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An unexpected gene cluster for downstream degradation of alkylphenols in *Sphingomonas* sp. strain TTNP3

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Abstract In silico analysis of nucleotide sequences flanking the recently found hydroquinone dioxygenase in *Sphingomonas* sp. strain TTNP3 revealed a gene cluster that encodes a hydroquinone catabolic pathway. In addition to the two open-reading frames encoding the recently characterized hydroquinone dioxygenase, the cluster consisted of six open-reading frames. We were able to express the three open-reading frames, *hqdC*, *hqdD*, and *hqdE*, and demonstrated that the three gene products, HqdC, HqdD, and HqdE had 4-hydroxymuconic semialdehyde dehydrogenase, maleylacetate reductase, and intradiol dioxygenase activity, respectively. Surprisingly, the gene cluster showed similarities to functionally related clusters found in members of the β- and γ-proteobacteria rather than to those found in other members of the genus *Sphingomonas* sensu latu.

Keywords Hydroquinone · Degradation · Sphingomonas · Nonylphenol · Bisphenol A

Introduction

Bisphenol A (BPA) and nonylphenol (NP) are well-known environmental contaminants that exert adverse effects on the reproductive system and the development of organisms (vom Saal and Hughes 2005; Soares et al. 2008). Both compounds have been widely detected in surface waters in concentrations up to several microgram per liter, i.e. levels that already may cause biological effects (Soares et al. 2008; Klecka et al. 2010; vom Saal and Hughes 2005; Raecker et al. 2011). Fortunately, under aerobic conditions, BPA and NP may both be degraded by various microorganisms (Kolvenbach et al. 2007; Gabriel et al. 2007a, 2008; Kang et al. 2006; Corvini et al. 2006b). Besides further restricting the use of BPA and nonylphenol polyethoxylates, optimization of biological wastewater treatment techniques, based on a better understanding of biodegradation mechanisms, may hence contribute to reduce current pollutant levels. We

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recently have characterized an *ipso*-substitution pathway that is used by several sphingomonads, i.e., members of the genus *Sphingomonas* sensu *latu*, to degrade nonylphenols and bisphenol A and that yields hydroquinone as an intermediate (Gabriel et al. 2005b, 2007a, b); Corvini et al. 2006a; Kolvenbach et al. 2007.

Sphingomonads are frequently isolated from the environment as potent degraders of a wide range of xenobiotics, such as PAH (Zhao et al. 2008; Schuler et al. 2009), γ-HCH (Manickam et al. 2008), pentachlorophenol (Yang et al. 2006), herbicides, such as 2,4-D (Shimojo et al. 2009), isoproturone (Hussain et al. 2011), and alkylphenolic disruptors (Gabriel et al. 2005a; Sakai et al. 2007; Sasaki et al. 2005). Among bacteria isolated from the environment, sphingomonads have a special status as their genes show a relatively low degree of sequence homology with homologous genes from other *Proteobacteria*. This indicates that flow of genetic information between sphingomonads and other genera is restricted (Stolz 2009).

In sphingomonads, hydroquinone is formed as a metabolite during degradation of 4-nitrophenol and, as a minor metabolite, during that of γ-hexachlorohexane (Leung et al. 1997, 1999; Endo et al. 2005). The hydroquinone dioxygenases found in these strains are of the LinE or PcpA type and are encoded by a single open-reading frame (Miyauchi et al. 1999; Ohtsubo et al. 1999). In the corresponding gene clusters, the open-reading frame encoding for the hydroquinone dioxygenase is located near that of maleylacetate reductase, which is transcribed in the reverse direction. The two genes are separated by a sequence assigned to a putative LysR-type transcriptional regulator (Endo et al. 2005). Other hydroquinone dioxyenases have also been found clustered with sequences coding for glutathione S-transferases associated with upstream steps of the lindane degradation pathway (Nagata et al. 2006).

In β- and γ-proteobacteria, hydroquinone was found as a metabolite in the degradation of 4-hydroxyacetophenone (Moonen et al. 2008) and 4-nitrophenol (Wei et al. 2010; Shen et al. 2010). The identified clusters contained open reading frames (putatively) coding for the two subunits of a heterotetrameric hydroquinone dioxygenase, a 4-hydroxymuconic semialdehyde dehydrogenase, a maleylacetate reductase, an intradiol dioxygenase and a ferredoxin (Shen et al. 2010; Wei et al. 2010; Moonen et al. 2008; Zhang et al. 2009).

Recently, the hydroquinone dioxygenase from *Sphingomonas* sp. strain TTNP3 has been purified. Unlike all other hydroquinone dioxygenases identified in sphingomonads so far, the enzyme is an $\alpha_2\beta_2$ heterotetramer and is encoded by two open-reading frames (*hqdA* and *hqdB*; Kolvenbach et al. 2011).

Here, we present the partial characterization of a *Sphingomonas* sp. strain TTNP3 gene cluster involved in hydroquinone degradation. Besides containing the genes coding for the hydroquinone dioxygenase, i.e. *hqdA*, *hqdB*, and additional open-reading frames encoding functional enzymes of the hydroquinone degradation pathway. Remarkably, in terms of arrangement and sequence of genes, the TTNP3 hydroquinone dioxygenase cluster resembles functionally related clusters contained in β- and γ-proteobacteria and is dissimilar to those in α-proteobacteria, such as sphingomonads.

Materials and methods

Genomic data and sequence accession numbers

Partial genomic data of *Sphingomonas* sp. strain TTNP3 has been as described elsewhere (Kolvenbach et al. 2011). The nucleotide and amino acid sequences reported here have been deposited at GenBank under accession number JF440299.

Chemicals

Tris, nicotinamide adenine dinucleotide (NAD) and the reduced salt of NAD (NADH) were obtained from Applichem (Axonlab, Switzerland). A technical nonylphenol mixture, hydroquinone, catechol, and hydroxyhydroquinone were obtained from Sigma (Switzerland).

In silico analysis of genomic sequences of *Sphingomonas* sp. strain TTNP3

Open-reading frames were identified using the GeneMark. hmm for Prokaryotes algorithm, version 2.4 (Lukashin and Borodovsky 1998). Comparisons of amino acid or nucleotide sequences were performed with the respective Basic Local Alignment Search Tool (BLAST) programs on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/; Altschul et al. 1997).

Bacterial strains and culture conditions for *Sphingomonas* sp. strain TTNP3

Sphingomonas sp. strain TTNP3 (Table 1) (deposited at BCCM under accession number LMG 21268) was grown on Standard I Medium as described previously (Corvini et al. 2004). Cells were harvested at an OD_{550} of about 3.0. If not stated otherwise, enzymatic activity was induced by addition of 0.5 mM technical grade nonylphenol 16 h prior to harvesting. Cultures were then centrifuged at 4,500×*g* for 15 min and resuspended in 50 mM Tris buffer (pH 7.0) at 4°C. The washing procedure was repeated twice before the

Table 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Characteristics	Source or reference
Strains		
Sphingomonas sp. strain TTNP3	Wild-type, nonylphenol degrader	Tanghe et al. (1999)
E. coli		
DH5α	Amplification of plasmids	NEB
BL21	Expression strain	Invitrogen
Plasmids		
pGEM-T easy	T-A clone vector	Promega
pGEM-hqdC	pGEM derivative bearing hqdC	This work
pGEM-hqdD	pGEM derivative bearing hqdD	This work
pGEM-hqdE	pGEM derivative bearing hqdE	This work
pGEM-con	pGEM derivative bearing the Promega control insert	This work

cells were resuspended to attain a dry weight concentration of 33 mg/mL and stored at −20°C.

Cloning and expression of hqdC, hqdD, and hqdE from Sphingomonas sp. strain TTNP3

hqdC, hqdD, and hqdE were amplified from Sphingomonas sp. strain TTNP3 by colony polymerase chain reaction (PCR) using high-fidelity polymerase Elongase (Invitrogen, Switzerland) and the primers listed in Tables 2 and 3. Products were purified by electrophoresis and subsequent extraction from agarose gels with the Nucleospin Extract II extraction kit (Macherey-Nagel, Switzerland), before adenylation and ligation into the pGEM-T easy vector (Promega, USA) by means of T4 DNA ligase (New England Bioscientific via Bioconcept, Switzerland) to yield plasmids designated pGEM-hqdC, pGEM-hqdD, and pGEM-hqdE, respectively. As a control, the control insert enclosed in the pGEM-T easy kit was ligated into the vector, designated pGEM-con (Table 1). Plasmids were individually transformed into 5-alpha high efficiency competent Escherichia coli (NEB, USA) for

plasmid amplification. The correct orientation of the respective insert in the vector was confirmed by PCR using the M13 reverse primer as forward primer (as it is oriented opposite to the desired insert) and the respective reverse primer used for amplification of the insert. Clones were grown on SOC medium containing 100 mg/L ampicillin, and plasmids were recovered by the Qiaprep Spin Miniprep Kit (Qiagen, Switerland) and transformed into BL21-DE3 pLysS competent E. coli (Invitrogen via Lubioscience, Switzerland). For protein expression, cells were grown on LB medium containing 100 mg/L ampicillin and 34 mg/L chloramphenicol to an OD_{600} of 0.6 and induced for 4 h at 37°C by addition of 1 mM IPTG, before harvesting the cells by centrifugation for 15 min at $4,500 \times g$. Overexpression was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Preparation of cell extracts and enzyme assays

Thawed cells of Sphingomonas sp. strain TTNP3 were disrupted by means of sonication on ice (8 min at 60% intensity, 0.6 s/s duty cycle using a Labsonic M sonicator by B. Braun Biotech, equipped with a 3 mm probe).

E. coli cells were harvested after induction, washed twice with Tris buffer (50 mM, pH 7.5 at 4°C) and disrupted by sonication on ice (30 s at 60% intensity, 0.6 s/s duty cycle using a Labsonic M sonicator by B. Braun Biotech, equipped with a 3-mm probe). The obtained crude extracts were separated from cells and debris by centrifugation ($21,500 \times g$ for 15 min).

Both formation and degradation of 4-hydroxymuconic semialdehyde in the wild-type strain were monitored photometrically at 320 nm (Spain and Gibson 1991). Of the sample, 35 μL (either crude cell extract or 50 mM Tris buffer at pH 7.0 for abiotic controls) was added to 280 μL of Tris buffer and reactions were started by adding 35 μL of a freshly prepared solution of hydroquinone in Tris buffer (final hydroquinone concentration of 100 μM).

4-Hydroxymuconic semialdehyde dehydrogenase activity of 4-hydroxymuconic semialdehyde dehydrogenase (HqdC)

Table 2 Primers used in this study

Primer	Oligo sequence	Purpose	Position in the sequence
hqdCF	ATGGCCCTCGACGACC	Amplification of hqdC	4153–4168
hqdCR	TCAGCGCTTGTAGAACGG	Amplification of hqdC	5632–5649
hqdDF	ATGCAGGATTTCATTTACTGCGG	Amplification of hqdD	5655–5677
hqdDR	TCATGATGCCGGACGCTC	Amplification of hqdD	6696–6713
hqdEF	ATGAAAGGTGCGATCTTCCC	Amplification of hqdE	6710–6729
hqdER	TTATGCTCCAGCCAAGCG	Amplification of hqdE	7520–7537
M13 reverse	CAGGAAACAGCTATGAC	Screening for correctly oriented inserts	None

Table 3 Comparison of open-reading frames of the hqd gene cluster in *Sphingomonas* sp. strain TTNP3 with homologous sequences by BLAST analysis

Gene product						Closest uncharacterized/characterized homologs			
ORF	Frame	Position in sequence (nt)	No. of amino acids	Mass (kDa)	Function	Description	GenBank accession no.	Overall sequence identity (%)[a]	Organism
hqdR	+2	1–846	282	31.9	Transcription regulator	AraC-type transcriptional regulator	EFN09601	39	*Sphingobium chlorophenolicum* L-1
hqdA	+2	1105–1617	171	18.6	Hydroquinone dioxygenase small subunit	Hydroquinone dioxygenase small subunit	NP_927509	51	*Photorhabdus luminescens subsp. laumondii* TTO1
						Hydroquinone dioxygenase small subunit	ADB81386	47	*P. fluorescens*
hqdB	+2	1663–2688	342	38.2	Hydroquinone dioxygenase large subunit	Hydroquinone dioxygenase large subunit	NP_927510	65	*Photorhabdus luminescens subsp. laumondii* TTO1
						Hydroquinone dioxygenase large subunit	ACA50458	62	*P. fluorescens*
orf1	+3	2753–3886	378	39.7	Hypothetical protein	Hypothetical protein	YP_003760983	13	*Nitrosococcus watsoni* C-113
						Assimilatory nitrate reductase	YP_981639	4	*P. naphthalenivorans* C12
hqdC	+2	4153–5649	499	53.5	Hydroxymuconic semi-aldehyde dehydrogenase	Putative aldehyde dehydrogenase	AAS87583	68	*Burkholderia* (*Ralstonia*) sp. SJ98
						Hydroxy-muconic semialdehyde dehydrogenase	ACA50459.1	68	*P. fluorescens* ACB
hqdD	+1	5655–6713	353	37.1	Maleylacetate reductase	Maleylacetate reductase	YP_003450967	63	*Azospirillum* sp. B510
hqdE	+3	6710–7537	276	29.7	Intradiol ring-cleavage dioxygenase	Intradiol ring-cleavage dioxygenase	ZP_02891065	56	*Burkholderia ambifaria* IOP40-10
						Intradiol ring-cleavage dioxygenase	ACN43573.2	52	*Pseudomonas putida* DLL-E4
hqdF	+3	7544–7939	132	15.0	Putative ferredoxin	Hypothetical protein	NP_927517	31	*Photorhabdus luminescens subsp. laumondii* TTO1
						Ferredoxin subunit of nitrite reductase and ring-hydroxylating dioxygenase	ZP_00055242.2	28	*Magnetospirillum magnetotacticum* MS-1

[a] Overall sequence identity was calculated by multiplication of the percentage of sequence coverage with the amino acid identity within aligned sequences, both of which were calculated by the BLAST algorithm

was assayed photometrically by monitoring the decrease of substrate concentration at 320 nm, in addition to subsequent gas chromatography–mass spectrometry (GC-MS) analysis as described below. Reaction mixtures contained 0.5 mM NAD, 200 μM 4-hydroxymuconic semialdehyde, and 175 μL cell extract brought to a final volume of 350 μL with 50 mM Tris buffer (pH 7.0). Reactions were started by addition of 4-hydroxymuconic semialdehyde, which was freshly produced by incubation of purified hydroquinone dioxygenase from *Sphingomonas* sp. strain TTNP3 with 400 μM hydroquinone for 10 min. The purification of hydroquinone dioxygenase of *Sphingomonas* sp. strain TTNP3 has been recently described (Kolvenbach et al. 2011).

For maleylacetate reductase activity of HqdE, reaction mixtures contained 2 mM NADH, 0.5 mM maleylacetate, and 175 μL cell extract, brought to a total volume of 350 μL with 50 mM Tris buffer (pH 7.0). Maleylacetate was freshly produced by hydrolysis of 5 mM *cis*-butenolide in water containing 15 mM NaOH (Kaschabek and Reineke 1995). For HqdE, 175 μL of cell extract and 140 μL of 50 mM Tris buffer (pH 7.0) were incubated with 0.5 mM hydroxyhydroquinone or catechol (both substrate solutions were prepared as 5 mM stock solutions in water instantly before the experiment), respectively, before incubating for 30 min at room temperature. The samples incubated with catechol also contained 1 mM of EDTA.

Cell extract of the strain bearing pGEMcon was used as a negative control for all enzyme assays.

After 30 min of incubation at room temperature, all reaction solutions for the respective enzyme assays were worked up for GC-MS analysis.

Analytical methods

Samples were acidified with a drop of 6 M HCl and extracted twice with two volumes of ethyl acetate; the organic phase was dried over Na_2SO_4 before evaporation under a gentle nitrogen stream. Extracts were redissolved in acetonitrile/N,O-bis(trimethylsilyl)trifluoroacetamide (90:10 *v/v*) for derivatization at 75°C for 15 min. Samples were analyzed in an Agilent 7890A series gas chromatograph (Agilent Technologies, Basel, Switzerland) equipped with a Zebron ZB-5MS column, (30 m×0.25 mm, 0.25 μm film thickness, Phenomenex) coupled to an Agilent 5975 C series mass spectrometer. The mass selective detector was operated in the scan mode (mass range *m/z* 50–600) with an electron energy of 70 eV. The oven temperature program was 70°C for 3 min, 8°C per minute to 250°C; the injector temperature was 200°C; the interface temperature 280°C. The injection volume was 1 μL (split 1:30). The carrier gas was helium (1 mL/min).

SDS-PAGE was carried out with 10% Tris-glycine minigels according to a standard protocol (Laemmli 1970) in a Mini-PROTEAN Tetra Cell (BioRad), using Precision plus unstained protein standards (Biorad).

Results

Hydroquinone metabolism in the wild-type strain

In degradation experiments with hydroquinone and crude extracts of *Sphingomonas* sp. strain TTNP3, we observed an initially rapid increase in absorption of the reaction mixture at 320 nm. The increase came to a halt after about 2 min and was followed by a decrease, indicating that the putative 4-hydroxymuconic semialdehyde intermediate had been degraded to further metabolites. Crude cell extracts from uninduced cells had an initial apparent hydroquinone dioxygenase specific activity of 5 mU/mg protein, whereas activities of extracts from cells induced with 0.5 mM hydroquinone and 0.5 mM technical nonylphenol were 96 and 297 mU/mg, respectively. The apparent degradation rates of 4-hydroxymuconic semialdehyde in the later course of the reaction were 0.6, 2.5, and 3.1 mU/mg protein for the uninduced, hydroquinone-induced, and nonylphenol-induced cells, respectively. GC-MS analysis of trimethyl silylated samples of the reaction mixture taken after 15 min of incubation led us to identify 4-hydroxymuconic semi-aldehyde and 3-oxoadipate as products. 4-Hydroxymuconic semialdehyde produced several chromatographic peaks that corresponded to different conformational isomers with mass spectra similar to one another and consistent with literature data (Miyauchi et al. 1999). 3-Oxoadipate was identified by the mass spectrum of its N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA)-derivatized form (Rieble et al. 1994).

Catechol and hydroxyhydroquinone metabolism in the wild-type strain

Crude extract of strain TTNP3 contained enzyme activities for turnover of catechol as well as hydroxyhydroquinone. GC-MS analysis of trimethyl silylated samples of incubation mixtures with catechol led to the detection of two meatbolites. One of the metabolites showed a retention time and a mass spectrum identical to those of the trimethyl silylated derivative of authentic *cis,cis*-muconic acid (Table 4) and the other metabolite showed a retention time and a mass spectrum identical to those of the trimethyl silylated derivative of authentic 3-oxoadipate (Table 4; Rieble et al. 1994). GC-MS analysis of trimethyl silylated samples of incubation mixtures with hydroxyhydroquinone also showed the formation of two metabolites. Here, one showed the retention time and

Table 4 Mass spectra of identified metabolites

Compound, TMS derivatized	Retention time(s) min	Mass spectrum m/z (relative intensity)
Maleylacetate	20.6	374 (0.8), 359 (2.6), 315 (1.8), 257 (67), 241 (1.3), 197 (4.9), 153 (11), 147 (100), 123 (4.4), 109 (3.2), 95 (4.5), 75 (44), 73 (45)
3-Oxoadipate	20.9	376 (3.8), 361 (23), 317 (3.7), 286 (11), 259 (8), 243 (14), 231 (13), 169 (58), 147 (84), 73 (100)
cis,cis-Muconate	18.5	286 (2.6), 271 (9), 243 (3.0), 169 (100), 147 (87), 73 (83)

respective mass spectrum identical to that of the trimethyl silylated derivative of maleylacetate (Table 4) and the other one had a mass spectrum identical to that reported for trimethylsilylated 3-oxoadipate.

hqd Gene cluster of *Sphingomonas* sp. strain TTNP3

In silico analysis of the sequences flanking *hqdA* and *hqdB*, which encode the small and large subunit of hydroquinone dioxygenase in *Sphingomonas* sp. strain TTNP3, revealed the presence of a gene cluster that contained, besides *hqdA* and *hqdB*, six additional open-reading frame (ORF; see Fig. 1). BLAST searches for four of these ORF (*hqdC*, *hqdD*, *hqdE*, and *hqdF*) revealed 68%, 63%, 56%, and 31% sequence similarities, respectively, to nucleic acid sequences coding for enzymes

functionally related to hydroquinone degradation (Table 3). The ORF termed *hqdR* bore sequence similarities to a putative AraC-type transcriptional regulator from *Sphingobium chlorophenolicum* L-1 (39% sequence identity), and the one termed *orf1* showed 4% sequence identity to an assimilatory nitrate reductase from *Polaromonas naphthalenivorans* strain CJ2 (Table 3).

Qualitative determination of enzyme activities in cell extract of *E. coli* BL21-DE3 overexpressing *hqdC*, *hqdD*, and *hqdE*

Incubation of cell extract of *E. coli* BL21-DE3 expressing HqdC with 4-hydroxymuconic semialdehyde and NAD$^+$ led to a decrease of absorption at 320 nm in the incubation mixture. GC-MS analysis of trimethylsilylated samples of
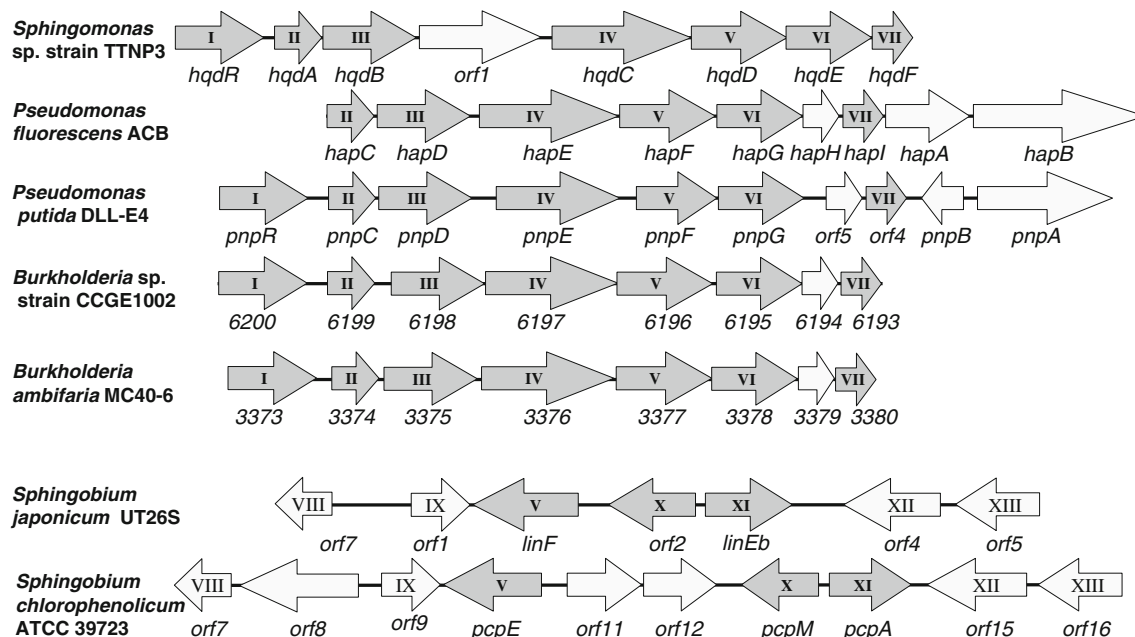

Fig. 1 Organization of the *hqd* gene cluster on a 8.0-kb fragment of the genome of *Sphingomonas* sp. strain TTNP3 (for detailed explanation, see Table 3), aligned with the *hap* gene cluster of *P. fluorescens* strain ACB (GenBank accession number AF355751), the *pnp* gene cluster of *Pseudomonas putida* DLL-E4 (GenBank accession number FJ376608) and putative hydroquinone degradation gene clusters of *Burkholderia* sp. strain CCGE1002 (NCBI reference sequence NC_014119.1; chromosome 3; e.g., 6200 corresponds to BC1002_6200) and *Burkholderia ambifaria* MC40-6 (NCBI reference sequence NC_010552.1; chromosome 2; 3373 corresponds to BamMC406_3373, e.g.). Organization of clusters attributed to HQ degradation in two sphingomonad strains are shown for comparison, i.e., *Sphingobium japonicum* UT26 (NCBI reference sequence AB177985) and *Sphingobium chlorophenolicum* ATCC 39723 (NCBI reference sequence AF512952). *Roman numbers* gene homologies, *dark gray arrows* genes attributed to hydroquinone degradatino, and *light gray arrows* other genes. *The genes indicated with I and X code* for (putative) transcriptional regulators
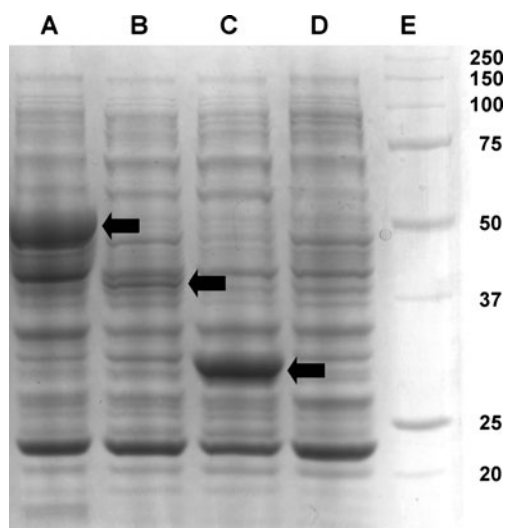
Fig. 2 SDS-PAGE analysis of cell extracts of *E. coli* strains after induction with 1 mM IPTG. *A*, *B*, *C*, and *D* show cell extract of the strains bearing, pGEM-hqdC, pGEM-hqdD, pGEM-hqdE, and pGEM-con, respectively; *E* marker proteins. *Arrows* indicate the bands attributed to the respective heterologously expressed enzymes

such incubation mixtures revealed the formation of a peak with respective retention time and mass spectrum corresponding to that of trimethylsilylated maleylacetate. Addition of NADH and maleylacetate to cell extract of the

E. coli strain expressing maleylacetate reductase (HqdD) yielded a product whose mass spectrum was identical to that of the 3-oxoadipate product detected in crude cell extracts of *Sphingomonas* sp. strain TTNP3. GC-MS analyses showed that cell extract of the *E. coli* strain expressing HqdE was capable of degrading both catechol and hydroxyhydroquinone to metabolites with retention times and mass spectra identical to those of *cis,cis*-muconic acid and maleylacetate, respectively. Expression of *hqdC*, *hqdD*, and *hqdE*, respectively, has been verified by SDS-PAGE (Fig. 2).

Discussion

Previous incubations of crude cell extracts of *Sphingomonas* sp. strain TTNP3 with the substrates NP and BPA, respectively, showed formation of hydroquinone as an intermediate. Hydroquinone was further degraded to 4-hydroxymuconic acid semialdehyde by a hydroquinone dioxygenase (Kolvenbach et al. 2011). In this work, we could show that 4-hydroxymuconic semialdehyde is transformed to maleylacetate and then to 3-oxoadipate in subsequent steps (Fig. 3). We could also show that the DNA sequences surrounding those sequences coding for the subunits of the already characterized hydroquinone dioxygenase form a gene cluster containing several open reading
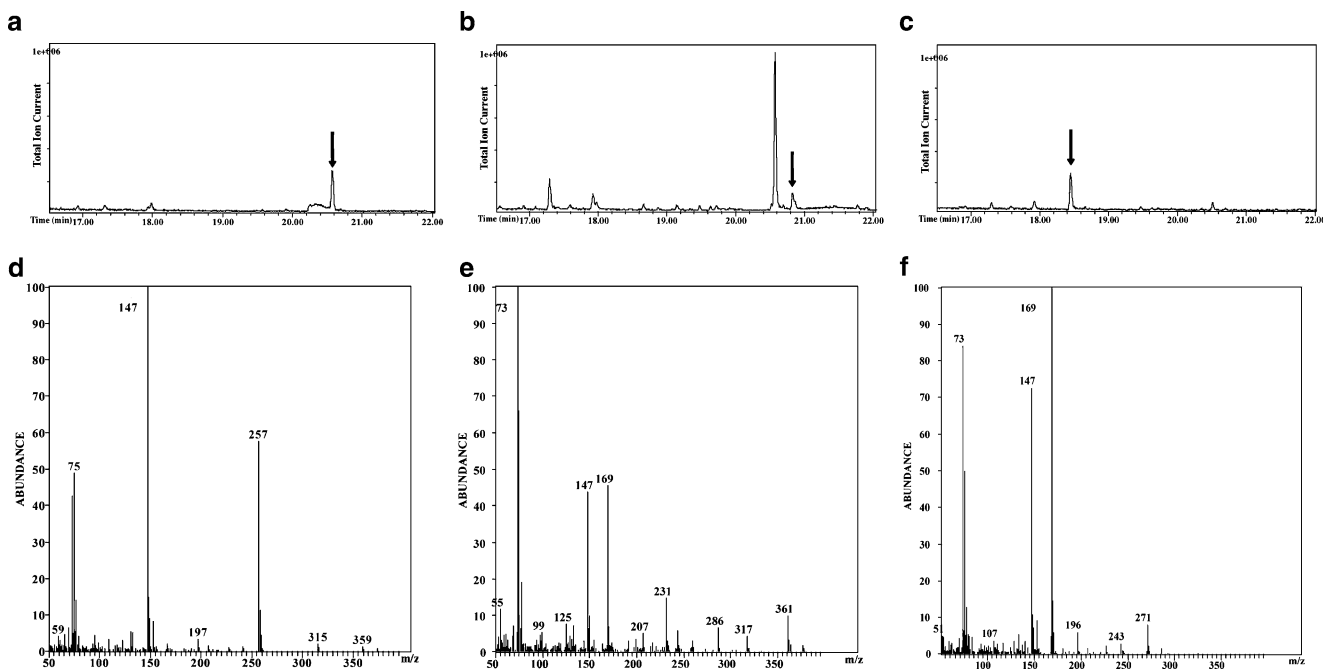

Fig. 3 Chromatograms of incubations with cell extracts of *E. coli* strains and mass spectra of metabolites formed by enzymes encoded in the *hqd* gene cluster. **a** GC-MS chromatogram of BSTFA-derivatized extract of the incubation of HqdC with 4-hydroxymuconic semi-aldehyde and NAD, derivatized maleylacetate is indicated by the *arrow*; **b** GC-MS chromatogram of BSTFA-derivatized extract of the incubation of HqdD with maleylacetate and NADH, derivatized 3-oxoadipate is indicated by the *arrow*; **c** GC-MS chromatogram of BSTFA-derivatized extract of the incubation of HqdE with catechol, derivatized *cis,cis*-muconate is indicated by the *arrow*; **d** mass spectrum of maleylacetate from **a**; **e** mass spectrum of 3-oxoadipate from **b**; **f** mass spectrum of *cis,cis*-muconate from **c**

Fig. 4 Proposed pathway for the degradation of hydroquinone in *Sphingomonas* sp. strain TTNP3. Enzyme activities that were successfully annotated to open-reading frames in the hqd cluster are labeled with the respective names. Open-reading frames that were functionally verified in this work are framed
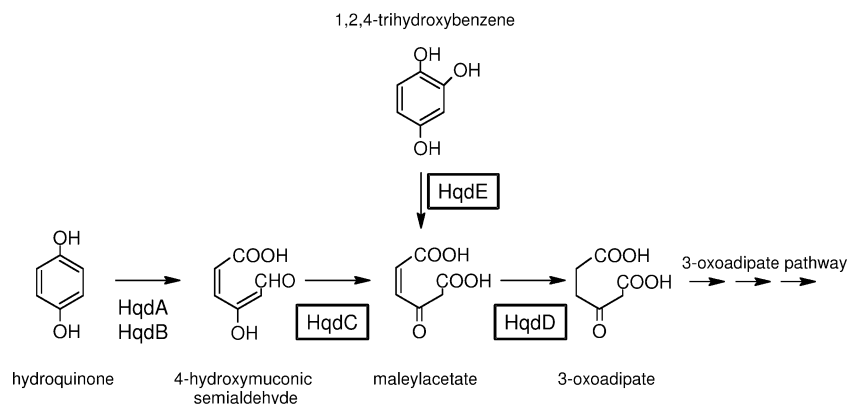
frames possibly associated to hydroquinone degradation in *Sphingomonas* sp. strain TTNP3. For two open reading frames, i.e. *hqdC* and *hqdD*, this association could be shown.

Furthermore, we demonstrated hydroquinone dioxygenase activity to be inducible by hydroquinone and technical NP, although it is possible that induction by NP was actually mediated by the hydroquinone intermediate formed during degradation of NP (Corvini et al. 2006a). We observed lower hydroquinone dioxygenase activities in extracts of cells grown in the presence of hydroquinone than in extracts of cells grown with technical NP. However, further research is needed to establish whether induction after incubation with NP may also be caused by the hydroquinone intermediate formed during degradation of NP, as NP is transformed by a constitutively expressed *ipso*-hydroxylase (Corvini et al. 2006a).

This contrast between the inducibility of hydroquinone dioxygenase and constitutive expression of nonylphenol *ipso*-hydroxylase, the enzyme catalyzing the initial step in NP degradation, is a further example of the complex regulation mechanisms operating in sphingomonads (Stolz 2009). Whether or not the genes *hqdC*, *hqdD*, *hqdE*, and *hqdF* are co-transcribed with *hqdA* and *hqdB* will need to be examined in further experiments.

Three of the ORFs in the *hqd* gene cluster of *Sphingomonas* sp. strain TTNP3 were cloned and heterologously expressed in *E. coli*. We confirmed that the putative HqdC and HqdD held the annotated function (see Fig. 4). The *E. coli* clone expressing HqdE was able to cleave both catechol and hydroxyhydroquinone to the respective intradiol ring-cleavage products, whereas the control strain only containing the vector with a control insert was not. In the wild-type strain, incubations with catechol also led to the formation of small amounts of 3-oxoadipate, indicative of the strain's ability to further metabolize the ring cleavage product. As hydroxyhydroquinone was not found as a metabolite in incubations of hydroquinone with cell extract of *Sphingomonas* sp. strain TTNP3, the role of HqdE in the degradation of hydroquinone still remains unclear in this strain.

As 40–60% sequence identity have been shown to be sufficient for protein function transfer (for enzymes, third-level to full enzyme commission numbers could be inherited within this threshold, as shown by Addou et al. (2009)), it is tempting to speculate that the ORF termed *hqdR* may be involved in the expression regulation of the cluster. However, its exact role remains elusive. The sequence-derived gene product showed 39% and 38% amino acid identity to putative *AraC*-type transcription regulators found in the shotgun genome sequence *S. chlorophenolicum* strain L-1 (GenBank accession number EFN09601.1) and in the genome sequence of *Sphingobium japonicum* strain UT26S (GenBank accession number BAI95789.1), respectively. Both putative regulators are located upstream of putative intradiol ring-cleavage dioxygenases and are transcribed in the same direction as the dioxygenases. Nevertheless, their exact role has not been elucidated yet.

The possible gene product of *hqdF* was identified by sequence identity as a putative [2Fe-2S] ferredoxin, with 28% overall sequence identity to a known ferredoxin (Table 3). It was recently shown that a group of 16 ferredoxins belonging to ring-hydroxylating enzymes had quite homogenous amino acid sequences, with sequence identities of as little as 29% (Kweon et al. 2008). Previously, it was shown that this type of ferredoxins might participate in the reactivation of catechol 2,3-dioxygenases (Hugo et al. 1998, 2000; Polissi and Harayama 1993) after suicide deactivation by halogenated or methylated catechol derivatives (Bartels et al. 1984; Cerdan et al. 1994). An analog reactivation mechanism has been recently proposed for heterotetrameric hydroquinone dioxygenases such as the ones identified in *Sphingomonas* sp. strain TTNP3 and *Pseudomonas fluorescens* strain ACB (Kolvenbach et al. 2011; Moonen et al. 2008). Future studies will show if *hqdF* codes for a ferredoxin involved in reactivation of hydroquinone dioxygenase.

As the amino acid sequence of the translated gene product of *orf1* showed only weak similarities to known

proteins (i.e., 4%, compare Table 3), its function in *Sphingomonas* sp. strain TTNP3 remains unknown.

In terms of arrangement of open-reading frames, the hqd gene cluster of *Sphingomonas* sp. strain TTNP3 resembles gene clusters associated with hydroquinone degradation of *Pseudomonas* (Moonen et al. 2008; Shen et al. 2010; Wei et al. 2010) and putative gene clusters of *Burkholderia* strains (Fig. 1) and does not resemble gene clusters associated with chlorohydroquinone degradation in downstream pathways of pentachlorophenol and lindane degrading sphingomonads (Cai and Xun 2002; Nagata et al. 2007). Moreover, BLAST searches for the gene products of the hqd cluster in all but one case (HdqR) showed that the sequences had higher similarities to sequences from nonsphingomonads than to those form other sphingomonads (Table 3). We conclude that strain TTNP3 may have acquired the hqd cluster at least partially from β-or γ-proteobacteria by horizontal gene transfer (Table 4).

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