Purification, Cloning, and Primary Structure of an Enantiomer-Selective Amidase from Brevibacterium sp. Strain R312: Structural Evidence for Genetic Coupling with Nitrile Hydratase

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An enantiomer-selective amidase active on several 2-aryl and 2-aryloxy propionamides was identified and purified from Brevibacterium sp. strain R312. Oligonucleotide probes were designed from limited peptide sequence information and were used to clone the corresponding gene, named amdA. Highly significant homologies were found at the amino acid level between the deduced sequence of the enantiomer-selective amidase and the sequences of known amidases such as indoleacetamide hydrolases from Pseudomonas syringae and Agrobacterium tumefaciens and acetamidase from Aspergillus nidulans. Moreover, amdA is found in the same orientation and only 73 bp upstream from the gene coding for nitrile hydratase, strongly suggesting that both genes are part of the same operon. Our results also showed that Rhodococcus sp. strain N-774 and Brevibacterium sp. strain R312 are probably identical, or at least very similar, microorganisms. The characterized amidase is an apparent homodimer of M, 2 × 54,671 which exhibited under our conditions a specific activity of about 13 to 17 μmol of 2-(4-hydroxyphenoxo)propionic acid formed per min per mg of enzyme from the racemic amide. Large amounts of an active recombinant enzyme could be produced in Escherichia coli at 30°C under the control of an E. coli promoter and ribosome-binding site.

Nitriles are extensively used in organic synthesis by the chemical industry as precursors to produce compounds such as amides and organic acids. However, the chemical conversion of nitriles presents several disadvantages, such as the need for strongly acidic or basic conditions, a high energy consumption, or the formation of unwanted by-products. More recently developed biochemical procedures in which microorganisms are used as catalysts look promising because temperature and pH conditions are less severe and because very pure products can be formed (23). In addition, bioconversions can be stereospecific and can lead to the production of a single enantiomer, which might be a crucial aspect in the manufacturing of active new drugs (5). However, the use of very efficient microbial strains is necessary to develop a biochemical process at the industrial scale. For instance, an efficient process for the production of acrylamide from acrylonitrile using resting cells of Pseudomonas chlorophosphor is B23 has recently been developed (21).

Many microorganisms can use nitriles as a sole carbon and/or nitrogen source. Among them, the potential use of Brevibacterium sp. strain R312 was recognized several years ago by Arnaud and colleagues (1). This gram-positive coryneform strain was shown to contain two types of enzyme activities for the hydrolysis of nitriles: a nitrile hydratase that hydrates nitriles into amides (6) and probably several amidases which transform amides into the corresponding organic acids (13). The bioconversion of water-soluble amides in a continuous immobilized cell reactor was also demonstrated (3). Such experiments led to the view that this type of corynebacteria may be viewed as “bags,” preventing enzyme loss without introducing important limitations to the diffusion of products.

This is the first report concerning the characterization and cloning of an amidase from Brevibacterium sp. strain R312 active on several 2-aryl or 2-aryloxy propionamides. This stereospecific enzyme is significantly homologous to several amidases from different microorganisms. In addition, we showed that the structural gene encoding this enzyme is located immediately upstream of the gene coding for nitrile hydratase, suggesting that both enzymes are coordinately expressed in the cell. Finally, our results, compared with other data (11), strongly suggest that two studied microorganisms in the field, Brevibacterium sp. strain R312 (1) and Rhodococcus (Corynebacterium) sp. strain N-774 (28), are probably identical, or very closely related.

MATERIALS AND METHODS

Abbreviations. HPLC, High-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; HPPAmide, 2-(4-hydroxyphenoxo)propionamide; HPPAcid, 2-(4-hydroxyphenoxo)propionic acid; RBS, ribosome-binding site.

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are described in Table 1. Brevibacterium and Escherichia coli strains were routinely grown on LB plates or in liquid LB medium at 30 and 37°C, respectively. Ampicillin was used at 100 μg/ml to maintain E. coli plasmids. Conditions used for the expression of heterologous proteins in E. coli have been described previously (8, 14).

Materials. T4 DNA ligase and restriction enzymes were purchased from New England BioLabs; T4 polynucleotide kinase and DNA polymerase I (Klenow fragment) were

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obtained from Boehringer Mannheim Biochemicals and used as recommended by the manufacturer.

**General methods.** Standard procedures for DNA isolation, manipulation, analysis, and amplification were used (18). Brevibacterium genomic DNA was prepared as described previously (9). The Sequenase Version 2.0 (United States Biochemical Corp.) was used for DNA sequencing with [α-35S]dATP (Amersham Corp.). 7-Deaza-dGTP was substituted for dGTP to reduce compression caused by the high G+C percentage. Polymerase chain reaction experiments were performed using the Taq polymerase from Cetus and the Perkin-Elmer Cetus DNA thermal cycler as recommended by the manufacturer. Proteins were fractionated by PAGE under denaturing conditions (18).

**Enzyme assay.** The enzyme activity on HPPAmide (20 mM) was monitored during stirring at 25°C in 4 ml of 50 mM sodium phosphate buffer (pH 7.0). The reaction was stopped by the addition of a 90:10 (vol/vol) mixture of acetonitrile and 1 N HCl, respectively. After homogenization and centrifugation, the supernatant was applied to an HPLC reverse-phase column (Hibar-Merck RP-18; 5 μm). The elution at 1 ml/min by an 85:15 (vol/vol) mixture of phosphoric acid (0.05 M) and acetonitrile, respectively, was monitored by the optical density at 280 nm, and the respective concentrations of HPPAmide and HPPAcid in the analyzed sample were measured from the peak positions and areas of standards.

**Enantiomer selectivity assays.** The enantiomer excess (ee), defined as the ratio \([R - S]/(R + S)\) × 100 (%), where \(R\) and \(S\) are, respectively, the concentrations of the \(R\(+\) and \(S\(-\) HPPAcid enantiomers, was deduced from HPLC on a chiral column (Resolvisol BSA 7; Macherey-Nagel) with the following elution buffer: sodium phosphate (pH 6.8)–10 mM isopropanol (95/5, vol/vol). Under these conditions, the retention times of the \(R\(+\) and \(S\(-\) forms were 7.2 and 13 min, respectively.

**Purification of enantiomer-selective amidase activity.** All procedures were done at 4°C.

(i) **Step 1. Preparation of crude enzyme solution.** Frozen cell paste (40 g [wet weight]) of Brevibacterium sp. strain R312 was thawed and suspended in 300 ml of buffer A (50 mM sodium phosphate [pH 7.0], 5 mM β-mercaptoethanol). Cells were broken by ultrasonic treatment, and cell debris was separated by centrifugation at 20,000 × g for 30 min. A 25-ml portion of a 10% solution of streptomycin sulfate was then slowly added under gentle agitation to 310 ml of the supernatant. After 45 min, the solution was clarified as described above and the supernatant was submitted to the following steps.

(ii) **Step 2. Ammonium sulfate fractionation.** The protein fraction precipitating between 30.8 and 56.6% saturation with ammonium sulfate was collected by centrifugation and dissolved in 35 ml of buffer A. This solution was further desalted by extensive dialysis against the same buffer.

(iii) **Step 3. Phenyl-Sepharose CL-4B chromatography.** The protein fraction was brought to 20% saturation with ammonium sulfate, clarified by centrifugation, and applied onto a column of phenyl-Sepharose CL-4B (Pharmacia) equilibrated with buffer A at 20% saturation with ammonium sulfate. Protein fractions containing the enzyme activity were eluted with the same buffer, pooled, and concentrated by ultrafiltration with an Amicon Diaflo PM10 cell to a volume of 18 ml.

(iv) **Step 4 and 5. Gel filtration.** Glycerol (10%) was added to the concentrated protein fraction, and the solution was loaded on an Ultragel AcA 44 (IBF-biotechnics, Villeneuve-la-Gareivne, France) column which had been equilibrated in 50 mM Tris hydrochloride (pH 7.0)–100 mM NaCl. Protein fractions containing the highest specific activity (about 32% of the total activity loaded on the column) were collected, concentrated, and submitted to an additional filtration step on the same gel. Once again, fractions (about 30% of applied proteins) exhibiting the highest specific activity were analyzed by SDS-PAGE and pooled. The enantiomer selectivity of the purified protein obtained by this procedure was also checked (ee > 95%).

**Protein sequencing.** About 3 nmol of the purified amidase preparation was reduced and carboxymethylated. The major protein component was then desalted and purified to homogeneity by RP C4 reverse-phase HPLC. The N-terminal amino acid sequence was determined by the sequential automated Edman degradation method with an Applied Biosystems model 470 A apparatus. The sequence NH2-ATIRPDKAIDAAARHYGTLDKTARL... was determined. To obtain an additional internal sequence, we submitted the same quantity of the protein to complete trypsin digestion. The reduced and carboxymethylated fragments were then separated by RP C8 reverse-phase HPLC (2.1 by 10 mm; flow rate, 0.2 ml/min), eluting with a gradient of 0 to

**TABLE 1. Bacterial strains and plasmids used**
50% (vol/vol) acetonitrile in 0.07% (vol/vol) trifluoroacetic acid. A peptide eluted in a well-separated peak at 40.8% acetonitrile was sequenced. The sequence NH₂-LEWPAL IDGALSYDVQDLQL . . . was obtained.

Oligonucleotide probes. Oligodeoxynucleotides were synthesized with a Biosearch 8600 automatic DNA synthesizer and purified by PAGE under denaturing conditions as previously described (8). Probe A was derived from part of the internal peptide sequence (IDGALSYDVQDL) on the noncoding strand of EcoRI (the following oligonucleotide 28-mer: 5'-ACGT GTAGGAGGCC(G,C)AG(G,C,T)CGCGGCTGAT-3'). An exact probe from the N-terminal sequence was derived by polymerase chain reaction as follows. Briefly, two highly degenerated oligonucleotides corresponding to nearly all coding possibilities of the first and last five amino acids of the N-terminal sequence and flanked with a restriction site were synthesized: 5'-CCGAATTCGACNACAT (T,C,A)GNCC NGA-3' (direct orientation, EcoRI site) and 5'-CCAGAATTCGACNACAT (G,T,A)TNCA(G,A) NGT-3' (reverse orientation, HindIII site), where N corresponds to an equimolar mixture of the four bases. These oligonucleotides were used to prime 30 cycles of enzymatic amplification by the Taq polymerase on the total genomic DNA of Brevibacterium sp. strain R312 as described previously (10). The amplified DNA was then digested with EcoRI and HindIII and cloned between the corresponding sites of M13mp19. Several recombinant clones were sequenced and were found to contain a DNA fragment encoding the N-terminal peptide sequence. In particular, the unique DNA sequence between both primers was used to design the exact 40-mer synthetic probe B (noncoding strand): 5'-GATGCGGTAATGCTTT GTGGGGCAGCTTATGGTTG-3'.

Southern blots and colony hybridization. Biodyne A nylon transfer membranes (Pall Industrie, St. Germain-en-Laye, France) were used for both Southern and colony hybridizations according to the manufacturer's specifications and protocols. For Southern blotting experiments, 5 to 10 μg of genomic DNA was digested to completion and fragments were separated on 0.8 to 1% agarose gels. Because oligonucleotide probes were subsequently used, depurination of DNA was avoided by omitting acid treatment of the gel. Oligonucleotides (100 to 500 ng) were 5' labeled with [γ-32P]ATP (3,000 Ci/mmol; Amersham), using T4 polynucleotide kinase as described previously (18), and were used without further purification. Standard hybridization buffer was 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-5× Denhardt solution-50 mM sodium phosphate (pH 6.5)-0.1% SDS-250 μg of salmon sperm DNA per ml. For probe A, prehybridization and hybridization (4 h and overnight, respectively) were done at 55°C in hybridization buffer. Filters were then washed several times in 3× SSC-0.5% SDS at 55°C, allowed to air dry, and exposed overnight to Kodak X-Omat XAR-5 films at ~70°C between intensifying screens. For probe B, hybridization buffer containing 50% formamide was used at 45°C, and the filter was washed twice for 1 h at room temperature in 2× SSC-0.1% SDS and for 5 min at 45°C in 0.1× SSC-0.1% SDS. Similar conditions were used for colony hybridization, except that autoradiography was only for 2 to 3 h without intensifying screens.

Plasmid constructions. The BamHI-NcoI (538-bp) and Ncol-EcoRI (1,760-bp) fragments from the 5.4-kbp insert of pXLI650 (Fig. 1) were inserted between the BamHI and EcoRI sites of pUC18 to give pXLI1675. The 2.3-kbp XbaI fragment of pXLI1675 containing the amidease gene with its natural RBS was then inserted in the correct orientation, under the control of the E. coli trp promoter, between the unique XbaI sites of plasmid pXL1599 to give pXLI1724. Plasmid pXL1599 is derived from pXL534 (16a) by substitution of the HindIII fragment containing the RBS and coding sequence for human serum albumin for the sequence 5'-. A AGCTTCTAGAGCTCTAGCTTTTG-3', which introduces the unique overlapping sites XbaI-SacI-XbaI. An NdeI (CATATG) site containing the ATG initiation codon of the amidease structural gene was created, and the corresponding 83-bp NdeI-XhoI fragment containing the first 26 codons of the amidease was obtained by polymerase chain reaction enzymatic amplification of the pXL1724 sequence with the two primers 5'-CAGGAGCACACTTTCATATGGCGACAATC-3' and 5'-CGGCCATCGAGCCGGCTGTTTTG-3' and subsequent digestion by NdeI and XhoI. This fragment was then ligated to the 5.25-kbp XhoI-EcoRI vector fragment of pXLI1724 containing the 494 last codons of the amidease gene and to an EcoRI-NdeI fragment containing a promoter and an RBS derived from the λ cII gene. pXL1751 was derived by using the 121-bp EcoRI-NdeI fragment of pXL534 containing the trp promoter, whereas pXL1752 contains the 1,228-bp EcoRI-NdeI fragment of pXL820 (14) containing both the λ pr promoter and clts.

sequence accession number. The sequence shown in Fig. 2 has been submitted to GenBank (accession number M32282).

RESULTS

Identification and purification from Brevibacterium sp. strain R312 of an enantiomer-selective amidase active on several 2-aryl or 2-aryloxy propionamides. Following the experimental protocols described in Materials and Methods, Brevibacterium sp. strain R312 (CBS 717.73) (1) was found to contain an amidase active ability to catalyze the stereospecific hydrolysis of several racemic 2-aryl propionamides such as 2-phenylpropionamide or 2-aryloxy propionamides such as HPPAmide into the corresponding S or R acid, respectively (E. Cerbelaud and D. Pétré, European Patent Application EP 330-529A, 1988). The enantiomer excess of the acid product was found to be consistently higher than 93 to 95% on several substrates, demonstrating the enantiomer selectivity of the amidation reaction. Using HPPAmide as a substrate, we found specific activities of 0.25 and 0.40 U/mg of bacterial proteins on whole cells and soluble cell extracts, respectively (1 unit defined as 1 μmol of HPPAcid formed per min under the conditions described in Materials and Methods). HPPAmide was subsequently used to monitor the reaction during the purification of the investigated enzymatic activity. Through the purification steps described in Materials and Methods, the enantiomer-selective enzyme was purified 31-fold up to a maximal purity of about 80% (Table 2, and see Fig. 4, lanes b and d). The global yield and the maximum specific activity were 6.3% and 15.6 U/mg, respectively, and were not optimized, since the objective was essentially to obtain enough protein for N-terminal sequencing. At that stage, a prominent band of apparent Mr, 59,000 ± 5,000, corresponding to about 80% of total proteins, was detected on an SDS-polyacrylamide gel. That this protein was indeed responsible for the measured activity was further confirmed by the SDS-PAGE analysis of the protein fractions characterized by the highest specific activity during the second Ultrogel AcA 44 gel filtration (data not shown).

Moreover, we observed in subsequent experiments (data not shown) using HPLC gel filtration that the amidase was eluted at a position corresponding to an apparent Mr, of 120,000 ± 5,000, suggesting that the enzyme behaves as a homodimer.
Cloning of the enantiomer-selective amidase. The N-terminal peptide sequence (27 residues) and the sequence of an internal fragment (21 residues) of the major polypeptide species in the purified preparation were determined as described in Materials and Methods. From these peptide sequences, two different oligonucleotide probes were designed. Probe A is a six-fold-degenerated 29-mer derived from the internal fragment, taking into account the codon usage in the tryptophan operon of Brevibacterium lactofermentum (19). For the other probe, the polymerase chain reaction procedure described by Girgis et al. (10) was performed to generate an exact 40-mer nucleotide probe (probe B) from the N-terminal sequence. These synthetic DNAs were then 32P labeled and used as hybridization probes. Southern hybridization with these probes against several digestions of the Brevibacterium sp. chromosomal DNA revealed that, at least for several restriction enzymes, probes A and B hybridized with the same fragment (data not shown). In particular, both probes hybridized with a single 5.4-kb PstI fragment. The PstI-digested chromosomal fragments of 4.8 to 6.3 kb were purified by agarose gel electrophoresis, ligated with PstI-digested pUC19 plasmid, and introduced into E. coli DH5a. About 500 ampicillin-resistant white transformants on X-gal medium obtained in this way were individually screened by colony hybridization with probe B. Two colonies showing a strong positive hybridization to both probes A and B were found. Restriction analysis of corresponding plasmid DNAs (pXL1650 and pXL1651) indicated that they had inserted the same 5.4-kb PstI fragment in either of the two possible orientations. A restriction map of this fragment is shown in Fig. 1. Supercoil DNA sequencing with both probes as primers and additional Southern analysis of the plasmids confirmed that the correct chromosomal fragment had been cloned, indicated that the two previously determined peptide sequences were next to each other in the protein, and showed that the studied gene was contained on a 2.3-kb BamHI-PstI fragment (Fig. 1).

Nucleotide sequence of enantiomer-selective amidase gene and structural evidence for coupling with the gene coding for nitrile hydratase. The nucleotide sequence of the BamHI-PstI fragment was determined as detailed in Fig. 1. The 2,447-bp-long sequence is shown in Fig. 2. The overall G+C composition of this sequence is 61.5%. An open reading frame coding for a 521-amino-acid protein (M, 54,671) containing the amino acid sequences determined from the presumed enantiomer-selective amidase is found between nucleotides 245 and 1807. In this coding sequence, the mean G+C composition of positions 1, 2, and 3 of the codons is 65.8, 52.5, and 70%, respectively. Such a pattern in codon usage has already been observed for high-G+C-containing organisms (4). An additional open reading frame coding for at least 188 amino acids is found 73 nucleotides downstream.

FIG. 1. Upper: Restriction map of the 5.4-kbp PstI fragment, the insert of plasmids pXL1650 and pLX1651, is shown with the positions (arrows) of the coding regions for the enantiomer-selective amidase (amdA) and the α subunit of nitrile hydratase (NHase). The sequenced BamHI-PstI region is indicated by the dashed arrow. Lower: More precise structure of the BamHI-PstI region. Unique sites are underlined. The sequencing strategy on both strands is shown by the arrows. Arrows with filled squares indicate sequencing from specific oligonucleotide primers. Fragments subcloned in M13 are indicated by shaded boxes. The region common with the Rhodococcus sp. strain N-774 fragment independently sequenced by Ikehata et al. (11) is shown by the black bar.
amidase

\[\text{BamHI} \]

\[\text{S.D.} \]

\[\text{R312} \]

\[\text{HindIII} \]

\[\text{PstI} \]

\[\text{FIG.} 2. \text{ Nucleotide and amino acid sequences of the BamHI-PstI region shown in Fig. 1 (lower panel). S.D., Potential RBS. The sequenced peptides from the purified \textit{Brevibacterium} sp. strain R312 enantiomer-selective amidase and nitrile hydratase \alpha subunit are underlined.} \]
from the above sequence. During a systematic search for homologies with published amino acid sequences, we found that the protein sequence deduced from this open reading frame was identical with the recently determined primary structure of the nitrile hydratase subunit from Rhodococcus sp. strain N-774 (11). Furthermore, a close inspection demonstrated that the nucleotide sequence of the 1,238-bp fragment between SphI and PstI was strictly identical with the published nucleotide sequence of the nitrile hydratase fragment from Rhodococcus sp. strain N-774, with a single nucleotide difference, a C instead of a G at position 1664 (changing Val to Leu in the amide sequence (Fig. 2), which may reflect either a sequence error or a minor allelic variation. The identity at the DNA level between the sequences of such long fragments from the two strains strongly argues in favor of Rhodococcus sp. strain N-774 (28) and Brevibacterium sp. strain R312 (1) being identical, or at least very closely related, microorganisms. An additional proof was provided by the cloning of the 3' overlapping 3.9-kbp HindIII-SphI genomic fragment from Brevibacterium sp. strain R312 (data not shown). The restriction map of this fragment was found to be in total agreement with the structure of the corresponding Rhodococcus sp. strain N-774 fragment (11). It was also verified that the structural genes for the two subunits of nitrile hydratase were identical for both microorganisms (data not shown). It can thus be concluded that the genes coding for the enantiomer-selective amidease and nitrile hydratase, two enzymes catalyzing successive reactions from nitriles to the corresponding acids, are closely linked on the bacterial genome.

**Sequence homology with known amidasies.** A systematic search of the NRBF and GENPRO protein data bases for good local homologies with the amidease sequence (15) gave known amidasies as the best matches. Highly significant homologies were found with indoleacetamide hydrolase from Pseudomonas syringae (29) and Agrobacterium tumefaciens (24), also known as the protein encoded by the rns2 locus of plasmid Ti. These amidasies are used by their phytopathogenic hosts to produce indoleacetic acid from indoleacetamide, indoleacetic acid being recognized as the principal auxin of higher plants. As shown in Fig. 3A by dot matrix analysis, the homology is clustered in the N-terminal half of the proteins (≈40% match with the P. syringae enzyme), in particular between residues 150 and 220 of the strain R312 amidease. Two stretches of 10 and 12 consecutive identical residues between the P. syringae and the R312 enzymes are found between residues 168 and 204 of the R312 amidease. This region is also highly conserved in both the indoleacetamide hydrolase of A. tumefaciens and the acetamide of Aspergillus nidulans (7) (Fig. 3B), strongly suggesting that it is part of the active site of these amidasies. Finally, we noted that the homology between the enantiomer-selective amidease of Brevibacterium sp. strain R312 and the indoleacetamide hydrolase of P. syringae is of the same order as the homology between the indoleacetamide hydrolases of P. syringae and A. tumefaciens.

**Expression of Brevibacterium sp. strain R312 amide activity in E. coli.** The expression of amidease was first analyzed in the recombinant E. coli DH5a strain containing either of the original clones pXL1650 and pXL1651. These plasmids carry the same 5.4-kbp chromosomal PstI fragment (Fig. 1) cloned in either orientation in the polylinker of plasmid pUC19. Even under conditions known to derepress the lac promoter, we could not detect by Coomassie blue staining any expression of the cloned gene (data not shown). In another experiment, the 2.26-kbp BamHl-PstI fragment containing the complete amide gene and the 58 bp upstream of the ATG codon was subcloned into a pT7 expression vector (8, 16a) to give pXL1724. Once again, no detectable synthesis of the presumed amidease protein could be evidenced by Coomassie blue staining, whatever the conditions (data not shown). The intrinsic expression capacity of these vectors could not be questioned since a protein of the expected size was specifically synthesized in an in vitro coupled transcription-translation assay (Amersham) when pXL1650, pXL1724, and, to a much weaker extent, pXL1651 were used as templates (data not shown). All these results strongly suggested that neither the amidease promoter, if present in the 3 kb 5' to the gene, nor the translation initiation signals of the gene were efficient in E. coli. Two other constructions were then made in which the structural gene for amidease was linked to both a strong E. coli promoter, such as the pLH or PRB promoter, and an efficient RBS, such as that derived from the E. coli 1 gene (8). Expression of amidease from these vectors, pXL1751 (pLH) and pXL1752 (PRB), was studied in E. coli B and E103S, respectively, two host strains known to give a high level of expression of heterologous proteins. Results shown in Fig. 4 demonstrate the specific high-level expression of an M, 55,000 protein, corresponding to at least 20% of total protein and comigrating with the main protein species of the purified amide preparation from Brevibacterium sp. strain R312 (lanes b and c). Subsequent analysis revealed that in fact only a minor proportion (<10%) of this protein was expressed as a soluble enzyme (Fig. 4, lanes g and i or n and p) when the recombinant strain was cultivated at 37 or 42°C, indicating that it was mainly produced as an aggregated, denatured protein as already observed for nitrile hydratase (11). However, when E. coli B(pXL1751) was grown at 30°C, similar amounts of the enzyme were synthesized but mainly (∼80 to 90%) as a soluble protein (data not shown), suggesting that the temperature is a major parameter for the correct folding of this protein. That the cloned protein was indeed responsible for the observed enantiomer-selective amide activity in Brevibacterium sp. strain R312 was demonstrated by activity measurements of the recombinant E. coli strains, compared with strictly isogenic strains expressing recombinant human interleukin-1β (14) as a control (Table 3). Only strains expressing the 55,000-M protein could hydrolyze HPPAmide. It was verified that this reaction was highly enantiomer selective, as described above. With or without sonication of the cells, specific amidease activities on HPPAmide were at least 20-fold higher for E. coli recombinants grown at 30°C compared with those grown at 37°C. In the best expression conditions (30°C in minimal medium

| TABLE 2. Purification of enantiomer-selective amidease |
|---------------------------------|--------|--------|--------|--------|
| Purification step               | Vol (ml) | Amt of protein (mg) | Sp act (U/mg) | Recovery (%) | Purification (fold) |
| Crude extract                   | 325     | 1.918   | 0.44    | 100          | 1        |
| Ammonium sulfate precipitate    | 29.5    | 613     | 1.04    | 75           | 2.4      |
| Phenyl-Sepharose eluate         | 77      | 200     | 3.30    | 78           | 7.5      |
| First gel filtration eluate     | 6       | 27      | 7.62    | 24.4         | 17.3     |
| Second gel filtration eluate    | 3       | 3.9     | 13.58   | 6.3          | 31       |

a One unit (U) is 1 μmol of HPPAcid formed per min under the conditions described in Materials and Methods.

b From 40 g of wet cells after streptomycin sulfate precipitation.
A

FIG. 3. Amino acid homologies with known amidases. (A) Dot matrix analysis (25) of the comparison between the amino acid sequences of indoleacetamide hydrolase from P. syringae (29) (residues 1 to 455) and R312 enantiomer-selective amidase (residues 1 to 521) showing the level of homology between the N-terminal parts of the proteins. (B) Amino acid sequence comparisons between region 150 to 220 of R312 amidase (upper sequence) and the corresponding sequences (lower sequences) of indoleacetamide hydrolase (TMS2 protein) from A. tumefaciens (a), indoleacetamide hydrolase from P. syringae (b), and acetamidase from A. nidulans (c). Identical and similar residues are indicated by = and −, respectively. Residues strictly conserved in all four sequences are underlined in row a.

without tryptophan), recombinant E. coli strains with up to threefold-higher specific activities than the original Brevibacterium sp. strain R312 were obtained.

DISCUSSION

This is the first report of the cloning and analysis of an amidase gene from a coryneform species. The R312 strain was isolated by Arnaud and co-workers (1) from a natural earth sample among strains able to utilize acetonitrile as the sole nitrogen source and was shown to hydrolyze a wide variety of nitrile compounds. It was then demonstrated that the metabolism of nitriles did not operate via a direct nitrilase reaction but that an amide intermediate was involved (12). The first enzymatic step, namely, the hydration of nitriles into amides, is performed by an enzyme named nitrile hydratase, as first proposed by Asano et al. (2) for Arthrobacter sp. strain J-1. That this enzyme is unique in Brevibacterium sp. strain R312 is suggested by two types of results. Only one biochemical entity was detected during the purification steps of the enzymatic activity, and a mutant strain, Brevibacterium sp. strain 19, was isolated which had completely lost its ability to hydrate nitriles, while retaining
enantiomer-selective amidase from Brevibacterium sp.

The expression of R312 amidase in E. coli. Coomassie blue staining of SDS-polyacrylamide gels. An 8.5% gel and a 12.5% gel were used for lanes a to e and f to q, respectively. The positions of amidase (55 kDa) and interleukin-1β (17 kDa) are indicated by the arrowheads. Lanes: a and k, molecular size standards; b and d, purified amidase from R312; c and e, total cell extracts of E103S(pXL1752) after promoter induction at 42°C and incubation at 30°C, respectively; f, h, and j, soluble proteins, total cell extract, and insoluble proteins, respectively, of control E103S(pXL1029) expressing recombinant soluble interleukin-1β; g and i, soluble and insoluble proteins, respectively, E103S(pXL1752) expressing amidase; m, o, and q, soluble proteins, total cell extract, and insoluble proteins, respectively, of control E. coli B(pXL906) expressing interleukin-1β under the control of the trp promoter; l, n, and p, same extracts as in lanes m, o, and q, respectively, of the isogenic strain E. coli B(pXL1751) expressing amidase.

A wild-type amidase activity (6). Nitrile hydratase from Brevibacterium sp. strain R312, characterized by a wide substrate spectrum (6), has been purified to homogeneity and is composed of two types of subunits of apparent Mr near 27,000 (22). The enzyme biosynthesis is not influenced by the nature of the carbon or nitrogen source but can be repressed by amides and amide analogs (27). On the other hand, it has been demonstrated that Brevibacterium sp. strain R312 harbors, as other microorganisms, several different types of amidase activities. For instance, an acylamidase amidohydrolase (EC 3.5.1.4) with a wide activity spectrum (wide-spectrum amidase) has been characterized (17). The purified enzyme is apparently a tetramer of four identical subunits (Mr, 43,000), is able to hydrolyze a large number of amides into their corresponding organic acids, and also possesses an acyl transferase activity (26). In a mutant strain, called A4, in which this enzyme is inactivated, another amidase activity able to hydrolyze a large number of α-amino amides has also been identified.

An interesting property of this second candidate enzyme, name α-aminoamidase, is its stereospecificity. Contrary to the wide-spectrum amidase, this enzyme is able to produce only L α-amino acids from α-amino amides (16).

We described in the present work the preliminary characterization and the cloning of a stereospecific amide which was identified by its ability to hydrolyze racemic 2-aryl or 2-arylxy propionamides (R312 amidase in this discussion). It is clear from the structures of the purified proteins that this enzyme is different from the wide-spectrum amidase. In addition, we recently performed N-terminal sequencing analysis of the purified wide-spectrum amidase (provided by A. Arnaud, Montpellier, France) and confirmed that the two proteins are indeed different. However, we still cannot exclude that the R312 amidase described here may be identical to the previously identified α-aminoamidase, although a preliminary study of the latter seemed to indicate a very different structure (16). More work on the substrate
specificity of these enzymes is clearly needed to clarify this point.

We found important homologies between the R312 amidase and both the A. nidulans acetamidase and the indoleacetamide hydrolases of two different plant pathogens. The significance of these homologies in terms of substrate specificity is not clear. For instance, we found a similar, or even higher, homology between R312 amidase and the P. syringae indoleacetamide hydrolase (32.1%) than between both indoleacetamide hydrolases from P. syringae and A. tumefaciens (26.4%) (29), although the latter ones are supposed to carry the same enzymatic functions. However, the very high conservation of nearly 40 residues around position 190 in all these amidases (between 57 and 75% identity) strongly suggests that this portion of the protein constitutes the active catalytic site responsible for the hydrolysis of the amide bond.

Another important conclusion from this work is the colocalization of the amdA gene coding for the stereospecific amidase with the gene for nitrile hydratase. The identification of the latter gene is based on its total identity at the nucleotide level with the corresponding gene of Rhodococcus sp. strain N-774. In fact, it was already known that enzymes from both microorganisms had similar structures (11, 22), and we recently found (data not shown) that the N-terminal peptide sequences of the two subunits of the purified Brevibacterium enzyme (kindly provided by A. Arnaud, Montpellier) were identical to those reported for the enzyme from Rhodococcus sp. (the sequenced N terminus of the α subunit is indicated in Fig. 2). We also take the identity at the nucleotide level of the common sequenced fragment from Brevibacterium sp. strain R312 and Rhodococcus sp. strain N-774 (one nucleotide difference in more than 1.55 kb) as a strong indication that both microorganisms should be at least very closely related, if not identical. It seems plausible from the above observations that nitrile hydratase can be expressed from a polycistronic mRNA initiated upstream from amdA since no sequence reminiscent of a transcription terminator can be found in the intercistronic region between the two genes; moreover, it has been shown, at least in E. coli (11), that nitrile hydratase can be expressed from a promoter inserted at the SphI site, located in amdA. We interpret this gene arrangement as an indication that the bacterial cell needs to coregulate, at least under some conditions, the gene expression of both enzymes.

The lack of significant expression of the R312 amidase in E. coli either from the PstI fragment (native promoter and RBS) or from the BamHI-PstI fragment (native RBS and E. coli promoter) is difficult to interpret in the context of previous results suggesting that the transcriptional and translational machineries could recognize similar signals in E. coli and Corynebacterium bacteria (at least Corynebacterium glutamicum). Work is in progress to map the promoter and to check whether R312 amidase can be readily expressed from native signals in prototype coryneform bacteria such as C. glutamicum or B. lactofermentum. It is also interesting to observe that, when expressed from E. coli signals, the solubility of the overexpressed protein is very dependent on the growth temperature between 30 and 37°C, although similar expression levels are obtained. That amidase cannot properly refold and reach its active three-dimensional conformation in vivo at 37°C appears to be the most obvious explanation. Moreover, specific activities measured on whole cells are well correlated to the observed solubilities. These results suggest not only that the production of an active Brevibacterium amidase can be obtained in E. coli but also that the whole recombinant E. coli cells can indeed be used in the bioconversion of amides.

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LITERATURE CITED


