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ORIGINAL PAPER

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Functional analyses of genes involved in the metabolism of ferulic acid in *Pseudomonas putida* KT2440

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Abstract *Pseudomonas putida* KT2440 is a physiologically extremely versatile non-pathogenic bacterium that is applied as a "biosafety strain" in biotechnological processes, as authorized by the USA National Institute of Health. Analysis of the *P. putida* KT2440 whole-genome sequence revealed the genetic organization of the genes *fcs*, *ech*, and *vdh*, which are essential for ferulic acid conversion to vanillic acid via vanillin. To confirm the physiological function of these structural genes as feruloyl-CoA synthetase (Fcs), enoyl-CoA hydratase/aldolase (Ech), and vanillin dehydrogenase (Vdh), respectively, they were cloned and expressed in *Escherichia coli*. Recombinant strains harboring *fcs* and *ech* were able to transform ferulic acid to vanillin. The enzyme activities of Fcs and Vdh were determined in protein extracts of these cells. The essential involvement of *fcs*, *ech* and *vdh* in the catabolism of ferulic acid in *P. putida* KT2440 was proven by separately inactivating each gene by insertion of Ω-elements. The corresponding mutant strains KT2440*fcs*ΩKm, KT2440*ech*ΩKm, and KT2440*vdh*ΩKm were not able to grow on ferulic acid. The potential application of *P. putida* KT2440 and the mutant strains in biotechnological vanillin production process is discussed.

Introduction

Soil bacteria belonging to the species *Pseudomonas putida* have been applied in technical biodegradation processes (Nusslein et al. 1992; Ramos et al. 1994; Ronchel et al. 1995), taking advantage of their extremely metabolic versatility. The strain *P. putida* KT2440 used in this study is a plasmid-cured, restriction-deficient derivative of the toluene degrader *P. putida* mt-2 (ATCC 33015) (Franklin et al. 1981; Ramos et al. 1994, 1997; Stanier et al. 1966). It is regarded as a biosafety strain for biotechnological applications, and has been authorized as such by the USA National Institute of Health in 1981. *P. putida* KT2440 colonizes the rhizosphere of many plants and has been extensively characterized both physiologically and genetically (Mermod et al. 1984; Ramos et al. 1995, Molina et al. 1998). To exploit the biotechnological potential of this strain, its genome sequence has recently been determined (www.tigr.org) in order to serve as the basis for functional genomic studies.

Since *P. putida* KT2440 is able to use a wide range of aromatic molecules as sole carbon sources, we decided to study its potential use in the biotechnological production of the flavor compound vanillin from ferulic acid. Ferulic acid, which is a known precursor for vanillin production using other microorganisms (Falconnier et al. 1994; Lesage-Meessen et al. 1996, 2002; Gasson et al. 1998; Achterholt et al. 2000), is an abundant aromatic residue found in the cell walls of woods, grasses, and corn husks (Escott-Watson and Marais 1992; Ishii 1997; Oosterveld et al. 2000).

In this study, we investigated the catabolism of ferulic acid in *P. putida* KT2440 and identified and functionally characterized those genes essential for ferulic acid degradation in this strain. The potential application of *P. putida* KT2440 and derived mutants in biotechnological vanillin production process is discussed.

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Materials and methods

Bacterial strains and plasmids

The strains of *Pseudomonas putida* KT2440 and *Escherichia coli* and the plasmids used in this study are listed in Table 1.

Growth of bacteria

E. coli was cultivated at 37 °C in Luria-Bertani (LB) or in M9 mineral salts medium (Sambrook et al. 1989). *P. putida* KT2440 strains were cultivated at 30 °C either in a nutrient broth (NB)

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Bacteria		
Pseudomonas putida KT2440		
Wild-type	Wild-type, ferulate-positive, kanamycin-sensitive	DSM 6125
KT2440*ech*ΩKm	Ferulate-negative, vanillin-positive, vanillate-positive, kanamycin-resistant	This study
KT2440*fcs*ΩKm	Ferulate-negative, vanillin-positive, vanillate-positive, kanamycin-resistant	This study
KT2440*vdh*ΩKm	Ferulate-negative, vanillin-positive, vanillate-positive, kanamycin-resistant	This study
Escherichia coli		
XL1-Blue	*recA1, endA1, gyrA96, thi, hsdRl7* (rk–, mk^+), *supE44, relA1,* λ^-, *lac*$^-$ [F', *proAB*, *lac*I^QZ△M15, Tn*10*(Tc^T)]	(Bullock et al. 1987)
S17-1	*recA*; harboring the *tra* genes of plasmid RP4 in the chromosome, *proA, thi-1*	(Simon et al. 1983a)
Plasmids		
pBluescript SK$^-$	Ap^r, *lacP0Z,* T7 and T3 promoter	Stratagene, San Diego, Calif.
pSUP202	Tc^r, Ap^r, Cm^r, *mob*	(Simon et al. 1983b)
pSKsymΩKm	pSKsym harboring ΩKm in the *SmaI* site of the MCS	(Overhage et al. 1999a)
pSK*ech*EK	pBluescript SK$^-$ harboring the PCR product comprising *ech* as EcoRI-*Kpn*I fragment	This study
pSY*fcs*BE	pBluescript SK$^-$ harboring the PCR product comprising *fcs* as *Bam*HI-*Eco*RI fragment	This study
PSK*fcs/ech*BEK	pBluescript SK$^-$ harboring the PCR products comprising *fcs* as *BamHI-EcoRI* fragment and *ech* as *Eco*RI-*Kpn*I-fragment downstream from and colinear with the *lac* promoter	This study
pSK*vdh*BE	pBluescript SK$^-$ harboring the PCR product comprising *vdh* as *Bam*HI-*Eco*RI fragment	This study
pSK*vdh*EE	pBluescript SK$^-$ harboring the PCR product comprising *vdh* as *Eco*RI fragment	This study
pSK*ech*ΩKm	pBluescript SK$^-$ harboring the ΩKm-disrupted *ech* gene	This study
pSK*fcs*ΩKm	pBluescript SK$^-$ harboring the ΩKm-disrupted *fcs* gene	This study
pSK*vdh*ΩKm	pBluescript SK$^-$ harboring the ΩKm disrupted *vdh* gene	This study
pSUP*ech*ΩKm	pSUP202 harboring the ΩKm-disrupted *ech* gene	This study
pSUP*fcs*ΩKm	pSUP202 harboring the ΩKm-disrupted *fcs* gene	This study
pSUP*vdh*ΩKm	pSUP202 harboring the ΩKm-disrupted *vdh* gene	This study

medium (0.8%, w/v; Bacto Difco) or in a mineral salts medium (MM) (Schlegel at al. 1961) supplemented with carbon sources as indicated. Ferulic acid, vanillin, and vanillic acid were dissolved in dimethyl sulfoxide and added to the medium at a final concentration of 0.1% (w/v). Tetracycline and kanamycin were used at final concentrations of 25 μg/ml and 100 μg/ml, respectively, for selection of recombinant *P. putida* KT2440 strains. Growth of bacteria was monitored with a Klett-Summerson photometer.

Analytical methods

Culture supernatants were analyzed for excreted intermediates chromatographically, without prior extraction, using an HPLC apparatus (Fa. Knauer, Berlin, Germany) as described previously (Overhage et al. 1999b).

Preparation of the soluble fractions of crude protein extracts

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

Enzyme assays and specific staining for vanillin dehydrogenase activity in polyacrylamide gels

Feruloyl coenzyme A (feruloyl-CoA) synthetase (Fcs) and vanillin dehydrogenase (Vdh) were assayed as described previously (Overhage et al. 1999a). The amount of soluble protein was determined as described by Bradford (1976).

Proteins were separated under non-denaturing conditions in 7.4% (w/v) polyacrylamide gels as described by Stegemann et al. (1973). To stain gels for Vdh activity, the gels were incubated at 30 °C in the dark in 100 mM potassium phosphate buffer (pH 7.0) containing 0.08% (w/v) NAD^+, 0.04% (w/v) *p*-nitroblue tetrazolium chloride, 0.003% (w/v) phenazine methosulfate, and different concentrations of vanillin.

Isolation, manipulation and transfer of DNA

Plasmid DNA and DNA restriction fragments were isolated and analyzed by standard methods (Sambrook et al. 1989). Competent cells of *E. coli* were prepared and transformed using the $CaCl_2$ procedure (Hanahan 1983). Conjugations of *E. coli* S17-1 (donor) harboring hybrid plasmids and of *P. putida* KT2440 (recipient) were carried out on solidified NB medium as described by Friedrich et al. (1981).

Computer-assisted localization of fcs, ech, and vdh in the genome sequence of P. putida KT2440

To identify genes involved in ferulic acid catabolism in *P. putida* KT2440, translated BLAST Search (blastx) from the National Center for Biotechnology Information (NCBI) was applied with sequences of corresponding genes from *Pseudomonas* sp. HR199, previously described (Overhage et al. 1999a), using a database with the genome sequence of strain KT2440 (generously provided by the Institute for Genomic Research, TIGR).

Amplification and cloning of fcs, ech, and vdh from genomic DNA of P. putida KT2440

The genes *fcs*, *ech,* and *vdh* were amplified in separate PCRs with genomic DNA of *P. putida* KT2440 as template DNA. To amplify *fcs*, oligonucleotides PCR2440*fcs*uB (5'-AAAAGGATCCCAAGAACAAGGAGCAACCTGCGTG-3') and PCR2440*fcs*dE (5'-AAAAGAATTCCAGGTCGATGGGCGAGATCGCGGC-3') were used as primers in the PCR (introduced restriction enzyme sites are underlined). To amplify *ech*, oligonucleotides PCR2440*ech*uE (5'-AAAAGAATTCCCCGTTAGCGTGGCCCCATCA CC-3') and PCR2440*ech*dK (5'-AAAAGGTACCGTCGTTATAGAGAAATACCCGCGG GC-3') were used as primers, and to amplify *vdh*, oligonucleotides PCR2440*vdh*uB (5'-AAA AGGATCCCGACAACAAGAGGAATGAGCATGTTG-3') and PCR2440*vdh*dE (5'-AAA AGAATTCCTAGATGGGATAGTGACGCGGGCCG-3') were used. The PCR products were isolated and digested with *Eco*RI and *Bam*HI (in the case of *fcs* and *vdh*) or *Eco*RI and *Kpn*I (in the case of *ech*) and ligated to correspondingly digested pBluescript SK- DNA. *E. coli* XL1-Blue was transformed with the ligation mixtures, and transformants harboring the hybrid plasmid pSK*vdh*BE, pSK*fcs*BE, or pSK*ech*EK were obtained.

Expression of ech, fcs, and vdh from P. putida KT2440 in E. coli

The cloned *ech* and *fcs* PCR products were recovered from pSK*fcs*BE and pSK*ech*EK after *Bam*HI/*Eco*RI (*fcs*), and *Eco*RI/*Kpn*I (*ech*) digestion, respectively, and ligated to *Bam*HI/*Kpn*I digested pBluescript SK⁻. *E. coli* XL1-Blue was transformed with the ligation mixture, and transformants harboring the hybrid plasmid pSK*fcs*/*ech*BEK with the genes *ech* and *fcs* colinear to and downstream of the *lacZ* promoter were obtained. *E. coli* XL1-Blue harboring plasmid pSK*fcs*/*ech*BEK was grown overnight in 50 ml of LB medium containing 12.5 μg of tetracycline per ml, 100 μg of ampicillin per ml, and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation, washed twice in 100 mM potassium phosphate buffer (pH 7.0), and resuspended in 50 ml of MM containing 5.2 mM ferulic acid. Bioconversion of ferulic acid with these resting cells was monitored by HPLC analysis of cultural supernatants. In addition, feruloyl-CoA synthetase activity was determined in soluble fractions of crude protein extracts of the corresponding cells. *E. coli* XL1-Blue harboring the hybrid plasmid pSK*vdh*BE was grown overnight in 50 ml of LB medium containing 12.5 μg of tetracycline per ml, 100 μg of ampicillin per ml, and 1 mM IPTG. Soluble fractions of crude protein extracts of harvested and washed cells exhibited vanillin dehydrogenase, which was determined by the corresponding enzyme assay and by specific activity staining.

Inactivation of fcs and ech by replacement with ΩKm

For inactivation of the *fcs* gene by replacement with ΩKm, a region upstream and downstream of *fcs* was amplified from genomic DNA of *P. putida* KT2440. To obtain the upstream region of *fcs*, oligonucleotides PCR*fcs*u1EB (5'-AAAAGGATCCGAATTCCCAA GGGCGCGCGCCTGGTCAGCG-3') and PCR*fcs*u2EV (5'-AAAAGATATCGCGCAGCAC GTCGTCGACGTGACTG-3') were used as primers in the PCR. To obtain the downstream region of *fcs*, oligonucleotides PCR*fcs*d1EV (5'-AAAAGATATCGGCCGGGCTGTGCACC GTCTTCGAG-3') and PCR*fcs*d2E (5'-AAAAGAATTCCCTCGTCGAACCAGCCCTGCAC CTG-3') were used as primers in the PCR. The isolated upstream and downstream PCR products were digested with *Bam*HI/*Eco*RV and *Eco*RI/*Eco*RV, respectively, and ligated with *Bam*HI/*Eco*RI-digested pBluescript SK⁻ (Stratagene, San Diego, Calif.) and ΩKm, recovered from *Sma*I-digested pSKsymΩKm (Overhage et al. 1999a). *E. coli* XL1-Blue was transformed with the ligation mixture, and transformants harboring the hybrid plasmid pSK*fcs*ΩKm were obtained. Fragment *fcs*ΩKm was isolated from *Eco*RI-digested pSK*fcs*ΩKm and ligated to *Eco*RI-digested vector pSUP202, which harbors a tetracycline resistance gene. Resulting pSUP*fcs*ΩKm was transferred from tetracycline- and kanamycin-resistant recombinant *E. coli* S17-1 strains to *P. putida* KT2440 by conjugation. Due to homologous recombination, the complete functional *fcs* gene was replaced with *fcs*ΩKm by a double crossover event, resulting in a kanamycin-resistant but tetracycline-sensitive phenotype of the mutant *P. putida* KT2440 *fcs*ΩKm.

Ech was inactivated in a similar manner, using oligonucleotides PCR*ech*u1EB (5'-AAAAGGATCCGAATTCCCTTTCGTCATCCGAGAGGTTGGATG-3') and PCR*ech*u2EV (5'-AAAAGATATCGGTTCTGCACTCTTGTTGTTCGAGGTG-3') to amplify the upstream region, and oligonucleotides PCR*ech*d1EV (5'-AAAAGATATCGCTG AGTCGCACCGCGGGCGGCGG-3') and PCR*ech*d2E (5'-AAAAGAATTCGCCGATGGA CCGCCGGGCTCAGCTC-3') to amplify the downstream region. The isolated upstream and downstream PCR products were combined with pBluescript SK⁻ and ΩKm to obtain the hybrid plasmid pSK*ech*ΩKm. *Ech*ΩKm was isolated and cloned in pSUP202. The resulting pSUP*ech*ΩKm was transferred to *P. putida* KT2440 as aforementioned to replace the complete functional *ech* by *ech*ΩKm and to obtain mutant *P. putida* KT2440*ech*ΩKm.

The genotypes of mutant strains KT2440*fcs*ΩKm and KT2440*ech*ΩKm were confirmed by amplification of the corresponding genes from genomic DNA of these mutants by PCR and sequencing of the obtained single PCR products.

Inactivation of vdh by insertion of ΩKm

For inactivation of *vdh* by insertion of ΩKm, the gene was first amplified from genomic DNA of *P. putida* KT2440. To obtain a gene flanked by *Eco*RI sites, the aforementioned oligonucleotide PCR2440*vdh*dE and the oligonucleotide PCR2440*vdh*uE (5'-AAAAGAATT CCGACAACAAGAGGAATGAGCATGTTG-3') were used as primers in the PCR. The isolated PCR product was digested with *Eco*RI and ligated to *Eco*RI-digested pBluescript SK⁻ to obtain hybrid plasmid pSK*vdh*EE. pSK*vdh*EE was digested with *Stu*I and ligated with ΩKm, resulting in plasmid pSK*vdh*ΩKm. Subsequently, *vdh*ΩKm was recovered from *Eco*RI-digested pSK*vdh*ΩKm, and the functional *vdh* gene was exchanged with the inactivated gene *vdh*ΩKm in *P. putida* KT2440 by homologous recombination as described for the exchange of *fcs* with *fcs*ΩKm. The success of the gene replacement in mutant *P. putida* KT2440*vdh*ΩKm was confirmed as described before.

DNA sequence determination

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-labeled primer cycle-sequencing kit with 7-deaza-dGTP (Amersham Life Science, Little Chalfont, UK) was used as specified by the manufacturer, together with synthetic fluorescently labeled oligonucleotides as primers.

Results

Growth of *Pseudomonas putida* KT2440 with ferulic acid as carbon source

P. putida KT2440 is able to utilize ferulic acid as sole carbon source for growth. A complete degradation of 4.5 mM ferulic acid by *P. putida* KT2440 cells was observed during the 7-h lag growth phase (Fig. 1). After ferulic acid was depleted from the medium, cells entered the exponential growth phase. Vanillin and vanillic acid

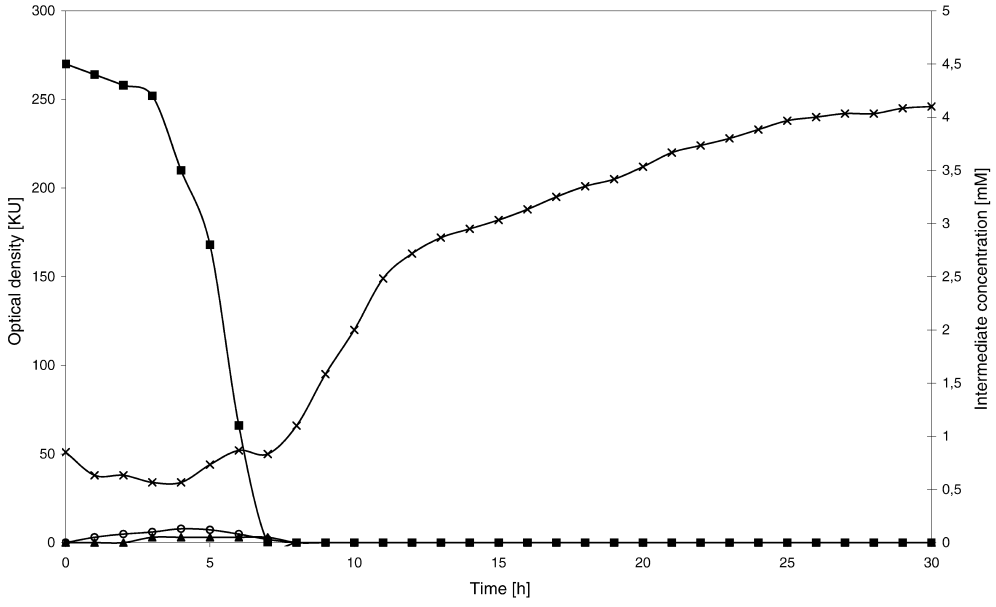

Fig. 1 Growth of *Pseudomonas putida* KT2440 in 50 ml MM with 0.1% (v/v) ferulic acid, and intermediates detected in the culture supernatant. Growth was monitored with a Klett-Summerson photometer. Cultures were incubated at 30 °C, samples were taken from the cultures, and supernatants were analyzed by HPLC as described in Materials and methods. × Absorbance, ■ ferulic acid, ◯ vanillic acid, ▲ vanillin

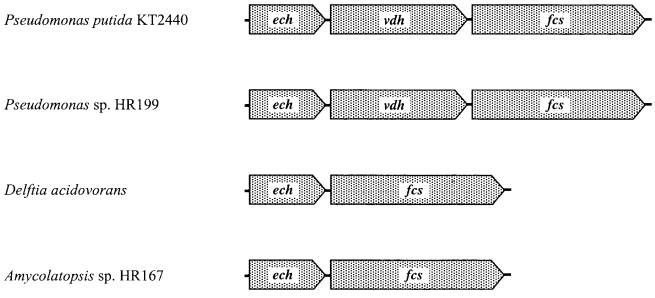

Fig. 2 Organization of the structural genes of the enoyl-CoA hydratase/aldolase (*ech*), feruloyl-CoA synthetase (*fcs*), and vanillin dehydrogenase (*vdh*) in different bacteria

were identified as intermediates that were excreted into the culture medium transiently in trace amounts.

Identification of the genes *ech*, *fcs*, and *vdh*

The nucleotide sequences of *fcs*, *ech*, and *vdh* from *Pseudomonas* sp. HR199 (fcs_{HR199}, ech_{HR199}, vdh_{HR199}) (Overhage et al. 1999b) were compared with the genome sequence of *P. putida* KT2440, which revealed the arrangement of three ORFs on fragment 10767 (length=128,524 bp) of strain KT2440 exhibiting highest homologies to fcs_{HR199}, ech_{HR199} and vdh_{HR199} (Fig. 2). The amino acid sequence deduced from ech_{HR199} exhibited 88% identity to the corresponding gene product from *P. putida* KT2440 (ech_{KT2440}) in a 276-amino-acid overlap. The translational start codon ATG of vdh_{KT2440} was located at a distance of 76 bp downstream of the translational stop codon TGA of ech_{KT2440}. The gene product of vdh_{KT2440} exhibited 80% identity with the Vdh of strain HR199 in a 482-amino-acid overlap. The translational start codon GTG of fcs_{KT2440} was located at a distance of 92 bp downstream of the translational stop codon TAG of vdh_{KT2440}. The gene product of fcs_{KT2440} exhibited 75% identity with the Fcs of strain HR199 in a 584-amino-acid overlap. Typical ribosomal binding sites (AAGGAG, AAGAGG, and AAGAG) preceded the translational start codons of *fcs*, *vdh*, and *ech* at distances of 8, 7, and 9 nucleotides, respectively. *Ech* and *fcs*, which are located in close proximity in a variety of other bacteria, were separated by the *vdh* gene in *P. putida* KT2440 (Fig. 2). Thus, the organization of these genes in strain KT2440 resembles that in *Pseudomonas* sp. HR199.

Heterologous expression of ech_{KT2440}, fcs_{KT2440}, and vdh_{KT2440} in *E. coli* and biotransformation of ferulic acid to vanillin by recombinant strains

To assign enzymatic functions to the products of *ech*, *fcs*, and *vdh* of *P. putida* KT2440, these genes were heterologously expressed in *E. coli*. *Ech*, *fcs*, and *vdh* were amplified and cloned colinear to and downstream of the *lacZ* promoter of pBluescript SK^- as described in Materials and methods. Resulting recombinant strains of *E. coli* XL1-Blue harboring pSK*fcs*/*ech*BEK exhibited feruloyl-CoA synthetase activity (Table 2). Recombinant strains harboring pSK*vdh*BE exhibited vanillin dehydrogenase activity (Table 3). The occurrence of feruloyl-CoA and vanillic acid as the products of the Fcs- and Vdh-catalyzed reactions, respectively, was proven by HPLC-analyses of the incubated enzyme assay reaction mixtures.

With resting cells of *E. coli* XL1-Blue harboring plasmid pSK*fcs*/*ech*BEK, a conversion of 4.3 mM ferulic acid to vanillin was obtained with a maximum rate of about 1.2 mmol per ml per h (data not shown). Beside vanillin, vanillyl alcohol was detected in the medium as a result of vanillin reduction by the *E. coli* cells. This reduction was also observed in a control experiment in

Table 2 Feruloyl-CoA synthetase activities in mutant strains of *Pseudomonas putida* KT2440 and in recombinant strains of *Escherichia coli* XL1-Blue. Cells of *P. putida* KT2440 (wild-type) and mutant strains of *P. putida* KT2440 were grown to the late-exponential phase at 30 °C in H16-MM containing 0.5% (w/v) sodium gluconate and 0.1% (v/v) ferulic acid. Recombinant strains of *E. coli* were grown for 12 h at 37 °C in LB in the presence of 1 mM IPTG. Feruloyl-CoA synthetase was assayed as described in Materials and methods. Specific activities are given as units per milligram of protein. Data represent the mean of three determinations

Strain	Specific activity of feruloyl-CoA-synthetase [U/mg protein]
Pseudomonas putida KT2440	0.09
Pseudomonas putida KT2440 *ech*ΩKm	<0.01
Pseudomonas putida KT2440 *fcs*ΩKm	<0.01
Pseudomonas putida KT2440 *vdh*ΩKm	<0.01
E. coli XL1-Blue (pSK*fcs/ech*BEK)	0.20
E. coli XL1-Blue (pBluescript SK⁻)	<0.01

Table 3 Vanillin dehydrogenase activities in *P. putida* KT2440, *P. putida* KT2440 *vdh*ΩKm and in recombinant strains of *E. coli* XL1-Blue. Cells of *P. putida* KT2440 (wild-type) and *P. putida* KT2440*vdh*ΩKm were grown to the late-exponential phase at 30 °C in H16-MM containing 0.5% (w/v) sodium gluconate and 0.1% (v/v) ferulic acid or 0.1% (v/v) vanillin, respectively (as indicated).Recombinant strains of *E. coli* were grown for 12 h at 37 °C in LB in the presence of 1 mM IPTG. Vanillin dehydrogenase was assayed as described in Materials and methods. Specific activities are given as units per milligram of protein. Data represent the mean of three determinations

Strain	Specific activity of vanillin dehydrogenase [U/mg protein]
Pseudomonas putida KT2440 (gluconate/ferulic acid)	0.09
Pseudomonas putida KT2440 (gluconate/vanillin)	0.01
Pseudomonas putida KT2440*vdh*ΩKm (gluconate/ferulic acid)	<0.01
Pseudomonasputida KT2440*vdk*ΩKm (gluconate/vanillin)	0.01
E. coli XL1-Blue (pSK*vdh*BE)	0.30
E. coli XLl-Blue (pSK*vdh*ΩKm)	<0.01
E. coli XL1-Blue (pBluescript SK⁻)	<0.01

which cells of *E. coli* XL1-Blue harboring only the vector pBluescript SK- were incubated in MM in the presence of 2 mM vanillin (data not shown).

Extracts of *E. coli* XL1-Blue harboring pSK*vdh*BE or only the vector pBluescript SK⁻ were analyzed in polyacrylamide gels together with an extract of ferulic acid-grown *P. putida* KT2440 cells by specific activity staining for vanillin dehydrogenase. Protein bands of similar migration were detected in extracts of *E. coli* XL1-Blue (pSK*vdh*BE) and *P. putida* KT2440, whereas no band occurred in the extract of *E. coli* XL1-Blue (pBluescript SK⁻) (Fig. 3).

Construction and characterization of *ech*-, *fcs*-, and *vdh*-deficient mutants of *P. putida* KT2440

To confirm the essential involvement of *ech*, *fcs,* and *vdh* in the catabolism of ferulic acid of *P. putida* KT2440, each gene was deleted separately and exchanged with an ΩKm-element. The homogenote genotypes of the resulting kanamycin-resistant and tetracycline-sensitive mutant strains KT2440*ech*ΩKm, KT2440*fcs*ΩKm, and KT2440*vdh*ΩKm were confirmed by PCR analysis and DNA sequencing. The phenotypes of the aforementioned mutants were investigated on solidified MM with ferulic acid, vanillin, vanillic acid, or sodium gluconate as sole carbon sources. All mutants were unable to grow on ferulic acid, but retained the ability to grow on vanillin or vanillic acid.

For further physiological characterization, cells of the mutant strains KT2440*ech*ΩKm, KT2440*fcs*ΩKm, and KT2440*vdh*ΩKm were precultured overnight in MM containing 0.5% (w/v) gluconate as sole carbon source. Cells were harvested, washed twice with MM and used for inoculation of 50 ml MM containing 5.2 mM ferulic acid as sole carbon source. The cultures were incubated for 30 h at 30 °C, and 1-ml samples were taken and analyzed by HPLC with respect to the appearance or consumption of catabolic intermediates. Cells of the wild type were incubated under identical conditions as a control. In wild type cultures, ferulic acid was completely depleted from the medium within 7 h and the occurrence of traces of the intermediates vanillin and vanillic acid was observed. In cultures of the mutant strains KT2440*ech*ΩKm, KT2440*fcs*ΩKm, and KT2440*vdh*ΩKm, no decrease of the ferulic acid concentration and no appearance of intermediates were observed within 30 h (data not shown).

The effect of the inactivation of *ech*, *fcs*, and *vdh* by Ω-element insertion in *P. putida* KT2440 was also investigated by enzyme assays for feruloyl-CoA-synthetase (Fcs) and vanillin dehydrogenase (Vdh) activity. Cells of the mutants and the wild type were grown in MM containing 0.5% (w/v) sodium gluconate plus 0.1% (w/v) ferulic acid, harvested in the late exponential growth phase, and soluble fractions of crude extracts were

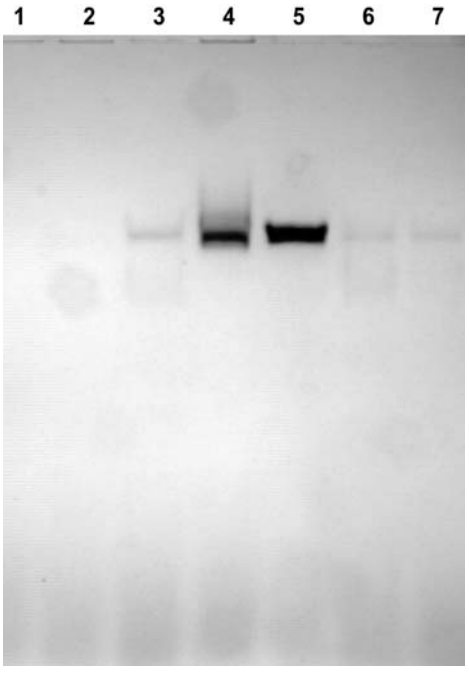

Fig. 3 Detection of vanillin dehydrogenase in gluconate-, ferulic acid-, and vanillin-grown cells of different strains of *P. putida* KT2440, and in recombinant *E. coli* strains, respectively. Cytoplasmic fractions were separated under non-denaturating conditions in a 7.4% (w/v) polyacrylamide gel as described in Materials and methods, and were stained for vanillin dehydrogenase. *Lanes: 1 E. coli* XL1-Blue (pBluescript SK⁻), *2 E. coli* XL1-Blue pSK*vdh*ΩKm, *3 P. putida* KT2440 (gluconate/vanillin), *4 E. coli* XL1-Blue pSK*vdh*BE, *5 P. putida* KT2440 (gluconate/ferulic acid), *6 P. putida* KT2440*vdh*ΩKm (gluconate/vanillin), *7 P. putida* KT2440*vdh*ΩKm (gluconate/ferulic acid)

analyzed for Fcs and Vdh activity. In contrast to the wild type, no Fcs activity was detectable in extracts of the mutants (Table 2). In mutant strain KT2440*fcs*ΩKm, this indicated successful inactivation of the feruloyl-CoA synthetase gene. In mutant strains KT2440*ech*ΩKm and KT2440*vdh*ΩKm the lack of Fcs activity might be attributed to a "downstream" effect of the Ω-element insertions in the *ech* or *vdh* gene, respectively, both of which are located upstream of the *fcs* gene (Fig. 2). With respect to the Vdh activities, it was remarkable that extracts of the mutant strain KT2440*vdh*ΩKm still exhibited about 10% of the Vdh activity obtained with extracts of the wild type (Table 3). To confirm the complete inactivation of Vdh in *P. putida* KT2440*vdh*ΩKm, extracts of this mutant were separated under non-denaturing conditions in a polyacrylamide gel together with an extract of the wild type, and the gel was specifically stained for vanillin dehydrogenase (Fig. 3). In extracts of the mutant, only a faint band was observed, which co-migrated with the Vdh band obtained with the extract of wild type cells grown in the presence of ferulic acid. However, this band was due to an unspecific reaction, since similar bands were observed in all *P. putida* KT2440-derived extracts in control experiments, omitting the substrate vanillin in the staining solution (data not shown in detail).

Remarkably, the mutant *P. putida* KT2440*vdh*ΩKm retained the ability to grow on vanillin as sole carbon source despite the lack of a functional *vdh* gene. To investigate this phenotype, cells of the mutant and the wild type were grown in MM containing 0.5% (w/v) sodium gluconate plus 0.1% (w/v) vanillin, harvested in the late exponential growth phase, and soluble fractions of crude extracts were analyzed for vanillin dehydrogenase activity by enzyme assay and activity staining. The extract derived from the mutant as well as the extract of the wild type showed similar, unexpected low Vdh activity (Table 3) and gave rise to only faint bands of similar migration in the activity staining (Fig. 3). These data indicated that vdh_{KT2440} was only expressed during growth of *P. putida* KT2440 in the presence of ferulic acid, but a different enzyme was responsible for vanillin oxidation during growth in the presence of vanillin.

Discussion

Due to their high adaptation capacity and their catabolic potential to metabolize an enormous range of natural organic compounds, pseudomonads play a crucial role in degradation processes of organic matter in nature (Stanier et al. 1966) and are frequently applied to the biotechnological production of valuable chemicals. One of the pseudomonads with current applications in many areas of biotechnology is the nonpathogenic, Gram-negative, biosafety microorganism *P. putida* KT2440. Sequencing of its genome, and the body of knowledge accumulated in the past on its genetics, physiology and biochemistry render this bacterium as a model organism for functional genomic studies of metabolic versatility.

In the present study, we analyzed the growth behavior of *P. putida* KT2440 with ferulic acid as an aromatic precursor for biotechnological vanillin production. Ferulic acid is very abundant in nature, since it is the oxidation product of the major aromatic constituent of lignin coniferyl alcohol (Crawford and Crawford 1980). It was found to be an excellent precursor for the production of vanillin that can be used for flavoring purposes (Muheim and Lerch 1999; Achterholt et al. 2000; Priefert et al. 2001). Four major pathways of ferulic acid degradation can be distinguished with respect to the initial reaction: (1) non-oxidative decarboxylation, (2) side-chain reduction, (3) coenzyme-A-independent deacetylation, and (4) coenzyme-A-dependent deacetylation (reviewed in Priefert et al. 2001).

The genome sequence of *P. putida* KT2440 was compared with known genes of the ferulic acid degradation pathway of *Pseudomonas* sp. HR199 (Overhage et al. 1999b), and the presence of the genes fcs_{KT2440}, ech_{KT2440}, and vdh_{KT2440} in the genome of strain KT2440 was revealed. The enzymatic functions of the corresponding gene products from *P. putida* KT2440 as feruloyl-CoA synthetase, enoyl-CoA hydratase/aldolase, and vanillin

dehydrogenase, respectively, were physiologically confirmed by expression of the corresponding genes in *E. coli*. Resting cells of the recombinant strain of *E. coli* expressing the genes *fcs* and *ech* simultaneously could be used for a biotransformation of ferulic acid to vanillin. However, the efficiency of this biotransformation was limited due to further disproportionation of vanillin, as observed before (Labuda et al. 1994; Overhage et al. 1999). Because of their rather high chemical reactivity, aldehydes like vanillin are often toxic and their accumulation is seldom observed (Lindahl et al. 1992). Actually, vanillin was found to inhibit cell metabolism and thus, most microorganisms either quickly oxidize or reduce the compound for detoxification.

To confirm the assumption that the ferulic acid catabolism in *P. putida* KT2440 proceeds via the same coenzyme A-dependent, non-β-oxidative pathway as in *Pseudomonas* sp. HR199, *fcs*, *ech,* and *vdh* were separately inactivated in *P. putida* KT2440 and the corresponding mutants were characterized physiologically, revealing the essential involvement of the corresponding enzymes in ferulic acid catabolism.

With respect to obtain a promising strain for a biotechnological process for the production of vanillin, investigation of the *vdh* knock-out mutant *P. putida* KT2440*vdh*ΩKm was of special interest, since it was expected to accumulate vanillin in the medium during growth on ferulic acid. However, this mutant was unable to grow with ferulic acid as sole carbon source and thus vanillin did not accumulate. Results obtained with this phenotype were in notably surprising contrast to those obtained with *Pseudomonas* sp. HR199. Although the arrangement of the ferulic acid degradation genes is essentially the same in both pseudomonads (Fig. 2), inactivation of *vdh* in *Pseudomonas* sp. HR199 did not influence its ability to degrade ferulic acid (Overhage et al. 1999a, b). However, like the corresponding mutant of *Pseudomonas* sp. HR199, mutant strain KT2440*vdh*ΩKm with inactivated *vdh* was still able to grow on vanillin as sole carbon source, just as the wild type strain. Oxidation of vanillin by *Pseudomonas* sp. HR199*vdh*ΩKm is due to a side activity of the *calB*-encoded coniferyl aldehyde dehydrogenase (Overhage et al. 1999a), which catalyzes a reaction in the eugenol degradation pathway in *Pseudomonas* sp. HR199 leading from eugenol to ferulic acid (Priefert et al. 1999; Achterholt et al. 1998). However, this pathway is missing in *P. putida* KT2440, which is not able to grow with eugenol as sole carbon source (data not shown). Thus, degradation of vanillin by the mutant *P. putida* KT2440*vdh*ΩKm must be attributed to a different enzyme exhibiting vanillin dehydrogenase activity in this strain. Surprisingly, even the wild type *P. putida* KT2440 exhibited only very low Vdh activity in extracts of cells grown in the presence of vanillin and did not show the Vdh band after activity staining, characteristic for cells grown in the presence of ferulic acid. Thus, an enzyme different from Vdh seems to be responsible for the oxidation of vanillin during growth in the presence of this substrate and *vdh* expression occurred only during growth in the presence of ferulic acid.

The present work will be the basis for the rational development of an efficient biocatalyst for the production of vanillin. This might be achieved by introducing a pathway leading from eugenol to ferulic acid in *P. putida* KT2440*vdh*ΩKm in order to extend the substrate range of KT2440 for the exploitation of this cheaper and more abundant vanillin precursor.

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