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SHORT COMMUNICATION

Purification, characterization and gene cloning of isoeugenol-degrading enzyme from *Pseudomonas putida* IE27

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Abstract An isoeugenol-degrading enzyme was purified to homogeneity from *Pseudomonas putida* IE27, an isoeugenol-assimilating bacterium. The purified enzyme was a 55 kDa monomer and catalyzed the initial step of isoeugenol degradation, the oxidative cleavage of the side chain double-bond of isoeugenol, to form vanillin. Another reaction product of isoeugenol degradation besides vanillin was identified to be acetaldehyde. The values of *K*m and k_{cat} for isoeugenol were 175 μM and 5.18 s^{-1}, respectively. The purified enzyme catalyzed the incorporation of an oxygen atom from either molecular oxygen or water into vanillin, suggesting that the isoeugenol-degrading enzyme is a kind of monooxygenase. The gene encoding the isoeugenol-degrading enzyme and its flanking regions were isolated from *P. putida* IE27. The amino acid sequence of the enzyme was similar to those of lignostilbene-α,β-dioxygenases, carotenoid monooxygenases and 9-*cis*-epoxycarotenoid dioxygenases.

Keywords Isoeugenol · Vanillin · *Pseudomonas putida* IE27 · Oxygenation · Double-bond cleavage

Introduction

Lignin-related phenylpropanoides such as eugenol and isoeugenol have attracted attention as natural renewable sources for the production of useful chemicals (Robenhorst 1996). Eugenol has great potential as a starting material for the synthesis of aromatic flavorings and aromas. *Pseudomonas* and *Corynebacterium* strains degrade eugenol to vanillin via coniferyl alcohol, coniferyl aldehyde and ferulic acid (Takada 1977; Takada and Kayahara 1983). The initial step of eugenol degradation was confirmed to be the double-bond-transferring hydroxylation catalyzed by eugenol dehydrogenase (Furukawa et al. 1998; Wieser et al. 1999). The biotransformation of eugenol to ferulic acid was demonstrated, in which *Ralstonia eutropha* H16, carrying the genes encoding eugenol hydroxylase, coniferyl alcohol dehydrogenase and coniferyl aldehyde dehydrogenase was used (Overhage et al. 2002). The biotransformation using isoeugenol-degrading bacteria has recently been reported. *Bacillus* sp. cells degraded isoeugenol to vanillic acid via vanillin, and its cell-free extract transformed isoeugenol to vanillin (Shimoni et al. 2000). We demonstrated that the whole cells of *P. putida* I58 transformed isoeugenol into vanillic acid, without the accumulation of vanillin (Furukawa et al. 2003). We also reported that *P. putida* IE27 cells, a different soil isolate, produced vanillin from isoeugenol, with a high yield (Yamada et al. 2007). The initial step of isoeugenol degradation to vanillin is of interest because of oxidative cleavage of the side chain double bond of isoeugenol. However, the enzymes catalyzing the oxidative cleavage of isoeugenol have not been purified from isoeugenol-degrading microorganisms yet. In the present study, to clarify the reaction mechanism of the double-bond cleavage of isoeugenol, we described the first purification and characterization of isoeugenol-degrading enzyme from *P. putida* IE27, and performed the cloning and analysis of the enzyme gene and its flanking regions.

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Materials and methods

Microorganism and culture conditions

Pseudomonas putida IE27 was isolated from soils though the enrichment culture using isoeugenol as a sole carbon source and cultivated under the optimal conditions described previously (Yamada et al. 2007). The cells were harvested by centrifugation at 12,000*g* for 30 min, followed by washing once with 0.15 M NaCl, and then suspended in 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol and 10% (v/v) glycerol.

Enzyme assay

The reaction mixture contained 7 mM isoeugenol, 100 mM potassium phosphate buffer (pH 7.0), 10% (v/v) ethanol and an appropriate amount of enzyme, in a total volume of 1 ml. The reaction was started, by adding isoeugenol as ethanol solution, carried out at 30°C for 10 min with reciprocal shaking (160 strokes min^{-1}) and stopped with 1 ml of methanol. After centrifugation at 13,000*g*, the supernatant was analyzed by HPLC for the determination of isoeugenol and vanillin. One unit of enzyme was defined as the amount of enzyme catalyzing the formation of 1 μmol of vanillin per minute.

Acetaldehyde was determined after the derivatization using 2,4-dinitrophenylhydrazine as follows. The reaction mixture (100 μl) and 100 μl of 15 mM 2,4-dinitrophenylhydrazdine dissolved in 6 N HCl were mixed and incubated at 30°C for 10 min. After adding 0.6 ml of methanol to dissolve acetaldehyde 2,4-dinitrophenylhydrazone, the solution was analyzed by HPLC for the determination of acetaldehyde 2,4-dinitrophenylhydrazone.

Effect of pH was investigated using 100 mM of the following buffers: citrate–sodium citrate (pH 3.0–6.0), acetate–sodium acetate (pH 4.0–5.5), potassium phosphate (pH 6.0–8.0), tris–HCl (pH 7.5–9.0) and glycine–NaOH (pH 9.0–11.0). Effect of metal ions and coenzymes on enzyme activity was examined under the standard assay conditions containing the following compounds at 1 mM: LiOH, $MgCl_2$, $CaCl_2$, $MnCl_2$, $FeSO_4$, $FeCl_3$, $CoCl_2$, $NiCl_2$, $CuCl_2$, $ZnSO_4$, $NaMoO_4$, $AgNO_3$, $CdCl_2$, $BaCl_2$, Na_2WO_4, $HgCl_2$, NAD, NADH, NADP and NADPH. FMN and FAD were tested at 0.1 mM. The following compounds were investigated at 1 mM as to their inhibitory effects on the enzyme activity: *N*,*N*-diethyldithiocarbamate, EDTA, 1,10-phenanthroline, 2,2′-bipyridyl, Tiron, 8-hydroxyquinoline, cuprizone, NaN_3, ascorbic acid, semicarbazide, diethylamine, cysteamine, hydroxylamine, 3-(aminomethyl) prydine, phenylhydrazine, *N*-ethylmaleimide, iodoacetic acid, *p*-chloromercuribenzoic acid, *R*-cycloserine, 5,5-dithiobis, diethyl phosphoramidate and PMSF.

Enzyme purification and amino acid sequence

Potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol and 10% (v/v) glycerol (PDG buffer) was used unless otherwise specified. *Pseudomonas putida* IE27 cells (5.0 g dry weight) from 1.8 l of culture broth were suspended in 60 ml of 50 mM PDG buffer and disrupted with an ultrasonic oscillator (Insonator 201M, Kubota, Japan) at 100 W for 10 min. Cell-free extract was obtained after the removal of cell debris by ultracentrifugation with a Beckman Ultracentrifuge model L8-70M (Beckman Instruments, USA) at 50,000*g* for 120 min, and subjected to ammonium sulfate fractionation at 30–45% saturation. The precipitate was dissolved in 10 mM PDG buffer and dialyzed against the same buffer, and loaded on a CIM DEAE-8 tube monolithic column equilibrated with 10 mM PDG buffer. The enzyme activity was eluted with 75 mM PDG buffer. The active fractions were combined and placed on a second CIM DEAE-8 column equilibrated with 40 mM PDG buffer. After the column was washed with 60 mM PDG buffer, the enzyme activity was eluted with 75 mM PDG buffer. The active fractions were pooled, and ammonium sulfate was added to give 70% saturation. The resulting precipitate was dissolved in 10 mM PDG buffer containing 0.2 M KCl and applied to a Sephacryl-S200 HR column equilibrated with 10 mM PDG buffer containing 0.2 M KCl. The enzyme was eluted with the same buffer, and the active fractions were combined.

The purified enzyme was digested with *Achromobacter* proteinase I (lysyl endopeptidase). The resulting peptides were purified by reverse phase HPLC and analyzed by a protein sequencer (model 476A, PE Biosystem, Tokyo).

Identification of reaction products

Vanillin produced by *P. putida* IE27 cells was identified previously (Yamada et al. 2007). Another reaction product, acetaldehyde, was isolated as acetaldehyde 2,4-dinitrophenylhydrazone from the reaction mixture using purified isoeugenol-degrading enzyme. After the removal of vanillin and residual isoeugenol by extraction with chloroform, acetaldehyde was converted into its hydrazone derivative as described earlier. The hydrazone formed was collected by filtration, dissolved in ethyl acetate, and applied to a silica gel column using a 8:1 (v/v) mixture of *n*-hexane and ethyl acetate as the

eluent. The purified hydrazone derivative was dried and identified by ^{1}H-NMR and ^{13}C-NMR with the authentic acetaldehyde 2,4-dinitrophenylhydrazon as a reference. Acetaldehyde 2,4-dinitrophenylhydrazone was obtained as a yellow solid, ^{1}H-NMR (500 MHz, $CDCl_3$): δ 2.14 (3H, d, $J = 5.7$ Hz), 7.58 (1H, q, $J = 5.3$ Hz, CH), 7.93 (1H, d, $J = 9.1$ Hz), 8.29 (1H, dd, $J = 2.3, 9.7$), 9.11 (1H, d, $J = 2.3$), and 11.04 (1H, s) and ^{13}C-NMR (125 MHz, $CDCl_3$): δ 18.5, 116.4, 123.4, 128.7, 129.9, 137.7, 145.1, and 148.5.

Isolation of a genomic clone for isoeugenol-degrading enzyme from *P. putida* IE27

The PCR was performed to obtain a nucleotide fragment encoding the *P. putida* IE27 isoeugenol-degrading enzyme gene. An upstream primer, 5′-GC(ACGT)GG(ACGT)AC(ACGT)ATGTT(CT)CC(ACGT)AC-3′, was designed from the *N*-terminal amino acid sequence (corresponding to AGTMFPT) of the enzyme. A downstream primers, 5′-CC(ACGT)GT(CT)TG(ACGT)GG(AG)TC(AG)AA(CT)TT-3′, was designed from an internal amino acid sequence [corresponding to (K)FDPQTG] of the enzyme. A genomic DNA from *P. putida* IE27 was subjected to amplification with the above two primers. Cycling was performed as follows: denaturation at 94°C for 1 min, annealing at 55°C for 90 s and extension at 72°C for 60 s, for a total of 30 cycles. The PCR product with a size of 491 bp was purified and utilized as probe for Southern blot analysis and colony hybridization. *Pseudomonas putida* IE27 genomic DNA from was digested with *Pst*I or *Acc*I, and Southern blot hybridization was performed with the probe DNA labeled by the random-priming method using a luminescent detection kit (Roche Diagnostics). A *Pseudomonas putida* IE27 partial genomic library was prepared with pBluescript SK(+) on the basis of the results of Southern blot analysis, and recombinants were screened by colony hybridization. DNA sequencing was performed by the dideoxy chain-termination method using an automated DNA sequencer (Amersham Biosciences, Long-Read Tower).

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with accession number AB291707 as isoeugenol monooxygenase gene of *P. putida* IE27.

Analytical methods

Vanillin, isoeugenol and acetaldehyde 2,4-dinitrophenylhydrazone were determined by HPLC under the conditions described previously (Yamada et al. 2007). Protein concentration was determined by the methods of Bradford (1976), with bovine serum albumin as the standard. Molecular mass of the native enzyme was estimated by gel-permeation HPLC with a TSK G-3000 SW column (0.75 × 60 cm) at 0.7 ml min^{-1} with 0.1 M potassium phosphate buffer (pH 7.0) containing 0.2 M NaCl as the eluent. The molecular mass of the purified enzyme was calculated from the line of regression obtained from the mobilities of glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), adenylate kinase (32 kDa) and cytochrome *c* (12.4 kDa).

Materials

Isoeugenol and vanillin were obtained from Wako Pure Chemical Industries (Japan). CIM DEAE-8 tube monolithic column was purchased from Sanwa Tsusho (Japan). Molecular marker proteins for HPLC were purchased from Oriental Yeast (Japan). Low molecular weight markers for SDS-PAGE and Sephacryl-S200 HR were obtained from Amersham Pharmacia Biotech (Japan). TSK G-3000 SW column were from Tosoh (Japan). $^{18}O_2$ (99%) and $H_2^{18}O$ (95–98%) were obtained from Taiyo Toyo Sanso (Japan) and Cambridge Isotope Laboratories (USA), respectively. All other reagents were of analytical grade and available from commercial sources.

Results and discussion

Physicochemical properties of *P. putida* IE27 isoeugenol-degrading enzyme

When a cell-free extract of *P. putida* IE27 was stored in the absence of sulfhydryl-protecting reagents or glycerol, 70% of the initial activity was lost after a 7-day storage at 4°C. The enzyme activity was stabilized by adding 1 mM dithiothreitol and 10% (v/v) glycerol to some extent. Although the enzyme purification was performed in a short period, it was not easy to carry out without any loss of enzyme activity (Table 1). The overall purification of the enzyme was 19-fold with a yield of 8%. The purified enzyme gave one band with molecular a mass of 55 kDa on SDS-PAGE (Fig. 1). The molecular mass of the enzyme was estimated to be 69 kDa by gel-permeation TSK G-3000 SW column, indicating that *P. putida* IE27 isoeugenol-degrading enzyme is a monomer. The *N*-terminal amino acid sequence obtained for the purified enzyme was ATFDRNDPQLAGTMFPTRIEAN.

Table 1 Purification of isoeugenol-degrading enzyme from *P. putida* IE27

Step	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1})	Purification (fold)	Yield (%)
Cell-free extract	956	284	0.297	1	100
Ammonium sulfate fractionation	313	146	0.468	1.6	52
First CIM DEAE-8 tube	63.2	130	2.06	6.9	46
Second CIM DEAE-8 tube	10.4	47.5	4.59	15	17
Sephacryl-S200	4.0	22.1	5.53	19	8

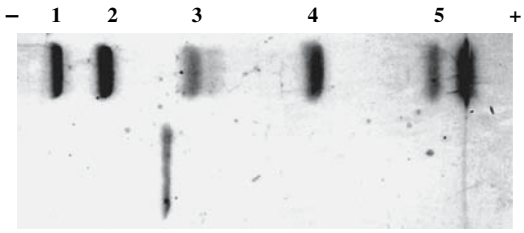

Fig. 1 SDS-PAGE of the purified isoeugenol-degrading enzyme. SDS-PAGE was carried out on 10% (w/v) polyacrylamide slab gels, which were strained with Coomassie brilliant blue R-250 (Laemmli 1970). Molecular mass maker proteins are phosporylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20.1 kDa). *Bottom*, 20 μg of the purified isoeugenol-degrading enzyme from *P. putida* IE27

The effects of temperature and pH on the enzyme were investigated. When the enzyme reaction was performed at various temperatures and under various buffer conditions (100 mM), the maximum activity was observed at 30°C and pH 9.0 (glycine–NaOH buffer). After the enzyme had been incubated at temperatures up to 10°C for 30 min, the activity was barely decreased. Treatment at 20, 30, 40 and 50°C caused 11, 20, 73 and 99% losses of the initial activity, respectively. The enzyme was stable during the incubation in a pH range of 6.0–10.5 at 20°C for 30 min.

Effect of various compounds on the enzyme activity was examined. The addition of $HgCl_2$, $AgNO_3$ and $CuCl_2$ severely inhibited the enzyme activity, 87, 73 and 56% of the activity being lost, respectively. Phenylhydrazine, 8-hydroxyquinoline, *R*-cycloserine and *p*-chloromercuribenzoic acid also inhibited the activity, the inhibition levels being 95, 90, 48 and 44%, respectively. The enzyme activity was not significantly affected by the addition of other tested compounds, including NAD, NADH, NADP, NADPH, FMN and FAD.

The purified enzyme catalyzed the oxidative cleavage of the double-bond of isoeugenol and 2-methoxy-4-vinylphenol. The activity toward 2-methoxy-4-vinylphenol was 1% of that toward isoeugenol. The values of *K*m and k_{cat} for isoeugenol were 175 μM and 5.18 s^{-1}, respectively. The k_{cat}/Km calculated to be 29.8 mM^{-1} s^{-1}. In our previous experiments using the whole cells of *P. putida* IE27, *trans*-anethole was transformed into *p*-anisaldehyde (Yamada et al. 2007). However, the present purified enzyme did not act on *trans*-anethole. The *trans*-anethole-degrading activity was also detected in the *P. putida* cells grown on nutrient medium not containing isoeugenol (data not shown). Thus, the double-bond cleavage of *trans*-anethole is probably catalyzed by other constitutive enzyme(s). The following compounds were inert as substrates: eugenol, coniferyl alcohol, coniferyl aldehyde, ferulic acid, 3,4-dihydroxycinnamic acid, 3,4-dimethoxycinnamic acid, 4-methoxycinnamic acid, 4-hydroxycinnamic acid, 3-pyridinepropionic acid, α-methoxycinnamic acid, styrene, *trans*-β-methylstyrene, *trans*-stilbene, *trans*-stilbene oxide, acrylic acid and *trans*-2-hexenoic acid.

Acetaldehyde formation and oxygen incorporation

Another reaction product besides vanillin, acetaldehyde, was isolated and identified as described in the "Materials and methods". After the enzyme was incubated with isoeugenol for 30 min, approximately equal moles of vanillin and acetaldehyde were formed by HPLC analysis (0.41 and 0.35 mM, respectively). Next, to verify the incorporation of oxygen atoms from molecular oxygen, isoeugenol conversion was examined anaerobically. Under nitrogen-exchanged conditions, no formation of vanillin was observed. When the enzyme reaction was performed in a sealed reaction vessel containing $^{18}O_2$, the mass spectrum of the vanillin formed was shifted by +2 *m*/*z* (Fig. 2b). In the reaction mixture containing $H_2^{18}O$, it was also shifted by +2 *m*/*z* (Fig. 2c). This indicates that the incorporation of an oxygen atom into vanillin is possible from either $^{18}O_2$ or $H_2^{18}O$. Lignostilbene oxygenase isozymes of *S. paucimobilis* TMY1009, the enzymes catalyzing the cleavage of C_α=C_β bond have been considered as dioxygenases, without examining the incorporation of oxygen atom from water (Habu et al. 1989). Carotenoid oxygenase is precisely named β-carotene 15,15′-monooxygenase, formerly also considered dioxygenase. Woggon et al. (2002) confirmed the reaction mechanism of oxidative cleavage of β-carotene by elegant experiments using $^{17}O_2$ and $H_2^{18}O$, in which β-carotene

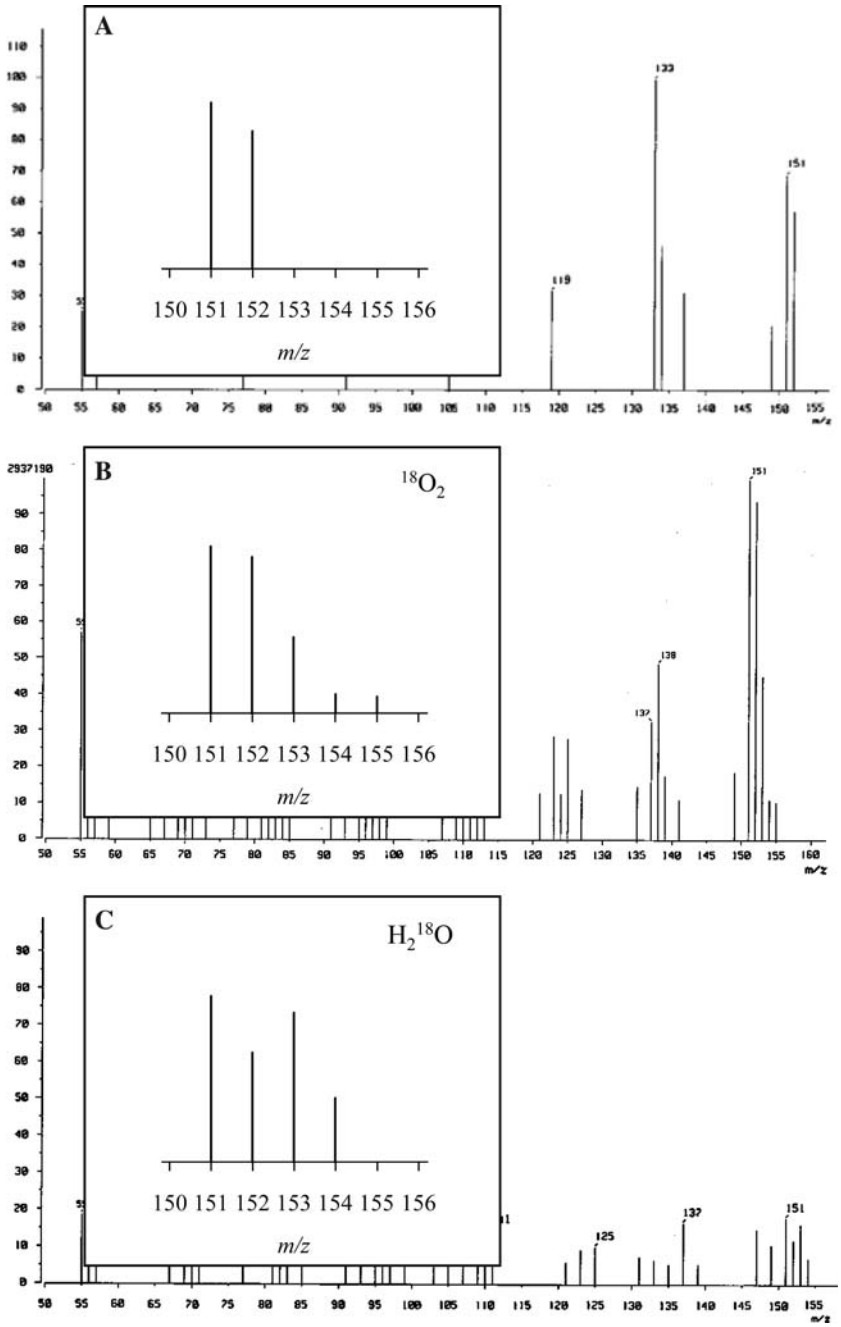

Fig. 2 Incorporation of oxygen atoms into vanillin. To verify the incorporation of oxygen atom from molecular oxygen and water into vanillin, the vanillin formed were isolated and analyzed by mass spectrometry. The enzyme reaction was performed under standard conditions (**a**). The reaction was performed in a sealed reaction vessel containing $^{18}O_2$ (**b**), or in the reaction mixture containing $H_2^{18}O$ at 87% (v/v) (**c**)

was cleaved into two molecules of retinal via the epoxide and diol by the monooxygenase. Recently, in the study on microbial degradation of isoeugenol by *Bacillus subtilis* HS8, Zhang et al. (2006) detected the intermediate as isoeugenol-diol, indicating that the epoxide-intermediate might be transiently formed and then hydrolyzed to isoeugenol-diol. On the basis of these observations, probably, isoeugenol degradation by *P. putida* IE27 is initiated by isoeugenol monooxygenase catalyzing epoxidation of the side chain double-bond and subsequent hydrolysis to form isoeugenol-diol, which can be cleaved into vanillin and acetaldehyde (Fig. 3). With respect to the side chain double-bond cleavage of isoeugenol by dioxygenation, Li et al. (2005) reported the biotransformation of isoeugenol into vanillin by soybean lipoxygenase, in which the intermediate might be its hydroperoxide (Bugg 2003) as shown in Fig. 3.

Cloning and nucleotide sequence of *P. putida* IE27 isoeugenol-degrading enzyme gene

A nucleotide fragment of the *P. putida* IE27 isoeugenol-degrading enzyme gene was amplified by PCR. On

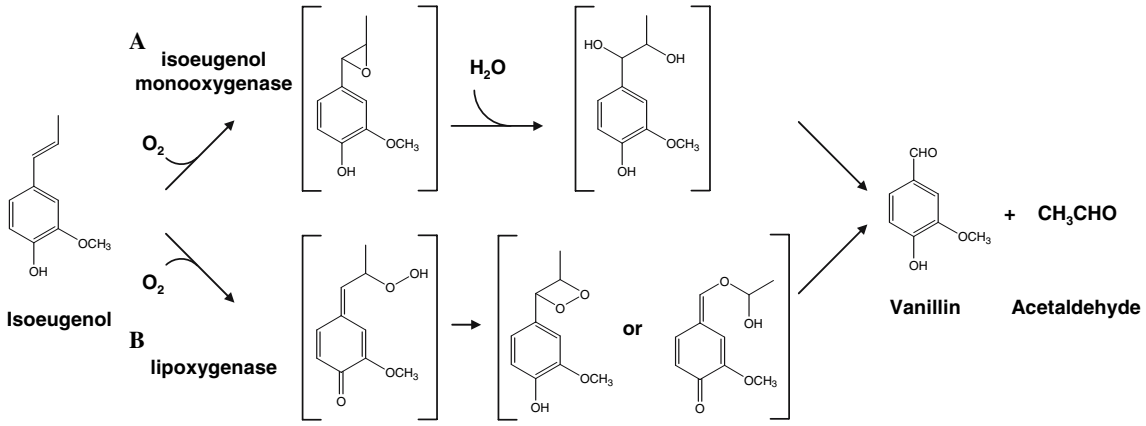

Fig. 3 Proposed mechanism for the cleavage of the side chain double-bond by isoeugenol monooxygenase (**a**) or lipoxygenase (**b**). In the reaction by lipoxygenase, isoeugenol hydroperoxide formed might be converted to vanillin via a dioxetane intermediate or by Criegee rearrangement (Bugg 2003)

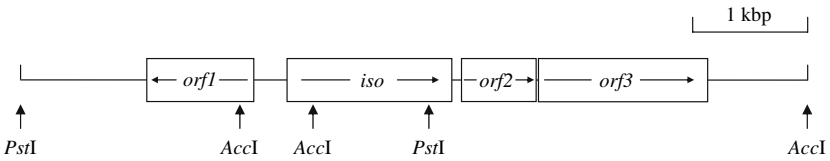

Fig. 4 Physical map of the chromosomal region containing isoeugenol-degrading enzyme gene and its flanking regions. The open reading flames found upstream and downstream of the isoeugenol-degrading enzyme gene (*iso*) are named as *orf1*, *orf2* and *orf3*, respectively

the basis of the results of Southern blot hybridization using the amplified gene fragment as a probe DNA, about 3.6 kb-*Pst*I and 4 kb-*Acc*I fragments were ligated to pBluescript SK (+), and the clones containing the isoeugenol-degrading enzyme gene were screened by colony hybridization. From the positive clones, the plasmid DNA was isolated and characterized. The 3.6 kb-*Pst*I fragment overlapped the 4 kb-*Acc*I fragment as shown in Fig. 4. The isoeugenol-degrading enzyme gene (*iso*) was composed of 1,434 bp, and coded for a protein of 478 amino acids with a molecular mass of 54,808 Da. This value is in agreement with the molecular mass determined on SDS-PAGE. The amino acid sequence of the enzyme exhibited 42, 42, 35, 42, 40 and 28% identities with those of lignostilbene-α,β-dioxygenase isozymes I (Kamoda and Saburi 1993) and III (Kamoda and Saburi 1995) of *Sphingomonas paucimobilis* TMY1009, lignostilbene-α,β-dioxygenase of *S. paucimobilis* SYK-6 (Hara et al. 2003), carotenoid oxygenases of *Rhodopseudomonas palustris* BisB18 (Q218T8) and *Novosphingobium aromaticivorans* DSM12444 (Q2GA76), and 9-*cis*-epoxycarotenoid dioxygenase of *Persea americana* (Chernys and Zeevaart 2000), respectively. The results of the homology search indicate that the isoeugenol-degrading enzyme of *P. putida* IE27 belongs to a polyene chain dioxygenase family described by Redmond et al. (2001). In the studies on microbial degradation of lignin-related compounds using *S. paucimobilis* TMY1009, a dehydrodivanillic acid-degrading bacterium, Kamoda et al. (1989) reported that lignostilbene-α,β-dioxygenase isozyme I catalyzed the conversion of isoeugenol with a low activity. Probably, the present isoeugenol-degrading enzyme is physiologically distinguishable from lignostilbene-α,β-dioxygenase isozymes I in its substrate specificity and enzyme induction, although its primary structure showed 42% identity with that of *S. paucimobilis* TMY1009 lignostilbene-α,β-dioxygenase isozyme I. In *S. paucimobilis* TMY1009 cells, lignostilbene-α,β-dioxygenases are constitutive enzymes. The isoeugenol-degrading enzyme of *P. putida* IE27 is strongly induced by isoeugenol (Yamada et al. 2007).

On analysis of the flanking regions of the isoeugenol-degrading enzyme gene of *P. putida* IE27, three open-reading frames (*orf1*, *orf2* and *orf3*) were found (Fig. 4). In the 5′-upstream region of isoeugenol-degrading enzyme gene, probable consensus sequences for the promoter were observed upstream of the transcription start site, i.e., -35 region, -10 region and Shine-Dalgarno sequence for the ribosomal binding site. The predicted amino acid sequence of *orf1* (936 bp) showed 30% identity with Cad gene transcriptional regulator of *Bradyrhizobium* sp. HW13 (Kitagawa et al. 2002). The gene product of *orf2* (648 bp)

showed 36% identity with aldolase of *Polaromonas naphthalenivorans* CJ2 (Jeon et al. 2006). The primary structure deduced from *orf3* (1,473 bp) showed 70, 67 and 42% identities with *p*-hydroxybenzaldehyde dehydrogenase of *P. putida* NCIMB9866 (Cronin et al. 1999), aldehyde dehydrogenase of *P. aeruginose* PAO1 (Stover et al. 2000) and vanillin dehydrogenase of *Caulobacter vibrioides* ATCC19089 (Nierman et al. 2001), respectively. Immediately downstream of isoeugenol-degrading enzyme gene of *P. putida* IE27, the gene for probable vanillin dehydrogenase (*orf3*) was found, suggesting that the genes for isoeugenol assimilation are co-regulated.

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