Cloning and Heterologous Expression of an Enantioselective Amidase from *Rhodococcus erythropolis* Strain MP50

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The gene for an enantioselective amidase was cloned from *Rhodococcus erythropolis* MP50, which utilizes various aromatic nitriles via a nitrile hydratase/amidase system as nitrogen sources. The gene encoded a protein of 525 amino acids which corresponded to a protein with a molecular mass of 55.5 kDa. The deduced complete amino acid sequence showed homology to other enantioselective amidas from different bacterial genera. The nucleotide sequence approximately 2.5 kb upstream and downstream of the amidase gene was determined, but no indications for a structural coupling of the amidase gene with the genes for a nitrile hydratase were found. The amidase gene was carried by an approximately 40-kb circular plasmid in *R. erythropolis* MP50. The amidase was heterologously expressed in *Escherichia coli* and shown to hydrolyze 2-phenylpropionamide, α-chlorophenylacetamide, and α-methoxyphenylacetamide with high enantioselectivity; mandeloamide and 2-methyl-3-phenylpropionamide were also converted, but only with reduced enantioselectivity. The recombinant *E. coli* strain which synthesized the amidase gene was shown to grow with organic amides as nitrogen sources. A comparison of the amidase activities observed with whole cells or cell extracts of the recombinant *E. coli* strain suggested that the transport of the amides into the cells becomes the rate-limiting step for amidase hydrolysis in recombinant *E. coli* strains.

Acylamid amidohydrolases (amidas) are used in biocatalysis for the chemoselective, regioselective, or enantioselective hydrolysis of various amides (17, 59). The chemo- and regioselectivities of amidas are utilized for the production of antibiotics (penicillin acylase), the hydrolysis of C-terminal amidase gene groups in peptides (peptide amidas), the analysis of glycoproteins [peptide-N^4-(N-acetyl-β-D-glucosaminyl)asparagine amidase F], or the transformation of cyclic imides (half-amidase, amidase) (5, 25, 50, 57, 59). Enantioselective amidas are used for the production of optical active D- or L-α-amino acids, hydroxycarboxylic acids, or α-methylarylactetic and α-methoxyarylactetic acids. L-specific aminomidas have been reported for *Pseudomonas putida*, *Mycobacterium neou- rnum*, and *Stenotrophomonas maltophilia*, and a D-specific aminoacid amidase has been found in *Ochrobactrum anthropi*. These enzymes usually also convert certain peptides and are therefore referred to as aminopeptidases (3, 21, 22, 41).

An evolutionarily different group of amidas has been found which enantioselectively converts 2-methylphenylacetamide (2-phenylpropionamide) and other α-methylarylactec-amides. This group of amidas has been found in different rhodococci but also in gram-negative organisms, such as *Pseudomonas chlororaphis* B23 or *Agrobacterium tumefaciens* d3 (6, 31, 44, 45, 49).

One of the best-characterized amidas with the ability to enantioselectively hydrolyze various α-methylarylactacemides that has been described is from *Rhodococcus erythropolis* MP50. This isolate was obtained from an enrichment with naproxen nitrile as sole nitrogen source and produced almost pure S-naproxen [S-2-(6-methoxy-2-naphthyl)propionic acid] from racemic naproxen nitrile [2-(6-methoxy-2-naphthyl)propionitrile] or racemic naproxen amide [2-(6-methoxy-2-naphthyl)propionamide] (37, 39). The conversion of racemic naproxenamide to S-naproxen with this strain was also studied with immobilized whole cells in the presence of organic solvents (15, 16). The strain converted naproxen nitrile and other nitriles by the combined action of a nitrile hydratase and an amidase. The amidase was purified, characterized, and shown to be responsible for the high degree of enantioselectivity. The purified enzyme converted racemic 2-phenylpropionamide, naproxenamide, and ketoprofen amide [2-3′-benzoylphenylpropionamide] to the corresponding S-acids with enantiomeric excess of >99% at almost 50% conversion of the racemic amides (23, 38, 39). The amidase also demonstrated enantioselective acyl-transferase activity in the presence of hydroxylamine and was used to produce optical active 2-phenylpropion-hydroxamate from racemic 2-phenylpropionamide (24). In order to allow a genetic manipulation of this interesting biocatalyst in the present study, the corresponding gene was cloned and characterized.

**MATERIALS AND METHODS**

**Bacterial strains, media, and plasmids.** *R. erythropolis* MP50 (DSMZ 9675) (37, 39) cells were routinely grown at 30°C in a mineral medium with succinate (10 mM), phenylacetonitrile (1 mM), Nutrient Broth (NB; Difco) (240 mg/liter), and NaCl (150 mg ml^-1\). For the isolation of genomic DNA, the strain was cultivated in Luria–Bertani (LB) medium with glycerine (1.5% [wt/vol]) and 1.5% (wt/vol) saccharose. *E. coli* DH5α and *E. coli* JM1109 cells were used as host strains for recombinant DNA work. *E. coli* strains were routinely cultured at 37°C in LB medium which was supplied with ampicillin (100 μg/ml), if appropriate.

The plasmid pBluescript II KS(+) (1) was used for most cloning experiments, and the λ-ramose-inducible plasmid vector pJO2702 was used for high levels of expression (62).

**Analytical methods.** Amides and acids were analyzed by high-pressure liquid chromatography (HPLC) as previously described (60).
Preparation of cell extracts. The cells of *R. erythropolis* MP50 were harvested by centrifugation (30 min, 8,000 rpm), resuspended in Tris-HCl buffer (30 mM, pH 7.5), and disintegrated by being ground with glass beads (0.3-mm diameter) in a Dyno-Mill type KD1 homogenizer (Fa Willy A. Bachofen, Basel, Switzerland). The cells of recombinant *E. coli* strains were disrupted by using a French press as previously described (60). Unbroken cells and cell debris were removed by centrifugation at 100,000 × g for 30 min at 4°C. Protein was determined by the method of Bradford (10) using bovine serum albumin as a standard.

Expression of amidase in *E. coli*. *E. coli* JM109(pST1WT) cells were grown in LB medium (120 ml) plus ampicillin (100 μg/ml) in 1-liter Erlenmeyer flasks at 37°C. When an optical density at 600 nm (OD600) of 0.2 to 0.3 was reached, 0.2% (wt/vol) rhamnose was added and the cells were cultivated at 30°C for another 6 h before the cells were harvested by centrifugation.

For the comparison of the amidase activities of cell extracts and whole cells of *E. coli* JM109(pST1WT), a bacterial culture was grown as described above and harvested by centrifugation and the cells were resuspended in Na+K+ phosphate buffer (30 mM, pH 7.4). This cell suspension was split into two equal parts, and from one of them a cell extract was prepared. The amidase activities of both preparations were determined. The protein content of the cell extract was determined using the Bradford method. The protein concentration of the whole-cell suspension was estimated by assuming that the protein content which was determined for the cell extract was identical to the protein content of the whole cells from which the cell extract was prepared.

Standard assay for determination of enzyme activities with cell extracts and purified enzyme preparations. The amide hydrolyzing activity was assayed routinely in reaction mixtures (0.5 ml) composed of 15 μmol of Tris-HCl buffer (pH 7.5) or 25 μmol of sodium potassium phosphate buffer (pH 7.4), 2.5 μmol of phenylacetamide (stock solution: 100 mM in methanol), and different amounts of protein (1 to 400 μg). The reaction was performed at room temperature in a plastic reaction tube. After different time intervals, aliquots were taken (100 μl) and the reaction was stopped by the addition of 10 μl of 1 M HCl, and the precipitated protein was removed by centrifugation (5 min, 20,800 × g). The hydrolysis of amides and the formation of the corresponding acids was determined by HPLC. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of product per min.

Enzyme purification. The amidase from *R. erythropolis* MP50 was purified by dialysis against pH 7.5, followed by dialysis against pH 8.3 (50 mM, NaHCO3). The purified enzyme was assayed by HPLC. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of product per min.

Cloning of amidase gene. Amidase was purified from cell extracts of *R. erythropolis* MP50 basically as described previously by Hirrlinger et al. (23), and the amino-terminus amidase amino acid sequence was determined (Table 1). The purified amidase was digested with trypsin, several peptides were isolated by HPLC, and the sequences of three fragments were determined (Table 1). The amino-terminus amino acid sequence and the sequences of the peptides P5329 and P6616 served for the design of oligonucleotide primers (Table I) for PCR experiments. Using genomic DNA of strain MP50 as template and primers derived from the amino-terminus sequence and one of the internal peptides P6616 or P5329, DNA fragments with sizes of 0.7 or 1.5 kb, respectively, were amplified. The amplified 1.5-kb fragment was DIG labeled and used as a probe to identify an approximately 4-kb fragment from the total DNA of strain MP50, which was subsequently cloned into pBluescript II KS(+). This plasmid was designated pRAM1. The sequence of the inserted DNA fragment in pRAM1 demonstrated that the amidase gene was at one end of the cloned DNA fragment. In order to identify genes located downstream of the amidase gene in the genome of strain MP50, a probe was constructed by PCR from the amidase gene carried on plasmid pRAM1 and used to clone an about-3.5-kb BamHI fragment from the genomic DNA into pBluescript II KS(+). This construct was designated pRAM2.

Nucleotide sequence accession number. The sequence data reported in this article will appear in the GenBank nucleotide sequence database under the number AY026386.

RESULTS

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### Table 1. Sequences of the amino terminus, tryptic peptides, and deduced oligonucleotides

<table>
<thead>
<tr>
<th>Protein or peptide</th>
<th>Amino acid sequence*</th>
<th>Deduced oligonucleotide sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino terminus</td>
<td>MRPNRPFGHVPRPPTAEOLQOEYSARHRHFDLD</td>
<td>GC(GC)GAGCAG(CT)TGCTCAAG(AAG)TA</td>
</tr>
<tr>
<td>P5329</td>
<td>FEESTLYR</td>
<td>TA(GC)AG(GC)GT(GC)CATGTCCTGTCAGAA</td>
</tr>
<tr>
<td>P6616</td>
<td>LPEPENYSGALGEVGGLR</td>
<td>CCGTAgTGC(TC)GCTGG(TC)AGCTTG</td>
</tr>
<tr>
<td>P8137</td>
<td>V(N/S)PL?TAAWPQSGVM</td>
<td></td>
</tr>
</tbody>
</table>

* Segments used for the design of oligonucleotides for PCR are underlined.
mass of 55.5 kDa. This value agreed sufficiently with the molecular mass of the amidase subunits (61 kDa) determined earlier by sodium dodecyl sulfate (SDS) gel electrophoresis (23). The deduced complete amino acid sequence showed the highest degree of sequence identity (34%) to an amidase from P. chlororaphis B23 (49).

The DNA fragment which was inserted in pRAM1 contained one more complete putative ORF (ORF2) and one fragmentary putative ORF (ORF1) which were transcribed in the opposite direction as amdA (Fig. 1). These ORFs showed the highest degree of sequence similarities to a regulatory protein from the GntR family and a transposase from the insertion element ISRh1, which was previously found in Rhizobium "hedysari" (Table 2).

The sequence analysis of the DNA fragment which was cloned in pRAM2 demonstrated that both sequences overlapped for 984 bp. Apart from the fragmentary amdA gene, three more putative ORFs were identified downstream from amdA (Fig. 1). The proteins encoded by ORF3 and ORF4 showed the highest degree of sequence identities to proteins with unknown functions from Propionibacterium acidipropionici and E. coli (Table 2). Surprisingly, it was found that the incomplete ORF1 which had been identified on pRAM1 was part of ORF5, which was identified on pRAM2. The protein encoded by ORF5 showed % sequence identity with the transposase from ISRh1. This suggested that amdA was part of a transposable element which was flanked by two direct repeats of an insertion element resembling ISRh1. This was further substantiated by the observation that ORF5 (the gene for the putative transposase) was flanked by two identical inverted repeats of 15 bp (5'-GGCACTGTCACGTTG-3'), which were very similar to the inverted repeats described for ISRh1 (both sequences contained one additional base pair each at different positions, but were otherwise identical). The same 15-bp repeat was also identified upstream of ORF1. No direct repeats were observed at the ends of the putative insertion elements containing ORF1 and ORF5. This has also been described for ISRh1 (46).

The presence of only a single copy of the amidase gene amdA in the genome of strain MP50 was confirmed by the results of the hybridization experiments which were performed with a 1.5-kb probe of amdA and preparations of genomic DNA digested with different restriction enzymes. In these experiments, consistently only one hybridizing band was observed with all restriction enzymes that did not cut within the amidase gene.

### TABLE 2. Putative genes and gene products from sequenced DNA fragments

<table>
<thead>
<tr>
<th>Gene or ORF</th>
<th>Position in sequence</th>
<th>Probable function of product</th>
<th>Size (amino acids)</th>
<th>Source</th>
<th>Region of sequence homology (as according to BLAST search)</th>
<th>% Identity</th>
<th>Reference for homologous proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1–267</td>
<td>Transposase</td>
<td>89</td>
<td>Rhizobium &quot;hedysari&quot;</td>
<td>ND</td>
<td>AAB81600</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1279–2028</td>
<td>Regulator (GntR family)</td>
<td>249</td>
<td>Streptomyces coelicolor A3(2)</td>
<td>22–235</td>
<td>32</td>
<td>CAB61304</td>
</tr>
<tr>
<td>amdA</td>
<td>2306–3883</td>
<td>Amidase</td>
<td>525</td>
<td>Pseudomonas chlororaphis B23</td>
<td>13–513</td>
<td>34</td>
<td>P27765</td>
</tr>
<tr>
<td>3</td>
<td>4171–4740</td>
<td>Hypothetical</td>
<td>189</td>
<td>Propionibacterium acidipropionici</td>
<td>2–183</td>
<td>33</td>
<td>CAB88395.1</td>
</tr>
<tr>
<td>4</td>
<td>4936–5193</td>
<td>Hypothetical</td>
<td>85</td>
<td>Escherichia coli</td>
<td>1–85</td>
<td>43</td>
<td>P46147</td>
</tr>
<tr>
<td>5</td>
<td>5206–5220</td>
<td>Transposase</td>
<td>236</td>
<td>Rhizobium &quot;hedysari&quot;</td>
<td>13–230</td>
<td>55</td>
<td>AAB81600</td>
</tr>
</tbody>
</table>

a Percentage of amino acids that are identical when sequences were aligned with sequences listed in the GenBank database of the NCBI facilities, ND, not determined.

b Accession number in the GenBank format.
TABLE 3. Hydrolysis of different amides by enantioselective amidases from R. erythropolis MP50 and A. tumefaciens d3

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Structure</th>
<th>Relative activity (%)</th>
<th>Enantiomeric ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Phenylpropionamide</td>
<td><img src="image" alt="Structure" /></td>
<td>100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2-Methyl-3-phenylpropionamide</td>
<td><img src="image" alt="Structure" /></td>
<td>104 ± 21</td>
<td>15</td>
</tr>
<tr>
<td>2-Methoxyphenylacetamide</td>
<td><img src="image" alt="Structure" /></td>
<td>20 ± 1</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2-Chlorophenylacetamide</td>
<td><img src="image" alt="Structure" /></td>
<td>112 ± 6</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Mandeloamide</td>
<td><img src="image" alt="Structure" /></td>
<td>25 ± 6</td>
<td>8</td>
</tr>
</tbody>
</table>

*The reaction mixtures were 0.5 ml and contained 15 μmol of Tris-HCl (pH 7.5), 0.25 μmol of the respective amide, and purified amidase (1.4 to 3.2 mg) from R. erythropolis MP50. The reference data for the amidase from A. tumefaciens d3 were determined in 25 μM Na phosphate or K phosphate buffer (pH 7.4) with cell extracts (0.01 to 0.6 mg) from E. coli JM109 (pST2WT) (60). The specific activities of the purified amidase from strain MP50 and the cell extract from E. coli JM109 pST2WT with 2-phenylpropionamide as substrate were 3.4 ± 0.7 and 0.39 U/mg of protein, respectively. The tests were performed in duplicate, and the standard deviations are given.

a Data were taken from a previous report (60), with permission.

Location of amidase gene on plasmid in strain MP50. It was previously shown that the enantioselective amidase of A. tumefaciens d3 is encoded by a plasmid (60). The analyses of cell lysates from strain MP50 by pulsed-field gel electrophoresis demonstrated the presence of two (presumably linear) plasmids with sizes of 180 and 230 kb. (Two linear plasmids with similar sizes had been previously identified in this strain [36]). After an additional S1 nuclease treatment of the cell lysates, two more plasmid bands with masses of about 5 and 40 kDa were detected. Because the S1 nuclease treatment linearizes cyclic plasmids, it can be assumed that these two smaller plasmids are present in a cyclic form in strain MP50. The plasmid DNAs were blotted and hybridized with the labeled 1.5-kb fragment of the amidase gene initially obtained by PCR (see above). This resulted in an hybridization signal with the 40-kb plasmid.

Expression of amidase gene in E. coli. The amidase gene was amplified by PCR from plasmid pRAM1 using a set of primers which created new NdeI and HindIII restriction sites and also replaced the GTG start codon with an ATG start codon. The amplified fragment was then ligated into the l-rhamnose-inducible expression vector pJOE2702 (62) to yield plasmid pST1WT, which was used to transform E. coli JM109. The amidase gene was induced in the recombinant E. coli cells at 30°C by the addition of l-rhamnose. The amidase activity of these cell extracts with phenylacetamide (5 mM) as substrate was 0.69 U/mg.

Conversion of different α-substituted phenylacetamides by amidase from strain R. erythropolis MP50. The purified amidase was incubated with 2-phenylpropionamide, mandeloamide, O-acetylmandeloamide, α-methoxyphenylacetamide, α-chlorophenylacetamide, or 2-methyl-3-phenylpropionamide. The amidase showed good to excellent enantioselectivities with α-methylphenylacetamide, α-methoxyphenylacetamide, α-chlorophenylacetamide, and 2-methyl-3-phenylpropionamide (Table 3). These experiments were performed at a substrate concentration of 0.5 mM to allow a comparison with the results obtained earlier for the amidase from A. tumefaciens d3. In order to obtain some more information about the enzyme kinetics, different concentrations (0.5 to 8 mM) of the respective substrates were converted with the purified amidase. The enzyme obeyed traditional Michaelis-Menten kinetics in this substrate range only during the conversion of 2-phenylpropionamide and 2-methyl-3-phenylpropionamide (Fig. 2). In contrast, more or less-pronounced substrate inhibition effects were observed with mandeloamide, 2-methoxyphenylacetamide, and 2-chlorophenylacetamide.

Growth of recombinant E. coli strains with amides as nitrogen source. The availability of cloned enantioselective amidas should allow the improvement of these enzymes by evolutionary strategies, which require potent selection or screening techniques. The release of ammonia from the organic amides in the course of the amidase reaction should exert a strong selective pressure for the selection of amide-converting strains. It was therefore tested if the presence of the cloned
amidase gene allowed the recombinant *E. coli* strains to grow with amides and if the growth rate was dependent from the substrate specificity of the amidase for the respective amides. These growth experiments demonstrated that the presence of the amidase gene indeed enabled *E. coli* JM109 cells to grow with different amides which were not used by the parental strain (Fig. 3). The growth rates of the recombinant *E. coli* strain did not correlate with the known substrate specificity of the purified amidase previously determined (23). These experiments suggested that in the recombinant strain, the activity of the amidase was not the growth-limiting factor in vivo. It was therefore tested if the uptake of the amides could be the rate-limiting step. Therefore, the conversion of 2-phenylpropionamide (0.5 mM) was compared with resting cells and a cell extract prepared thereof and it was found that the amidase activity of the cell extract (4 U mg of protein \(^{-1}\)) was more than 10 times higher than the activity observed with resting cells (0.3 U mg of protein \(^{-1}\)). In a control experiment with the wild-type strain *R. erythropolis* MP50, the cell extract demonstrated less than twice as much activity as the whole cells. These results suggested that the uptake of the amides may be the rate-limiting step in the recombinant organism and furthermore indicated that the genes encoding putative amide transport systems which have been found adjacent to the amidase genes in *A. tumefaciens* d3, *Methylophilus methylotrophus*, *Pseudomonas aeruginosa*, or *Rhodococcus* sp. strain R312 (12, 47, 60, 68) may indeed be necessary for the optimal uptake of amides into the bacterial cells.

**DISCUSSION**

The ability of amidases to enantioselectively hydrolyze \(\alpha\)-methylphenylacetamides and \(\alpha\)-methoxyphenylacetamides was first demonstrated by Mayaux et al. for the enzymes from two rhodococcus strains (26, 44, 45). This ability was later shown for some other bacterial amidases from different gram-positive and gram-negative isolates (13, 32, 60). The sequence information which was obtained during these earlier studies, and the present work clearly demonstrated that these enantioselective amidases form a group of evolutionarily related enzymes, which differ according to their sequences significantly from other acylamide amidohydrolases, such as indolacetamide hydrolases, acetamide hydrolases, formamide hydrolases, and the so-called wide-spectrum amidases, which hydrolyze short-chain amides (basically acetamide and similar compounds) (11) (Fig. 4). Furthermore, these enantioselective amidases are, according to their amino acid sequences, fundamentally different from the aminopeptidases, which are used for the enantioselective synthesis of \(d\)- or \(l\)-amino acids from the corresponding racemic amides (3, 21, 22, 41). The BLAST searches for related enzymes surprisingly demonstrated that the subunit A of the glutamyl-tRNA(Gln)-amidotransamidase from *Bacillus subtilis* (and presumably various other organisms) also clustered within the group of the enantioselective amidases. This enzymatic activity is involved in gram-positive bacteria (and also *Archaea* and cyanobacteria) in the transamination of misacylated Glu-tRNA\(^{\text{Glu}}\) to Gln-tRNA\(^{\text{Glu}}\) which functionally replaces the lack of a glutaminyl-tRNA synthetase in these organisms (14, 27).

The genetic localization of the amidase from *R. erythropolis* MP50 resembled the situation observed earlier for the amidase from *A. tumefaciens* d3 and clearly differentiated both enzymes from other microorganisms, because previously no indications for the localization of amidase genes on plasmids have been found. Furthermore, all other genes encoding \(S\)-specific enantioselective amidases form a group of evolutionarily related enzymes (11) (Fig. 4). Furthermore, these enantioselective amidases are, according to their amino acid sequences, fundamentally different from the aminopeptidases, which are used for the enantioselective synthesis of \(d\)- or \(l\)-amino acids from the corresponding racemic amides (3, 21, 22, 41). The BLAST searches for related enzymes surprisingly demonstrated that the subunit A of the glutamyl-tRNA(Gln)-amidotransamidase from *Bacillus subtilis* (and presumably various other organisms) also clustered within the group of the enantioselective amidases. This enzymatic activity is involved in gram-positive bacteria (and also *Archaea* and cyanobacteria) in the transamination of misacylated Glu-tRNA\(^{\text{Glu}}\) to Gln-tRNA\(^{\text{Glu}}\) which functionally replaces the lack of a glutaminyl-tRNA synthetase in these organisms (14, 27).

**Fig. 3.** Growth of *E. coli* JM109 (pST1WT) with succinate as carbon source and different amides as nitrogen sources. The growth experiments were performed in 100-ml Erlenmeyer flasks with baffles in a medium which contained in 25 ml of Na phosphate or K phosphate buffer (50 mM, pH 7.4), succinate (25 mM), proline (0.5 mM), thiamine (1 \(\mu\)M), ampicillin (100 \(\mu\)g/ml), and acetamide (\(\bullet\)), hexanamide (\(\square\)), isovalerianamide (\(\triangledown\)), pivalamide (\(\triangledown\)), or mandeloamide (\(\bigcirc\)) (5 mM each) or no additional nitrogen source (\(\square\)). The flasks were inoculated with 100 \(\mu\)l of a preculture grown in the same growth medium with succinate and hexanamide. The bacterial cultures were grown at 37°C and growth monitored photometrically at 546 nm.
Enantioselective amidases

Subunit A of the glutamyl-tRNA\(^\sim\)-amidotransamidase

Entanioselective amidases

Indolacetamide hydrolases

6-Aminohexanoate-cyclic-dimer hydrolases

Acetamidase

Formamidase

Wide spectrum amidase

Aliphatic amidases

FIG. 4. Dendrogram resulting from pairwise alignments of amino acid sequences by using the program CLUSTAL. Agrobacterium tumefaciens d3 (AF315580 [60]); Agrobacterium tumefaciens (P03868 [30]); Bacillus subtilis 168 (O06491 [14]); Comamonas acidovorans KPO-2771-1 (20); Flavobacterium sp. strain K172 (P13397 [61]); Helicobacter pylori 85P (CA472932 [53]); Methylphilus methylothrophus IS05228 [67]; Mycobacterium smegmatis NCTC 8159 (Q07838 [42]); Pseudomonas aeruginosa (P11436 [2]); Pseudomonas chlororaphis (Q50228 [67]); Pseudomonas putida 5B (O69768 [67]); Pseudomonas sp. strain NK87 (P13398 [61]); Pseudomonas syringae EW2009 (P06618 [69]); Rhodococcus rhodochrous J1 (P3270 [32]); Rhodococcus sp. (A41326 [45]); Rhodococcus sp. strain R312 (enantioselective amidade) (P22984 [44]); and Rhodococcus sp. strain R312 ("wide-spectrum" amidade) (Q01360 [56]).

tioselective amidases were physically connected to nitrile hydratase genes (32, 34, 44, 45, 49, 51, 67). The reason(s) for these differences is currently unclear, but it may be connected with the different enrichment conditions which were applied for the isolation of the respective organisms: R. erythropolis MP50 and A. tumefaciens d3 have been enriched with 2-arylpropionitiles. In contrast, all other well-studied strains possess nitrile hydratase activity have been enriched with small aliphatic nitriles.

A further peculiarity of the localization of the amidase gene from R. erythropolis MP50 was the observation that it was surrounded by two copies of a putative insertion element. Recently, some examples for the presence of insertion elements in rhodococci have been described, and there is also one example known for the presence of an insertion element within a gene cluster which contains nitrile hydratase and amidase genes (33). Surprisingly, the sequence alignments demonstrated that the putative transposase from strain MP50 was much more closely related to the transposase from a gram-negative Rhizobium strain (IsRh1: 53% sequence identity) than to known insertion elements from other rhodococci (as IS1415, IS676, IS1164, or IS2112; < 22% sequence identity) (33, 35, 40, 46, 48). The presence of two copies of the putative insertion element, which surround the amidase gene (and two putative regulatory genes) suggest that the amidase gene is part of a transposon structure. This hypothesis was further substantiated by the observation that according to Southern blotting experiments, the amidase gene was lost from the genome of the strain after growth under nonselective conditions and that these mutant strains still maintained a plasmid with a size of approximately 40 kb (unpublished results).

Amidases with the ability to enantioselectively hydrolyze 2-arylaylamides (such as 2-phenylpropionamide or 2-phenylbutyramide) have been found in several rhodococci and pseudomonads (8, 13, 18, 20, 23, 29, 32, 44, 45). The major aim in these previous studies was the preparation of S-2-arylpropionic acids (such as S-inbuprofen, S-naproxen, and S-ketoprofen), which are the pharmacologically active enantiomers in these nonsteroidal anti-inflammatory drugs produced in large quantities by the pharmaceutical industry (54). Only recently, some information was accumulating which suggested that this group of amidases is also able to enantioselectively convert phenylacetamide derivatives which carry substituents other than methyl groups in the α-position of the phenylacetamide core structure. Thus, it has been found that racemic α-amino-phenylacetamide (phenylglycinamide) and also α-amino-phenylacetnitrile (phenylglycinnitrile) can be converted to L-phenylglycin and D-phenylglycinamide with rather large enantiomeric excesses by various bacteria with nitrile hydratase/amidase activities (9, 37, 58, 63, 64, 65). The results of the present study about the enantioselective amidase from R. erythropolis MP50 and our previous study about the enzyme from A. tumefaciens d3 clearly demonstrated that other substrates in the α-position of phenylacetamide are also able to induce a highly enantioselective conversion by this group of amidases. This significantly increases the possible applications of this group of enzymes for biotransformation reactions.

REFERENCES


