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Identification of a homogentisate-1,2-dioxygenase gene in the fungus *Exophiala lecanii-corni*: analysis and implications

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Abstract *Exophiala lecanii-corni* is a dimorphic fungus capable of degrading several volatile organic compounds (VOCs) including ethylbenzene, which has been classified as a hazardous air pollutant by the Environmental Protection Agency. In contrast to bacterial species, little is known about the mechanisms of fungal degradation of VOCs. The results described herein suggest a potential pathway for ethylbenzene degradation in *E. lecanii-corni* via styrene, phenylacetate and homogentisate. Consistent with this proposed pathway, a full-length homogentisate-1,2-dioxygenase gene (*EIHDO*) has been identified, cloned and sequenced. The nucleotide sequence of *EIHDO* consists of a 1,452-bp open reading frame encoding a protein with 484 amino acids. The expression of the gene product increases when grown on ethylbenzene, further suggesting that it could be involved in ethylbenzene degradation and may be responsible for the aromatic ring cleavage reaction. In addition, a 907-bp fragment isolated upstream from this gene shares 78% sequence identity at the amino acid level with the amino acid sequences of two fungal phenylacetate hydroxylase genes. This observation suggests that the genes responsible for ethylbenzene degradation

may be clustered. This research constitutes the first step towards a better understanding of ethylbenzene degradation in *E. lecanii-corni*.

Introduction

Industrial processes such as petrochemical processing, surface coating, printing, and other operations release large quantities of volatile organic compounds (VOCs). In the presence of sunlight, VOCs can react with nitrogen oxides to form ground level ozone, a reactive oxidizing gas that can cause severe eye, nose and throat irritation (USEPA 1999). Examples of VOCs released include gasoline compounds (benzene, toluene, ethylbenzene and toluene), styrene, and chlorinated organic compounds (perchloroethylene, trichloroethylene and vinyl chloride). In 2004, at least 49 major metropolitan areas in the United States violated, or were in danger of violating, the Environmental Protection Agency (EPA) ambient air quality standard for ozone (USEPA 2004). Approximately 4.7 million tons of hazardous air pollutants (HAPs) are emitted annually in the United States, many of which are suspected or known to cause adverse human health effects (USEPA 2002). Ethylbenzene has been classified as a HAP by the EPA. Moreover, according to the International Agency for Research on Cancer (IARC Working Group 2000), ethylbenzene is a potential carcinogen that has been shown to be tumorigenic in rats and mice (Stott et al. 2003; NTP 1999).

Biofiltration, a treatment technology in which a polluted stream is passed through a biologically active packed bed, has been used to treat gas streams that contain low concentrations of hazardous VOCs. Most vapor-phase bioreactor (VPB) research to date has focused on degradation by bacterial biofilms. Recent data suggest that fungi can remove higher VOC loadings and remain at optimal activity levels for longer periods than bacteria, even under adverse treatment conditions (Woertz et al. 2001; Moe and Qi 2004). However, few studies have focused on the biological pathways responsible for fungal degradation of VOCs. In ad-

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dition, most bacterial and fungal VPB research has examined a relatively small number of VOCs, primarily toluene and styrene (Morales et al. 2003; Moe and Irvine 2001; Woo et al. 2000; du Plessis et al. 1998; Juteau et al. 1999; Acuna et al. 1999).

Exophiala lecanii-corni is a black dimorphic fungus that is capable of degrading aromatic compounds including toluene and ethylbenzene as well as some non-aromatic hydrocarbons such as methyl propyl ketone (Woertz et al. 2001; Qi et al. 2002). To better understand the mechanisms of fungal degradation in VPBs, it is necessary to obtain more knowledge about the key enzymes responsible for the fungal metabolism of a range of VOCs. Herein, a potential degradative pathway for ethylbenzene is proposed, a gene involved in the pathway is described and expression of the gene examined when ethylbenzene is the sole carbon source. These results set the stage for future studies on fungal degradation of aromatic hydrocarbons in biological systems.

Materials and methods

Strains and culture conditions

E. lecanii-corni CBS 102400 was used as the parent strain for all experiments. *Escherichia coli* XL-Blue (New England Biolabs, Beverly, Mass.) was used as the host strain for all vector propagations, pBluescript II KS (+/-) (Stratagene, La Jolla, Calif.) was used for the construction of the *E. lecanii-corni* subgenomic library and for deriving subclones for sequencing, and pGEM-T Easy (Promega, Madison, Wis.) was used for PCR product propagation.

Bottle studies

E. lecanii-corni was cultured on sporulating plate medium consisting of 0.5 g/l dextrose, 10 g/l potassium acetate, 1 g/l yeast extract and 20 g/l agar. Spores were harvested by gently washing the cultures using sterile basal medium and transferred to 250-ml bottles. Each bottle was inoculated with 5×10^4 cells/ml as determined by hemacytometer count. Cells were grown up in 20-ml basal medium consisting of 1.0 g/l $(\text{NH}_4)_2\text{SO}_4$, 1.01×10^{-3} mg/l $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.25 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.24×10^{-3} $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.02 g/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.10×10^{-3} g/l $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.17×10^{-3} g/l $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 1.36×10^{-3} g/l $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.24×10^{-3} g/l $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and 0.58×10^{-3} g/l $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. Each test chemical (styrene, phenylacetate or ethylbenzene) was added aseptically as a neat liquid (2 μl) to individual bottles, which were then sealed with Teflon tape and a Minninet cap. For each chemical, a triplicate set of bottles was used. An additional triplicate set of bottles inoculated with *E. lecanii-corni* was autoclaved and served as the killed control. All chemicals were obtained from Sigma-Aldrich (St. Louis, Mo.). After the chemical in the bottles was consumed, an additional 2 μl of the organic compound was added to each bottle. This was repeated three to seven times depending on

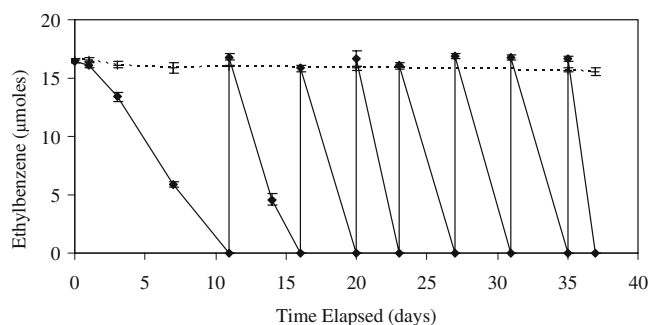


Fig. 1 Ethylbenzene depletion curve. *Solid lines* Bottles inoculated with *Exophiala lecanii-corni*, *dashed lines* killed controls. Each data point was obtained by averaging the amount of ethylbenzene remaining in each of three replicate bottles. An additional 2 μl ethylbenzene was added to each bottle upon depletion of the chemical. *Error bars* ± 1 SD

the depletion rate of the chemical. Headspace samples were collected regularly using a gas-tight syringe, and analyzed on a Hewlett Packard model 6890 gas chromatograph (GC) equipped with a flame-ionization detector and a HP-5 capillary column. Helium was used as the carrier gas at a flow rate of 1.8 ml/min. The make-up gas flow to the detector consisted of He (28.4 ml/min), H_2 (35 ml/min) and zero grade air (350 ml/min). The column temperature started at 75°C for 1 min, increased at a rate of 15°C/min to 130°C, and was maintained at 130°C for 1 min. The injector and detector temperatures were maintained at 250°C. The GC was calibrated using five bottles containing a known amount of each chemical to be analyzed. A 0.5-ml sample of each standard was injected into the GC, and a five-point calibration curve was produced.

Preparation and analysis of nucleic acids

E. lecanii-corni genomic DNA was isolated by ethanol precipitation after breaking the cells with glass beads as previously described for *Wangiella dermatitidis* (Liu et al. 2004). Southern blotting was performed using standard methods (Ausubel et al. 1989). *E. lecanii-corni* cells used for mRNA isolation were grown in a flow-through bubbler

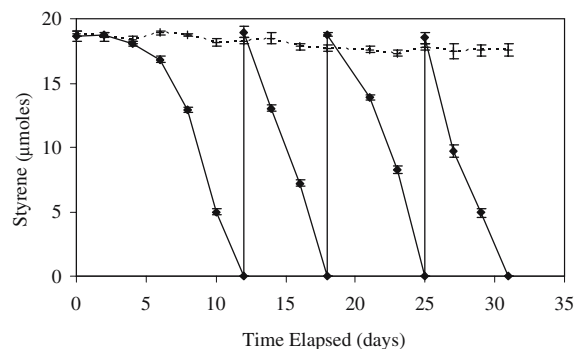


Fig. 2 Styrene depletion curve. *Solid lines* Bottles inoculated with *E. lecanii-corni*, *dashed lines* killed controls. Each data point was obtained by averaging the amount of styrene remaining in each of three replicate bottles. An additional 2 μl styrene was added to each bottle upon depletion of the chemical. *Error bars* ± 1 SD

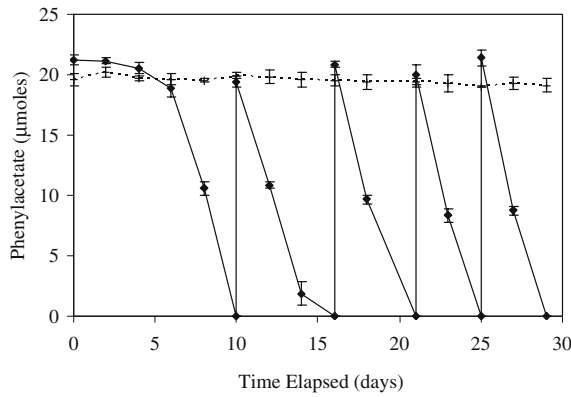


Fig. 3 Phenylacetate depletion curve. *Solid lines* Bottles inoculated with *E. lecanii-corni*, *dashed lines* killed controls. Each data point was obtained by averaging the amount of phenylacetate remaining in each of three replicate bottles. An additional 2 µl phenylacetate was added to each bottle upon depletion of the chemical. *Error bars* ±1 SD

system in the presence of 1,000 ppm (v/v) toluene or ethylbenzene. Cells were harvested in mid-log phase. mRNA was isolated using a Poly(A)Pure kit (Ambion, Bedford, Mass.). Equal amounts of mRNA (0.5 and 1 µg) were then transferred onto nylon membranes using a Slot Blot apparatus (Bio-Rad, Hercules, Calif.) and analyzed by northern hybridization using standard methods (Ausubel et al. 1989). A fragment obtained from PCR using degenerate primers was used to synthesize the probe, using a DECAprimeII Random Priming DNA labeling kit (Ambion). The PCR was performed on a GeneAmp PCR System 2700 (Applied

Biosystems, Foster City, Calif.) using primers designed from published, conserved amino acid regions identified in derived homogentisate dioxygenase proteins. The PCR fragment was cloned using a PGEM-T Easy Vector System (Promega). The fragment was then sequenced by the University of Texas Institute for Cellular and Molecular Biology Core Facility (Austin, Tex.) and sequence identity with other genes determined by a BLAST search of the NCBI nonredundant blastx database and aligned with other derived proteins using the DIALIGN2 (available at <http://www.genomatix.de/cgi-bin/dialign/dialign.pl>).

Construction and screening of a subgenomic library

E. lecanii-corni DNA was digested with the restriction enzyme *Xho*I to generate Southern blotting samples and probed with the *EIHD0* 500-bp PCR product to identify the region of DNA fragments that contained *EIHD0* using methods adapted from Cheng et al. (2004). Appropriately sized DNA fragments were collected from an agarose gel using a gel extraction kit (Qiagen, Valencia, Calif.). DNA fragments were then ligated into pBluescript vectors that had been digested by the same restriction enzyme used to generate the library, and then dephosphorylated. Ligation products (1–5 µg DNA) were introduced into *E. coli* XL-Blue competent cells by electroporation. Transformed cells were spread on LB agar medium containing ampicillin and the resulting library was subjected to screening using the *EIHD0* 500-bp PCR product as a probe.

Fig. 4 Alignment of full *Exophiala lecanii-corni* homogentisate dioxygenase amino acid sequence with published sequences from *Emericella nidulans* and *Magnaporthe grisea*. *Underlined sequence* Fragment obtained with the degenerate PCR primers. * Amino acids either identical or similar among the three species, *lower case letters* Amino acids considered not to be aligned, *upper case letters* amino acids considered as aligned

Exophiala	1	MPVTQFAVTD	TYEYLNFGfg	skd-EALKGA	LPLAANSPOK	CP1GLYTEKL
Emericella	1	MPVTEFSFKD	PYTYQNGFDS	YHESEALEGA	LPVGHNSPOK	APYGLYAEKL
Magnaporthe	1	MPATEFEFKE	KYRYQNGFDS	YLESEAVPGA	LPIAQNSPOK	PPHGLYAEKL
		**** * *	* * * * *	* * * * *	* * * * *	* * * * *
Exophiala	50	SGTAFATAPRD	ENQQSWLYRI	LPSASH----	-----	QNFEPVPDSS
Emericella	51	SGTAFATAPRH	ENKQTWVYRI	LPAAAH----	-----	ENFveedass
Magnaporthe	51	SGTAFATAPRN	ENKQSWLYRI	LPSCAHppfr	vkqgqagehdv	ENQPPTTPIP
		*****	** *****	** * *		* * *
Exophiala	86	PNSltsstpps	fgkhfna---	-----	IPNQLRWDPF	DFDRESSWIH
Emericella	87	yhtlsdak--	-----	KLQH	IPNQLRWDPF	DLDetvDWWH
Magnaporthe	101	PGQpltpgqq	pqhehkdemo	rfaplsRLHY	IPNQLRWDPF	DHDPQSDFFS
		*			*****	* * *
Exophiala	123	SLHLVAGAGD	PTMKQGLGMY	IYAAGSTMPD	KSAFYSADGD	FLIVPQHGVL
Emericella	119	GLHLVAGSGD	PTVKQGLGIL	LYAAGKDMg-	KEAFYSADGD	FLIVAQHGVL
Magnaporthe	151	GLHLIAGAGE	PTLKHGIGMF	VYAAGKSMST	SSAFYSADGD	LLIVAQSGVL
		*****	** *****	*****	*	*****
Exophiala	173	DIRTEFGKML	VRPNEICVIP	RGIRYHVALH	Dne-PVRGYI	MELYQGHFKL
Emericella	168	DIQTELGRL	VRPNEICVIP	RGVRYRVTLP	DG--PVRGYI	CELYQGHVQL
Magnaporthe	201	DIRTELGwLL	VRPLEIAVIP	RGIRFQVLLP	EGtgPARGYA	LELYQGHFAL
		** * * *	** * * * *	*****	* * *	*****
Exophiala	222	PELGPIGSNG	LANARDFQTP	VADFiEDHen	TEWTLYG---	-----KF
Emericella	216	PELGPIGSNG	LANARDFQAP	VAAFdDEEGP	TEYRLYS---	-----KF
Magnaporthe	251	PELGPIGSNG	LANARDFQAP	VACFsEDHGP	TAFsagpsap	rdgyevtaKF
		*****	*****	** * *	* * *	**
Exophiala	261	GGNLFPAKQS	HTPFDIVAWH	GTYYPKYDL	GRFNVPVGSIS	YDHPDPSIFT
Emericella	255	NNHLFSARQD	HTPFDIVAWH	GNYYPKYDL	GRFNTMGSVS	FDHPDPSIYT
Magnaporthe	301	NNTLFATRQA	HTPFDVVAWH	GNYYPFKYDL	GRFNTIGAIS	YDHPDPSIFT
		** *	*****	*****	*****	*****
Exophiala	311	VLTApsiphg	AGTAVADFVI	FPPRWLVAEN	TFRPPWYHRN	TMSEFMGLIM
Emericella	305	VLTApsidh--	AGTAVADFVI	FPPRWLVAEK	TFRPPWYHRN	TMSEFMGLIT
Magnaporthe	351	VLTApsidh--	AGTAVADFVI	FPPRWLVGED	TFRPPWYHRN	TMSEFMGLIT
		*****	*****	*****	*****	*****
Exophiala	361	GNYDAKggagk	G--GFQPAGA	SLHNMTSGHG	PDMQTFERAS	TMELAPQKVG
Emericella	353	GNYDAKTG--	G--GFQPAGA	SLHNIMSAGH	PDMHAFEGAS	NADLKPTKIG
Magnaporthe	399	GDYDAKKG--	GkgGFVPGGA	SLHNVMSSHG	PDAASYEAGAR	EAELKPAKVG
		* * * * *	* * * * *	* * * * *	* * * * *	* * * * *
Exophiala	409	EGSMAFMFES	CLMVGVTWEW	LKTCQKVQES	YNAHWSGPKL	VHFKRPEtsr
Emericella	399	DGSMAFMFES	SIMVGVSEWG	LKTCQKVQEE	YNEHWSQPKL	RHFkdpkraq
Magnaporthe	447	AGSCAFMFES	CFMVGVTDWG	LRTCQKVQEG	YSQESWGGVK	TWVKRPEgas
		*****	*****	*****	** * * *	** *

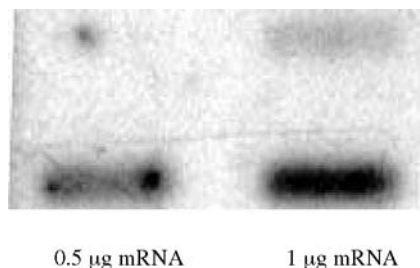


Fig. 5 Northern slot blot analysis of mRNA extracted from toluene- (*top*) and ethylbenzene- (*bottom*) grown cells using the homogentisate-1,2-dioxygenase PCR fragment as probe

Results

Batch reactor studies

As shown in Figs. 1, 2 and 3, the concentrations of ethylbenzene, styrene and phenylacetate decreased over a period of 2–20 days when incubated with *E. lecanii-corni*. By comparison, autoclaved controls showed no decrease in the

Fig. 6 Alignment of partial *Exophiala lecanii-corni* phenylacetate hydroxylase amino acid sequence with published sequences from *Penicillium chrysogenum* and *Emericella nidulans*. * Amino acids that are either identical or similar between the different species, lower case letters Amino acids considered not to be aligned, upper case letters amino acids considered as aligned

Exophiala	1	gspglqefdi	klidt-----	-----	-----	-----	-----
Penicillium	1	MAIQTLAVAV	ITVVYFVIRY	FNRTDIPKIK	GLPEIPGIPI	FGNLLQLGDQ	
Emericella	1	MSLQITIGIAA	VAVVYFLIRY	FNRTDIPKIK	GLPEVPVGIPI	FGNLLQLGDQ	
		* *	* *				
Exophiala	16	-----	-----	-----	-----	-----	-----
Penicillium	51	HATVTGKWKAK	KFGPVFQVRM	GNKRIVFANG	FDSVRQLWIK	DSSALISRPT	
Emericella	51	HATVAQKWKAK	KFGPVFQVRM	GNKRIVFANG	FDSVRQLWIK	DSSALISRPT	
Exophiala	16	-----	-----	-----	-----	-----	-----VDL
Penicillium	101	FHTFHSVSS	SQGFTIGTSP	WDDSCCKRRK	AAATALNRPA	VQSYMPIIDL	
Emericella	101	FHTFHSVSS	SQGFTIGTSP	WDDSCCKRRK	AAATALNRPA	TQSYMPIIDL	***
Exophiala	19	ESCVAIKDMY	KCMDGTVDL	DPRKYFHRFA	LNTSLTLNYG	IRIDGGIDQE	
Penicillium	151	ESNSSIKELY	RDSQNGKRDV	NPTAYFQRYA	LNTSLTLNYG	FRIEGNVDDT	
Emericella	151	ESMSSIRELL	RDSANGTMDI	NPTAYFQRYA	LNTSLTLNYG	IRIEGNVNDI	
		** ***** *	* ** * ** *	*****	*****	*****	*****
Exophiala	69	LLREgenhpr	icgclspvvh	VERVSNFRS	TSNNWQYIIP	LLRLpfisRQ	
Penicillium	201	LLHE-----	-----IVD	VERGVSNFRS	TSNNWQYIIP	LLRIFP--KM	
Emericella	201	LLRE-----	-----IVD	VERGVSNFRS	TSNNWQYIIP	LLRIFP--KM	
		****	**	*****	*****	**** *	
Exophiala	119	NKSAEYRAR	RDKYLTFPLD	MLKDRIAKGT	DRPCITGNII	KDPEETLNDI	
Penicillium	236	NNEAADFRGR	RDKYLTYLLD	MLKDRIAKGT	DKPCITGNIL	KDPEAKLNDI	
Emericella	236	NNEAEPRFR	RDKYLTYLLD	VLKDRIAKGT	DKPCITGNIL	KDPEAKLNDI	
		* * ***** *	***** *	*****	*****	*****	*****
Exophiala	169	ELKSICLTMV	SAGIDTVPGN	MIMGLGYLAS	PEGQHIQKKA	YDEVMKVYPn	
Penicillium	286	EVKSICLTMV	SAGLDTVPGN	LIMGIAYLAS	EDGQRIQKKA	YDAIMEVYP-	
Emericella	286	EIKSICLTMV	SAGLDTVPGN	LIMGIAYLAS	EDGQRIQKRA	HDEIMKVYP-	
		*****	*****	*****	*****	* ** **	
Exophiala	219	DGEAWEKCLV	EKVPYVITAL	TKEILRFWTV	IPVCLPRTSi	kdiklqrrxs	
Penicillium	335	DGDAWEKCLV	EKVPYVITAL	VKEVLRFWTV	IPICLPREST	KDIQWNGATF	
Emericella	335	DGDAWEKCLL	EKVPYVITAL	VKETLRFWTV	IPICLPRENT	KDIVWNGAVI	
		*****	*****	** *****	***** *	**** *	
Exophiala	269	yrAGSVFYMN	AW-----	-----	-----	-----	
Penicillium	385	P-AGTTFYMN	AWAADYDEDH	FkdadkFIPE	RYLEASE-GA	GTPHYAYGAG	
Emericella	385	P-KGTTFFMN	AYAADYDETH	FtnphaFEPE	RYLTASsdGS	GTPHYGYGAG	
		** ***** *					
Exophiala	281	-----	-----	-----	-----	-----	
Penicillium	433	SRMCAGSHLA	NRELFYAFIR	LVTAFNMHtA	KETADRPILN	AIECNLIPTA	
Emericella	434	SRMCAGSHLA	NRELFYAYVR	LITAFNMHtA	KRAEDRPILD	AIECNAIPTA	
Exophiala	281	-----	-----	-----	-----	-----	
Penicillium	483	LTTEPKPFKV	GFSARDPkk1	eQWIAESDER	TKDL-		
Emericella	484	LTTEPKPFKV	GFKPRDPv1v	rKWIAESEER	TKHLn		

concentration of these compounds. In addition, a significant increase in visible biomass was observed when each compound was provided as the sole carbon source. These observations suggest that *E. lecanii-corni* might initiate ethylbenzene degradation by transformation to styrene in the manner of *Pseudomonas* sp. NCIB 9816-4 (Lee and Gibson 1996), and further degrading styrene using reactions such as those described in *Pseudomonas putida* U and *P. putida* CA-3, both of which are capable of degrading styrene via phenylacetate and homogentisate (O'Connor et al. 1995; Olivera et al. 1994, 1998; O'Leary et al. 2001).

PCR amplification of an EIHO gene fragment

Published conserved regions of homogentisate dioxygenase gene sequences were aligned using DIALIGN, and sections with high sequence identity were used as templates to design degenerate primers. The genes used for this alignment were those from *Emericella nidulans* (NCBI accession number U30797), *Xanthomonas campestris* (NC003902),

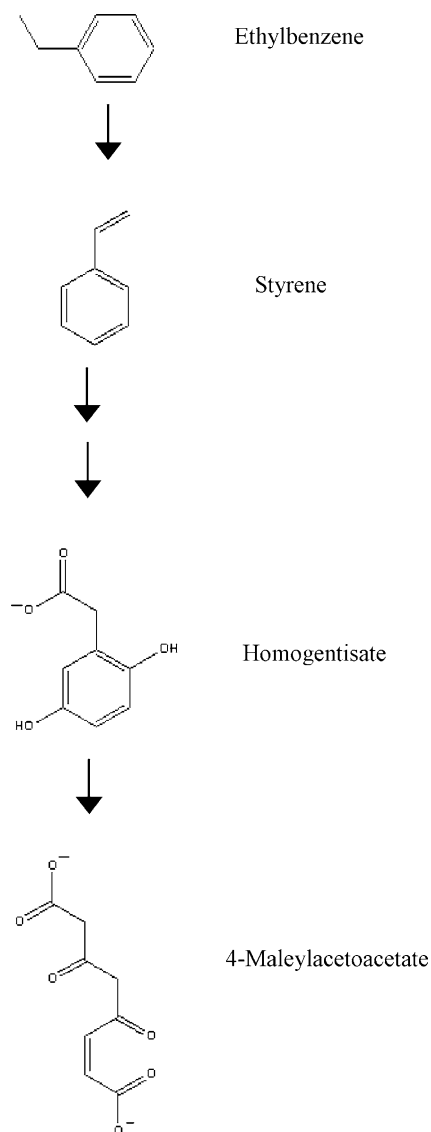


Fig. 7 Putative ethylbenzene degradation pathway in *E. lecanii-corni*

P. putida (AE016791) and *Vibrio vulnificus* (NC004459). The forward primer sequence used was 5'-GAT GGC GAY TTC YTG ATY GT-3' and the reverse primer sequence was 5'-CCA KCG YCG YGG AAA RAT RAC A-3', where Y, K and R represent equal amounts of G/T, C/T and A/G, respectively. A PCR product approximately 500 base pairs in length was obtained and cloned. This product contained a 360-bp fragment showing 83% similarity with the amino acid sequences coded by the *E. nidulans* homogentisate-1,2-dioxygenase gene (NCBI accession number U30797). The gene was therefore named *EIHD0*. The alignment is shown in Fig. 4.

Expression studies

To determine whether *E. lecanii-corni* ethylbenzene degradation might occur via styrene and homogentisate, an

expression study comparing levels of homogentisate-1,2-dioxygenase mRNA production was performed for toluene-grown and ethylbenzene-grown cells using the above PCR product as a probe. The results show that for identical mRNA levels, *EIHD0* is present at a higher concentration when *E. lecanii-corni* cells were grown on ethylbenzene as compared to toluene when all other growth conditions remained the same (Fig. 5). This observation indicates that ethylbenzene induces the production of homogentisate-1,2-dioxygenase and suggests that this enzyme is involved in ethylbenzene degradation, consistent with the results of the phenylacetate and styrene depletion study.

Gene isolation by subgenomic library screening

Several clones with sequence identity to the *EIHD0* probe were obtained. One clone, pCQHDO1, was selected and sequenced. It was found to contain an insert approximately 8-kb in length including the full *EIHD0* gene, which was cloned and sequenced. The sequence consists of a 1,452-bp open reading frame, encoding a protein with 484 amino acids, which is interrupted by a 59-bp intron in the 5' region and a 52-bp intron in the 3' region (positions 68–127 and 1,371–1,423, respectively). The complete amino acid sequence is shown in Fig. 4. The sequence information has been deposited with GenBank and assigned accession no. AY509194. In addition, a 907-bp fragment on the 5' side of the insert was sequenced and found to be similar to fungal phenylacetate hydroxylase genes (Fig. 6). The *E. lecanii-corni* fragment displayed 77 and 78% amino acid identity with the phenylacetate hydroxylase gene product in *Penicillium chrysogenum* (accession no. AAF21760) and *E. nidulans* (CAB43093), respectively.

Discussion

In general, bacterial aromatic compound degradation pathways are divided into an upper and lower pathway. The upper pathway consists of an initial oxidation of the aromatic ring catalyzed by either a monooxygenase or a dioxygenase and the subsequent transformation of the compound to a common intermediate such as catechol, protocatechuate, gentisate or homogentisate. In the lower pathway, the ring is cleaved by incorporating two oxygen atoms into the substrate. This reaction is generally mediated by a dioxygenase (Jenkins and Dalton 1985; Overhage et al. 1999).

Several ethylbenzene degradative pathways have been studied in *Pseudomonas* and *Rhodococcus* spp. (Shirai and Hisatsuka 1979; Warhurst et al. 1994). To date, ethylbenzene degradation in bacteria has been described to follow three different pathways. The first pathway proceeds through 2,3-dihydroxyethylbenzene, the second through styrene and the third through acetophenone. On the fungal side, studies of aromatic ring degradation have focused on the lower pathway's degradation mechanisms (Cox et al. 1996; Fernández-Cañón and Peñalva 1995). Of particular relevance to this research is the previous isolation of a homogentisate-1,

2-dioxygenase gene in *Emericella nidulans*, which is also one of the genes shown to be involved in one of the ethylbenzene bacterial degradation pathways (Fernández-Cañón and Peñalva 1995). Similarly, studies performed with *Pleurotus ostreatus* and with *Exophiala jeanselmei*, a fungus closely related to *Exophiala lecanii-corni*, showed that both fungi are capable of styrene degradation and utilize a pathway similar to the bacterial ethylbenzene degradation pathway, which proceeds via styrene, phenylacetate and homogentisate (Lee and Gibson 1996; Cox et al. 1996; Olivera et al. 1994; Hagedorn and Chapman 1985; Crawford 1976). However, in contrast to *E. lecanii-corni*, *E. jeanselmei* is not able to metabolize ethylbenzene. No information on the ability of *P. ostreatus* to degrade ethylbenzene has been published.

Homogentisate-1,2-dioxygenase is commonly linked to phenylalanine and tyrosine catabolism in eukaryotic organisms (Fernández-Cañón and Peñalva 1995, 2002; Granadino et al. 1997). The results from this study suggest that the enzyme might also be involved in the fungal metabolism of aromatic compounds. *E. lecanii-corni* was capable of degrading and growing on ethylbenzene and other metabolites (styrene and phenylacetate) when these substrates were supplied as the sole carbon sources. In addition, the homogentisate-1,2-dioxygenase gene was induced by the presence of ethylbenzene, suggesting that this enzyme is responsible for ethylbenzene ring cleavage in *E. lecanii-corni*. Figure 7 shows the key steps in the proposed ethylbenzene degradation pathway in *E. lecanii-corni*, proceeding via styrene, phenylacetate, and homogentisate. The presence of a DNA sequence similar to that of the gene for phenylacetate hydroxylase upstream from the homogentisate-1,2-dioxygenase sequence indicates that these genes may be clustered in the same way as in other fungi. Efforts are currently underway to identify these genes.

To our knowledge, this is the first report of a fungus having the capacity to degrade ethylbenzene being linked to a homogentisate-1,2-dioxygenase enzyme. Several fungi have been suggested to use a similar pathway to degrade styrene. According to Cox et al. (1996), *E. jeanselmei* degrades styrene via styrene oxide, phenylacetaldehyde, phenylacetate, 2-hydroxy phenylacetate and homogentisate. In addition, (Braün-Lulleman et al. 1997) found that 2-phenylethanol was an intermediate in styrene and ethylbenzene degradation for *P. ostreatus*. Because phenylacetate reacts abiotically to form 2-phenylethanol quite readily, it is possible that *P. ostreatus* also degrades ethylbenzene by a similar pathway. However, *E. jeanselmei* did not degrade ethylbenzene and no additional reports are available for the other fungi that degrade styrene.

Gene expression studies in environmental engineering are limited by the number of relevant genes that have been sequenced and characterized. The isolation of genes involved in contaminant metabolism and co-metabolism will ultimately lead to a better understanding of microbial degradation of VOCs and enable future monitoring of gene expression in environmental systems.

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