APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2002, p. 3279–3286 Vol. 68, No. 7
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.7.3279–3286.2002

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

Sandra Trott,† Sibylle Bürger, Carsten Calaminus, and Andreas Stolz*

Institut für Mikrobiologie, Universität Stuttgart, 70569 Stuttgart, Germany

Received 2 January 2002/Accepted 19 April 2002

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 amidases 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 amide hydrolysis in recombinant *E. coli* strains.

Acylamide amidohydrolases (amidases) are used in biocatalysis for the chemoselective, regioselective, or enantioselective hydrolysis of various amides (17, 59). The chemo- and regioselectivities of amidases are utilized for the production of antibiotics (penicillin acylase), the hydrolysis of C-terminal amide groups in peptides (peptide amidase), the analysis of glycoproteins [peptide-N^4-(*N*-acetyl-β-D-glucosaminyl)asparagine amidase F], or the transformation of cyclic imides (half-amidase, imidase) (5, 25, 50, 55, 57, 59). Enantioselective amidases are used for the production of optical active D- or L-α-amino acids, hydroxycarboxylic acids, or α-methylarylacetic and α-methoxyarylacetic acids. L-specific aminoamidases have been reported for *Pseudomonas putida*, *Mycobacterium neoaurum*, and *Stenotrophomonas maltophilia*, and a D-specific amino acid 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 amidases has been found which enantioselectively converts 2-methylphenylacetamide (2-phenylpropionamide) and other α-methylarylacetamides. This group of amidases 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 amidases with the ability to enantioselectively hydrolyze various α-methylarylacetamides 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, naproxen amide, and ketoprofen amide [2-(3′-benzoylphenyl) propionamide] 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-phenylpropionhydroxamate 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 glycine (1.5% [wt/vol]) and 1.5% (wt/vol) saccharose. *E. coli* DH5α and *E. coli* JM109 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 L-rhamnose-inducible plasmid vector pJOE2702 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).

* Corresponding author. Mailing address: Institut für Mikrobiologie, Universität Stuttgart, 70569 Stuttgart, Germany. Phone: 49-711-6855489. Fax: 49-711-6855725. E-mail: Andreas.Stolz@PO.Uni-Stuttgart.DE.

† Present address: Tyndall Air Force Base, FL 32403.

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

Protein or peptide	Amino acid sequence[a]	Deduced oligonucleotide sequences
Amino terminus	MRPNRPFGHVRPPTAEQLQEYSARHHFDLD	GC(GC)GAGCAG(CT)T(GCT)CA(AG)GA(AG)TA
P5329	FEESTLYR	TA(GC)AG(GC)GT(GC)(GC)(AT)(CT)TC(CT)TC(AG)AA
P6616	LPEPENYGSALGEGVSGLR	CCGTAGTT(CT)TC(GCT)GG(CT)TC(AGCT)GG
P8137	V(N/S)VPL?TAAWPIQSGVM	

[a] Segments used for the design of oligonucleotides for PCR are underlined.

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 KDL 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 (OD_{600}) 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 (50 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 each), 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 at room temperature by use of a fast-performance liquid chromatography system (FPLC) system as previously described (23).

Protein cleavage, isolation of peptides, and sequencing of peptides and N termini. The digestion of the purified amidase by trypsin and the subsequent separation of tryptic digests by reversed-phase HPLC were performed as described previously (60). The amino-terminal sequence of the amidase and the amino acid sequences of internal peptides from the amidase were determined by automated Edman degradation.

Isolation of genomic DNA. Cell suspensions (1.5 ml) from *R. erythropolis* MP50 grown on LB were centrifuged (14,000 rpm, 3 min), and the cells were resuspended in 1 ml of 10% (wt/vol) saccharose containing 10 mg of lysozyme. Cells were incubated for 2 h at 37°C and harvested by centrifugation. Genomic DNA was prepared as described by Ausubel et al. (4).

Isolation of plasmid DNA. Plasmid DNA from *E. coli* DH5α was isolated with the Flexi-Prep kit (Amersham Pharmacia Biotech) or the Qiaprep Spin Miniprep kit (Qiagen, Hilden, Germany). The preparation of plasmids for the detection of megaplasmids was basically performed as previously described (60).

DNA manipulation techniques. Digestion of DNA with restriction endonucleases (Gibco BRL, New England Biolabs), electrophoresis, and ligation with T4 DNA ligase (Gibco BRL) were performed according to the standard procedures (52). Transformation of *E. coli* was done by the method described by Inoue et al. (28). PCR, cloning of PCR products, DNA sequencing, and nucleotide sequence analysis were performed as described previously (43, 60).

Hybridization procedures. A digoxigenin (DIG) DNA labeling and detection kit was used according to the instructions of the supplier (Boehringer Mannheim). The hybridization temperature was set to 68°C.

Construction of plasmid pST1WT for overexpression of amidase in *E. coli*. For expression in *E. coli*, the amidase gene was inserted in the plasmid vector pJOE2702 (62) under the control of an L-rhamnose-inducible promoter by using a PCR amplification strategy with simultaneous introduction of *Nde*I and *Hin*dIII sites as described previously (60).

Chemicals. The synthesis and sources of all chemicals used have been described before (6, 7, 23, 60).

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

RESULTS

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-terminal 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-terminal amino acid sequence and the sequences of the peptides P5329 and P6616 served for the design of oligonucleotide primers (Table 1) for PCR experiments. Using genomic DNA of strain MP50 as template and primers derived from the amino-terminal 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 *Kpn*I 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 *Bam*HI fragment from the genomic DNA into pBluescript II KS(+). This construct was designated pRAM2.

Nucleotide sequence of amidase gene and surrounding DNA fragments. Plasmids pRAM1 and pRAM2 contained inserts of 3,902 and 3,524 bp, respectively. The GC contents of these inserts were 68.0 and 67.4%, respectively, and were therefore within the typical range for the chromosomal DNA of *R. erythropolis* (67 to 71%) (19). In the insert in pRAM1, three tentative open reading frames (ORFs) were identified. The deduced amino acid sequence of one of these ORFs contained all of the amino acid sequences determined for the amino terminus and the internal peptides of the amidase, and the gene was therefore designated *amdA*. The knowledge of the amino-terminal amino acid sequence unequivocally proved that the start codon was a GTG triplet. The gene encoded a protein of 525 amino acids which corresponded to a protein with a molecular

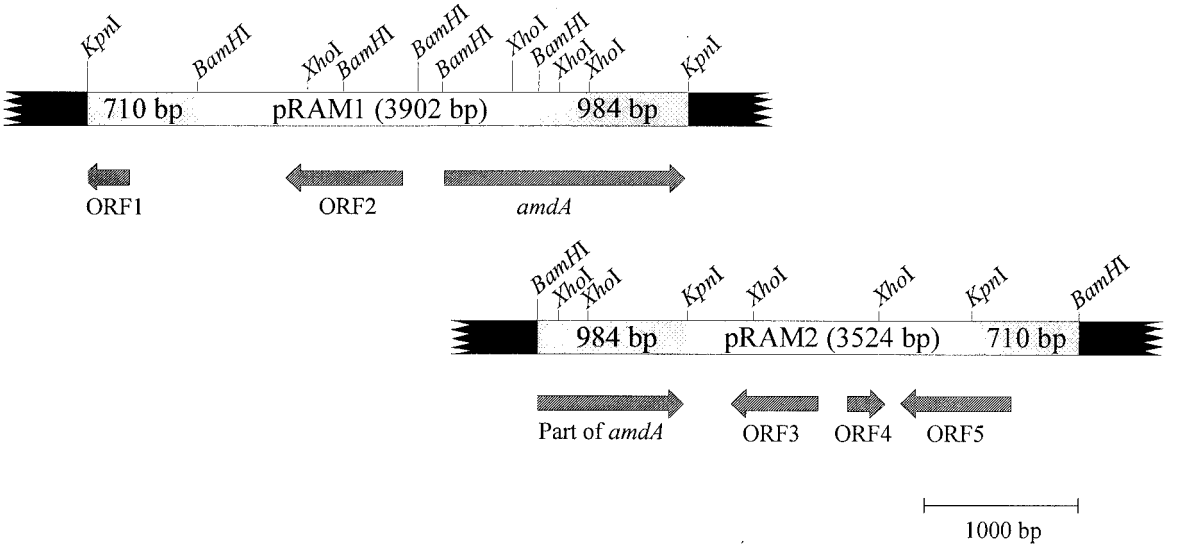


FIG. 1. Genetic organization of the 6.4-kb DNA fragment sequenced. Plasmids pRAM1 and pRAM2 carried the 3.9-kb *Kpn*I fragment and the 3.5-kb *Bam*HI fragment indicated. The positions and orientations of the different ORFs detected within the locus are shown by large arrows.

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 IS*Rh1*, 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 acidpropionici* 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 53% sequence identity with the transposase from IS*Rh*1. This suggested that *amdA* was part of a transposable element which was flanked by two direct repeats of an insertion element resembling IS*Rh1*. 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 IS*Rh1* (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 IS*Rh1* (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.

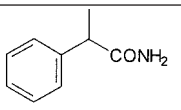
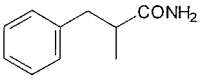
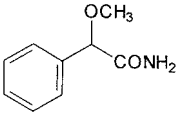
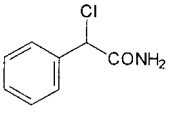
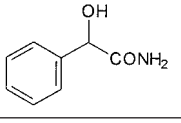
TABLE 2. Putative genes and gene products from sequenced DNA fragments

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

[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[a]

Substrate	Structure	Relative activity (%)		Enantiomeric ratio	
		MP50	d3[b]	MP50	d3[b]
2-Phenylpropionamide		100	100	>100	>100
2-Methyl-3-phenylpropionamide		104 ± 21	37	15	>100
2-Methoxyphenylacetamide		20 ± 1	3	>100	36
2-Chlorophenylacetamide		112 ± 6	56	>100	>100
Mandeloamide		25 ± 6	15	8	6

[a] 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.

[b] 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 *Nde*I and *Hin*dIII 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 amidases 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

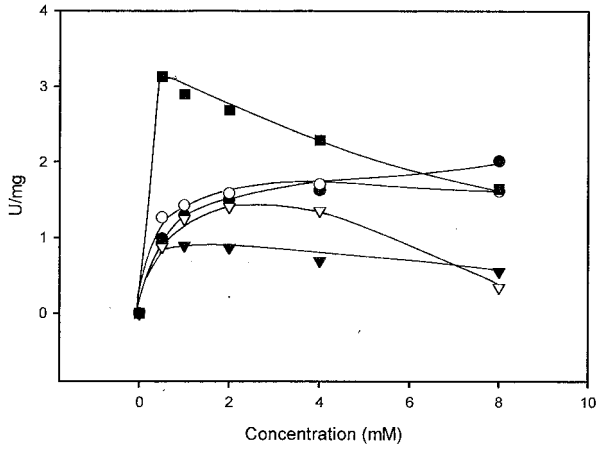


FIG. 2. Conversion of various concentrations of different amides by enantioselective amidase from *R. erythropolis* MP50. The reaction mixtures were 0.5 ml and contained 1.4 to 2.8 μg of the purified amidase, 15 μmol of Tris-HCl (pH 7.5), and a 0.5 to 8 mM concentration of 2-methyl-3-phenylpropionamide (●), 2-phenylpropionamide (○), 2-methoxyphenylacetamide (▼), mandeloamide (▽), or 2-chlorophenylacetamide (■). The reaction mixtures were incubated at room temperature. After 5, 10, 15, 25, and 35 min, aliquots (80 μl each) were taken and the reaction was stopped by the addition of 8 μl of 1 M HCl. The precipitated protein was removed by centrifugation (3 min at 15,800 × *g*), and the supernatants were reneutralized with 1 M NaOH and analyzed by HPLC for the formation of the respective acids.

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, 66, 68) may indeed be necessary for the optimal uptake of amides into the bacterial cells.

DISCUSSION

The ability of amidases to enantioselectively hydrolyze α-methylphenylacetamides and α-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 transamidation of misacylated Glu-tRNA^{Gln} to Gln-tRNA^{Gln} 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 enan-

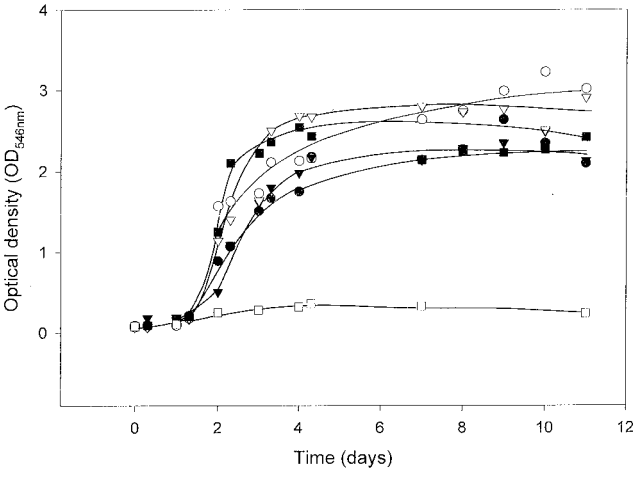


FIG. 3. Growth of *E. coli* JM109 (pST1WT) with succinate as carbon source and different amides as nitrogen sources. The growth experiments were performed in 100-ml Erlenmeyer flasks with baffles in a medium which contained in 25 ml of Na phosphate or K phosphate buffer (50 mM, pH 7.4), succinate (25 mM), proline (0.5 mM), thiamine (1 μM), ampicillin (100 μg/ml), and acetamide (●), hexaneamide (○), isovalerianamide (▼), pivalamide (▽), or mandeloamide (■) (5 mM each) or no additional nitrogen source (□). The flasks were inoculated with 100 μl 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.

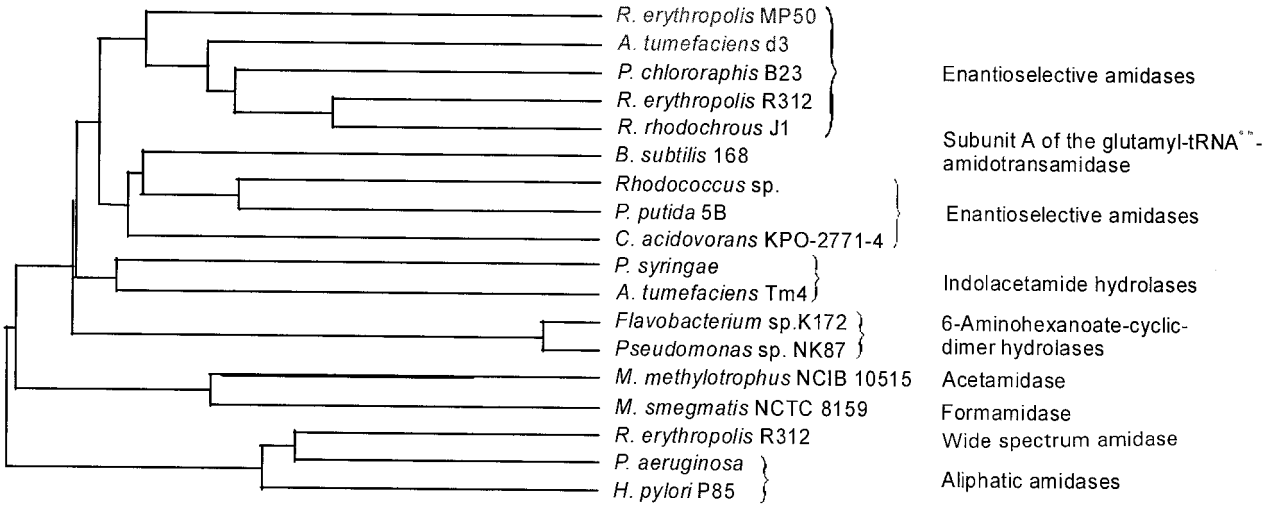


FIG. 4. Dendrogram resulting from pairwise alignments of amino acid sequences by using the program CLUSTAL. *Agrobacterium tumefaciens* d3 (AF315580 [60]); *Agrobacterium tumefaciens* (P03868 [30]); *Bacillus subtilis* 168 (O06491 [14]); *Comamonas acidovorans* KPO-2771-4 (20); *Flavobacterium* sp. strain K172 (P13397 [61]); *Helicobacter pylori* 85P (CAA72932 [53]); *Methylophilus methylotrophus* (Q50228 [67]); *Mycobacterium smegmatis* NCTC 8159 (Q07838 [42]), *Pseudomonas aeruginosa* (P11436 [2]), *Pseudomonas chlororaphis* B23 (P27765 [49]); *Pseudomonas putida* 5B (O69768 [67]); *Pseudomonas* sp. strain NK87 (P13398 [61]); *Pseudomonas syringae* EW2009 (P06618 [69]); *Rhodococcus rhodochrous* J1 (S38270 [32]); *Rhodococcus* sp. (A41326 [45]); *Rhodococcus* sp. strain R312 (enantioselective amidase) (P22984 [44]); and *Rhodococcus* sp. strain R312 ("wide-spectrum" amidase) (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-arylpropionitriles. In contrast, all other well-studied strains possessing 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 (Is*Rh1*: 53% sequence identity) than to known insertion elements from other rhodococci (as IS*1415*, IS*1676*, IS*1164*, or IS*2112*; < 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-arylacylamides (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*-ibuprofen, *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-aminophenylacetamide (phenylglycinamide) and also α-aminophenylacetonitrile (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 substituents 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.

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