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Potential of *Rhodococcus* strains for biotechnological vanillin production from ferulic acid and eugenol

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Abstract The potential of two *Rhodococcus* strains for biotechnological vanillin production from ferulic acid and eugenol was investigated. Genome sequence data of *Rhodococcus* sp. I24 suggested a coenzyme A-dependent, non-β-oxidative pathway for ferulic acid bioconversion, which involves feruloyl–CoA synthetase (Fcs), enoyl–CoA hydratase/aldolase (Ech), and vanillin dehydrogenase (Vdh). This pathway was proven for *Rhodococcus opacus* PD630 by physiological characterization of knockout mutants. However, expression and functional characterization of corresponding structural genes from I24 suggested that degradation of ferulic acid in this strain proceeds via a β-oxidative pathway. The vanillin precursor eugenol facilitated growth of I24 but not of PD630. Coniferyl aldehyde was an intermediate of eugenol degradation by I24. Since the genome sequence of I24 is devoid of eugenol hydroxylase homologous genes (*ehyAB*), eugenol bioconversion is most probably initiated by a new step in this bacterium. To establish eugenol bioconversion in PD630, the vanillyl alcohol oxidase gene (*vaoA*) from *Penicillium simplicissimum* CBS 170.90 was expressed in PD630 together with coniferyl alcohol dehydrogenase (*calA*) and coniferyl aldehyde dehydrogenase (*calB*) genes from *Pseudomonas* sp. HR199. The recombinant strain converted eugenol to ferulic acid. The obtained data suggest that genetically engineered strains of I24 and PD630 are suitable candidates for vanillin production from eugenol.

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Introduction

Bacteria belonging to the genus *Rhodococcus* are ubiquitous in the environment and have frequently been isolated from a large variety of ecosystems, including soil, marine habitats, groundwater, activated sludge, and the guts of insects (Bell et al. 1998; Larkin et al. 1998). These Gram-positive bacteria are closely related to the mycolic acid containing genera *Nocardia*, *Corynebacterium*, and *Mycobacterium* (Bell et al. 1998; Goodfellow et al. 1998). Since many *Rhodococcus* species are able to degrade xenobiotic compounds, interest for their use in various industrial and environmental applications has been inspired. Beside their significant role in bioremediation, rhodococci are promising candidates for a wide range of biotransformation processes due to their metabolic versatility (Bell et al. 1998; Warhurst and Fewson 1994; O'Brien et al. 2002).

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is quantitatively one of the most important flavor additives in the world. Since the demand for natural vanillin cannot be accommodated from vanilla pod extraction, biotechnological processes based on microorganisms become more and more important (Krings and Berger 1998). Potential substrates for these processes are eugenol (4-allyl-2-methoxyphenol), ferulic acid (4-hydroxy-3-methoxycinnamate), and lignin (Chen et al. 1982; Tadasa and Kayahara 1983; Toms and Wood 1970). For the development of economically feasible processes, biotransformations of these natural raw materials based on microorganisms are most suitable due to the rapid growth of the biocatalysts and their accessibility to molecular genetics (reviewed by Priefert et al. 2001).

In the present study, we describe the investigation of eugenol and ferulic acid catabolism in *Rhodococcus* sp. I24 and *Rhodococcus opacus* PD630, respectively. The obtained data suggest a new pathway for ferulic acid degradation in strain I24 that is different from the coenzyme A-dependent, non-β-oxidative pathway occurring in the Gram-positive *Amycolatopsis* sp. HR167 (Achterholt et al. 2000) and in pseudomonads (Gasson et al. 1998; Masai et al. 2002; Narbad and Gasson 1998; Overhage et al. 1999b; Plaggenborg et al. 2001, 2003). Since *Rhodococcus* sp. I24 exhibits eugenol-degradation ability, the potential application of genetically engineered derivatives of strain I24 and *R. opacus* PD630 as candidates for vanillin production from eugenol is discussed.

Materials and methods

Bacterial strains, plasmids, and cultivation conditions

The bacterial strains and plasmids used in this study are listed in Table 1 or described in the text. Strains of *Escherichia coli* were cultivated at 37°C in Luria–Bertani (LB) broth (Sambrook et al. 1989). *Pseudomonas* strains, *R. opacus* PD630, and *Rhodococcus* sp. I24 were cultured at 30°C either in LB, nutrient broth (NB) (0.8% w/v; Bacto, Difco), or mineral salts medium (MM) (Schlegel et al. 1961). Ferulic acid and vanillin were dissolved in dimethyl sulfoxide and were added to the medium at final concentrations of 5.15 and 6.57 mM, respectively. Eugenol was directly added to the medium at final concentrations of 0.61–4.26 mM. Kanamycin was used at a final concentration of 50 μg ml^{-1} for *Rhodococcus* strains. Tetracycline, ampicillin, and chloramphenicol were used at final concentrations of 12.5, 100, or 34 μg ml^{-1}, respectively, for recombinant strains of *E. coli*. Tetracycline was used at a final concentration of 25 μg ml^{-1} for *Pseudomonas* strains. Growth of bacteria was monitored by measuring the turbidity of the cultures at 600 nm (OD_{600nm}). Solid media were prepared by the addition of 1.5% (w/v) agar–agar. To investigate eugenol degradation by *Rhodococcus* sp. I24, precultures were grown in MM containing 4% (w/v) glucose as carbon source. Cells were harvested, washed twice with MM, and used for inoculation of MM containing 2.4 mM eugenol. For physiological characterization of *R. opacus* PD630 mutants, cells were precultured in MM with 0.5% (w/v) sodium gluconate as carbon source. Cells were harvested, washed twice with MM, and used for inoculation of MM containing 5.1 mM ferulic acid as sole carbon source. Eugenol biotransformation by recombinant strains of *R. opacus* PD630 was investigated by growing the strains at 30°C in MM containing 4% (w/v) glucose in the presence of kanamycin. When the culture reached an OD_{600nm} of 11, eugenol was added to a final concentration of 1.22 mM. The occurrence of intermediates in the culture supernatant was analyzed by high-performance liquid chromatography (HPLC).

Analytical methods

Culture supernatants were analyzed for catabolic intermediates chromatographically, without prior extraction,

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
Bacteria		
Rhodococcus opacus PD630	Wild type, eugenol-negative, ferulate-positive, vanillin-positive, kanamycin-sensitive, ampicillin-sensitive	DSM44193
PD630*fcs*ΩKm	Ferulate-negative, vanillin-positive, kanamycin-resistant	This study
PD630*vdh*ΩKm	Ferulate-negative, vanillin-positive, kanamycin-resistant	This study
Rhodococcus sp. I24	Wild type, eugenol-positive, ferulate-positive, vanillin-positive	Buckland et al. (1999)
Pseudomonas sp. SK6167	Ferulic acid-negative mutant of *Pseudomonas* sp. strain HR199	Overhage et al. (1999b)
Pseudomonas sp. SK6202	Ferulic acid-negative mutant of *Pseudomonas* sp. strain HR199	Overhage et al. (1999b)
Escherichia coli		
XL1-Blue	*recA1endA1gyrA96 thi-1hsdR17* (r_K^-, m_K^+) *supE44 relA1*, λ$^-$, *lac* F′ [*proAB lacI*^{q}*lacZΔM15*::Tn*10*(Tc^r)]	Bullock et al. (1987)
S17-1	*thi-1 proA hsdR17* (r_K^-, m_K^+) *recA1*; harboring the *tra* genes of plasmid RP4 in the chromosome	Simon et al. (1983)
Rosetta(DE3)pLysS	F$^-$*ompT hsdS*$_B$(r_B^-,wm_B^-) *gal dcm lacY1* (DE3) pLysSRARE (Cm^r)	Novagen, Madison, WI, USA
Plasmids		
pBluescript SK$^-$	*lacPOZ*′, T7, and T3 promoter, Ap^r	Stratagene, San Diego, CA, USA
pET23a	T7 promoter, Ap^r	Novagen
pGEM-T Easy	*lacPOZ*′, MCS, Ap^r	Promega, Madison, WI, USA
pVK100	Cosmid, *mob*, Tc^r, Km^r	Knauf and Nester (1982)
pBBR1-MCS5	*lacPOZ*′, *mob*, broad host range, Gm^r	Kovach et al. (1995)
pSKsymΩKm	pSKsym harboring ΩKm in the *Sma*I site of the MCS	Overhage et al. (1999a)
pBBRKmNC903	*E. coli*/*Rhodococcus* shuttle vector *mob*, *rep*, Km^r	Kalscheuer et al. (1999)

using an HPLC apparatus (Fa. Knauer, Berlin, Germany) as described previously (Overhage et al. 1999b). Proteins were separated under denaturing conditions in 11.5% (w/v) polyacrylamide gels [sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)] according to Laemmli (1970). Proteins in the gels were stained with Serva Blue R.

Electroporation

Plasmids for generating mutant strains by homologous recombination were introduced into strains of *Rhodococcus* by electroporation as described by Kalscheuer et al. (1999).

Preparation of soluble fractions of crude protein extracts

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

Enzyme assays

Feruloyl coenzyme A (feruloyl–CoA) synthetase (Fcs) and vanillin dehydrogenase (Vdh) were assayed as described previously by Overhage et al. (1999b) and Gasson et al. (1998), respectively. The amount of soluble protein was determined according to Bradford (1976).

Isolation, manipulation, and transfer of DNA

Genomic DNA, plasmid DNA, and DNA restriction fragments were isolated and analyzed by standard methods (Marmur 1961; Sambrook et al. 1989). Competent cells of *E. coli* were prepared and transformed by using the $CaCl_2$ procedure (Hanahan 1983). Conjugations of *E. coli* S17-1 harboring hybrid plasmids (donors) and of *Rhodococcus* strains (recipients) were performed on solidified NB medium as described by Friedrich et al. (1981).

Construction of a genomic library of *R. opacus* PD630

Partially *Eco*RI-digested genomic DNA of *R.opacus* PD630 was ligated with *Eco*RI-linearized cosmid pVK100 DNA. The ligation mixture was packaged in l particles and subsequently transduced into *E. coli* S17-1. About 3,000 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 of *Pseudomonas* sp. HR199 (Overhage et al. 1999b), respectively, to screen for complementation.

Computer-assisted localization of eugenol catabolism genes in the genome sequence of *Rhodococcus* sp. I24

Genes potentially involved in eugenol catabolism in *Rhodococcus* sp. I24 were identified by applying basic local alignment search tool (BLAST) programs (Altschul et al. 1997), using the amino acid sequences of EhyA, EhyB, Fcs, Ech, and Vdh of *Pseudomonas* sp. HR199 (Overhage et al. 1999b; Priefert et al. 1997, 1999) and of Fcs and Ech of *Amycolatopsis* sp. HR167 (Achterholt et al. 2000), respectively.

Amplification and cloning of fcs_{I24}, ech_{I24}, and vdh_{I24} from genomic DNA of *Rhodococcus* sp. I24

The genes *fcs*, *ech*, and *vdh* were amplified in separate polymerase chain reactions (PCRs) with genomic DNA of *Rhodococcus* sp. I24 as template DNA. To amplify fcs_{I24}, oligonucleotides fcs_{I24}-Bup and fcs_{I24}-Ed (Table 2) were used as primers. To amplify ech_{I24}, primers ech_{I24}-S/D-Eup and ech_{I24}-Hd (Table 2) were used to introduce a ribosomal binding site preceding the translational start codon at a distance of 5 nucleotides. To amplify vdh_{I24}, primers vdh_{I24}-Xup and vdh_{I24}-Ed (Table 2) were used. The PCR products were isolated and digested with *Bam*HI and *Eco*RI (for fcs_{I24}), *Eco*RI and *Hin*dIII (for ech_{I24}), or *Xba*I and *Eco*RI (for vdh_{I24}), and ligated to correspondingly digested pBluescript SK^- DNA, respectively. *E. coli* XL1-Blue was transformed with the ligation mixtures, and transformants harboring the hybrid plasmids pSKfcs_{I24}BE, pSKech_{I24}EH, or pSKvdh_{I24}XE were obtained. Fragment ech_{I24}EH was isolated from *Eco*RI-/*Hin*dIII-digested pSKech_{I24}EH and ligated to *Eco*RI-/*Hin*dIII-digested pSKfcs_{I24}BE, resulting in hybrid plasmid pSKfcs_{I24}/ech_{I24}BEH.

In addition, fcs_{I24} was amplified using primers fcs_{I24}-ENup and fcs_{I24}-Edown (Table 2). After isolation, the PCR product was digested with *Eco*RI and ligated to *Eco*RI-digested pBluescript SK^-, resulting in hybrid plasmid pSKfcs_{I24}EE. Fragment fcs_{I24}NE was isolated from *Nde*I-/*Eco*RI-digested pSKfcs_{I24}EE and ligated to *Eco*RI-/*Nde*I-digested pET23a DNA. *E. coli* Rosetta(DE3)pLysS was transformed with the ligation mixture, and transformants harboring the hybrid plasmid pETfcs_{I24}NE were obtained.

Amplification and cloning of fcs_{PD630} from genomic DNA of *R. opacus* PD630

The gene fcs_{PD630} was amplified by PCR with genomic DNA of *R. opacus* PD630 as template DNA. To amplify fcs_{PD630}, primers fcs_{PD630}-Bup and fcs_{PD630}-Ed (Table 2) were used to introduce *Bam*HI and *Eco*RI sites, respectively. The PCR product was isolated, digested with *Bam*HI and

Table 2 Oligonucleotide PCR primers used in this study

Name	Sequence (5′–3′)[a]
fcs$_{I24}$-Bup	AAAAGGATCCCCAAGGCATCGAGGAGATCCCGTGC
fcs$_{I24}$-Ed	AAAAGAATTCCGTGGCGGTCGGGTCAGTGGGCG
ech$_{I24}$-S/D-Eup	AAAAGAATTC*AAGGAG*ATATAATGGCCGACGAGTCCACCGTTACC
ech$_{I24}$-Hd	AAAAAAGCTTTCAGCGACCCTTCCAGACCGGCTG
vdh$_{I24}$-Xup	AAAATCTAGACCGTACACAAGGAGTTCGTGATG
vdh$_{I24}$-Ed	AAAAGAATTCGGTCGGCGGGGCCGCTCAGAAGG
fcs$_{I24}$-ENup	AAAAGAATTCCATATGCTCAATCAAGGTACCGGAACCTGGCC
fcs$_{I24}$-Edown	AAAAGAATTCTCAGTGGGCGTACTGCTCGCGCAAC
fcs$_{PD630}$-Bup	AAAAGGATCCCGAAGGAGAGCGCGGACAACGACC
fcs$_{PD630}$-Ed	AAAAGAATTCCGACGCCGTCGAACAACACGATCCC
del*fcs*$_{PD630}$upEI	AAAAGAATTCGGTGATCCTCGTCCTCCTCCGCG
del*fcs*$_{PD630}$upEV	AAAAGATATCCATGGTGACTCCTCGTTCTCTTCG
del*fcs*$_{PD630}$downK	AAAAGGTACCCCAGCCGCGTGTCGCTCTCCGT
del*fcs*$_{PD630}$downEV	AAAAGATATCCGCGCCTGTGAGTACTTCTTGACC
*vdh**$_{PD630}$-up	AARCCSGACCCSCGSACSGCS
*vdh**$_{PD630}$-down	GTCGATGATSGGNCCSAGSGC
*vdh**$_{PD630}$inverse-up	GTTCGAGGTGTGCCCGCGTGAGG
*vdh**$_{PD630}$inverse-down	CGGCGGCAACAACGCCCTCATCG

[a]Introduced restriction enzyme sites are underlined; introduced ribosomal binding sites are italicized

*Eco*RI, and ligated to correspondingly digested pBluescript SK$^-$ DNA to obtain hybrid plasmid pSK*fcs*$_{PD630}$BE.

Expression and functional characterization of *fcs*$_{I24}$, *ech*$_{I24}$, *vdh*$_{I24}$, and *fcs*$_{PD630}$

Recombinant strains of *E. coli* XL1-Blue harboring hybrid plasmids pSK*fcs*$_{I24}$BE (*fcs*$_{I24}$ downstream of and codirectional with the *lacZ* promoter of the vector), pSK*fcs*$_{I24}$/*ech*$_{I24}$BEH (*fcs*$_{I24}$ and *ech*$_{I24}$ downstream of and codirectional with the *lacZ* promoter), pSK*fcs*$_{PD630}$BE (*fcs*$_{PD630}$ downstream of and codirectional with the *lacZ* promoter), and pSK*vdh*$_{I24}$XE (*vdh*$_{I24}$ downstream of and codirectional with the *lacZ* promoter) were used to prepare protein extracts for enzymatic assays. Strains were grown overnight in LB medium containing 12.5 μg of tetracycline per milliliter, 100 μg of ampicillin per milliliter, and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation at 4,000×*g* for 20 min at 4°C and washed twice in 100 mM potassium phosphate buffer (pH 7.0). Soluble fractions of crude protein extracts were obtained as described above and were applied in the corresponding enzyme assays. Recombinant *E. coli* Rosetta(DE3)pLysS harboring the hybrid plasmid pET*fcs*$_{I24}$NE with *fcs*$_{I24}$ downstream of and codirectional with the T7 promoter of the vector was grown at 37°C in LB medium containing 34 μg of chloramphenicol per milliliter and 100 μg of ampicillin per milliliter. When the cultures reached an OD_{600nm} of 0.5, IPTG was added to a final concentration of 1 mM. The induced cultures were incubated at 37°C for 3 h. Cells were then harvested, and protein extracts were prepared as described above.

Whole cell biotransformation assay

Escherichia coli XL1-Blue (pSK*fcs*$_{I24}$/*ech*$_{I24}$BEH) was grown at 37°C in 50 ml LB medium containing 12.5 μg of tetracycline and 100 μg of ampicillin per milliliter. When the cultures reached an OD_{600nm} of 0.5, IPTG was added to a final concentration of 1 mM, and incubation was continued for 5 h. Alternatively, the strain was grown overnight for 12 h in the presence of 1 mM IPTG. Cells were then harvested and washed as described above, and cells were finally resuspended in 50 ml MM containing 5.1 mM ferulic acid. Cultures were further incubated at 37°C, and supernatants of these resting cell cultures were analyzed for intermediates of ferulic acid catabolism by HPLC.

Inactivation of *fcs*$_{PD630}$ in *R. opacus* PD630 by replacement with ΩKm

For inactivation of the *fcs* gene in *R. opacus* PD630 by replacement with ΩKm, a region upstream and downstream of *fcs*$_{PD630}$ was amplified from genomic DNA of strain PD630 by PCR. To obtain the upstream region, primers del*fcs*$_{PD630}$upEI and del*fcs*$_{PD630}$upEV (Table 2) were used. To obtain the downstream region, primers del*fcs*$_{PD630}$-downK and del*fcs*$_{PD630}$downEV (Table 2) were used. The isolated PCR products were digested with *Eco*RI/*Eco*RV and *Kpn*I/*Eco*RV, and ligated with *Eco*RI-/*Kpn*I-digested pBluescript SK$^-$ and ΩKm, recovered from *Sma*I-digested pSKsymΩKm. *E. coli* XL1-Blue was transformed with the ligation mixture, and transformants harboring the hybrid plasmid pSK*fcs*$_{PD630}$ΩKm were obtained. After linearization, pSK*fcs*$_{PD630}$ΩKm was introduced into the wild-type

strain *R. opacus* PD630 by electroporation. Due to homologous recombination, the complete functional fcs_{PD630} gene was replaced with fcs_{PD630}ΩKm by a double crossover event, resulting in a kanamycin-resistant phenotype of the mutant *R. opacus* PD630*fcs*ΩKm. The genotype of this mutant was confirmed by amplification of the corresponding gene from genomic DNA by PCR using primers fcs_{PD630}-Bup and fc_{PD630}-Ed.

Inactivation of vdh_{PD630} in *R. opacus* PD630 by disruption with ΩKm

For inactivation of vdh_{PD630} by insertion of ΩKm, a central part of vdh_{PD630} (vdh^*_{PD630}) was amplified from genomic DNA of *R. opacus* PD630 using primers vdh^*_{PD630}-up and vdh^*_{PD630}-down (Table 2) designed from a highly conserved region (Fig. 1). The isolated 459-bp PCR product was ligated into pGEM-T Easy to create hybrid plasmid pGEM-T Easy-vdh^*_{PD630}. This plasmid was used as a template in an inverse PCR using vdh^*_{PD630}inverse-up and vdh^*_{PD630}inverse-down (Table 2) as primers. The resulting blunt-ended PCR product was ligated with ΩKm, recovered from *Sma*I-digested pSKsymΩKm. *E. coli* XL1-Blue was transformed with the ligation mixture, and transformants harboring pGEM-TEasy-vdh^*_{PD630}ΩKm were obtained. From this plasmid, vdh^*_{PD630}ΩKm was excised as *Eco*RI fragment and inserted into the *Eco*RI site of pBBR1-MCS5. *E. coli* S17-1 was transformed with the ligation mixture, and transformants harboring the hybrid plasmid pBBR1-MCS5-vdh^*_{PD630}ΩKm were obtained. This plasmid was transferred from gentamicin- and kanamycin-resistant recombinant *E. coli* S17-1 strains to *R. opacus* PD630 by conjugation. Due to homologous recombination, ΩKm was inserted into the functional vdh_{PD630} gene, resulting in a kanamycin-resistant phenotype of the mutant *R. opacus* PD630*vdh*ΩKm. The genotype of this mutant was confirmed by amplification of the corresponding gene from genomic DNA by PCR using primers vdh^*_{PD630}ΩKm-up and vdh^*_{PD630}ΩKm-down.

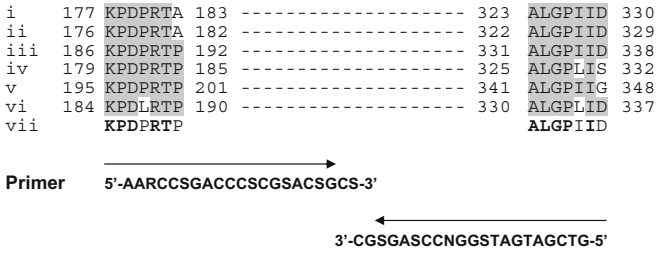

Fig. 1 Homologies of vanillin dehydrogenase of *Rhodococcus* sp. I24 and of different aldehyde dehydrogenases at specific amino acid positions and deduced primer sequences for *vdh* knockout construction in *R. opacus* PD630. Amino acid sequence of the *vdh* gene product from *Rhodococcus* sp. I24 (*i*; this study, accession number AAY98503), probable aldehyde dehydrogenase from *Streptomyces coelicolor* A3(2) (*ii*; Bentley et al. 2002, CAB76355), benzaldehyde dehydrogenase II from *Xanthomonas axonopodis* pv. *citri* 306 (*iii*; da Silva et al. 2002, AAM35246), benzaldehyde dehydrogenase from *Caulobacter crescentus* CB15 (*iv*; Nierman et al. 2001, AAK24368), benzaldehyde dehydrogenase II from *Xanthomonas campestris* pv. *campestris* ATCC 33913 (*v*; da Silva et al. 2002, AAM39673), putative benzaldehyde dehydrogenase oxidoreductase protein from *Ralstonia solanacearum* (*vi*; Salanoubat et al. 2002, CAD17380). Amino acid positions are indicated. Identical amino acid residues are *shaded*. The consensus sequence is given (*vii*). The deduced nucleotide sequence of primers used for the *vdh* knockout construction considering the *R. opacus* codon usage is shown (primer)

DNA sequence determination

DNA sequences were determined by the dideoxy chain termination method (Sanger et al. 1977) with a 4000L DNA sequencer (LI-COR Inc., Biotechnology Division, Lincoln, NE, USA).

Results

Growth of *R. opacus* PD630 and *Rhodococcus* sp. I24 in the presence of different eugenol concentrations

Since it is known that eugenol has antibacterial properties, the maximum concentrations of eugenol tolerated as cosubstrate by strains PD630 and I24 were determined. *R. opacus* PD630 tolerated eugenol concentrations up to 1.22 mM. At higher concentrations, growth was completely inhibited. In contrast, *Rhodococcus* sp. I24 tolerated eugenol concentrations up to 2.44 mM. However, when strain I24 was precultured with ferulic acid as carbon source, growth with eugenol as sole carbon source was observed up to a concentration of 3.04 mM.

To investigate eugenol degradation by *Rhodococcus* sp. I24, cells precultured in MM containing glucose were used for inoculation of MM containing eugenol. Within 32 h of incubation, eugenol was nearly exhausted from the medium (Fig. 2). Concomitantly, the OD_{600nm} increased from 0.3 to 1. Coniferyl aldehyde was the only detectable intermediate and was found only in trace amounts. Cultures were further incubated, and portions of eugenol were added as indicated in Fig. 2. After 50 h of growth, a final OD_{600nm} of 3.6 was reached, and a total of about 7.5 mM eugenol was metabolized.

Identification of eugenol and ferulic acid catabolism genes from *Rhodococcus* sp. I24

The amino acid sequences of feruloyl–CoA synthetase and enoyl–CoA hydratase/aldolase from *Amycolatopsis* sp. HR167 and *Pseudomonas* sp. HR199 (gene products of fcs_{HR167}, ech_{HR167}, fcs_{HR199}, and ech_{HR199}), and of eugenol hydroxylase and vanillin dehydrogenase from *Pseudomonas* sp. HR199 (gene products of $ehyAB_{HR199}$ and vdh_{HR199}) (Achterholt et al. 2000; Overhage et al. 1999b, Priefert et al. 1997, 1999), were compared with the genome sequence of *Rhodococcus* sp. I24. The arrangement of three open reading frames (ORFs), whose deduced amino acid sequences exhibited highest similarities to Fcs_{HR167}, Ech_{HR167}, and Vdh_{HR199} (Table 3), is depicted in Fig. 3. However, no sig-

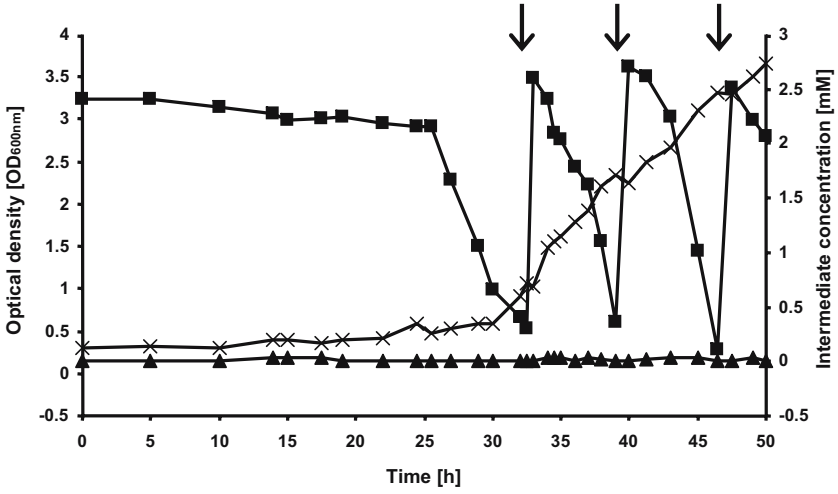

Fig. 2 Growth of *Rhodococcus* sp. I24 in MM with 2.44 mM eugenol as sole carbon source. Cultures were incubated at 30°C, and growth was monitored by measuring turbidity at 600 nm. Additions of 20-µl portions of eugenol (corresponding to a final concentration of 2.44 mM) are indicated (↓). Eugenol and intermediate concentrations were determined by HPLC. *X*, OD_{600nm}; ▪, eugenol ▲, coniferyl aldehyde

nificant similarities were obtained with the eugenol hydroxylase sequences, suggesting that the initial step of eugenol degradation in strain I24 is catalyzed by an enzyme that is different from bacterial eugenol hydroxylases known so far (Furukawa et al. 1998; Priefert et al. 1999).

The putative enoyl–CoA hydratase/aldolase gene (ech_{I24}) of strain I24 was identified using the amino acid sequence of Ech_{HR167}. However, when the amino acid sequence of Ech_{HR167} was applied in a "tblastn" search in the nr nucleotide sequence database of the National Center for Biotechnology Information (NCBI), highest identities were only found to classical enoyl–CoA hydratase (47%), and no aldolase or lyase function was annotated.

The calculated molecular masses of Fcs_{I24}, Ech_{I24}, and Vdh_{I24} were 54.7, 27.6, and 50.3 kDa, respectively. The identified genes fcs_{I24}, ech_{I24}, and vdh_{I24} putatively involved in the ferulic acid degradation in *Rhodococcus* sp. I24 are not clustered, but distributed in the genome (Fig. 3). Thus, the organization of these genes is different from known operons, where the genes *fcs*, *ech*, and *vdh* or at least *fcs* and *ech* are

Table 3 Similarities of ferulic acid catabolism genes from different bacterial sources

	Fcs_{I24} (AAY98502)	Fcs_{HR167} (CAC18323)	Fcs_{PD630} (AY685057)	Fcs_{KT2440} (AAN68960)	Fcs_{HR199} (CAB60226)	$Fcs_{Delftia}$ (CAC83622)	$FerA_{Sp}$ (BAC79255)
Fcs_{I24}		54	47	22	21	NSS	
Fcs_{HR167}			47	25	21		
Fcs_{PD630}				23	22		
Fcs_{KT2440}					75		
Fcs_{HR199}							
$Fcs_{Delftia}$							60
	Ech_{I24} (AAY98541)	Ech_{HR167} (CAC18324)	Ech_{KT2440} (AAN68962)	Ech_{HR199} (CAA72285)	$Ech_{Delftia}$ (CAC83621)	$FerB_{Sp}$ (BAC79254)	
Ech_{I24}		37	31	32	32	31	
Ech_{HR167}			64	60	50	46	
Ech_{KT2440}				90	52	48	
Ech_{HR199}					51	48	
$Ech_{Delftia}$						70	
	Vdh_{I24} (AAY98503)	Vdh_{PD630}[a] (this study)	Vdh_{KT2440} (AAN68961)	Vdh_{HR199} (CAA72286)			
Vdh_{I24}		63	40	40			
Vdh_{PD630}[a]			42	43			
Vdh_{KT2440}				80			

Results of BLAST of 2 sequences (Tatusova and Madden 1999); given as amino acid sequence identities (%). Indices indicate the bacterial sources. Accession numbers are given in parentheses

Fcs and *FerA* Feruloyl–CoA synthetase, *Ech* enoyl–CoA hydratase or enoyl–CoA hydratase/aldolase, *FerB* feruloyl–CoA hydratase/lyase, *Vdh* vanillin dehydrogenase, *I24 Rhodococcus* sp. I24, *HR167 Amycolatopsis* sp. HR167, *PD630 R. opacus* PD630, *KT2440 Pseudomonas putida* KT2440, *HR199 Pseudomonas* sp. HR199, *Delftia Delftia acidovorans*, *Sp Sphingomonas paucimobilis*, *NSS* no significant similarity

[a]Partial central sequence of vdh_{PD630} comprising 153 amino acid residues

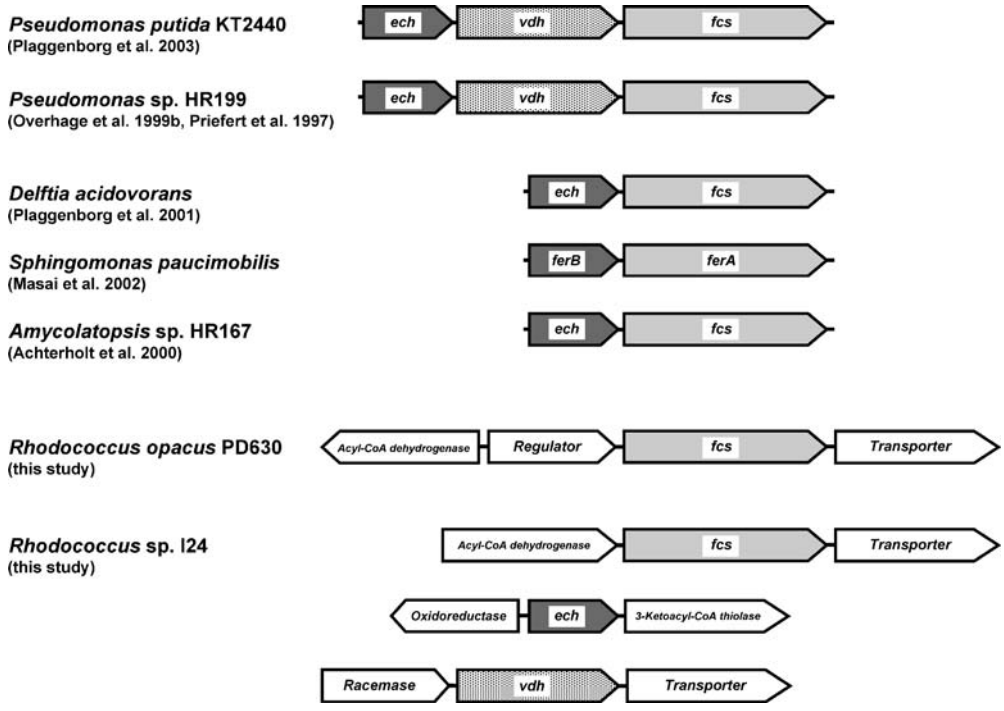

Fig. 3 Organization of structural genes involved in ferulic acid catabolism from different bacteria. *fcs* or *ferA*, feruloyl–CoA synthetase; *ech*, enoyl–CoA hydratase or enoyl–CoA hydratase/aldolase; *ferB*, feruloyl–CoA hydratase/lyase; *vdh*, vanillin dehydrogenase

located in close proximity (Fig. 3). However, this organization might be typical for *Rhodococcus* strains, since the *fcs* gene of *R.opacus* PD630 is also not accompanied by other ferulic acid catabolism genes (Fig. 3).

Heterologous expression of fcs_{I24}, vdh_{I24}, and ech_{I24} in *E. coli* and functional characterization of the corresponding gene products

To assign enzymatic functions to the gene products of fcs_{I24}, ech_{I24}, and vdh_{I24}, these genes were expressed in *E. coli* as described in "Materials and methods." Protein extracts derived from *E. coli* XL1-Blue (pSKfcs_{I24}BE) exhibited feruloyl–CoA synthetase activity (Table 4), and extracts derived from *E. coli* XL1-Blue (pSKvdh_{I24}XE) exhibited vanillin dehydrogenase activity (Table 4). The formation 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. The function of ech_{I24} was tested in a whole cell biotransformation assay as described in "Materials and methods." However, no conversion of ferulic acid to vanillin was obtained with resting cells of *E. coli* XL1-Blue (pSKfcs_{I24}/ech_{I24}BEH) (data not shown). Since the activity of the fcs_{I24} gene product was proven by an enzymatic assay (Table 4), and a protein band of 27±1 kDa corresponding to the size of Ech was detected by SDS-PAGE analysis of the corresponding protein extract (data not shown), this negative result was indicative for a missing aldolase activity of the ech_{I24} gene product.

Identification of the genes involved in ferulic acid catabolism in *R. opacus* PD630

A functional screen was applied to identify the genes involved in the ferulic acid degradation pathway of *R. opacus* PD630. We took advantage of the ferulic acid-negative mutants SK6167 and SK6202 of *Pseudomonas* sp. HR199 (Overhage et al. 1999b), which were chosen as recipients for the genomic library of *R.opacus* PD630. Transconjugants of mutant SK6167 were isolated, which were able to grow on ferulic acid and were thus complemented by the received hybrid cosmids. From complementing hybrid cosmids, a 14-kbp *Eco*RI fragment (E14) was subcloned in the vector pBBR1-MCS5, and complementing capacity of E14 was confirmed. Further subcloning assigned the complementing capacity to a 3.8-kbp *Pst*I fragment, which was sequenced. The obtained sequence exhibited four ORFs (Fig. 3) and is available under accession number AY685057. One of the ORFs was designated as fcs_{PD630} because its deduced amino acid sequence exhibited highest similarity with the feruloyl–CoA synthetase of *Amycolatopsis* sp. HR167 (Achterholt et al. 2000) (Table 3). Putative functions of the other identified ORFs are indicated in Fig. 3.

Heterologous expression of fcs_{PD630} in *E. coli* and functional characterization of the gene product

The enzymatic function of the fcs_{PD630} gene product was assigned by heterologous expression of the gene in *E. coli*

Table 4 Feruloyl–CoA synthetase and vanillin dehydrogenase activities in recombinant strains of *E. coli* XL1-Blue and *E. coli* Rosetta (DE3)pLysS

Strain	Specific activity of feruloyl–CoA synthetase[c] (U mg^{-1} protein)
E. coli XL1-Blue (pBluescript SK^-)	<0.01
E. coli Rosetta(DE3)pLysS (pET23a)	<0.01
E. coli XL1-Blue (pSK*fcs*$_{I24}$BE)[a]	0.09
E. coli XL1-Blue (pSK*fcs*$_{I24}$/*ech*$_{I24}$BEH)	0.09
E. coli Rosetta(DE3)pLysS (pET*fcs*$_{I24}$NE)[b]	0.11
E. coli XL1-Blue (pSK*fcs*$_{PD630}$BE)	0.16
	Specific activity of vanillin dehydrogenase[c] (U g^{-1} protein)
E. coli XL1-Blue (pBluescript SK^-)	<1
E. coli XL1-Blue (pSK*vdh*$_{I24}$XE)	30

[a]Recombinant strains of *E. coli* XL1-Blue were grown for 12 h at 37°C in LB in the presence of tetracycline and ampicillin at final concentrations of 12.5 or 100 μg ml^{-1}, respectively, and 1 mM IPTG

[b]Recombinant *E. coli* Rosetta(DE3)pLysS were grown in LB medium to an OD_{600nm} of 0.5 at 37°C in the presence of ampicillin and chloramphenicol at final concentrations of 100 or 34 μg ml^{-1}, respectively, before IPTG was added to a final concentration of 1 mM. The induced cultures were incubated at 37°C for 3 h

[c]Feruloyl–CoA synthetase and vanillin dehydrogenase were assayed as described in "Materials and methods." Data represent the mean of three independent determinations

as described in "Materials and methods." Protein extracts derived from *E. coli* XL1-Blue (pSK*fcs*$_{PD630}$BE) exhibited feruloyl–CoA synthetase activity (Table 4). Feruloyl–CoA was proven to be the product of this enzymatic reaction by HPLC analysis.

Construction and characterization of *fcs*- and *vdh*-deficient mutants of *R. opacus* PD630

To confirm the essential involvement of *fcs*$_{PD630}$ and *vdh*$_{PD630}$ in the catabolism of ferulic acid in *R. opacus* PD630, these genes were inactivated by insertion of an ΩKm element as described in "Materials and methods." The genotypes of mutants *R. opacus* PD630*fcs*ΩKm and *R. opacus* PD630*vdh*ΩKm were confirmed by PCR analyses. The corresponding phenotypes were investigated on solidified MM with ferulic acid, vanillin, or sodium gluconate as sole carbon sources. *R. opacus* PD630*fcs*ΩKm and *R. opacus* PD630*vdh*ΩKm were not able to grow on ferulic acid but retained the ability to grow on vanillin and sodium gluconate.

For further physiological characterization, both mutants were studied in ferulic acid biotransformation experiments as described in "Materials and methods." In the control cultures of the wild type, ferulic acid was completely depleted from the medium, and transient accumulation of vanillic acid and protocatechuic acid was observed (Fig. 4). In contrast, in cultures of the mutant strains *R. opacus* PD630*fcs*ΩKm and *R. opacus* PD630*vdh*ΩKm, no decrease of the ferulic acid concentration and no appearance of intermediates were observed (data not shown). However, when vanillin was applied as substrate in the biotransformation, *R. opacus* PD630*vdh*ΩKm was able to degrade vanillin (data not shown).

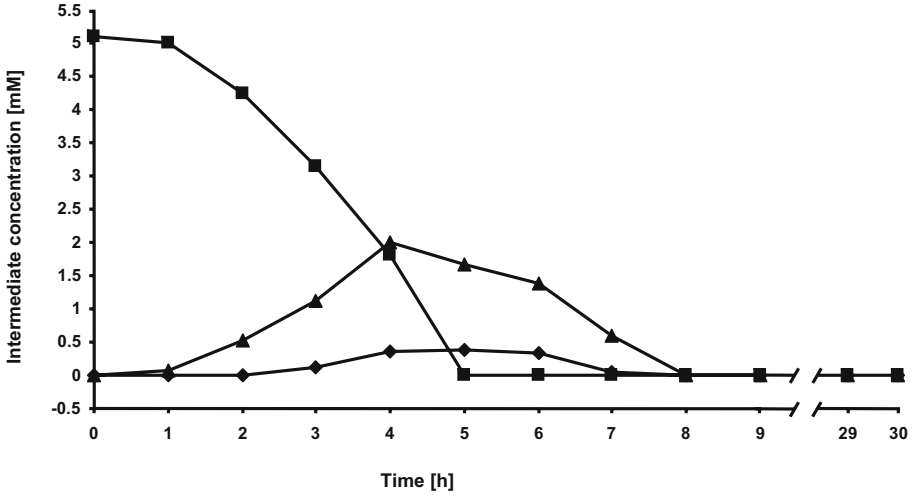

Fig. 4 Biotransformation of ferulic acid by resting cells of *R. opacus* PD630. Cells were precultured overnight in MM containing 0.5% (w/v) sodium gluconate as carbon source. The cells were harvested, washed, and resuspended in MM containing 5.15 mM ferulic acid. Incubation was performed at 30°C, and samples were taken and analyzed by HPLC. ▪, ferulic acid; ▲, vanillic acid; ♦, protocatechuic acid

Biotransformation of eugenol by a recombinant strain of *R. opacus* PD630

Since *R. opacus* PD630 was not able to degrade eugenol, we intended to establish the first biotransformation steps in this strain by genetic engineering. Hybrid plasmid pBBR KmNC903-SK*vao*mP*calA*m*calB*, harboring a gene cassette comprising the genes *vaoA* encoding vanillyl alcohol oxidase of *P. simplicissimum* CBS170.90 (which also converts eugenol to coniferyl alcohol), and *calA* and *calB* encoding coniferyl alcohol and coniferyl aldehyde dehydrogenase of *Pseudomonas* sp. HR199, respectively, under the control of the *lacZ* promoter was transferred to *R. opacus* PD630. One part of this hybrid plasmid (pSK*vao*mP*calA*m*calB*) has been successfully used for highly efficient biotransformation of eugenol to ferulic acid with recombinant strains of *E. coli* (Overhage et al. 2003). This plasmid was combined with the *Rhodococcus* vector pBBRK mNC903, and the resulting plasmid pBBRKmNC903-Sk*vao*mP*calA*m*calB* was mobilized from *E. coli* S17-1 to *R. opacus* PD630. Cells of the resulting transconjugant *R. opacus* PD630 (pBBRKmNC903-SK*vao*mP*calA*m*calB*) were analyzed in a biotransformation assay as described in "Materials and methods." Eugenol was converted with a rate of 1.22 mmol h^{-1} l^{-1} of culture (data not shown). The occurrence of the intermediates coniferyl aldehyde and ferulic acid in the culture supernatant was proven by HPLC analyses. When the wild-type strain *R. opacus* PD630 was treated in the same way (omitting kanamycin addition), no conversion of eugenol was observed.

Discussion

There is long-lasting experience in the application of members of the genus *Rhodococcus* for biotechnological processes (Warhurst and Fewson 1994). For example, acrylamide, which is one of the most important biotechnological products, is produced using *Rhodococcus* strains (Hughes et al. 1998). Thus, we were interested to investigate the potential of *Rhodococcus* strains for biotechnological vanillin production from ferulic acid and eugenol, which are suitable precursors for such a biotransformation. The degradation of these aromatic compounds has been studied in a variety of microorganisms, and a coenzyme A-dependent, non-β-oxidative pathway was identified as the most common pathway of ferulic acid degradation in bacteria (Priefert et al. 2001). The involved genes have been cloned and functionally characterized from Gram-negative bacteria (Gasson et al. 1998; Masai et al. 2002; Narbad and Gasson 1998; Overhage et al. 1999b; Plaggenborg et al. 2001, 2003; Priefert et al. 1997) and from a Gram-positive bacterium (Achterholt et al. 2000). These genes were applied in this study in an in silico approach to identify corresponding genes in *Rhodococcus* sp. I24 and *R. opacus* PD630. Candidate genes were amplified by PCR, cloned, and functionally characterized.

The results obtained for *R.opacus* PD630 suggest degradation of ferulic acid via the coenzyme A-dependent, non-β-oxidative pathway in this bacterium. Evidence is provided by (1) detection of feruloyl–CoA synthetase activity of the fcs_{PD630} gene product, (2) a ferulic acid-negative phenotype of the *fcs* knockout mutant *R. opacus* PD630*fcs*ΩKm, and (3) a ferulic acid-negative phenotype of the *vdh* knockout mutant *R. opacus* PD630*vdh*ΩKm. The inability of the vdh_{PD630}-negative mutant to degrade ferulic acid implies that vanillin is an obligate intermediate of ferulic acid catabolism, which is in agreement with the coenzyme A-dependent, non-β-oxidative pathway (Achterholt et al. 2000; Gasson et al. 1998; Masai et al. 2002; Narbad and Gasson 1998; Overhage et al. 1999b; Plaggenborg et al. 2001, 2003). However, mutant *R. opacus* PD630*vdh*ΩKm was still able to oxidize vanillin when provided as the substrate of the biotransformation. A similar effect has also been described for the *vdh* knockout mutants of *Pseudomonas* sp. HR199 (Overhage et al. 1999a) and *Pseudomonas putida* KT2440 (Plaggenborg et al. 2003) and was attributed to the activity of another aldehyde dehydrogenase. The latter is synthesized when vanillin is present in substrate concentration; however, it seems not to be induced during growth on ferulic acid, when vanillin is only transiently accumulated at low concentrations.

The in silico data obtained for *Rhodococcus* sp. I24 predicted the same pathway for ferulic acid degradation as in strain PD630 because the fcs_{I24} and vdh_{I24} gene products exhibited significant homologies to corresponding amino acid sequences from other bacteria (Table 3). However, Ech_{I24} exhibited in contrast only low similarities to known enoyl–CoA hydratases/aldolases but higher identities to classical enoyl–CoA hydratases with no aldolase or lyase function. This is remarkable because previous studies revealed high conservation of enoyl–CoA hydratases/aldolases among bacteria (Plaggenborg et al. 2001, 2003; Priefert et al. 2001), which is also obvious from Table 3. Functional analyses of Ech_{I24} provided further evidence that this enzyme is distinguished from previously described Echs. As expected, recombinant strains of *E. coli* harboring fcs_{I24} and ech_{I24} exhibited feruloyl–CoA synthetase activity. Moreover, proteins, whose sizes corresponded to the gene products of fcs_{I24} and ech_{I24}, were detected in the protein extracts by SDS-PAGE. However, these strains were not able to transform ferulic acid to vanillin, indicating lacking aldolase activity of the ech_{I24} gene product. Since feruloyl–CoA synthetase activity was biochemically proven for the fcs_{I24} gene product, we now propose a coenzyme A-dependent, β-oxidative pathway for ferulic acid degradation in strain I24, analogous to β-oxidation of fatty acids. This assumption is further strengthened by the localization of a gene directly downstream of ech_{I24} encoding a putative β-ketothiolase (Fig. 3), which is an essential enzyme in the β-oxidative pathway.

Current commercial processes for biotechnological vanillin production using *Amycolatopsis* sp. HR167 (Rabenhorst and Hopp 1997; Rabenhorst et al. 2003) and *Streptomyces setonii* (Muheim et al. 1998) suffer from the high price of natural ferulic acid, which is due to its limited accessibility from lignin by biological means. Eugenol, which is the main constituent of the essential oil of the

clove tree, represents a cheap alternative substrate (Priefert et al. 2001). In this study, we identified *Rhodococcus* sp. I24 as a potential biocatalyst for vanillin production due to its intrinsic capacity to degrade the vanillin precursor eugenol. The catabolism of eugenol, which proceeds via ferulic acid and vanillin, has been studied in detail in pseudomonads (Brandt et al. 2001; reviewed in Priefert et al. 2001). The initial conversion of eugenol to coniferyl alcohol is catalyzed by eugenol hydroxylase, which is encoded by *ehyAB* (Brandt et al. 2001; Furukawa et al. 1998; Priefert et al. 1999). However, analysis of the genome sequence of *Rhodococcus* sp. I24 revealed the absence of *ehyAB* homologous genes in this strain, indicating that a different enzyme is responsible for the hydroxylation of eugenol. In contrast to strain I24, *R. opacus* PD630 was not able to use eugenol as sole carbon and energy source. To establish the initial reactions leading from eugenol to ferulic acid in this strain, we took advantage of plasmid pSK*vao*mP*calA*m*cal*B, which had been successfully used for this purpose in *E. coli* (Overhage et al. 2003). By expression of the *vaoA* gene encoding vanillyl alcohol oxidase of *Penicillium simplicissimum* CBS 170.90 (which also converts eugenol to coniferyl alcohol), as well as the *calA* and *calB* genes encoding coniferyl alcohol and coniferyl aldehyde dehydrogenase of *Pseudomonas* sp. HR199, respectively, under the control of the *lac* promoter, the corresponding *E. coli* strain converted eugenol to ferulic acid with a molar yield of 91% (Overhage et al. 2003). For stable establishment of these genes in *R. opacus* PD630, plasmid pSK*vao*mP*calA*m*cal*B was combined with the *Rhodococcus* vector pBBRK mNC903. The resulting hybrid plasmid pBBRKmN C903-SK*vao*mP*calA*m*calB* was transferred to strain PD630, and the genes were expressed in the transconjugant, resulting in the production and transient accumulation of ferulic acid from eugenol. These results, and the successful inactivation of the vanillin dehydrogenase gene in mutant *R. opacus* PD630*vdh*ΩKm, are promising initial steps in the rational design of efficient biocatalysts for biotechnological vanillin production based on *Rhodococcus* strains.

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