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ORIGINAL PAPER

**S. Verseck · A. Bommarius · M.-R. Kula**

# Screening, overexpression and characterization of an *N*-acylamino acid racemase from *Amycolatopsis orientalis* subsp. *lurida*

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**Abstract** Thirty-one different actinomycete strains were used in a genetic screening using PCR and Southern hybridization methods to detect *N*-acetylamino acid racemases (AAR) in order to obtain enzymes with different properties. Cloning and sequencing of a 2.5 kb *Eco*RI DNA fragment from *Amycolatopsis orientalis* subsp. *lurida* revealed the coding gene of an *N*-acetylamino acid racemase, which had identities to the *aar* gene of *Amycolatopsis* sp. TS-1-60 [Tokuyama and Hatano (1995) Appl Microbiol Biotechnol 42:884–889] of 86% at the level of DNA, and 90% at the level of amino acids. The heterologous overexpression in *Escherichia coli* resulted in a specific activity of about 0.2 U/mg of this racemase. A two-step purification with heat treatment followed by anion-exchange chromatography led to almost homogeneous enzyme. The optimum pH of the enzyme was 8.0 and it was stable at 50°C for 30 min. The relative molecular mass of the native enzyme and the subunit was calculated to be 300 kDa and 40 kDa by gel filtration and SDS–PAGE, respectively. The isoelectric point (pI) of the AAR was 4.4. It catalyzed the racemization of optically active *N*-acetylamino acids such as *N*-acetyl-L- or -D-methionine and *N*-acetyl-L-phenylalanine. Further characterization of the racemase demonstrated a requirement for divalent metal ions ($Co^{2+}$, $Mn^{2+}$, $Mg^{2+}$) for activity and inhibition by EDTA and *p*-hydroxymercuribenzoic acid. AAR is sensitive to substrate inhibition at concentrations exceeding 200 mM.

S. Verseck · M.-R. Kula (✉)
Institute of Enzyme Technology of the
Heinrich-Heine-University Duesseldorf, 52426 Jülich, Germany
e-mail: M.-R.Kula@fz-juelich.de
Tel.: +49-2461-13716, Fax: +49-2461-612490

A. Bommarius
Degussa-Huels AG, Research,
Development and Application Technology, Fine Chemicals,
PO Box 1345, 63403 Hanau, Germany

## Introduction

Enantiomerically pure α-amino acids are increasingly important products with extended applications in the food and feed, pharmaceutical or agrochemical industries (Bommarius et al. 1998). Some L-amino acids, such as glutamate and lysine, are produced on very large scale by microbial synthesis, while others including D-enantiomers and non proteinogenic amino acids are obtained by enzymatic synthesis from readily available precursors (Jetten and Sinskey 1995; Sahm et al. 1996; Faber 1997).

For example, L-methionine is produced continuously from *N*-acetyl-D, L-methionine using L-aminoacylase in an enzyme membrane reactor (Wandrey and Flaschel 1979; Bommarius et al. 1996). In this process, only *N*-acetyl-L-methionine is stereospecifically deacetylated by L-aminoacylase and converted into L-methionine. After separating L-methionine from the mixture, the remaining *N*-acetyl-D-methionine is racemized by chemical methods under rather severe conditions (high temperature, low pH) to be used again as starting material for the next cycle of the process (Fig. 1). If *N*-acetylamino acids could be selectively racemized by an enzyme in the presence of an optically active amino acid, *N*-acetyl-D, L-methionine could be converted totally into L-methionine by the combined action of racemase with the L-aminoacylase without any intermittent separation step. Such an *N*-acetylamino acid racemase (AAR) activity was found by Tokuyama et al. (1994a, b) in various actinomycete strains. The gene for the *N*-acetylamino acid racemase from *Amycolatopsis* sp. TS-1-60 was cloned and the gene product characterized (Tokuyama and Hatano 1995a, b, 1996). The requirement for a high concentration of divalent metal ions (e.g. cobalt ions >10 mM) for enzyme activity, substrate inhibition at concentrations exceeding 50 mM (Palmer et al. 1999) and inhibition by L-methionine at less than 100 mM severely restrict the use of this enzyme in a commercial process.

In this work, we outline a DNA-based screening method using PCR and Southern hybridization methods

to detect *N*-acetylamino acid racemase genes in other actinomycetes to obtain enzymes with improved properties (Verseck et al. 1999). Since there is only one specific DNA sequence for a AAR gene available from *Amycolatopsis* sp. TS-1-60 (Tokuyama and Hatano 1995b), we amplified a 504 bp DNA fragment from chromosomal DNA of *Amycolatopsis orientalis* subsp. *lurida* (and other actinomycetes) using a polymerase chain reaction (PCR) approach with degenerated primers. One of the degenerated primer pool was derived from the N-terminal AAR amino acid sequence of *Amycolatopsis* sp. TS-1-60 and *Streptomyces atratus* Y-53 (Tokuyama and Hatano 1995a, b, 1996), and the other was deduced from the highly conserved KXK motive of the enolase superfamily (Babbitt et al. 1996). This superfamily of enzymes is related by their ability to catalyze the abstraction of the α-proton of a carboxylic acid to form an enolic intermediate. Both primers were adapted to the GC-rich codon usage of actinomycetes (Wright and Bibb 1992).

The resulting DNA fragment was used in hybridization experiments for probing genomic DNA from different actinomycete strains in Southern blots in order to identify homologous sequences. We detected a strong hybridization signal with genomic DNA from *A. orientalis* ssp. *lurida* and weaker signals in other actinomycetes.

Cloning of the gene coding for the *N*-acetylamino acid racemase from *A. orientalis* subsp. *lurida*, heterologous overexpression in *E. coli*, purification, and characterization of the racemase are described.

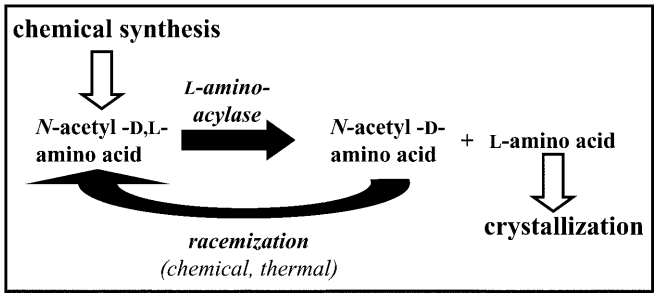


**Fig. 1** Industrial process for L-amino acid productions with an L-specific aminoacylase

## Materials and methods

### Chemicals

Diazoacetylnorleucine methyl ester, ethylenediaminetetraacetic acid (EDTA), hydroxylamine, *p*-hydroxymercuribenzoic acid, L-penicillamine, *o*-phenanthroline, phenylhydrazine and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma-Aldrich (Deisenhofen, Germany). Other chemicals, especially the amino acid derivatives, were from Degussa-Hüls (Hanau, Germany).

### Bacterial strains and growth conditions

The bacterial strains used in this study were *E. coli* BL21 (DE3) [*ompT*, *hsdS*$_B$ ($r_B^-m_B^-$), [F$^-$], λ prophage with T7 *rnp*], *E. coli* DH5α [ *j80d*, *lacZ Δ* M15, *endA1*, *recA1*, *hsdr17* ($r_K^-m_K^-$), *supE44*, *thi*-1, *gyrA96*, *relA1*, Δ(*lacZYA-argF*)U169], *A. fastidiosa* [DSM 43855], *A. mediterranei* [DSM 43304], *A. orientalis subsp. orientalis* [DSM 40040], *A. orientalis* subsp. *lurida* [DSM 43134], *A. rugosa* [DSM 43194], *A. sulphurea* [DSM 46092], *Chainia yaxianenesis* [ATCC 43139], *Micromonospora sagamiensis* [DSM 43912], *Saccharopolyspora erythraea* [DSM 40517], *Streptomyces abikoensis* [DSM 41491], *S. aminophilus* [DSM 40186], *S. cinereus* [DSM 43033], *S. coelicolor* [DSM 40233], *S. erythraeus* [DSM 41446], *S. felleus* [DSM 40130], *S. galbus* [DSM 40089], *S. griseofuscus* [DSM 40191], *S. griseus* [DSM 40236], *S. hygroscopicus* subsp. *hygroscopicus* [DSM 40578], *S. kanamyceticus* [DSM 40500], *S. levoris* [DSM 40202], *S. lividans* [DSM 46482], *S. nodosus* [DSM 40109], *S. noursei* [DSM 40635], *S. ornatus* [DSM 40307], *S. rimosus* [DSM 40760], *S. spectabilis* [DSM 40512], *S. variabilis* [DSM 40179], *S. vellosus* [DSM 931], *Streptoalloteichus hindustanus* [NRRL B-11280], *Streptosporangium roseum* [DSM 43021].

*E. coli* strains used for propagation of plasmids were grown at 37°C in Luria-Bertani (LB) medium (Sambrook et al. 1989) containing 100 µg/ml ampicillin (Promega-Serva, Heidelberg, Germany). LB medium containing 1.5% agar was used for cultivation on solid medium. *E. coli* BL21(DE3) carrying the plasmid for overexpression (pAAR3–21I) of the AAR protein (Table 1) was cultivated in LB supplemented with 100 µg/ml ampicillin at 37°C. Induction of gene expression was carried out as described by Studier et al. (1990). After reaching an $OD_{600\ nm}$ of 0.5–0.8, Isopropyl-β-D-thiogalactopyranoside (IPTG; Roth, Karlsruhe, Germany) was added to a final concentration of 1 mM, followed by incubation for 3–6 h at 37°C.

All actinomycetes strains were cultivated in TSB medium (Oxoid, Wesel, Germany) at 28°C, with the exception of *Amycolatopsis sulphurea*, which was cultivated in DSMZ-medium 426 (DSMZ, Braunschweig, Germany).

**Table 1** Plasmids used in this study

| Plasmids | Relevant properties | Reference |
|---|---|---|
| pUC18 | *bla*, *lacZ*-α | Yanisch-Perron et al. 1985 |
| pET11a | *bla*, *lacI*, T7-ϕ10, *lacO*, *s10p* | Studier et al. 1990 |
| pSAR4–7SF | 504 bp PCR fragment in pUC18 *Sma*I containing the N-terminal segment of *aar* from *A. orientalis* subsp. *lurida* | This work |
| pAAR1–21I, pAAR1–19IV | 2,5 kb *Eco*RI fragment in pUC18 *Eco*RI with *aar* in both orientations | This work |
| pAAR2–21I | 1,1 kb PCR fragment of *aar* with newly created *Nde*I (N-terminal) and *Bgl*II (C-terminal) sites in pUC18 *Sma*I | This work |
| pAAR3–21I | 1,1 kb *Nde*I/*Bgl*II fragment of pAAR2–21I in pET11a *Nde*I/*Bam*HI | This work |

### General DNA manipulation techniques, DNA sequencing and analysis

Chromosomal DNA of the actinomycete strains were prepared according to method B of Mehling et al. (1995), extraction of plasmid DNA by the alkaline lysis method and agarose gel electrophoresis as described by Sambrook et al. (1989). For sequencing purposes, recombinant plasmid DNA was prepared using plasmid kits (Qiagen, Hilden, Germany). Restriction enzymes (New England Biolabs, Bad Schwalmbach, Germany) and T4 DNA-ligase (Roche Diagnostics, Mannheim, Germany) were used according to the instructions of the suppliers. DNA fragments of agarose gels were isolated by using the JetSorb kit (Genomed, Bad Oeynhausen, Germany). The transformation of *E. coli* strains was carried out according to Hanahan (1983).

The 'Thermo sequenase fluorescent labeled primer cycle sequencing kit' (Amersham Pharmacia Biotech, Uppsala, Sweden) with mp13/pUC18 standard primers were used for DNA sequencing of pUC18 recombinant subclones. The DNA sequence was determined by an automated DNA sequence analyzer (ALF; Amersham Pharmacia Biotech, Freiburg, Germany). The computer-assisted analysis and comparison of nucleotide and protein sequences were performed utilizing the programs DNASIS (Hitachi) and BLAST (NCBI; Altschul et al. 1997).

### Amplification of DNA fragments by polymerase chain reaction (PCR)

The DNA fragments of the *aar* gene were amplified by PCR using chromosomal DNA from the corresponding bacterial strains (given above). All primers were synthesized by MWG-Biotech (Ebersberg, Germany).

The degenerate primer pools were derived from the N-terminal AAR amino acid sequence of *Amycolatopsis* sp. TS-1-60 (Tokuyama and Hatano 1995b), respectively *Streptomyces atratus* Y53 (Tokuyama et al. 1994b), and the highly conserved KXK-motive of the enolase superfamily (Babbitt et al. 1996). The primers contained the following sequences: AR1, 5′-(AG)TGAA-(AG)CT(GC)AG(GC)GG(GCT)GT(GC)GA(AG)CT(GC)CG(GC-CGA-3′ (position 1–29); AR5, 5′-CCA(GC)CC(GC)GG(GCT)-TCGATCTT(GC)AGCTTGAT(GC)-CG-3′ (position 475–504).

Each reaction mixture contained 1 U *Taq* DNA polymerase (Gibco BRL, Eggenstein, Germany), 10 nmol of each dNTP, 50 pmol of each primer and approximately 0.1 µg of genomic DNA in a final volume of 50 µl with the appropriate buffer. PCR amplification was performed using a programmable thermocyler (Personal Cycler; Biometra, Göttingen, Germany). Amplifications with degenerated primers were performed by a 'touch-down' PCR with different annealing temperatures: After predenaturing the genomic DNA at 98°C for 5 min, *Taq* DNA polymerase was added, and the denaturing temperature was changed to 95°C for 1 min. The first 12 cycles were performed by an annealing temperature of the calculated $T_m$ of the primers for 45 s with a gradually decreasing temperature of 1°C per cycle, followed by an elongation at 72°C for 70 s. Further 30 cycles were carried out changing again to the annealing temperature of the calculated $T_m$. The elongation times were conform to the '1 kb DNA=1 min' rule.

The *aar* gene was amplified by PCR using the forward primer 5′-CAAGGAGCA**CATATG**AAACTCAGCGGTGTGG-3′ with an additional *Nde*I recognition sequence (bold letters) covering the ATG start codon, and the reversed primer 5′-GA-ATTCGTA**AGATCT**TACGAACCGATCCACG-3′ with an additional *Bgl*II recognition sequence (bold letters) directly behind the TAA stop codon of the *aar* reading frame. The PCR was performed as described above, with the exception that the annealing temperature remained for 30 cycles at 67°C for 45 s.

### Southern blot and colony hybridization

The Southern blotting and the colony transfer (of cells from shotgun cloning) on Hybond $N^+$ nylon membranes (Amersham Pharmacia Biotech) and the hybridization followed the 'DIG system users guide' (Boehringer Mannheim, 1994). Southern blot membranes were prehybridized for 2 h in prehybridization solution and hybridization was performed overnight at 61°C. Stringency washes were carried out twice with 2×SSC, 0,1% SDS (w/v) for 5 min at room temperature, followed by two washes with 0.5×SSC, 0,1% SDS (w/v) for 15 min at 61°C. Chemiluminescent detections were performed with CSPD (Roche Diagnostics).

The 504 bp insert of pSAR4–7SF was used as *aar* probe in the hybridization experiments described above. The DNA fragment was labeled with digoxigenin-11-dUTP by the random primed method described in the 'DIG system users guide' (Boehringer Mannheim, 1994).

### Shotgun cloning of the *aar* gene

A partial genomic library from *A. orientalis* subsp. *lurida*, containing DNA fragments selected by Southern analysis of genomic DNA preparations with the *aar* probe, was obtained by complete digestion of total DNA with restriction enzyme *Eco*RI, followed by ligation of purified fragments of about 2.5 kb in size into the *Eco*RI site of pUC18, and subsequent transformation of *E. coli* DH5α cells with the ligation mixture. Positive clones were detected by colony hybridization with digoxygenin (DIG)-labeled probes as described above.

### Construction of plasmids for *aar* expression

All DNA amplificates were cloned blunt end into *Sma*I hydrolyzed pUC18 vector by the use of the Sure clone kit (Amersham Pharmacia Biotech), transformed into *E. coli* DH5α, and prepared for analysis of the DNA sequence of the PCR products. The properties of the recombinant plasmids used in this study are given in Table 1. The general strategy was to create an *Nde*I recognition sequence covering the start codon at the 5′-end and a *Bgl*II recognition site covering the stop codon of the PCR-amplified *aar* reading frame. To fuse the *aar* reading frame directly to the given expression cassette with a suitable promoter and ribosome binding site (RBS), the pUC18-cloned PCR fragments (*Nde*I/*Bgl*II) were subcloned into the *E. coli* vector pET11a (*Nde*I/*Bam*HI) under the control of the IPTG inducible T7-promoter (Studier et al. 1990).

### Enzyme assay

*N*-Acylamino acid racemase activity was determined in an assay containing 50 mM Tris/HCl (pH 8), 25 mM *N*-acetyl-D-methionine, 6 mM cobalt chloride and *N*-acetylamino acid racemase in a final volume of 200 µl. After the reaction mixture had been incubated at 30°C for 6–30 min, the mixture was boiled for 3 min to stop the reaction. The amount of substrate and product was determined by HPLC using Gyncotec equipment (Germering, Germany). The chiral stationary phase (CSP) in the respective HPLC column (150×4 mm) consisted of *tert*-butyl carbamoyl quinidine immobilized onto 5 µm Kromasil silica. This set of CSPs and respective columns has been developed by W. Lindner and co-workers (University of Vienna) and on request these columns can be made available (e-mail: Wolfgang.Lindner@univie.ac.at). The flow rate was 1 ml/min and the chromatograms were developed over an isocratic elution with 70% methanol, 30% ammonium acetate (0.01 M) and 0.5 ml acetic acid per litre (Degussa-Hüls) in 35 min. The absorbance of the effluent was recorded at 200 nm, the sample volume injected onto the column was 20 µl. If available, a calibration curve was generated with concentrations between 0.25 and 12.5 mM of the corresponding enantiomers. The retention times for different substrates were: *N*-acetyl-D-alanine, 15.4 min; *N*-acetyl-D-aminobutyric acid, 16.7 min; *N*-acetyl-D-methionine, 23.0 min; *N*-acetyl-D-naphthylalanine, 27.8 min; *N*-acetyl-D-phenylalanine, 25.4 min; *N*-acetyl-D-valine, 16.4 min; *N*-acetyl-L-*tert*-leucine, 12.5 min; *N*-acetyl-L-methionine, 18.4 min;

*N*-acetyl-L-phenylalanine, 18.8 min; *N*-acetyl-L-tyrosine, 22.0 min; *N*-acetyl-L-valine, 11.4 min; *N*-benzyloxycarbonyl-L-phenylalanine, 16.9 min; *N*-chloroacetyl-L-phenylalanine, 19.9 min.

### Polarimetric assay

Changes of optical rotation of *N*-acetyl-L or D-amino acids were monitored at 365 nm for 20 s of integration time with a Perkin-Elmer polarimeter 241 (Weiterstadt, Germany). The assay containing 50 mM Tris/HCl (pH 7.5), 6 mM $CoCl_2$, 25 mM substrate and *N*-acylamino acid racemase (0.3–0.5 U). The reaction mixture was incubated in a polarimeter cell (1 ml volume) at 30°C and monitored for 1–6 h.

### Cell disintegration

Recombinant *E. coli* cells were disrupted by wet milling (Hummel and Kula 1989), using a mixer mill (Retsch-Schwingmühle, Haan, Germany). Up to 16 ml of cell suspension [30% in 50 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 0.5 mM DTT] and 32 g glass beads (0.3 mm diameter) were treated in the mixer 15 min two times at maximal frequency. Cell debris and glass beads were removed by centrifugation at 18,000 *g* for 30 min.

### Purification of AAR from *A. orientalis* subsp. *lurida*

The supernatant after cell disruption was heated at 50°C for 20 min, centrifuged for 20 min at 18,000 *g* to remove insoluble proteins, and was used for the second step during purification. All following steps were carried out at room temperature. The cell extract was adjusted to an NaCl concentration of 300 mM and applied onto a Q-Sepharose FF column (1.6×10 cm, 20 ml volume) connected to a FPLC system (Amersham Pharmacia Biotech). The column was equilibrated with 50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 300 mM NaCl and operated with a flow rate of 4 ml/min. The unbound material was washed out with equilibration buffer and AAR was eluted in four steps (60 ml buffer each) of 340 mM, 420 mM, 500 mM and 1 M NaCl, within 300 ml. The eluates was monitored at 280 nm. Fractions of 6 ml were collected and assayed for AAR activity. The enzyme eluted at 420 mM NaCl. Fractions with AAR activity were pooled and stored at –20°C.

### Determination of the molecular weight of the AAR

A 1 ml solution of purified enzyme was applied to a Superdex G-200 (Amersham Pharmacia Biotech) column (56.5×1.6 cm, 113.6 ml volume) pre-equilibrated with Tris/HCl (pH 7.5) with 0.2 M NaCl and eluted with the same buffer at a flow rate of 1 ml/min. The absorbance of the eluates was recorded at 280 nm. A calibration curve was generated with the 'LMW and HMW' gel filtration calibration kit (Amersham Pharmacia Biotech).

### Substrate spectrum and inhibition studies

Substrate specificity of the AAR was tested using the HPLC and/or polarimetric assay described above and with different amino-acid derivatives as substrates. Inhibition studies were performed by the enzyme assays supplemented with different concentrations of various potential inhibitors. The reaction products were analyzed by HPLC.

### Protein determination

Protein concentrations were measured according to Bradford (1976) by comparison to a standard curve generated using bovine serum albumin.

### Polyacrylamide gel electrophoresis and protein blotting

SDS–PAGE analysis and the following silver staining were performed according to Laemmli (1970) and Rabilloud (1992), respectively. Electroblotting of proteins was described by Matsudaira (1987). Proteins were blotted from SDS gels onto a FluoroTrans PVDF membrane by use of a Multifor II NovaBlot Unit (Amersham Pharmacia Biotech). The desired proteins were excised from the membranes and were directly used for sequence analysis.

### Protein sequence analysis

The sequence analysis was performed using an automated pulsed liquid protein sequencer 477 A from Applied Biosystems (Weiterstadt, Germany) by the method of Edman (1967). The identification of amino acid phenylthiohydantoins was performed by an on-line HPLC system (PTH analyzer 120 A).

### Isoelectric point

The isoelectric point (pI) was determined by agarose gel electrophoresis with carrier Ampholite. Electrophoresis was conducted under constant power (2 W) for 90 min using a electrophoresis unit (Serva, Heidelberg, Germany) and Servalyt pH 3–10. The calibration curve for pI was obtained with Serva protein test mixture (pH 3–10).

### EMBL/GenBank accession number

The nucleotide sequence of the 1.3 kb *Bam*HI/*Eco*RI DNA fragment with the reading frame of the *aar* gene and the resulting protein sequence has been submitted to the 'EMBL nucleotide sequence database' with the accession number AJ292519.

## Results

### DNA-based screening for *N*-acetylamino acid racemases

The first goal of the DNA-based screening for *N*-acetylamino acid racemases was to provide a useful probe for detection of this gene in genomic DNA of different actinomycetes. For this purpose, we used the PCR technique to amplify larger DNA fragments of homologous DNA sequences, which served as probe after DIG labeling.

PCR with the AR1 and AR5 primers produced a DNA fragment of 504 bp with genomic DNA of *A. orientalis* subsp. *lurida* as template. Amplification of a corresponding PCR fragment of about 500 bp was also observed in *A. orientalis* subsp. *orientalis*, *S. coelicolor* and *S. lividans*. No specific PCR product could be detected with genomic DNA of the tested strains of *A. mediterranei*, *S. erythraeus*, *S. galbus*, *S. griseofuscus*, *S. griseus* and *S. ornatus* (data not shown).

The 504 DNA fragment of *A. orientalis* subsp. *lurida* was cloned in pUC18x*Sma*I resulting in the plasmid pSAR4–7SF. Sequence analysis of this DNA insert revealed an identity to the coding region of the N-terminus of the *aar* gene from *Amycolatopsis* sp. TS-1-60 of 83% (Tokuyama and Hatano 1995b). This 504 bp fragment was DIG-labeled and used as a probe in Southern blots to detect the presence of *aar* genes in the *Eco*RI digested

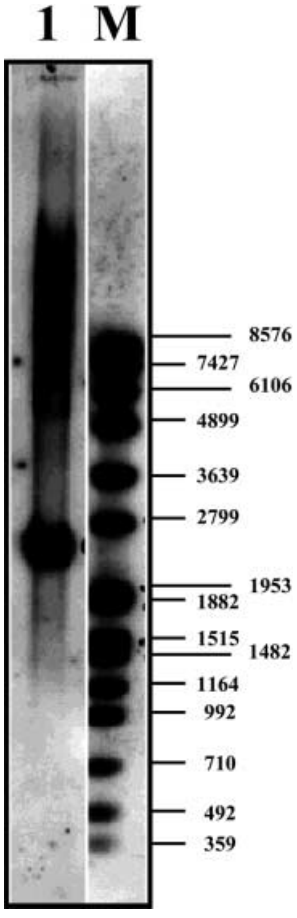


**Fig. 2** Hybridization signal with *Eco*RI digested genomic DNA of *Amycolatopsis orientalis* subsp. *lurida* (2.5 kb) with the DIG-labeled 504 bp DNA probe. *Lane M* DNA molecular weight marker VII, DIG-labeled (Roche Diagnostics), *the numbers* indicate the length of the marker fragments in base pairs

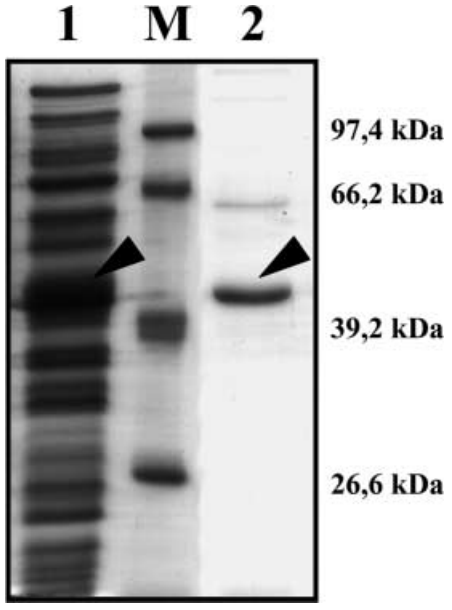


**Fig. 3** SDS–PAGE analysis of crude extract after heat treatment (*lane 1*) and of partial purified *N*-acylamino acid racemase (*arrow*s) from *A. orientalis* subsp. *lurida* after anion-exchange chromatography (*lane 2*). *Lane M* Low-range marker proteins (Roche Diagnostics)

genomic DNA of 31 different actinomycetes strains (given above). Typical results from the hybridization studies are shown in Fig. 2.

A strong hybridization signal was observed with genomic DNA of *A. orientalis* subsp. *lurida*. The 2.5 kb *Eco*RI fragment of *A. orientalis* subsp. *lurida* was ligated into the *Eco*RI restriction site of pUC18 and, transformed in *E. coli* DH5α. About 300 colonies from the partial DNA library were screened by colony hybridization using the DIG-labeled DNA fragment described above. Two positive colonies were obtained (data not shown) with the 2.5 kb *Eco*RI DNA fragment inserted in both orientations (pAAR1–21I and pAAR1–19IV). The subsequent DNA sequencing of a 1.3 kb *Bam*HI/*Eco*RI DNA fragment revealed a reading frame, which has identities to the *aar* gene of *Amycolatopsis* sp. TS-1-60 (Tokuyama and Hatano 1995b) of 86% at the level of DNA, and 90% at the level of amino acids.

## Heterologous overexpression of N-acylamino acid racemase from *A. orientalis* subsp. *lurida* in *E. coli*

For the heterologous overproduction of the *aar* gene of *A. orientalis* subsp. *lurida* in *E. coli*, pUC18-cloned PCR fragments (pAAR2–21I) were ligated into the *Nde*I/*Bam*HI restriction sites of the expression vector pET11a under the control of the IPTG inducible T7-promoter. To fuse the *aar* reading frame directly to the given expression cassette with the T7 promoter and a suitable ribosome binding site (RBS), an *Nde*I and a *Bgl*II recognition sequence covering the start and stop codons, respectively, were created by PCR. The resulting expression vector pAAR3–21I with a *Bam*HI/*Bgl*II hybrid site was transformed in *E. coli* BL21 (DE3), which carried the IPTG inducible T7 polymerase integrated into the chromosome (Studier et al. 1990). Transformants containing the pAAR3–21I plasmid were cultivated in 500 ml flasks with 200 ml LB medium for overproduction of the *N*-acylamino acid racemase. The IPTG-induced expression resulted in the appearance of a protein clearly visible on SDS–PAGE (Fig. 3). The apparent molecular mass of 40 kDa of this protein was in good agreement with the calculated value of 39.5 kDa. The amount of *N*-acylamino acid racemase in a soluble form was about 3% of the total soluble *E. coli* protein. The cell-free supernatant from *aar* overexpression clones catalyzed the racemization of *N*-acetyl-D-methionine with an specific activity of about 0.2 U/mg protein.

## Purification of *N*-acylamino acid racemase from *A. orientalis* subsp. *lurida*

The overexpressed *N*-acylamino acid racemase from *A. orientalis* subsp. *lurida* in *E. coli* was purified to near homogeneity (>90%) by two steps: heat treatment and anion-exchange chromatography using Q-Sepharose FF. The purification is summarized in Table 2.

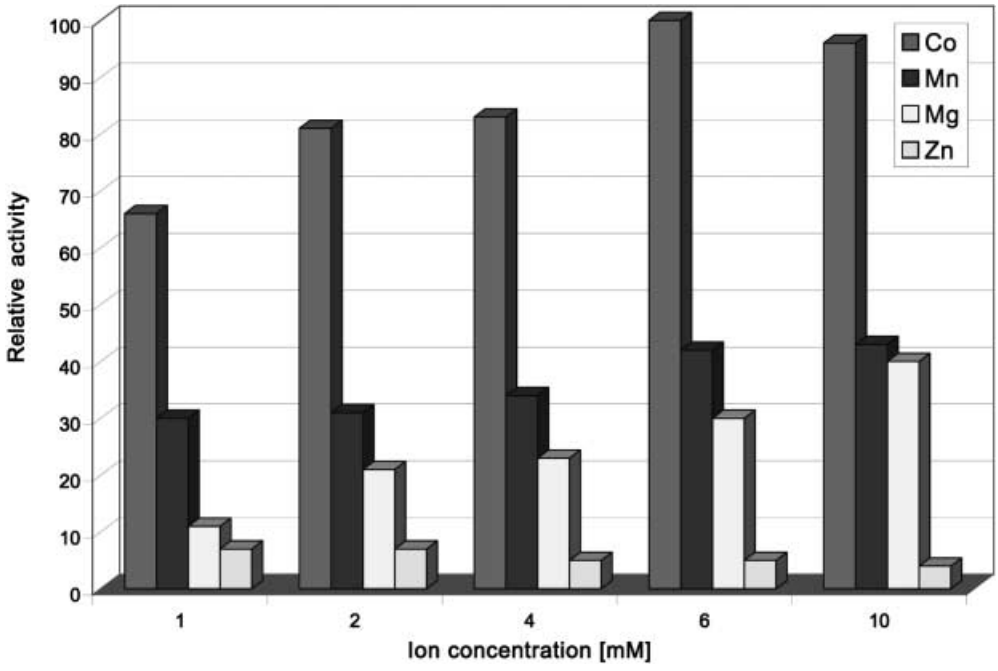


**Fig. 4** Effect of various metal ions in different concentrations on the relative enzyme activity of *N*-acylamino acid racemase from *A. orientalis* subsp. *lurida*. The standard assay was supplemented with different metal ions and incubated for 15 min at 30°C

**Table 2** Purification of the *N*-acylamino acid racemase from *Amycolatopsis orientalis* subsp. *lurida*

| Step | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Yield (%) | Purification factor |
|---|---|---|---|---|---|
| Crude extract: (from 7 g cells) | 700 | 70 | 0.1 | 100 | 1 |
| Heat treatment | 450 | 54 | 0.12 | 77 | 1.6 |
| Q-Sepharose FF | 11.9 | 52.4 | 4.4 | 75 | 59 |

The heat treatment was performed at 50°C for 20 min, reducing the total soluble protein to about 64%. The second purification was achieved by anion-exchange chromatography. The enzyme eluted at a salt concentration of 420 mM NaCl. Q-Sepharose FF chromatography resulted in 59-fold purification with 75% yield. The purified enzyme gave a dominant band on SDS-PAGE (Fig. 3) and the following N-terminal amino acid sequence was obtained:

N-Met-Lys-Leu-Ser-Gly-Val-Glu-Leu-Arg-Arg-Val-Arg-Met-Pro-Leu-Val-Ala-Pro-Phe-Arg-Thr-Ser-

The 22 amino acids match the deduced amino acid sequence from the DNA sequence determined. Therefore, we conclude that the overexpressed protein in *E. coli* BL21 (DE3) is the *N*-acylamino acid racemase from *A. orientalis* subsp. *lurida*.

## Physicochemical properties of *N*-acylamino acid racemase

The pH optimum of the *N*-acylamino acid racemase from *A. orientalis* subsp. *lurida* was around pH 8 in 50 mM Tris/HCl at 30°C and with *N*-acetyl-D-methionine as a substrate. The enzyme was stable up to 50°C for 30 min at pH 7.5. The molecular mass of the native enzyme was determined to be 300 kDa by gel filtration on a Superdex G-200 column. The pI value was 4.4 (data not shown).

## Effect of metal ions

Figure 4 summarizes the effect of various metal ions in different concentrations on the activity of racemase. *N*-Acylamino acid racemase activity was highest with $Co^{2+}$, followed by $Mn^{2+}$ and $Mg^{2+}$, whereas $Zn^{2+}$ resulted in only low level of activity.

## Substrate specificity of *N*-acylamino acid racemase

*N*-acylamino acid racemase catalyzed the racemization of various industrially important *N*-acylamino acids, which are listed in Table 3. As for many of the substrates investigated single enantiomers were not available for calibration in an HPLC assay, these substrates were tested in a polarimetric assay. Besides *N*-acetyl-D and L-methionine, the *N*-acyl derivatives of aromatic amino acids such as *N*-acetyl-D and L-phenylalanine, *N*-acetyl-L-tyrosine and *N*-chloroacetyl-L-phenylalanine were effective substrates. The derivatives *N*-acetyl-D-naphthylalanine, *N*-acetyl-L-*tert*-leucine and *N*-benzyloxycarbonyl-L-phenylalanine were not racemized. The $K_m$ value

**Table 3** Substrate specificity of *N*-acylamino acid racemase from *A. orientalis* subsp. *lurida*. The specific activities were investigated by the standard assay and analyzed. The specific activity with *N*-acetyl-D-methionine was taken as 100%

| Substrate | | Relative activity (%) |
|---|---|---|
| *N*-Acetyl-D-alanine | [b] | 3 |
| *N*-Acetyl-D-aminobutyric acid | [b] | 11 |
| *N*-Acetyl-D-methionine | [a,b] | 100 |
| *N*-Acetyl-D-naphthylalanine | [b] | 0 |
| *N*-Acetyl-D-phenylalanine | [a] | 76 |
| *N*-Acetyl-D-valine | [a] | 83 |
| *N*-Acetyl-L-*tert*-leucine | [b] | 0 |
| *N*-Acetyl-L-methionine | [a,b] | 130 |
| *N*-Acetyl-L-phenylalanine | [a] | 30 |
| *N*-Acetyl-L-tyrosine | [b] | 30 |
| *N*-Acetyl-L-valine | [a] | 22 |
| *N*-Benzyloxycarbonyl-L-phenylalanine | [b] | 0 |
| *N*-Chloracetyl-L-phenylalanine | [b] | 7 |

[a] HPLC analysis
[b] Polarimetric assay

and the $V_{max}$ calculated from Michaelis-Menten plots were 24 mM and 8 U/mg for *N*-acetyl-D-methionine and 35 mM and 13.7 U/mg for *N*-acetyl-L-methionine. The racemization reaction is subject to substrate inhibition with a $K_I$ of 457 mM or 398 mM for *N*-acetyl-D- and -L-methionine, respectively. In contrast, L-methionine was no substrate for *N*-acylamino acid racemase (data not shown). The $K_m$ value and the $V_{max}$ were 23 mM and 6.5 U/mg for *N*-acetyl-D-phenylalanine, and 5 mM and 1.5 U/mg for *N*-acetyl-L-phenylalanine, as well as, 148 mM and 27.3 U/mg for *N*-acetyl-D-valine, and 42 mM and 2.5 U/mg for *N*-acetyl-L-valine. The racemization of the latter substrates were not subject to substrate inhibition in the range of substrate concentrations tested (up to 250 mM).

### Effect of inhibitors on the activity of the *N*-acylamino acid racemase

The influence of potential inhibitors on *N*-acylamino acid racemase activity were tested by the standard assay with *N*-acetyl-L-methionine as substrate and supplemented with 1 mM of different inhibitors, with the exception of 10 mM EDTA. The serine protease inhibitor phenylmethyl sulfonyl fluoride (PMSF), as well as hydroxylamine and L-penicillamine, known inhibitors of pyrodoxal 5′-phosphate-dependent (PLP) enzymes, did not affect enzyme activity.

Phenylhydrazine, which interacts with carbonyl residues, causes a loss of 31% of racemase activity. The enzyme is strongly inhibited by the sulfhydryl reagent *p*-hydroxy mercuribenzoic acid, which implicates that the AAR needs reduced sulfhydryl residues for optimal catalytic activity. The sulfhydryl reagent diazoacetylnorleucin methylester affects acidic proteases (pH 2–5) and had no affect on the AAR activity. Addition of an excess of 10 mM of the metal chelating reagent EDTA (the enzyme assay contains 6 mM $CoCl_2$) strongly inhibited enzyme activity, suggesting that this racemase is a metalloenzyme. This conclusion is supported by the observation that in assays without supplemented metal ions no racemase activity was detected.

## Discussion

We amplified a 504 bp DNA fragment from *A. orientalis* subsp. *lurida* using a PCR approach with degenerated primer pools derived from the highly conserved KXK motive of the enolase superfamily (Babbitt et al. 1996) and from the N-terminus of two known *N*-acylamino acid racemases (Tokuyama and Hatano 1995a). With this DNA-fragment as probe in hybridization experiments, we could detect and isolate the whole *aar* gene from *A. orientalis* subsp. *lurida*. Furthermore, we could detect only weak or no signal in 30 other actinomycete strains. Interestingly, *S. spectabilis* showed *N*-acylamino acid racemase activity in a classical screening (Tokuyama et al. 1994a), but produced no hybridization signal in our experiments. Strain differences or lower sequence-similarities of the probe could be the reason for the negative result. For detection of other *N*-acylamino acid racemases in actinomycetes and related genera, we are going to use the whole *aar* gene as a probe.

Physicochemical characteristics of the heterologously overexpressed and purified enzyme of *A. orientalis* subsp. *lurida* were similar to those from *Amycolatopsis* sp. TS-1-60 and *S. atratus* (Tokuyama and Hatano 1995a), such as a pI of 4.4 and a pH optimum value of 8. The $M_r$ of about 300 kDa for the native enzyme and the $M_r$ of 40 kDa for the subunit suggests that the enzyme is a homooctamer. An important exception is, that the AAR from *A. orientalis* subsp. *lurida* exhibited substrate inhibition at concentrations of *N*-acetyl-D-methionine exceeding 200 mM in contrast with 50 mM for the racemase from *Amycolatopsis* sp. TS-1-60 (Palmer et al. 1999). This fact could be important for the use of AAR in an industrial racemization process.

A recent publication of Palmer et al. (1999) showed, that the *N*-acylamino acid racemases from *Amycolatopsis* sp. TS-1-60 catalyzes the *o*-succinylbenzoate synthase reaction more efficiently than the racemization of *N*-acetylamino acids. The *o*-succinylbenzoate synthase reaction may be the physiological role of the enzyme and the racemase activity only a side reaction, which explains the relatively high $K_m$ (24.1 mM and 34.8 mM) and low $V_{max}$ (8 U/mg and 13.7 U/mg) of the enzyme from *A. orientalis* subsp. *lurida.*

All known *N*-acylamino acid racemases require divalent metal ions such as $Co^{2+}$, $Mn^{2+}$ or $Mg^{2+}$ for activity and are inhibited by chelating agents such as EDTA. Therefore, they are all metalloenzymes. This characteristic is contrary to other known racemases, which require pyrodoxal 5′-phosphate (PLP) or no cofactor for their activity (Tokuyama and Hatano 1995a). Sequence similarity of *N*-acylamino acid racemases to the enolase super-

family and their metal ion requirement, makes a similar catalytic mechanism plausible for all members of this family (Babbitt et al. 1996; Palmer et al. 1999). It would be interesting to determine and to compare the X-ray crystal structure of *N*-acylamino acid racemases with other members of the enolase superfamily.

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