from C. glutamicum
- pSV73: Shuttle vector E. coli-corynebacteria, pUC and pSR1
- pYG822: Ap’, Km’, amdA under control of E. coli Prp, on pSV73

* 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 protoplasting 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; 10⁵ transformants per microgram of DNA were routinely obtained. Conditions used for the expression of recombinant amidas 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 (3). 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-phenylpropionic 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 PPAamide (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 H₂PO₄ and CH₃CN [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 CH₂CN–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)} × 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)} × 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 Centrprep 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-Cl–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-Cl–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: hydroxypatite chromatography. The solution was injected onto a Bio-Gel HPHT column (Bio-Rad) equilibrated with 85 mM Tris-Cl–0.5 mM DTT–10 μM CaCl2–15% 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 CaCl2–15% 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 NaH2PO4–50 mM Na2SO4 (pH 6.8). The apparent Mr of the enzyme was estimated by using GPC/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: 4% (v/v) at 0 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, NHE-ADPATDVPPVDPYAAD TGDVR; fraction 124, NHE-TFLEAGELVPATDYIK; and fraction 162, NHE-ELFDIDVLIAPTVSPPALP.

Oligonucleotide probe, Southern blots, and colony hybridization. The 32-mer degenerated oligonucleotide probe 5’-GCACIGCTGAT(T,G)GTCCIGTCCIGA(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 30°C for 5 min.

Plasmid constructions. (i) E. coli expression plasmids. An NdeI 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’-CGGGGTTGTCGGATCATATG... and 3’-AGATCTTCGGTTCGAATC... The 114-bp NdeI-BglII PCR fragment obtained after digestion with these enzymes was then ligated to either of the two EcoRI-NdeI fragments carrying a promoter and the lambda cII-derived ribosome-binding site, between the EcoRI and the BglII sites of plasmid pC20H (14). pXL1891 was derived in this way with the 121-bp EcoRI-NdeI fragment of pXL534 (12) containing the Ptrp promoter, whereas pXL1892 contains the 1,228-bp EcoRI-NdeI 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 BglII-Sall fragment (containing the rest of the amidase gene) of pXL1835 and to the 3.1-kbp EcoRI-Sall 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 BglII site and cloned into the BamHI site of plasmid pUC8. The PstI fragment of plasmid pUC4K (Pharmacia) containing the aminoglycoside 3'-phosphotransferase gene from transposon Tn903 was then incorporated into the PstI site of the pSR1 or pUC8 hybrid plasmid to yield pSV73. Expression plasmid pYG822 (Fig. 6) was then obtained by ligating the 6.5-kbp SalI-BglII vector fragment of pSV73 to the 1,696-bp HindIII-Sall fragment of pXL1835 containing the whole amidase gene and to the 96-bp BamHI-HindIII fragment of pXL1891 containing the E. coli Ptrp promoter.

Nucleotide sequence accession number. The sequence of the 3.2-kbp PstI fragment of Rhodococcus sp. has been assigned in 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 amido lytic 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 about 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 Mr of the purified native enzyme was estimated at 118,000 by HPLC...
Activities were normalized per milligram of total soluble proteins in the reaction. Activities were normalized per milligram of total soluble proteins in the cell sample. ND, not determined.

**TABLE 2.** Comparison of amida specific activities of *Brevibacterium* sp. strain R312 and *Rhodococcus* sp.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Brevibacterium sp. strain R312</th>
<th>Rhodococcus sp.</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPAImide</td>
<td>0.016 (68)</td>
<td>0.333 (311)</td>
<td>21</td>
</tr>
<tr>
<td>HPPAmide</td>
<td>0.242 (92)</td>
<td>0.040 (ND)</td>
<td>0.17</td>
</tr>
</tbody>
</table>

* 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.

Cloning of the *Rhodococcus* amidaase. 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 32P, 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 BamHI, KpnI, SplI, SstI, SmaI, SalI, and PstI digestions, a single genomic band strongly hybridizing to the probe was found. The hybridizing band corresponding to a PstI fragment of approximately 3.2 kb was chosen for further cloning. The ≈3- to ≈4-kb PstI 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 PstI. 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 stringent 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 PstI 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.

**Nucleotide sequence of the PstI fragment from *Rhodococcus* sp.:** evidence that the gene coding for the enantiomer-selective amidaase is coupled to a gene coding for a nitrile hydratase. The complete nucleotide sequence of both strands of the 3.2-kbp PstI 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 (Mr, 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 amidaase 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 Mr 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 PstI fragment and because such a genetic organization had
<table>
<thead>
<tr>
<th>Codon</th>
<th>Amino Acid</th>
<th>Position</th>
</tr>
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<tbody>
<tr>
<td>ATG</td>
<td>Met</td>
<td>1</td>
</tr>
<tr>
<td>TAA</td>
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<td>812</td>
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<tr>
<td>TGA</td>
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<tr>
<td>CCA</td>
<td>Pro</td>
<td>130</td>
</tr>
</tbody>
</table>

**Note:**
- The presumed n-terminal amino acid is shown in **bold**.
- The table represents a portion of a larger dataset, focusing on the amino acid sequence and its corresponding codons.
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 *amidA* (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*, of 26,000 (26K). This is further suggested by the presence of the Shine-Dalgarno sequence AGGAG six nucleotides

![Image](http://jb.asm.org/Downloaded from http://jb.asm.org/con...en June 20, 2017 by guest)
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 \( \alpha \) 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 acetylamin 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-cyclodimer 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 \( NdeI \) site was created by PCR at the presumed ATG initiation codon (position 210 in Fig. 2). The fragment between this site and the Sacl 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 \( \text{Prp} \) and \( p_{\text{R}} \)) and translation (ribosome-binding site of the lambda \( cII \) gene) initiation. The vectors thereby obtained, pXL1893 (\( \text{Prp} \)) and pXL1894 (\( p_{\text{R}} \)), 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 Tn903. 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 (residues 197 to 253). Consensus residues in block letters are boxed, underlined, presented in normal letters, and indicated by hyphens represent positions that are conserved in seven of the sequences, six to four of seven, and three of seven sequences and nonconserved positions, respectively. Consensus residues in line 8 (putative amidase) are simply underlined.

sequence (positions 1 to 1683) with its own translation initiation region under the control of the *E. coli* *Ptrp* 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-
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 GGSSSG 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 ElI ACDH enzymes from *Flavobacterium* sp. strain K172, products of the *nlaA* and *nlaB* 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 EI and El enzymes suggest that Ser-174 of El, a strictly conserved position in the consensus, could be essential for the enzymatic activity; in addition, *p*-chloromercuribenzoate is an inhibitor of the El 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. 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.]

[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, 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 hydrazel several nitriles to the corresponding amidates (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 (nhaA and nhaB, respectively), the cistrans encoding the two subunits are in the reverse order compared with the situation found in Brevibacterium sp. strain R312 (amda-ntbB-ntaA instead of amda-ntaA-ntB). This is not a unique situation since, for instance, the order of the thr genes in B. subtilis, thrA-thrB-thrC, whereas in E. coli (17, 18); however, this is to our knowledge the first instance of such an inversion in a relative 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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