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# Identification of *Amycolatopsis* sp. strain HR167 genes, involved in the bioconversion of ferulic acid to vanillin

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**Abstract** The gene loci *ech*, encoding enoyl-CoA hydratase/aldolase, and *fcs*, encoding an unusual feruloyl-CoA synthetase, which are involved in the bioconversion of ferulic acid to vanillin by the Gram-positive bacterium *Amycolatopsis* sp. strain HR167, were localized on a 4,000 bp *Pst*I fragment (P40). The nucleotide sequence of P40 was determined, revealing open reading frames of 864 bp and 1,476 bp, representing *ech* and *fcs*, respectively. The deduced amino acid sequences of *ech* exhibited 62% amino acid identity to the enoyl-CoA hydratase/aldolase from *Pseudomonas* sp. strain HR199 and the enoyl-CoA hydratase/lyase from *P. fluorescens* strain AN103. The deduced amino acid sequences of *fcs* exhibited up to 37% amino acid identity to long-chain fatty acid coenzymeA ligases but no significant similarity to the feruloyl-CoA synthetase of *Pseudomonas* sp. strain HR199. Fragment P40 was cloned in pBluescript $SK^-$ and *fcs* and *ech* were expressed in *Escherichia coli*. Recombinant strains were able to transform ferulic acid to vanillin. In crude extracts of these recombinant strains, feruloyl-CoA synthetase and enoyl-CoA hydratase/aldolase activities were detected by photometric assay and high-performance liquid chromatography. The obtained data suggest that ferulic acid degradation in the Gram-positive *Amycolatopsis* sp. strain HR167 proceeds via a pathway similar to that recently described for the Gram-negative *P. fluorescens* strain AN103 and *Pseudomonas* sp. strain HR199.

## Introduction

The Gram-positive bacterium *Amycolatopsis* sp. strain HR167 is able to utilize ferulic acid as sole carbon and energy source for growth. This strain was isolated during a screening for microorganisms which were able to convert ferulic acid to vanillin; and it is used for the biotechnological production of vanillin (Rabenhorst and Hopp 1997). Vanillin is frequently used for the production of flavors for foods and there is a growing interest to produce "natural" vanillin from natural substrates by biotransformation (Hagedorn and Kaphammer 1994; Krings and Berger 1998; Rabenhorst 1996). Since vanillin occurs as an intermediate in the degradation routes of eugenol, ferulic acid and lignin (Chen et al. 1982; Overhage et al. 1999a; Priefert et al. 1999; Steinbüchel et al. 1998; Toms and Wood 1970), these compounds can be used as vanillin precursors in biotransformation processes. We are currently investigating the physiological and genetic basis of the vanillin production process based on the biotransformation of ferulic acid by *Amycolatopsis* sp. strain HR167 (Rabenhorst and Hopp 1997).

Many microorganisms are able to degrade ferulic acid. In many cases the initial step is catalyzed by a decarboxylase; and the formation of 4-hydroxy-3-methoxystyrene is observed (Rosazza et al. 1995). With respect to biotechnological approaches a second mechanism, initiated by shortening the ferulic acid side chain for a $C_2$ unit, is of greater interest. A deacetylation to yield vanillin was proposed for *Comamonas* (formerly *Pseudomonas*) *acidovorans* (Toms and Wood 1970), *Bacillus subtilis* (Gurujeyalakshmi and Mahadevan 1987), *Burkholderia cepacia* (Andreoni et al. 1984) and *Streptomyces setonii* (Muheim and Lerch 1999; Sutherland et al. 1983), but detailed biochemical investigations of the mechanism are not available. Only recently, a novel CoA-dependent, non-$\beta$-oxidative pathway for ferulic acid cleavage was identified in *P. fluorescens* AN103 (Gasson et al. 1998; Narbad and Gasson 1998) and *Pseudomonas* sp. strain HR199 (Overhage et al. 1999a). An accumulation of vanillin, which is the product of this reaction, was not observed in *Pseudomonas* sp. strain HR199, but was observed in mutants with an inactivated vanillin dehydrogenase gene (Overhage et al. 1999b).

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In the present study, we investigated ferulic acid catabolism in the Gram-positive *Amycolatopsis* sp. strain HR167, which is most probably also initiated by the CoA-dependent, non-$\beta$-oxidative cleavage of ferulic acid and proceeds via vanillin, vanillate and guaiacol (Fig. 1).

## Materials and methods

### Bacterial strains and plasmids

The strains of *Amycolatopsis*, *Pseudomonas* and *Escherichia coli* as well as the plasmids used in this study are listed in Table 1.

### Growth of bacteria

Cells of *E. coli* were cultivated at 37 °C in Luria-Bertani (LB) or in M9 mineral salts medium (Sambrook et al. 1989). Cells of *Amycolatopsis* sp. HR167 were cultivated at 42 °C in YMG medium (0.4% w/v yeast extract, 1% w/v malt extract, 0.4% w/v glucose, pH 7.2) or TYN medium (1.0% w/v tryptone, 0.25% w/v yeast extract, 0.5% w/v NaCl, pH 7.2). Cells of *Pseudomonas* sp. strains were cultivated at 30 °C either in a nutrient broth (NB) medium (0.8% w/v; Bacto Difco) or in MM (Schlegel et al. 1961) or HR-MM (Rabenhorst 1996) mineral salts media supplemented with carbon sources as indicated. Ferulic acid and vanillin were dissolved in dimethyl sulfoxide and were added to the medium at final concentrations of 0.1% w/v. Tetracycline and kanamycin were used at final concentrations of 25 µg/ml or 300 µg/ml for *Pseudomonas* sp. strains, respectively. Growth of the bacteria was monitored with a Klett-Summerson photometer.

### Qualitative and quantitative determination of catabolic intermediates

Culture supernatants obtained by centrifugation were analyzed for excreted intermediates of ferulic acid catabolism by liquid chromatography without prior extraction, using a high-performance liquid chromatography (HPLC) apparatus (Knauer, Berlin, Germany). Separation was achieved by reversed-phase chromatography on Nucleosil-100 $C_{18}$ (5 µm particle size, 250 mm × 4.0 mm column) with a gradient of 0.1% v/v formic acid (eluant A) and acetonitrile (eluent B) in a range of 20–100% v/v eluant B and at a flow rate of 1 ml/min. For quantification, all intermediates were calibrated with external standards. The compounds were identified by their retention times and the corresponding spectra were obtained with a diode array detector (WellChrom Diodenarray-Detektor K-2150, Knauer).

### Preparation of the soluble fractions of crude extracts

Cells were disrupted either by a two-fold French press passage at 96 MPa, or by sonication (1 min/ml cell suspension with an amplitude of 40 µm) with a Bandelin Sonopuls GM200 ultrasonic disintegrator. Soluble fractions of crude extracts were obtained by centrifugation at 100,000 *g* at 4 °C for 1 h.

### Enzyme assays

Feruloyl coenzyme A (feruloyl-CoA) synthetase was assayed spectrophotometrically at 30 °C by a modified method described by Zenk et al. (1980). The reaction mixture (1 ml) contained 100 mM

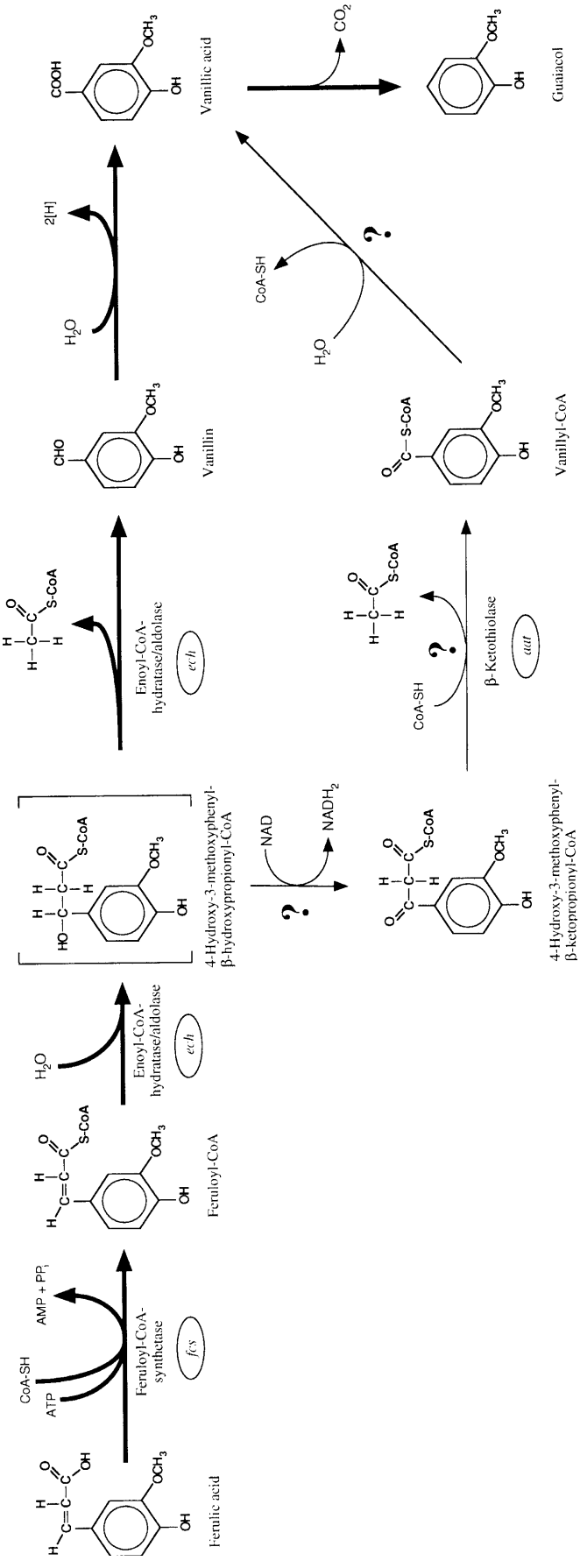



**Fig. 1** Proposed route for the catabolism of ferulic acid in *Amycolatopsis* sp. strain HR167. *Fine arrows* indicate $\beta$-oxidation analogous to that of fatty acid catabolism

**Table 1** Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| *Amycolatopsis* sp. | | |
| HR167 | Wild type, ferulate positive, vanillin positive, vanillate positive | (Rabenhorst and Hopp 1997) |
| Mutants of *Pseudomonas* sp. Strain HR199 | | |
| SK6167 | Ferulate negative, vanillin positive, vanillate positive, protocatechuate positive | (Overhage et al. 1999a) |
| SK6202 | Ferulate negative, vanillin positive, vanillate positive, protocatechuate positive | (Overhage et al. 1999a) |
| *Escherichia coli* | | |
| XL1-Blue | *recA1 endA1 gyrA96 thi hsdR17* ($rk^-$, $mk^+$) *supE44relA1*, $\lambda^-$, *lac*$^-$ [F′ *proAB lacI*$^q$ZΔM15, Tn*10*(tet)] | (Bullock et al. 1987) |
| S17-1 | *recA*, harboring the *tra* genes of plasmid RP4 in the chromosome, *proA*, *thi-1* | (Simon et al. 1983) |
| Plasmids | | |
| pVK100 | $Tc^r$, $Km^r$, cosmid, broad host range | (Knauf and Nester 1982) |
| pBluescript $SK^-$ | $Ap^r$ lacPOZ′, $T_7$ and $T_3$ promoter | Stratagene, San Diego, Calif. |

potassium phosphate buffer (pH 7.0), 2.5 mM $MgCl_2$, 0.7 mM ferulic acid, 2 mM ATP, 0.4 mM coenzyme A and an appropriate amount of extract. The assay was started by the addition of ATP and the initial absorbancy increase due to the formation of feruloyl-CoA ($\varepsilon = 10\ cm^2\ \mu mol^{-1}$) was measured at 345 nm.

The activity of the enoyl-CoA hydratase/aldolase was determined by a modified method described by Gasson et al. (1998). Extracts were incubated at 30 °C in a reaction mixture (0.5 ml) containing 90 mM sodium phosphate buffer (pH 7.0), 3 mM $MgCl_2$ and an appropriate amount of 4-hydroxy-3-methoxyphenyl-β-hydroxypropionyl-CoA (HMPHP-CoA). HMPHP-CoA was prepared according to Gasson et al. (1998). The conversion of HMPHP-CoA to vanillin was confirmed by HPLC analysis. Since the concentration of HMPHP-CoA was not determined, only qualitative determination of enoyl-CoA hydratase/aldolase activity was performed.

The soluble protein was determined as described by Bradford (1976).

### Isolation and manipulation of DNA

Plasmid DNA and DNA restriction fragments were isolated and analyzed by standard methods (Sambrook et al. 1989).

### Transfer of DNA

Competent cells of *E. coli* were prepared and transformed by using the $CaCl_2$ procedure (Hanahan 1983). Conjugations of *E. coli* S17–1 (donor) harboring hybrid plasmids with *Pseudomonas* strains (recipient) were performed either on solidified NB medium (Friedrich et al. 1981), or by a minicomplementation method (Priefert et al. 1997).

### Construction of a genomic library of *Amycolatopsis* sp. strain HR167

Partially *Eco*RI-digested genomic DNA of *Amycolatopsis* sp. strain HR167 was ligated with *Eco*RI-linearized cosmid pVK100. The ligation mixture was packaged in λ particles and subsequently transduced into *E. coli* S17–1. About 5,000 transductants were selected on LB-Tc agar plates and the hybrid cosmids of these strains were conjugatively transferred to the ferulic acid-negative mutants SK6167 and SK6202, respectively.

### DNA sequence determination and analysis

DNA sequences were determined by the dideoxy chain termination method (Sanger et al. 1977) with a 4000L DNA sequencer (LI-COR Biotechnology Division, Lincoln, Neb., USA). A Thermo Sequenase fluorescence-labelled primer cycle-sequencing kit with 7-deaza-dGTP (Amersham Life Science, Little Chalfont, UK) was used as specified by the manufacturer, together with synthetic fluorescence-labelled oligonucleotides as primers. Nucleotide and amino acid sequences were analyzed with the Genetics Computer Group sequence analysis software package (GCG package, version 6.2, June 1990; Devereux et al. 1984) or with the "Blast" programs (Altschul et al. 1997).

### Chemicals

Restriction endonucleases, T4 DNA ligase, λ DNA and enzymes or substrates used in the enzyme assays were obtained from Roche Molecular Biochemicals (Mannheim, Germany) or from GibcoBRL Life Technologies (Karlsruhe, Germany). Agarose type NA, radioisotopes, chromatography columns and chromatography matrices were purchased from Amersham Pharmacia Biotech (Freiburg, Germany). Synthetic oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany). All other chemicals were from Haarmann & Reimer (Holzminden, Germany), Merck Eurolab (Darmstadt, Germany), Serva Feinbiochemica (Heidelberg, Germany), or Sigma-Aldrich Fine Chemicals (Deisenhofen, Germany).

### Nucleotide sequence accession number

The nucleotide and amino acid sequence data reported in this paper have been submitted to the EMBL, GenBank and DDBJ nucleotide sequence databases and are listed under accession no. AJ290449.

## Results

### Biotransformation of ferulic acid by *Amycolatopsis* sp. strain HR167

*Amycolatopsis* sp. strain HR167 is able to degrade ferulic acid. A complete conversion of 5.1 mM ferulic acid by *Amycolatopsis* sp. strain HR167 cells pregrown in YMG medium was observed within 6.5 h (Fig. 2). Vanillic acid was the first intermediate detectable in the culture medium after 1 h incubation. Vanillin was accumulated to a maximum of about 2.6 mM after 6.5 h, but was

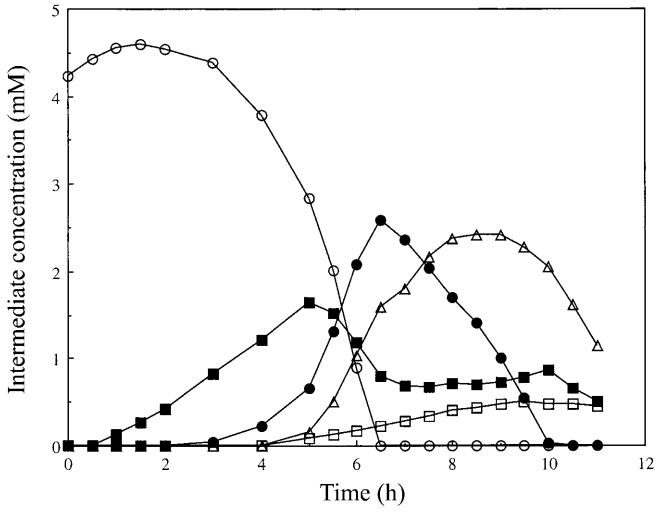



**Fig. 2** Biotransformation of ferulic acid by *Amycolatopsis* sp. strain HR167. Cells were cultivated for 15 h at 42 °C in 50 ml YMG medium. Cells were harvested, washed and resuspended in 50 ml mineral salts medium containing 5.1 mM ferulic acid. Incubation was performed at 42 °C, and samples were taken and analyzed by high-performance liquid chromatography. ○ Ferulic acid, ● vanillin, □ vanillyl alcohol, ■ vanillic acid, △ guaiacol

completely degraded within a further 4 h. Guaiacol was accumulated in the medium to comparable concentrations as vanillin but with delay. Vanillyl alcohol was only accumulated to less than 0.4 mM during the incubation (Fig. 2).

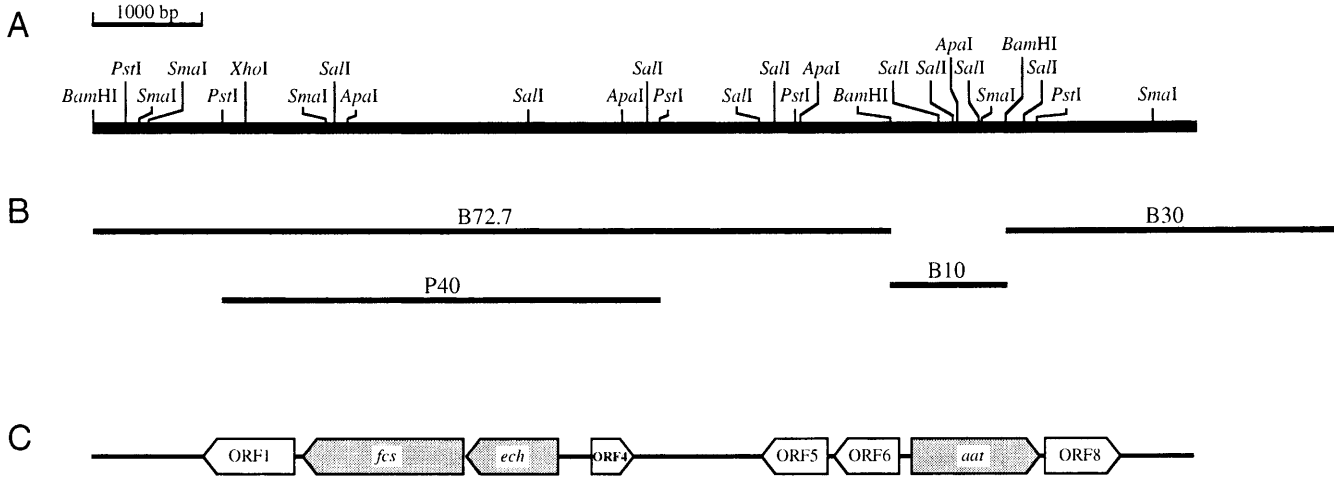
## Cloning of the genes involved in ferulic acid catabolism of *Amycolatopsis* sp. strain HR167

To identify the genes involved in the ferulic acid degradation pathway of *Amycolatopsis* sp. strain HR167, we took advantage of recently isolated nitrosoguanidine-induced ferulic acid-negative mutants (SK6167 and SK6202) of the ferulic acid-utilizing *Pseudomonas* sp. strain HR199 (Overhage et al. 1999a), which were chosen as recipients for the genomic library of *Amycolatopsis* sp. HR167. Four transconjugants of mutant SK6167 and two transconjugants of mutant SK6202 were isolated, which were complemented by the received hybrid cosmids and thus were able to grow again on ferulic acid. One of the corresponding hybrid cosmids (pVK15–1) harbored only one 20-kbp *Eco*RI fragment (E200), which also occurred in all other complementing hybrid cosmids together with different *Eco*RI fragments. E200 was isolated and cloned in the vector pBluescript SK$^-$, resulting in the plasmid pSKE200.

## Subloning of the genes *ech*, *fcs* and *aat*

Fragment E200 was isolated from *Eco*RI-digested plasmid pSKE200 and was digested with different restriction endonucleases. Resulting fragments were cloned in the vector pBluescript SK$^-$ and the obtained hybrid plasmids were used as template DNAs in sequencing reactions together with universal and reverse primers. Using synthetic oligonucleotides as primers, the nucleotide sequences of the fragments B72.7, B10 and a part of B30 were determined and eight open reading frames (ORF) were identified (Fig. 3). The putative translational product of ORF2 exhibited significant similarities with different coenzymeA ligases from various sources (Fig. 4) and was thus a candidate for the feruloyl-CoA synthetase (Fcs) gene *fcs*. The translational start codon GTG of *fcs* overlapped with the TAG stop codon of ORF3, which was referred to as *ech* since its putative translational product exhibited significant similarities with the enoyl-CoA hydratase/aldolase (Ech) of *Pseudomonas* sp. strain HR199 and the enoyl-SCoA hydratase/lyase of *P. fluorescens* strain AN103 (Fig. 5). At a distance of 3,247 bp upstream of the translational

**Fig. 3A–C** Localization of *fcs*, *ech* and *aat*. **A** Restriction map of a part of *Eco*RI restriction fragment E200 from hybrid cosmid pVK15–1. **B** relevant subfragments used in this study. **C** Structural genes of the feruloyl-CoA synthetase (*fcs*), enoyl-CoA hydratase/aldolase (*ech*), β-ketothiolase (*aat*) and open reading frames (*ORF*) localized on fragment E200



start codon ATG of *ech*, ORF7 was detected, which was transcribed in the opposite direction and was referred to as *aat*, since its putative translational product exhibited significant similarities with β-ketothiolases (Aat) from various sources. Typical Shine–Dalgarno sequences (each GGAG) preceded the translational start codons of *fcs*, *ech* and *aat* at distances of 5, 6 or 6 nucleotides, respectively. The G + C contents of *fcs*, *ech* and *aat* were 74.1, 70.3 and 72.9 mol%, respectively; and the codon usages of *fcs*, *ech* and *aat* were very similar.

The putative translational products of the other identified ORFs (Fig. 3) exhibited highest similarities only with some hypothetical proteins from various sources.

### Properties and putative functions of the *ech*, *fcs* and *aat* gene products

The relative molecular masses of Ech, Fcs and Aat calculated from the amino acid sequences deduced from *ech*, *fcs* and *aat*, were 31,915, 51,883 and 41,540 Da, respectively.

The amino acid sequences deduced from the genes *ech*, *fcs* and *aat* were compared with those collected in GenBank. With the *ech* gene product, highest similarity was obtained with the enoyl-SCoA hydratase/lyase of *P. fluorescens* strain AN103 [62% identity in 267 amino acid (aa) overlap; Gasson et al. 1998]. The relationship of the Ech from *Amycolatopsis* sp. strain HR167 to other enzymes of the enoyl-CoA hydratase/isomerase family is shown in Fig. 5. With the *fcs* gene product, highest similarity was obtained with the *fadD5* gene product from *Mycobacterium tuberculosis* (41% identity in 491 aa overlap; Cole et al. 1998), whereas it exhibited only low similarity with the Fcs of *Pseudomonas* sp. strain HR199 (Fig. 4b). The relationship of the Fcs from *Amycolatopsis* sp. strain HR167 to other coenzymeA ligases is shown in Fig. 4. With the *aat* gene product, highest similarity was achieved with the *fadA6* gene product of *M. tuberculosis* (49% identity in 386 aa overlap; Cole et al. 1998).

### Heterologous expression of *ech* and *fcs* in *E. coli*

Since there was only weak similarity of the *fcs* gene product of *Amycolatopsis* sp. strain HR167 to the feruloyl-CoA synthetase of *Pseudomonas* sp. strain HR199, this enzymatic function was assigned to the *fcs* gene product of *Amycolatopsis* sp. strain HR167 by heterologous expression of the gene in *E. coli*. Fragment P40 (Fig. 3) was isolated from *Pst*I-digested pSKE200 and cloned in pBluescript SK$^-$ with the genes *ech* and *fcs* colinear to and downstream of the *lacZ* promoter. The resulting hybrid plasmid pSKP40c conferred Fcs (Table 2) and Ech activity to recombinant strains of *E. coli* XL1-Blue, which was revealed by the enzyme assays described in Materials and methods. Even recombinant strains of *E. coli* XL1-Blue harboring a hybrid plasmid (pSKP40a) with the genes *ech* and *fcs* antilinear to the *lacZ* promoter exhibited Fcs activity (Table 2). Thus, most probably a DNA sequence on fragment P40 was recognized by the *E. coli* RNA polymerase and functioned as a weak promoter.

### Biotransformation of ferulic acid to vanillin by resting cells of *E. coli* XL1-Blue (pSKP40c)

*E. coli* XL1-Blue, which harbored hybrid plasmid pSKP40c, was cultivated overnight in 50 ml LB medium containing 12.5 μg tetracycline/ml and 100 μg ampicillin/ml. Cells were harvested by centrifugation, washed twice in 100 mM potassium phosphate buffer (pH 7.0) and resuspended in 50 ml HR-MM containing 5.15 mM ferulic acid. After an incubation of 6 h, 2.3 mM vanillin was obtained and after 23 h a maximum of 3.1 mM vanillin was obtained in the culture supernatant. Beside vanillin, vanillyl alcohol was detected in the medium, which was due to a reduction of vanillin by the *E. coli* cells. This reduction was also observed in a control experiment, when cells of *E. coli* XL1-Blue harboring only the vector pBluescript SK$^-$ were incubated in HR-MM in the presence of 2 mM vanillin.

## Discussion

*Amycolatopsis* sp. strain HR167 is able to utilize ferulic acid as sole carbon source for growth. Ferulic acid is converted to the intermediates vanillin, vanillate and guaiacol, which are excreted into the medium (Fig. 2). The genes involved in ferulic acid catabolism were identified on DNA fragment E200. The genes *fcs* and *ech*, which encoded proteins with similarities to thiokinases and enoyl-CoA hydratase/aldolases, respectively, are most probably constituents of one operon, since the translational start codon of *fcs* overlapped with the translational stop codon of *ech*. At a distance of about 3,250 bp, the *aat* gene was identified, whose deduced amino acid sequence exhibited similarities to β-ketothiolases. The function of the *fcs* gene product as a feruloyl-CoA synthetase was concluded from the similarities found for the corresponding amino acid sequence to fatty acid-CoA synthetases. Like these enzymes, Fcs possessed an amino acid sequence (aa 154–163) proposed to be the AMP-binding domain (Fig. 4a; Babbitt et al. 1992). However, since the amino acid sequence similarity of Fcs from *Amycolatopsis* sp. strain HR167 to the corresponding protein from *Pseudomonas* sp. strain HR199 was rather low (Fig. 4b), further evidence for the function of Fcs from *Amycolatopsis* sp. strain HR167 was achieved by expressing the corresponding gene in *E. coli*. Fcs activity was detected in extracts of the recombinant strain by spectrophotometric assay and the formation of feruloyl-CoA was directly proven by HPLC analysis. Since only ORF4, *fcs* and *ech* were

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  i     1                      MRNQGLGSWPVR-RARMSPHATAVRHGGT--ALTYAELSRRVARLANGLRAA-GVRPGDRVAYLGPNHPAYLETLFACGQAGAVFV   82
 ii     1                      MKNIG---WMLRQRATVSPRLQAYVEPSTDVRMTYAQMNALANRCADVLTAL-GIAKGDRVALLMPNSVEFCCLFYGAAKLGAVAV   82
iii     1  MTAQLASHLTRALTLAQQQPYLARRQNWVNQLERHAMMQPDAPALRFVGN--TMTWADLRRRVAALAGALSGR-GVGFGDRVMILMLNRTEFVESVLAANMIGAIAV  104
 iv     1                    MVFTEDTIGEFFEKQVERYADKEFIVYPDRDLRFTYREFNERVNLLAKGLLSI-GIGKGDHVGIWATNVPDWLTFLFATAKIGAVLV   86
  v     1     MKKVWLNRYPADVPTEINPDRYQSLVDMFEQSVARYADQPAFVNMGE--VMTFRKLEERSRAFAAYLQQGLGLKKGDRVALMMPNLLQYPVALFGILRAGMIVV  102
 vi                                  e  a   pd  A v  g     Tya l  Rv  lA  L    G  kGDRVa lmpN       lfa    GAv V

  i    83  PLNFRLGVPELDHALADSGASVL-----IHTPEHAE-----------TVAAL-------------------AAG--------RLLR---------VPAGEL---  131
 ii    83  PINTRLAAPEVSFILSDSGSKVV-----IYGAPSAP-----------VIDAI-------------------------------RAQA---------DPPGTVTDW  131
iii   105  PLNFRLTPTEIAVLVEDCVAHVM-----LTEAALAPVAIGVRNIQPLLSVIVV-------------------AGG--------SSQD---------SVFGYE---  165
 iv    87  TVNTAYKSHELEYVMKQSDMKALAIIDGFRDVDYVQ------------TLYEL-------------------VPELKTHERGHLRSER---------FPELRS-VI  151
  v   103  NVNPLYTPRELEHQLNDSGASAI-----VIVSNFAH------------TLEKVVDKTAVQHVILTRMGDQLSTAKG--------TVVNFVVKYIKRLVPKYHL---  180
 vi        p N rl   El     DSga v            A             t                        a g         r             P g

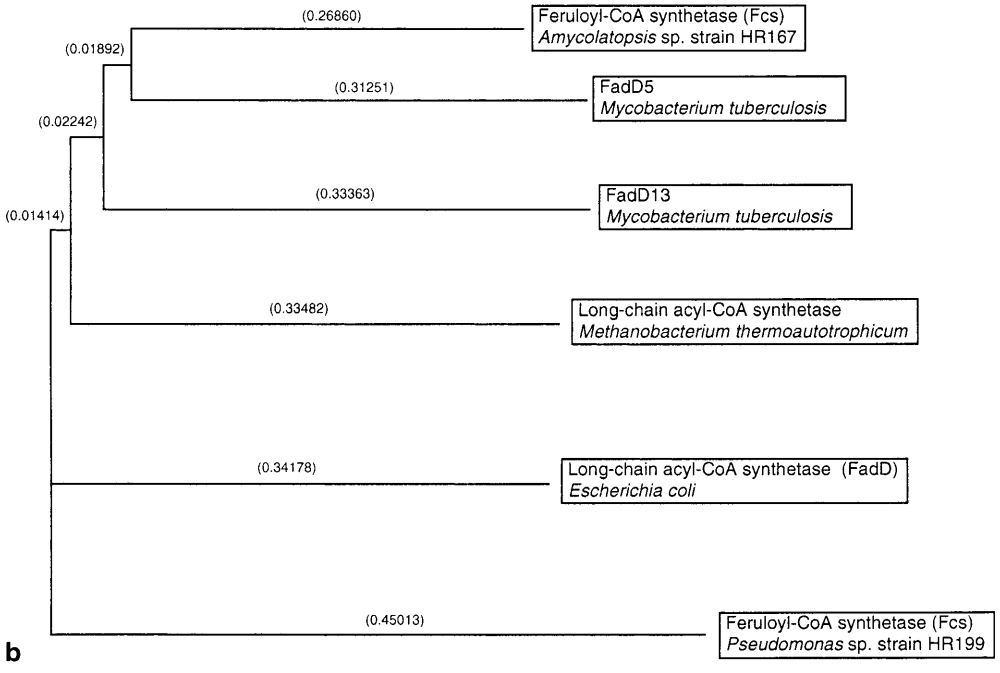
  i   132  ----------DAADD-----EPPDLPVGL----------DDVCLLMYTSGSTGRPKGAMLTHGNLTWNC--VNVLVETDL-ASDERALVAA-PLFHAAALGMVCL  207
 ii   132  IGADSLAERLRSAAAD-----EPA-VECGG----------DDNLFIMYTSGTTGHPKGVVHTHESVHSAA--SSWASTIDV-RYRDRLLLPL-PMFHVAALTTVIF  217
iii   166  ----------DLLNEAGDVHEPVDIPN-----------DSPALIMYTSGTTGRPKGAVLTHANLTGQA--MTALYTSGANINSDVGFVGV-PLFHIAGIGNMLT  245
 iv   152  YIGAQKHRGMYNTNELMLLGKHVPDTELRTVMSTLK----NTDVINMQYTSGTTGFPKGVMLTHRNILNNG--YYIGERQRF-TEEDRLCLPV-PLFHCFGIVLGVL  250
  v   181  ----------PDAIS-----FRSALHNGYRMQYVKPELVPEDLAFLQYTGGTTGVAKGAMLTHRNMLANLEQVNATYGPLL-HPGKELVVTALPLYHIFALTINCL  270
 vi                  a       ep d   g           dD    mYTSGTTG PKGamLTH N   n               drl v   PLFH aal    l

  i   208  PTLLKGG-TVILH-SAFDPGAVLSAVEQERVTLVFGVPTMYQAIAAHPRWRSADLSSLRTLLCGGAPVPADLASRYL-DRGLAFVQ---GYGMTEAAPGVLVLDRAH  308
 ii   218  -SAMRGV-TLISM-PQFDATKVWSLIVEERVCIGGAVPAILNFMRQVPEFAELDAPDFRYFITGGAPMPEALIKIYA-AKNIEVVQ---GYALTESCGGGTLLLSED  317
iii   246  GLLLGLP-TVIYPLGAFDPGQILDVLEAEKVTGIFLVPAQWQAVCTEQQARPRDLRLR-VLSWGAAPAP-DALLRQM-SATFPETQILAAFGQTEMSPVTCMLLGED  348
 iv   251  ALLTHGG-TLVMI-ELFDPLLVLAAVEKERCTALYGVPTMFIAEFTHPMFDMFDLSSLRTGIMAGSPCPIEAMKRVMNDMNMKEVTI--AYGLTEASPVFTQTSVDD  353
  v   271  LFIELGGQNLLIT-NPRDIPGLVKELAKYPFTAITGVNTLFNALLNNKEFQQLDFSSLHLSAGGGMPVQQVVAERWVKLTGQYLLE---GYGLTECAPLVSV-NPYD  372
 vi        l  Gg Tli     FDp  vl   e ErvT   gVPt   A    p f   Dlsslr    GGaP P     R         vq   gYG TE  P    l   D

  i   309  VAEK-IGSAGVPSPFTDVRLAGP-SGEPVPPGEKGEIVVSGPNVMKGYWGRPEATAEVL-RDGWFHSGDVATVDGDGYFHVVDRLKDMIISGGENIYPAEVENELYG  412
 ii   318  ALRK-AGSAGRATMFTDVAVRGD-DGVIREHGE-GEVVIKSDILLKEYWNRPEATRDAF-DNGWFRTGDIGEIDDEGYLYIKDRLKDMIISGGENVYPAEIESVIIG  419
iii   349  AIAK-RGSVGRVIPTVAARVVDQ-NMNDVPVGEVGEIVYRAPTLMSCYWNNPEATAEAF-AGGWFHSGDLVRMDSDGYVWVVDRKKDMIISGGENIYCAELENVLAS  452
 iv   354  PIEKRVETVGTPLPHIEVKIVDPETGEELGPGEPGEICCRGYNVMKGYYKMPEMTAEAIDEDGWLHSGDLAVMDEDGYYSIVGRIKDMIIRGGENIYPREIEEFLHT  460
  v   373  IDYH-SGSIGLPVPSTEAKLVDD-DDNEVPPGQPGELCVKGPQVMLGYWQRPDATDEII-KNGWLHTGDIAVMDEEGFLRIVDRKKDMILVSGFNVYPNEIEDVVMQ  476
 vi          K  GS G p p t v  vd   g   ppGE GEiv  gp vMkgYW rPEATaEa    GWfHsGD a mD dGY  iVDR KDMIisGGENiYPaEiE vl

  i   413  YPGVEACAVIGVPDPRWGEVGKAV-VVPADGSRIDGDELLAWLRTRLAGYKVPKSVEFTDRLPITGSGKILKGEVRRRFG                          491
 ii   420  VPGVSEVAVIGLPDEKWGEIAAAI-VV-ADQNEVSEQQIVEYCGTRLARYKLPKKVIFAEAIPRNPTGKILKTVLREQYSATVPK                      503
iii   453  HPDIAEVAVIGRADEKWGEVPIAVAAVTNDDLRI--EDLGEFLTDRLARYKHPKALEIVDALPRNPAGKVLKTELRLRYGACVNVERRSASAGFTERRENRQKL  554
 iv   461  MPGVKDVQVVGIPDEKYGEIVGAF-IIREDGADILEEDVRDYAIQRIARYKVPKHVFFVDEFPLTASGKVQKFKLREMAVELLKKKEQP                  548
  v   477  HPGVQEVAAVGVPSGSSGEAVKIF-VVKKDPS-LTEESLVTFCRRQLTGYKVPKLVEFRDELPKSNVGKILRRELRDEARGKVDNKA                      562
 vi        PGV eVAViG PDekwGE   A  vV  D   i ee l      RLArYKvPK VeF D lP    GKiLK eLR      v
```

**a**




**b**

◀

**Fig. 4a, b** Homologies of *Amycolatopsis* sp. strain HR167 feruloyl-CoA synthetase with CoA ligases from various sources. **a** The amino acid sequence of the feruloyl-CoA synthetase from *Amycolatopsis* sp. strain HR167 deduced from the *fcs* gene (*i*) was aligned with: (*ii*) the amino acid sequence of the *fadD13* gene product from *Mycobacterium tuberculosis* (Cole et al. 1998), (*iii*) the *fadD5* gene product from *M. tuberculosis* (Cole et al. 1998), (*iv*) the long-chain fatty acid-CoA ligase from *Methanobacterium thermoautotrophicum* (Smith et al. 1997) and (*v*) the the long-chain fatty acid-CoA ligase from *Escherichia coli* (Black et al. 1992). Amino acids are specified by standard one-letter abbreviations. *Dashes* indicate gaps introduced into the sequences to improve the alignment. Amino acid residues which are identical to the *Amycolatopsis* sp. strain HR167 *fcs* gene product at one particular sequence position are *shaded*. The consensus sequence is given (*vi*). The signature sequence of the AMP-binding domain (Babbitt et al. 1992) is *boxed*. **b** The relationship between the feruloyl-CoA synthetases of *Amycolatopsis* sp. strain HR167 and *Pseudomonas* sp. strain HR199 (Overhage et al. 1999a) and the proteins in panel **a** is displayed as a dendrogram, which was constructed using the CLUSTAL program from pairwise similarity scores generated by the method of Wilbur and Lipman (1983) with the following parameters: k-tuple length = 1, gap penalty = 3, number of diagonals = 5, diagonal window size = 5, gap opening penalty = 10, gap extension penalty = 0.10 and protein weight matrix is blosum. Relatedness is represented by the branch length (distances are given as 0.01% divergence in parentheses)

completely localized on the cloned fragment P40 used for these experiments (and ORF4 exhibited similarities only with some hypothetical proteins) it was most probable that the *fcs* gene product was responsible for the detected Fcs activity. Thus, this is the first report of the cloning and expression of an *fcs* gene from a Gram-positive bacterium.

During our investigations of ferulic acid catabolism of *Amycolatopsis* sp. strain HR167, the isolation and characterization of a gene of the enoyl-SCoA hydratase/isomerase superfamily from *P. fluorescens* strain AN103 was reported (Gasson et al. 1998) encoding an enzyme for the hydration and non-oxidative cleavage of feruloyl-SCoA to vanillin and acetyl-SCoA. From the amino acid sequence comparison (Fig. 5) and from the expression of the corresponding gene in *E. coli*, it was obvious that the *ech* gene product of *Amycolatopsis* sp.

**Fig. 5** Homologies of *Amycolatopsis* sp. strain HR167 enoyl-CoA hydratase/aldolase to members of the enoyl-CoA hydratase/isomerase family from different sources. The amino acid sequence of the enoyl-CoA hydratase/aldolase from *Amycolatopsis* sp. strain HR167 deduced from the *ech* gene (*i*) was aligned to: (*ii*) the amino acid sequence of the enoyl-CoA hydratase/aldolase from *Pseudomonas* sp. strain HR199 (Priefert et al. 1997), (*iii*) the *p*-hydroxycinnamoyl CoA hydratase/lyase from *Pseudomonas fluorescens* (Gasson et al. 1998), (*iv*) the ferulic acid hydratase from *Pseudomonas putida* (Venturi et al. 1998), (*v*) the enoyl-CoA hydratase from *Escherichia coli* (Ferrandez et al. 1997), (*vi*) the human mitochondrial enoyl-CoA hydratase (Kanazawa et al. 1993) and (*vii*) the enoyl-CoA hydratase (*phaA* gene product) from *Pseudomonas putida* (Olivera et al. 1998). Amino acids are specified by standard one-letter abbreviations. *Dashes* indicate gaps introduced into the sequences to improve the alignment. Amino acid residues which are identical to the *Amycolatopsis* sp. strain HR167 *ech* gene product at one particular sequence position are *shaded*. The consensus sequence is given (*viii*)

```
   i     1                                MSTAVGNGRVRTEPWGETVLVEFDEG--IAWVMLNRPDKRNAMNPTLNDEMVRVLDHLEGDDRCRVLVLTGAGES   73
  ii     1                                      MNSYDGRWSTVDVKVEEG--IAWVTLNRPEKRNAMSPTLNREMVEVLEVLEQDADARVLVLTGAGES   65
 iii     1                                      MSTYEGRWKTVKVEIEDG--IAFVILNRPEKRNAMSPTLNREMIDVLETLEQDPAAGVLVLTGAGEA   65
  iv     1                                                                MSPTLNREMRDVLETVEQDADARVLVLTGEGSA   33
   v     1                                    MSELIVSRQQQ----VLLLTLNRPAARNALNNALLTQLVNELEAAATDSSISVCVITGNARF   58
  vi     1          MAALRVLLSCARGPLRPPVRCPAWRPFASGANFEYIIAEKRGKNNTVGLIQLNRPKALNALCDGLIDELNQALKIFEEDPAVGAIVLTGGDKA   93
 vii     1  MHILTPPGRDPSSRPCKQFQEMPAHDVPCVQPEEP-DMPRYLDVQAPENG--VQLITLQRPEALNALCTELLAELATALDAAARDDQIGVVVLTGSRKA   96
viii                                                  v     g       LNRP  rNAm ptLn Em  vLe  E D    VlVLTG g a

   i    74  FSAGMDLKEYFREVDATGSTAVQIKVRRASAEWQWKRLANWSKPTIAMVNGWCFGGAFTPLVACDLAFADEDARFGLSEVNWGIPPGGVVSRALAATVP  172
  ii    66  WTAGMDLKEYFRETDAG-PEILQEKIRREASTWQWKLLRMYTKPTIAMVNGWCFGGGFSPLVACDLAICADEATFGLSEINWGIPPGNLVSKAMADTVG  163
 iii    66  WTAGMDLKEYFREVDAG-PEILQEKIRREASQWQWKLLRMYAKPTIAMVNGWCFGGGFSPLVACDLAICADEATFGLSEINWGIPPGNLVSKAMADTVG  163
  iv    34  WTAGMDLKEYFREVDAG-PEILQERFRRDASEWQWKLLRFISKPTIAMVNGWCFGGGFSPLVACDLAICADEATFGLSEINWGIPPGNLVSKAMADTVG  131
   v    59  FAAGADLNEMAEKDLAA--------TLNDTRPQLWARLQAFNKPLIAAVNGYALGAGCELALLCDVVVAGENARFGLPEITLGIMPGAGGTQRLIRSVG  149
  vi    94  FAAGADIKEMQNLSFQD--------CYSSKFLKHWDHLTQVKKPVIAAVNGYAFGGGCELAMMCDIIYAGEKAQFAQPEILIGTIPGAGGTQRLTRAVG  184
 vii    97  FAAGADIREMAERDLVG--------ILNDPRVAHWQRIAAFAKPLIAAVNGYALGGGCELVMCADIVIAGSDARFGQPEINLGIIPGAGGTQRLLRAVG  187
viii        f AGmDLKEyfre dAg     q   rr    wqWk L    KPtIAmVNGwcFGGGf plvaCDlaia   A FGLsEINwGIpPG  vs ala tVG

   i   173  QRDALYYIMTGEPFDGPPRAEMRLVNEALPADRLRERTREVALKLASMNQVVLHAAKTGYKIAQEMPWEQAEDYLYAKLDQSQFADKAGARAKGLTQFL  271
  ii   164  HRESLYYIMTGKTFGGQQAAKMGLVNQSVPLAELRSVTVELAQNLLDKNPVVLRAAKIGFKRCRELTWEQNEDYLYAKLDQSRLLDPEGGREQGMKQFL  262
 iii   164  HRQSLYYIMTGKTFGGQKAAEMGLVNESVPLAQLREVTIELARNLLEKNPVVLRAAKHGFKRCRELTWEQNEDYLYAKLDQSRLLDTEGGREQGMKQFL  262
  iv   132  HRESR-IIMTGKTFDGQKAAQMGLVNKSVPVAQLRDEVVLLAQDLLDKNPVVLRAAKNGFKRCRELTWEQNEDYLYAKLR-----DPEP-RAQGLKQFL  223
   v   150  KSLASKMVLSGESITAQQAQQAGLVSDVFPSDLTLEYALQLASKMARHSPLALQAAKQALRQSQEVALQAGLAQERQLFT--LLAATED-RHEGISAFL  245
  vi   185  KSLAMEMVLTGDRISAQDAKQAGLVSKICPVETLVEEAIQCAEKIASNSKIVVAMAKESVNAAFEMTLTEGSKLEKKLFY--STFATDD-RKEGMTAFV  280
 vii   188  KPLAMQMVLTGEAITARHAQQAGLVSEITQPEFTVERAMQIARNIAAKAPLAVRLAKEALLKAGDTDLASGLRFERHAFT--LLAGTAD-RDEGIQAFQ  283
viii        r a   imTG  f gQ AaqmGLVn   P   LrE    lA  la knPvVLrAAK g k   E tweq edylyakl    l dte  R  G  qFL

   i   272  DQKSYRPGLSAFDPEK  287
  ii   263  DEKSIKPGLQTYKR    276
 iii   263  DDKSIKPGLQAYKR    276
  iv   224  DDKSIKPGLQAIKR    237
   v   246  QKR--TPDFKGR      255
  vi   281  EKR--KANFKDQ      290
 vii   284  EKR--PARFQGR      293
viii        d ks kPglq
```

**Table 2** Feruloyl-CoA synthetase activities in *Amycolatopsis* sp. HR167 and in recombinant strains of *E. coli* XL-1 Blue

| Strain[a,b] | Specific activity of feruloyl-CoA synthetase[c] (U/g protein) |
|---|---|
| *Amycolatopsis* sp. strain HR167 (gluconate) | < 0.5 |
| *Amycolatopsis* sp. strain HR167 (ferulic acid) | 105 |
| *E. coli* XL-1 Blue (pSKP40c) (+IPTG) | 92 |
| *E. coli* XL-1 Blue (pSKP40c) (−IPTG) | 99 |
| *E. coli* XL-1 Blue (pSKP40a) (−IPTG) | 6 |
| *E. coli* XL-1 Blue (pBluescript SK$^-$) | < 0.5 |

[a] Cells of *Amycolatopsis* sp. strain HR167 were grown to the late exponential phase at 42 °C in HR-MM mineral salts medium containing either 0.5% w/v gluconate or 0.5% w/v ferulic acid
[b] Cells of recombinant strains of *E. coli* were grown for 12 h at 37 °C in Luria-Bertani medium either in the presence of 1 mM IPTG or without IPTG as indicated
[c] The feruloyl-CoA synthetase activities were determined in soluble fractions of crude extracts at 30 °C by a modified method of Zenk et al. (1980) as described in Materials and methods

strain HR167 had the same function as the enzyme reported by Gasson et al. (1998) and the Ech of *Pseudomonas* sp. strain HR199 (Overhage et al. 1999a). As reported for these enzymes, Ech of *Amycolatopsis* sp. strain HR167 also exhibited high similarity to the "ferulic acid deacetylase" of *P. putida* strain WCS358 (Venturi et al. 1998), which most probably represents an Ech (Overhage et al. 1999a).

The occurrence of an *aat* gene close to *fcs* and *ech* may suggest that ferulic acid degradation in *Amycolatopsis* sp. strain HR167 proceeds via a mechanism analogous to the β-oxidation pathway of fatty acid catabolism, which had been proposed earlier for the degradation of substituted cinnamic acids in plants (Vollmer et al. 1965). This pathway would include the thioclastic cleavage of 4-hydroxy-3-methoxyphenyl-β-ketopropionyl-CoA to yield acetyl-CoA and vanillyl-CoA, catalyzed by the *aat* gene product (β-ketothiolase) (Fig. 1). However, genes encoding a 4-hydroxy-3-methoxyphenyl-β-hydroxypropionyl-CoA dehydrogenase or a vanillyl-CoA hydrolase, which would catalyze the subsequent reactions, were not identified in *Amycolatopsis* sp. strain HR167. In addition, from the expression experiments it was obvious that the gene products of *fcs* and *ech* catalyzed the conversion of ferulic acid to vanillin, which is further oxidatively metabolized in *Amycolatopsis* sp. strain HR167. Therefore, the involvement of the *aat* gene product in ferulic acid metabolism seemed to be unlikely.

In conclusion, ferulic acid degradation in the Gram-positive *Amycolatopsis* sp. strain HR167 proceeds via feruloyl-CoA, which is hydrated and cleaved by the action of the *ech* gene product. This CoA-dependent, non-β-oxidative mechanism of ferulic acid cleavage was hitherto described only in the Gram-negative *P. fluorescens* strain AN103 (Gasson et al. 1998), *P. putida* strain WCS358 (Venturi et al. 1998) and *Pseudomonas* sp. strain HR199 (Overhage et al. 1999a).

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