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ORIGINAL PAPER

# *Sphingobium* sp. HV3 degrades both herbicides and polyaromatic hydrocarbons using *ortho*- and *meta*-pathways with differential expression shown by RT-PCR

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**Abstract** *Sphingobium* sp. HV3 described as an herbicide degrader harbours the pSKY4 plasmid, encoding an aromatic *meta*-pathway. The function of the plasmid was studied by Tn*5* transposon mutagenesis and plasmid isolation and the degradation capacities of the HV3 strain were re-evaluated. Transcription of the *tfdC* from *ortho*-pathway was contrasted to the *xylE* and *bphC* of *meta*-pathway using real-time PCR. Cloning of the Tn*5*-insertion sites from the megaplasmid revealed genes for both aromatic and polyaromatic degradation. In the mutant Km24 strain the transposon was inserted to an ORF similar to the large subunit of ring hydroxylating dioxygenase, in the Km383 to a *cis*-biphenyl dihydrodiol dehydrogenase and in the Km187 and Km42 to a reductase component of a dioxygenase. A chlorocathecol *ortho*-pathway (10 kb) was amplified from the HV3 strain. The transcription of the *tfdC* was induced by 2,4-dichlorophenoxyacetic acid herbicide and *m*-xylene caused highest induction of both upper and lower aromatic *meta*-pathway genes. The detected novel degradation capacities (*m*-xylene, toluene, biphenyl, fluorene and phenanthrene) can be explained by the presence of functional *meta*-pathway genes in the pSKY4 megaplasmid. The characterization of the *Sphingobium* sp. HV3 improves our understanding of versatile catabolic bacteria unveiling roles of degradation pathways and plasmids in biodegradation.

**Keywords** Tn*5* transposon · Extradiol dioxygenase · Intradiol dioxygenase · Phenanthrene · Toluene · Xylene · Biodegradation · Gene expression · Real-time PCR · *Sphingomonas*

## Introduction

Various chloroaromatic-, polyaromatic- and BTEX-compounds (Benzene, Toluene, Ethylbenzene and Xylene) in soil are many times degraded by microorganisms if the environmental conditions are favourable. Special constraints on biodegradation are set when the three types of pollutants simultaneously are present in soil. The chlorinated aromatic compounds are degraded through a different pathway than those not containing chlorines (Reineke 1998). Aromatic degrading bacterial communities have increasingly been depicted from hydrocarbon polluted soils by genetic techniques to reveal bacterial species present or functional genes encoding pollutant degrading

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enzymes (Kleinsteuber et al. 2006; Sipilä et al. 2006; 2008). To learn about the regulation of pathways and connect genes to function isolated bacterial species are studied. Some bacteria have developed astonishingly diverse capacities for degradation of hydrocarbon pollutants and these strains are of biotechnological interest since they show adaptation to different pollutants and interesting catabolic pathways (Denef et al. 2006).

The *Sphingomonas* genus is comprised by gram-negative, glycosphingolipid-containing bacteria that belong to the α-4-subgroup of Proteobacteria (Takeuchi et al. 2001). They have frequently been isolated from polluted sites and display diverse degradation capacities of pollutants (Leys et al. 2004; Peng et al. 2008; Pinyakong et al. 2003b; White et al. 1996) and their abundance in polluted sites is also confirmed by cultivation independent methods (Leys et al. 2004; Kleinsteuber et al. 2006). With the advent of polyphasic taxonomy in the 1990s this group became more recognized (Khan et al. 1996; Yrjälä et al. 1998; Zipper et al. 1996). Before that many strains of this group were not even recognized to group with Alphaproteobacteria (Yrjälä et al. 1998). Taxonomically *Sphingomonas* are identifiable by a sphingosine-sugar in the membrane (White et al. 1996). It was evident that the *Sphingomonas* genus is heterogeneous and it was amended by introduction of new genera (Takeuchi et al. 2001) but this was not confirmed by Yabuuchi et al. (2002).

Catabolic pathways for the degradation of environmental pollutants are frequently encoded by catabolic plasmids like the TOL (Williams and Murray 1974) and NAH plasmids (Yen and Serdar 1988) that are the best-known ones isolated from Gammaproteobacteria. Other types of catabolic plasmids have more recently been characterized from the Alphaproteobacteria; (Fredrickson et al. 1995; Basta et al. 2004; Keck et al. 2006), but large plasmids have not been much studied. They are cumbersome to isolate and often difficult to distinguish from the chromosomal DNA. The pNL1 megaplasmid isolated from *Novosphingobium aromaticivorans* F119 contains a multifunctional degradation pathway that serves in breakdown of both mono- and polyaromatic compounds. In *Sphingomonas* the catabolic genes are seemingly not organised in same type of operons as in Gammaproteobacteria. The genes are more randomly spread in several operons encoding enzymes of several different biochemical pathways (Pinyakong et al. 2003a). The knowledge of the function and regulation of *Sphingomonas* catabolic pathways is limited. It is still not known, for instance, how the expression of the multiple oxygenase subunits and the specific gene operons are regulated and expression studies of aromatic degradation genes are rare (Stolz 2009).

The chlorocatechol *ortho*-pathway is the central pathway for break down of chloroaromatics which produces chlorocatechols that are further degraded to yield tricarboxylic acid derivatives (Reineke 1998). Chloroaromatic herbicides like 2,4-D are degraded through the chlorocatechol tfd-pathway, which has been well studied in Beta- and Gammaproteobacteria (Lang et al. 2005) but only recently isolated from Alphaproteobacteria (Thiel et al. 2005). The Alphaproteobacterium *Sphingomonas herbicidivorans* is a herbicide degrader with a documented chromosomal *ortho*-pathway (Kohler 1999).

*Sphingobium* sp. HV3 was isolated from agricultural field soil (sandy clay) maintained with standard agricultural practices including addition of herbicides (Kilpi 1980). *Sphingobium* sp. HV3 was previously described as a herbicide degrader (2,4-D and MCPA) (Kilpi et al. 1980, 1983) and found to harbour the pSKY4 megaplasmid with a catechol *meta*-pathway (Yrjälä et al. 1994, 1997). The HV3 strain, first isolated and typed as a *Pseudomonas,* was then reclassified as a *Sphingomonas* species (Yrjälä et al. 1998). The strain was together with a *Nocardia* sp. found to degrade polychlorinated biphenyls (Kilpi et al. 1988). Only the lower catechol *meta*-pathway genes were cloned and characterised (Yrjälä et al. 1994, 1997) from the HV3 strain. The role of the pSKY4 megaplasmid in the upper pathway degradation was uncertain as well as the extent of the degradation capacities of the strain. We studied the pSKY4 megaplasmid with regard to upper pathway catabolic genes and their function, quantified transcripts of *ortho*- and *meta*-ring-cleavage dioxygenases and re-evaluated the degradation ability of the strain in order to describe the catabolic capacities of this biotechnologically interesting strain.

## Materials and methods

### Bacterial strains, culture conditions and plasmid isolation

The *Sphingobium* sp. HV3 isolated from field soil (Kilpi 1980) was routinely maintained on minimal salt

solution plate (Horvath and Alexander 1970), containing a few naphthalene crystals on the lid. The strain has been deposited to the University of Helsinki Hambi culture collection (http://www.mm.helsinki.fi/mmkem/hambi/) with the Hambi code 2249. Minimal medium was also used for liquid cultures with 0.05% *m*-toluate as a carbon source. *Sphingobium* sp. HV3 was cultured at 28°C and maintained in room temperature. *E. coli* XL2 Blue/DH5α cells from cloning experiments were grown on Luria broth plates at 37°C containing 150 μg $ml^{-1}$ ampicillin. Kanamycin, 15 μg $ml^{-1}$, was added for cloning of Tn5 transposition sites.

For the induction experiment *Sphingobium* sp. HV3 was first cultivated in minimal medium (Horvath and Alexander 1970) with 0.1% w/v glucose as carbon source usually 1–2 days in 25°C until the $A_{550}$ was 0.7. The cells were pelleted by centrifugation and washed with minimal salt solution and the washing step was repeated twice. Washed cells were resuspended in minimal media containing different carbon sources: *m*-xylene from the gas phase, 2,4-dichlorophenoxy acetic acid (2,4-D) 0.05% w/v, biphenyl 0.05% w/v, phenanthrene 0.05% w/v, benzoate 0.05% w/v or glucose 0.05% w/v. The aromatic catabolism of HV3 was induced at 25°C with constant shaking 150 rpm 24 h for *m*-xylene, glucose, benzoate; 72 h for 2,4-D and 96 h for biphenyl and phenanthrene. The induction time was experimentally defined and set according to length of lag phase of growth of *Sphingobium* sp. HV3 for each compound. The lag phase was measured by the Klett Summerson photoelectric colorimeter as in the induction experiment.

The pSKY4 megaplasmid was isolated from *Sphingobium* strains (Table 1; Wheatcroft and Williams 1981). SDS-NaOH lysates were purified by sucrose gradient (15–40%) density centrifugation to separate chromosomal DNA from plasmid DNA (Fig. S1A—Supplementary material). Collected fractions were analysed on agarose gel (0.6%), plasmid-containing fractions were ethanol-precipitated and subjected to *Eco*RI restriction enzyme analysis. The cleavage product was run on an agarose gel (0.6% agarose, 30 V and 15 h).

## Tn*5*-transposon mutagenesis

*Escherichia coli* S17-1 and the plasmid pSUP2021 or pSUP5011 were used for transposon mutagenesis (Simon et al. 1989). The donor *E. coli* S17-1, and the recipient *Sphingobium* sp. HV3, were grown in 50 ml media with 120 rpm shaking to exponential phase and the cells were centrifuged and washed with phosphate buffer pH 7.2. The cell-pellet was resuspended in growth media and donors/recipients were mixed in the ratio 1/3 on filter membranes with vacuum suction. The filter membranes were put on peptone yeast extract (PYE) Petri dishes in 28°C over night and the bacterial mass was collected from the filter, diluted as needed and plated on minimal salt solution plates with benzoate and kanamycin (50 μg $ml^{-1}$). The kanamycin resistant transformant strains were screened for their ability to grow on salicylate and naphthalene. Four strains were selected for further studies (Km24, Km42, Km187 and Km383).

## Localisation of Tn*5* transposition sites

The genotypes of Km24, Km42, Km187 and Km383 strains were characterised. Total DNA was isolated and the genomic location of transposition was verified using southern hybridisation (Data not shown). For cloning of transposition sites isolated megaplasmids were digested by the *Bam*HI restriction enzyme. The Tn*5*-transposon sequence contains only one *Bam*HI restriction site (Simon et al. 1989) in position 3061 and *Bam*HI restriction leaves the neomycin phosphotransferase gene and its promoter region intact within the 3061 bp fragment of the Tn*5* transposon. Restricted megaplasmids were cloned into *Bam*HI-cleaved and dephosphorylated pUC19 vector and the transformants were screened using kanamycin and ampicillin selection. Two clones from each library were selected for plasmid isolation with Wizard miniprep kit (Promega, Madison, USA). *Bam*HI restriction analysis was performed to determine the size of the insert. The exact position of the Tn*5*-transposon was determined by sequencing with the Tn5rev primer.

## Genetic characterization of chlorocatechol gene cluster

A partial chlorocatechol 1,2-dioxygenase was amplified from the genomic DNA of HV3 by using the primers CCDb and CCDe (Leander et al. 1998). The PCR fragment was sequenced with the CCDe primer

**Table 1** Plasmids, primers, and bacterial strains used in the study

| Primers, strains and plasmids | Description | Sequence/Genbank accession number | Reference |
|---|---|---|---|
| Plasmids | | | |
| pSKY4 | Megaplasmid from *Sphingobium* sp. HV3 | – | Yrjälä et al. (1997) |
| pSUP2021/ pSUP5011 | Suicide vector containing Tn*5*; 11.7 kb; ColE1 Ori; Apr, Cmr, Kmr | – | Simon et al. (1989) |
| pUC19 | Cloning vector, pMB1, AMPr, lacZ | L09137 | Yanisch-Perron et al. (1985) |
| ClKm187 | Plasmid from isolation of Tn*5* mutantion site | AM490255 | This study |
| ClKm383 | Plasmid from isolation of Tn*5* mutantion site | AM490256 | This study |
| ClKm24 | Plasmid from isolation of Tn*5* mutantion site | AM490257 | This study |
| ClKm42 | Plasmid from isolation of Tn*5* mutantion site | – | |
| Strains | | | |
| E. coli S17-1 | Donor of Tn*5* mutagenesis | – | |
| E. coli DH5a | Host in cloning assay of transposition mutants | – | |
| *Sphingobium* sp. HV3 | Herbicide degrading strain isolated from agricultural field soil | – | Kilpi (1980) |
| *Sphingobium* sp. Km24 | Tn*5* mutant strain of *Sphingomonas* sp. HV3 | – | This study |
| *Sphingobium* sp. Km42 | Tn*5* mutant strain of *Sphingomonas* sp. HV3 | – | This study |
| *Sphingobium* sp. Km187 | Tn*5* mutant strain of *Sphingomonas* sp. HV3 | – | This study |
| *Sphingobium* sp. Km383 | Tn*5* mutant strain of *Sphingomonas* sp. HV3 | – | This study |
| Primers | | | |
| Tn5rev | Sequencing of transposition site | GCCGAAGAGAACACAGATTTAG | This study |
| CCDb | Amplification of partial chlorocatechol 1,2-dioxygenase (TfdC) | GTITGGCAYTCIACICCIGAYGG | Leander et al. (1998) |
| CCDe | Amplification of partial chlorocatechol 1,2-dioxygenase (TfdC) | TCGAAGTAGTAYTGIGT | Leander et al. (1998) |
| Or1 | Amplification of *ortho*-pathway | GCCATGATCAGAGCGGAATC3 | This study |
| Or2 | Amplification of *ortho*-pathway | GTCCATCAAATGCACGAATG | This study |
| Or3 | Amplification of *ortho*-pathway | TATGTTGCGATGAACGATGG | This study |
| Or4 | Amplification of *ortho*-pathway | CCTCCCTCGGTACCATATCC3 | This study |
| Chloroseq1 | Sequencing of chlorocatechol 1,2-dioxygenase (TfdC) | GGTGGTCAACGTGTGGAATC | This study |
| Q1 | RT-PCR of chlorocatechol 1,2-dioxygenase transcript | CGCATGTCCATTACAAGGTTC | This study |
| Q2 | RT-PCR of chlorocatechol 1,2-dioxygenase transcript | TCAAAGTCGGTCTCGATCAC | This study |
| Q3 | RT-PCR of catechol 2,3-dioxygenase operon transcript | TTGCAGTGCATGAAGTAGGG | This study |
| Q4 | RT-PCR of catechol 2,3-dioxygenase operon transcript | TCAAGAACCCCGATGTCTG | This study |
| Q5 | RT-PCR of 2,3-dihydroxybiphenyl 1,2-dioxygenase operon transcript | TCAAGAACCCCGATGTCTG | This study |
| Q6 | RT-PCR of 2,3-dihydroxybiphenyl 1,2-dioxygenase operon transcript | GTTTCATCGACCAGTTCCTC | This study |

**Table 1** continued

| Primers, strains and plasmids | Description | Sequence/Genbank accession number | Reference |
|---|---|---|---|
| 16SqR | RT-PCR of 16S rRNA gene transcript | CTTTACGCCCAGTAATTCCG | Kouzuma et al. (2006) |
| 16SqF | RT-PCR of 16S rRNA gene transcript | GTGAGTGATGAAGGCCTTAG | Kouzuma et al. (2006) |

and found to be identical to the chlorocatechol 1,2-dioxygenase (*tfdC*) from *Sphingomonas* sp. TFD44 (Thiel et al. 2005). On the basis of the sequence information from *Sphingomonas* sp. TFD44 chlorocatechol catabolic gene clusters, primer pairs Or/Or2 and Or3/Or4 were designed (Table 1). Or1/Or2 and Or3/Or4 were used in amplification of 4800 and 3600 bp fragments, respectively, of a chlorocatechol gene cluster from genomic DNA of *Sphingobium* sp. HV3 using the PCR program: initial denaturation at 94°C for 1 min followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 52°C for 30 s, and elongation at 72°C for 3 min 30 s, followed by final elongation at 72°C for 7 min. The 50 µl PCR mixture contained 20 pmol of primers, 200 µM dNTP, 1.5 units of DNA polymerase (Biotools, B&M Labs, Madrid Spain) and 1× reaction buffer containing 200 µM $MgCl_2$. The complete chlorocatechol 1,2-dioxygenase gene was sequenced using CCDb and Chloroseq1 primers from Or1/Or2 PCR product.

## Growth tests

For growth tests with PAHs (phenanthrene, fluoranthene, anthracene, biphenyl, pyrene and fluorene) the *Sphingobium* sp. HV3 strain was maintained on minimal salt solution plate containing *m*-toluate and transferred to plates containing PAH as crystals on agarose minimal media. Growth tests were done in triplicate. Growth substrates *m*-xylene and toluene were offered in gas phase using sterile Erlenmeyer flask with a side arm. Agarose media was solidified in bottom of the Erlenmeyer flask and the liquid substrate was put in the side arm.

Growth tests of Tn*5* mutant strains were conducted using minimal salt solution plates supplemented with kanamycin 15 µg $ml^{-1}$ (Horvath and Alexander 1970). Different growth substrates were added to plates either as crystals (phenanthrene, biphenyl, 4-chloro-2-methylphenoxyacetic acid (MCPA), 2,4-D) in one corner of plate, or in case of naphthalene, toluene and *m*-xylene, substrates were supplemented via gas phase. Salicylate and benzoate were supplemented as 0.05% solution to the media. Growth tests were done in triplicate at 28°C. Results were first checked after 2 days of incubation and rechecked after 4 days. No growth was observed in negative controls containing only substrate or containing only the strain without the substrate.

## DNA/RNA isolation, sequencing and phylogenetic analysis

Plasmid purification from *E. coli* strains was done using the Montage Plasmid $Miniprep_{96}$ Kit (Millipore, Massachusetts, USA) and Wizard miniprep kit (Promega, Madison, USA) with ethanol precipitation if needed. Restriction enzyme digestions were performed using over night incubation to ensure complete digestion. The chromosomal DNA from *Sphingobium* sp. HV3 was isolated according to Ausubel et al. (1987). Total RNA was isolated from 20 ml cell culture (OD 0.5–0.8) using modified hot phenol method (Tyystjarvi et al. 2001). In the precipitation 2 M LiCl was used to reduce the genomic DNA carryover. Automated sequencing was performed using ABI 3130 with ABI Big Dye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems, Foster City, USA). Sequences were assembled using the Staden Sequence Analysis Package. Tn*5* mutant clones were sequenced by primer-walking strategy or using the Template Generation System™ II-kit (Finnzymes, Finland). Potential open reading frames (ORFs) were identified with manual inspection and comparison to Genbank sequences using the ORF Finder program. Identified ORFs were compared to Genbank sequences using the BLASTP 2.2.7 program. The sequences were deposited in the EMBL database with the accession numbers AM490255, AM490256 and AM490257.

Distance estimates for construction of phylogenetic trees were calculated using the Tajima and Nei (1984) algorithm. Phylogenetic dendrograms were built by the neighbour-joining method (Saitou and Nei 1987) and their construction done by the Treecon v. 1.3b program. The generated phylogenetic tree was rooted with *Zymomonas mobilis* ZM4.

### Real-time PCR

The expression of *tfdC*, *xylE*, *bphC* and 16S rRNA genes from *Sphingobium* sp. HV3 cells induced with different carbon sources was quantified. The primers are shown in Table 1. The annealing temperature and primer concentration was optimized to obtain specific products. Total RNA was treated by DNAase. An aliquot of the RNA samples was used to synthesize cDNA. From each sample 70 ng RNA was reverse transcribed (RevertAid™ M-MuLV, Fermentas, Canada) using random hexamers (Roche applied sciences, Germany,) according to manufacturers guidelines. Both RNA (negative control) and cDNA were quantified using LightCycler® 480 system (Roche applied sciences) with syber green chemistry (LightCycler® 480 SYBR Green I Master, Roche applied sciences). The real-time PCR was performed using initial denaturation at 95°C 10 min and 45 cycles of denaturation 95°C 10 s, annealing 61°C 15 s and extension 72°C 12 s. Melting curve analysis and agarose gel electrophoresis was performed to check the quality of the PCR product. Gel purified PCR products were quantified using the Qubit fluorometer (Invitrogen, USA). The qPCR results were analysed by absolute quantification and relative quantification method (Data not shown; Pfaffl 2001) using LightCycler® 480 software version 1.5 (Roche applied sciences). Absolute standardized mRNA equivalents (R′) were calculated according to Corbella and Puyet (2003). R′ used in this study was normalised with 16S rRNA gene expression levels from each cDNA extract.

$$R' = R^s \times \frac{R^S_{Glu\cos e16S}}{R^s_{16S}}$$

where $R^s$ is the absolute quantification result of *xylE*/*bphC*/*tfdC* transcription and $R^s_{\text{Glucose16S}}$ is the result of 16S rRNA gene transcription in glucose and $R^s_{16S}$ is the result of quantification of 16S rRNA gene transcription from same cDNA extract as from which the *xylE*/*bphC*/*tfdC* gene was quantified. Student's test (*t*-test) was used to evaluate significance of the expression level changes in different induction experiments.

## Results

### Phylogenetic position of the *Sphingobium* sp. HV3 strain

The heterogeneous *Sphingomonas* genus was, according to the 16S rRNA gene phylogeny, split into four phylogenetic clusters by Takeuchi et al. 2001 and three new genera, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis* were proposed. The *Sphingomonas* sp. HV3 is in present 16S rRNA phylogeny placed in the *Sphingobium* cluster with two close relatives, *Sphingomonas* MP24 and MP25 (Fig. S2—Supplementary material; Lim et al. 2004). The MP24 was similar to *Sphingobium* sp. HV3 according to 16S rRNA sequence (99% similarity), colony morphology and ability to degrade MCPA and 2,4-dichlorophenoxyacetic acid (2,4-D) and interestingly the MP24 strain also harbours large plasmids (Lim et al. 2004). The most accurate classification of the HV3 strain thereby is *Sphingobium* sp. HV3.

### Transposon mutagenesis of the *Sphingobium* sp. HV3

The catabolic functions of the pSKY4 megaplasmid were studied by Tn*5* transposon mutagenesis in three experiments. These experiments produced >4500 transconjugants and their growth with different aromatic carbon sources was analysed. Altogether 21 mutants with lost capacity to degrade naphthalene and/or salicylate were found. The pSKY4 plasmid was isolated from Tn*5* transposon mutant strains and a Tn*5* probe was hybridised to the plasmids. Of 11 strains with the transposon in the pSKY4 four were selected for further study, Km24, Km42, Km187 and Km383. The mutants Km187 and Km42 were chosen while they had lost the ability to degrade most of the test substrates (phenanthrene$^-$, biphenyl$^-$, toluene$^-$ *m*-xylene$^-$ and salicylate$^-$). Mutants Km24 (naphthalene$^-$, salicylate$^-$) and Km383 (naphthalene$^-$)

**Table 2** Phenotype of mutant strains received by Tn*5* mutagenesis and genetic localization of transposition sites

| Tn*5* mutant Strain | Gene with trans-Position site | Predicted function of the gene | Phenotype of the strain[a] |
|---|---|---|---|
| Km187 | *bphA4* | Reductase component of a dioxygenase | nah$^-$, sal$^-$, *m*-tol$^-$, phe$^-$, bph$^-$ |
| Km42 | *bphA4* | Reductase component of a dioxygenase | nah$^-$,sal$^-$, *m*-tol$^-$, phe$^-$, bph$^-$ |
| Km383 | *bphB* | *cis*-biphenyl dihydrodiol dehydrogenase | nah$^-$ |
| Km24 | *bphA1c* | Large subunit of a ring hydroxylating dioxygenase | nah$^-$,sal$^-$, |

*a* Substrates, *nah* = naphthalene, *sal* = salicylate, *m*-tol = *m*-toluate, *phe* = phenanthrene, *bph* = biphenyl

represented the most common phenotypes among mutants.

The Tn*5*-mutated megaplasmids from the four mutant strains (Table 2; Fig. 1) were isolated and the transpositions sites were localised. In mutant strains Km187 and Km42 the transposon was localised to an ORF encoding a reductase component of a dioxygenase (BphA4; Table 3). In strain Km383 (naphthalene$^-$) the Tn*5* transposon was localised to an ORF showing similarity to *cis*-biphenyl dihydrodiol dehydrogenase (BphB) from *Sphingomonas sp.* CHY-1 (Demaneche et al. 2004; Jouanneau and Meyer 2006; Table 3). The naphthalene- phenotype of the Km383 suggests that BphB functions as a cis-1,2-dihydro-1, 2-dihydroxynaphthalene dehydrogenase in *Sphingobium* sp. HV3. In the Km24 mutant the Tn*5* transposition was localised to a gene encoding a large subunit component of a ring hydroxylating dioxygenase homologous to the pNL1 *bphA1c* gene (Table 3).

In total 15.7 kb pSKY4 plasmid sequence was obtained from Tn*5* mutant clones (Fig. 2) revealing several *Sphingomonas meta*-pathway genes. The clone ClKm187 (5.6 kb) contained the genes from partial *bphA4* to partial *bphA1b*, the clone ClKm383 (5.5 kb) genes from partial *bphA1b* to partial *bphB* and the clone ClKm24 (4.6 kb) genes from partial *xylY* to partial *bphA1c*. The fourth transposon clone, ClKm42, contained only 37 bp pSKY4 plasmid sequence with 100% identity to the pNL1 *bphA4* gene which was also recovered from clone ClKm187. The overall gene organisation and comparison to pNL1 is displayed in Fig. 2. Clones Clkm187 and ClKm383 formed a single 11.1 kb fragment confirmed by PCR analysis and sequencing of the PCR product (data not shown). The sequence analysis of transposition clones revealed genes encoding enzymes involved in xylene, toluene, naphthalene and biphenyl degradation.

*Eco*RI restriction of the pSKY4 megaplasmid

The transposition sites in the megaplasmid could be visualised by restriction analysis of isolated mutant megaplasmids confirming the hybridisation results. Comparison of restriction banding patterns of mutant megaplasmids to the wild type showed insertions of the large 5 kb Tn*5* transposon (Fig. 1), which lacks *Eco*RI restriction sites (Simon et al. 1989). The megaplasmid from the Km187 mutant strain contained a 15.6 kb fragment that was missing from the wt *Eco*RI digest of the pSKY4 plasmid (Fig. 1). In

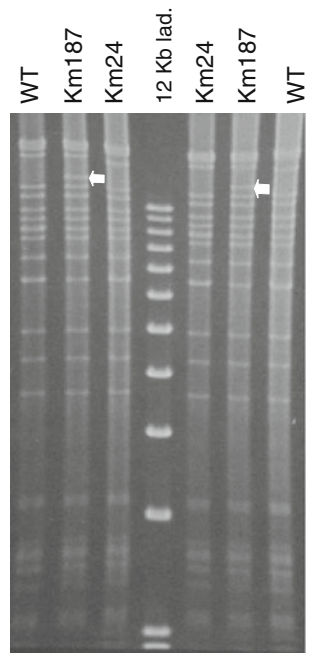


**Fig. 1** Agarose gel electrophoresis of *Eco*RI restricted pSKY4 megaplasmid from *Sphingobium* sp. HV3 and megaplasmids from the two Tn*5* mutant strains Km24 and Km187. *White arrows* point out the additional band in megaplasmid of the Km187 mutant strain

**Table 3** Genes found by cloning and sequencing of transposition sites from Tn*5* mutants Km24, Km42, Km187 and Km383

| Genes from Tn*5* clones | Functional description of closest relative | Tn*5* c lone | Ident. (%)* | Closest match in database | GenBank access no |
|---|---|---|---|---|---|
| *BphA4* partial | *BphA4*; ferredoxin reductase subunit aromatic oxygenase | ClKm187/ ClKm42 | 95 | *N. aromaticvorans* (pNL1) | NC_002033 |
| *BphR* | *BphR*; regulatory protein | ClKm187 | 95 | *N. aromaticvorans* (pNL1) | NC_002033 |
| *BphA1a* | *BphA1a*; large subunit aromatic a dioxygenase | ClKm187 | 98 | *N. aromaticvorans* (pNL1) | NC_002033 |
| *BphA2a* | *BphA2a*; small subunit aromatic oxygenase | ClKm187 | 96 | *N. aromaticvorans* (pNL1) | NC_002033 |
| *BphA1b* | *BphA1b*; large subunit aromatic a dioxygenase | ClKm187 | 99 | *N. aromaticvorans* (pNL1) | NC_002033 |
| *BphA2b* | *BphA2b*; small subunit aromatic oxygenase | ClKm187/ ClKm383 | 97 | *N. aromaticvorans* (pNL1) | NC_002033 |
| *Orf1217* | *orf1217*; Ligand gated channel | ClKm383 | 83 | *N. aromaticvorans* (pNL1) | NC_002033 |
| *XylM* | *XylM*; xylene monooxygenase hydroxylase subunit | ClKm383 | 98 | *N. aromaticvorans* (pNL1) | NC_002033 |
| *Xyl*A | *XylA*; xylene monooxygenase electron transfer subunit | ClKm383 | 99 | *N. aromaticvorans* (pNL1) | NC_002033 |
| *BphB* partial | *BphB*; putative inner membrane protein, dihydrodiol dehydrogenase | ClKm383 | 100 | *Sphingomonas* sp. CHY-1 | AM230449 |
| *XylY* partial | *saro2420*; small subunit toluate/benzoate dioxygenase (*xylY*) | ClKm24 | 98 | *N. aromaticvorans* (pNL1) | NC_002033 |
| *XylX* | *XylX*; large subunit toluate/benzoate dioxygenase | ClKm24 | 99 | *N. aromaticvorans* (pNL1) | NC_002033 |
| *BphC* | *BphC*; dihydroxy naphthalene/biphenyl dioxygenase | ClKm24 | 96 | *N. aromaticvorans* (pNL1) | NC_002033 |
| *BphA3* | *PhnR*; Rieske-type ferredoxing | ClKm24 | 100 | *S. chungbukensis* DJ77 | AF061802 |
| *BphA2c* | *PhnA2b*; Salicylate 1-hydroxylase beta subunit | ClKm24 | 100 | *Sphingomonas* sp. CHY-1 | AJ633552 |
| *BphA1c* partial | *PhnA1b*; salicylate 1-hydroxylase alpha subunit | ClKm24 | 100 | *Sphingomonas* sp. CHY-1 | AJ633552 |

* Amino acid similarity in alignment by BLASTP

Km187 the Tn*5* transposon was inserted into an approximately 10 kb *Eco*RI fragment.

Comparison of restriction fragment sizes of pSKY4 and pNL1 megaplasmids displayed different banding patterns in that pSKY4 contained seven *Eco*RI fragments larger than 10 kb and *in silico* digestion revealed only four in pNL1 (16.4, 15.6, 14.2 and 12.9 kb).

## Chlorocatechol *ortho*-pathway in *Sphingomonas* sp. HV3

*Sphingobium* sp. HV3 degrades phenoxy acid herbicides suggesting presence of the modified *ortho*-pathway in the strain. The amplified and sequenced chlorocatechol 1,2-dioxygenase gene from the HV3 was 100% similar to the *tfdC* gene in *Sphingomonas* sp. TFD44. The chlorocatechol 1,2-dioxygenase catalyses the intradiol aromatic ring-cleavage reaction of chlorinated aromatics. For characterization of the HV3 chlorocatechol pathway new primers were designed (Or1, Or2 and Or3, Or4; Table 1) that produced amplicons of expected size, 4800 and 3600 bp, respectively (Fig. S1B—Supplementary material). Since the chlorocatechol 1,2-dioxygenase gene in *Sphingobium* sp. HV3 was identical to that in the TFD44 strain, we assume that the rest of the genes also are highly similar, further supported by the similar size of amplicons.

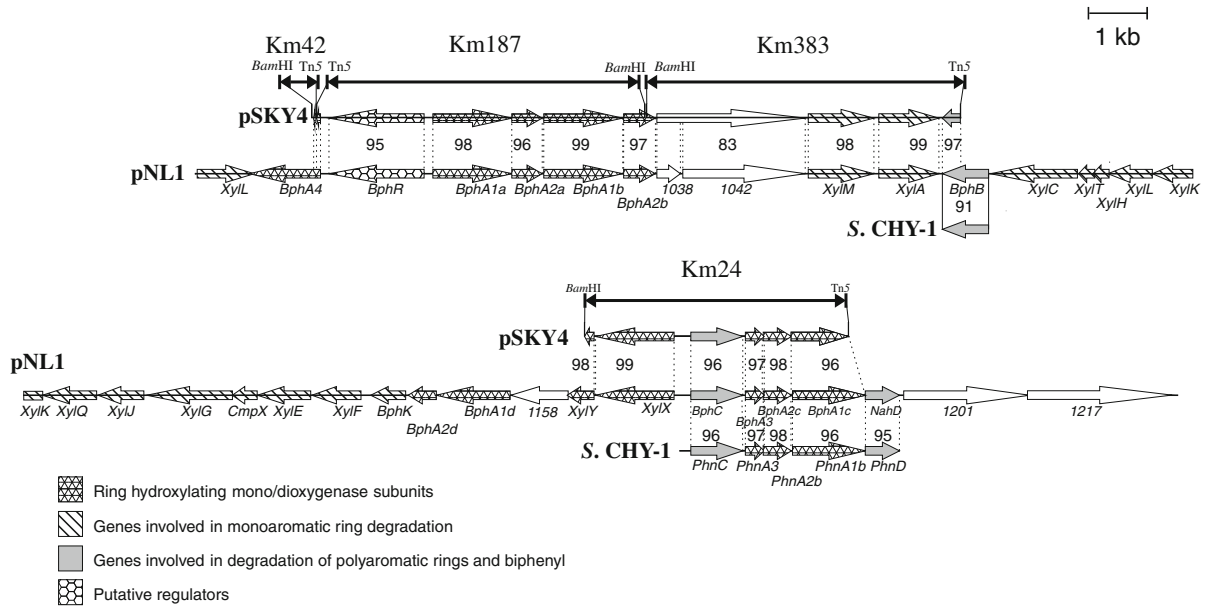


**Fig. 2** Graphical depiction of putative genes in Tn*5* transposon clones from mutant strains Km24, Km42, Km187 and Km383 with comparison to corresponding genes in the pNL1 megaplasmid from *Novosphingobium aromaticivorans*. Selected genes from *Sphigomonas* sp. CHY-1 showing similarity to pSKY4 are shown below the megaplasmid operons. Genes are presented as *arrows* describing direction of transcription. Amino acid similarities are shown in the space between the operons

## Expression of ring-cleavage dioxygenases

The expression of the intradiol dioxygenase *tfdC* from the modified *ortho*-pathway operon (Fig. 3) and the two extradiol dioxygenases (*xylE* and *bphC*) from the upper and lower *meta*-pathways operons (Fig. 4) were assayed using real-time PCR with the aim of studying the activity of the three pathways in presence of different growth substrates. The 16S rRNA gene was used as a reference gene as in similar previous studies (Kouzuma et al. 2006; Parnell et al. 2010). Similar results were obtained from both absolute quantification and relative quantification methods (Data not shown). The gene expression in *Sphingobium* sp. HV3 was induced using different carbon sources glucose, biphenyl, *m*-xylene, phenanthrene and 2,4-D.

The chlorocatechol intradiol dioxygenase (*tfdC*) operon was not significantly expressed by induction with biphenyl, *m*-xylene, phenanthrene, or even 2,4-dichlorophenoxy acetic acid (data not shown). This unexpected result lead us to study the temporal expression of *tfdC* induced with 2,4-dichlorophenoxy acetic acid (Fig. 3). The *tfdC* operon was immediately induced with the introduction of 2,4-D but the

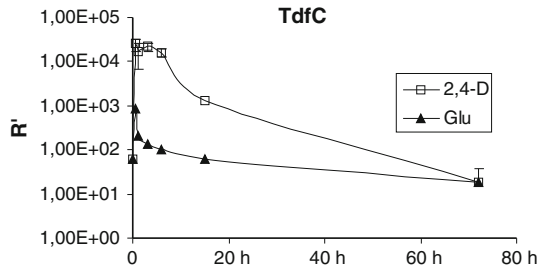


**Fig. 3** Temporal analysis of the chlorocatechol 1,2-dioxygenase (*tfdC*) gene expression in minimal growth media with 2,4-dichlorophenoxyacetic acid (2,4-D) and glucose (Glu) as carbon source. R′ value in *y*-axis notes standardized mRNA equivalents

expression levels decreased already after 6 h showing timely adjusted regulation.

The *xylE* operon was constitutively expressed in the HV3 strain grown on glucose ($R' = 5.22 \times 10^2$) and induction on biphenyl ($R' = 1.80 \times 10^4$), *m*-xylene ($R' = 4.87 \times 10^4$) and phenanthrene ($R' = 6.20 \times 10^3$) up-regulated the expression significantly ($P > 0.05$; Fig. 4A). Interestingly the *xylE* operon was significantly repressed upon growth on the 2,4-D herbicide ($P > 0.05$) showing that the induction of the *meta*-pathway is specific to certain

**Table 4** *Sphingobium* sp. HV3 degradation capacities

| Substrate | *Sphingobium* sp. HV3 | References |
|---|---|---|
| 2,4-Dichlorophenoxy acetic acid (2,4-D) | + | Kilpi (1980) |
| 2-Hydroxynaphthalene | + | This study |
| 2-Methyl-4-clorophenoxyacetic acid (MCPA) | + | Kilpi (1980) |
| 3-Chlorobenzoate | + | Kilpi et al. (1988) |
| 3-Methyl-salicylate | + | Kilpi et al. (1988) |
| 3-Methyl-salicylate | + | Kilpi et al. (1988) |
| 3-Phenyl-2-propenoic acid | − | This study |
| 4-Clorobenzoate | + | Kilpi et al. (1988) |
| 4-hydroxy-3-methoxybenzoate | − | Kilpi (1980) |
| Acetone | + | This study |
| Adipic acid | + | This study |
| Anthracene | m[a] | This study |
| Benzoate | + | Kilpi (1980) |
| Biphenyl | + | This study |
| Fluoranthene | m[a] | This study |
| Fluorene | +[b] | This study |
| 1-Hydroxynaphthalene | m[c] | This study |
| *m*-Toluate | + | Kilpi et al. (1988) |
| *m*-Xylene | + | This study |
| Naphthalene | + | Kilpi et al. (1988) |
| *p*-Cresol | - | This study |
| Phenanthrene | + | This study |
| Phenol | - | This study |
| *p*-Toluate | + | Kilpi (1980) |
| Pyrene | m[d] | This study |
| Salicylate | + | Kilpi (1980) |
| Toluene | + | This study |

*m* Colour changes in culture media indicating transformation of test compounds in absence of growth, + Growth on minimal salt agar plates with the substrate as sole source of carbon

[a] Light pink colour in liquid media

[b] Yellow colour accumulation in media

[c] Reddish colour in media

[d] Greenish colour

aromatics. In contrast to the *xylE* operon the *bphC* upper *meta*-pathway operon was not expressed constitutively ($R' = 1.10 \times 10^{1}$). The *bphC* operon was, however, up-regulated like the lower *meta*-pathway *xylE* operon and repression of the *bphC* operon was detected upon growth on 2,4-D (Fig. 4B). The highest induction of both *xylE* and *bphC* *meta*-pathway operons was achieved by *m*-xylene as inducer.

### Degradation capacities of the *Sphingobium* sp. HV3

The catabolic genes in the Tn*5* transposon clones indicated wider degradation capacities of HV3 than previously reported (Fig. 2). The transposon mutagenesis revealed plasmid-borne genes encoding enzymes for degradation of xylenes, toluene, biphenyls and polyaromatics. Phenanthrene was found to be a growth substrate (Table 4) and the strain even physically grew over the phenanthrene crystals on minimal medium. Growth tests with fluorene produced intensive yellow colour formation in the media and very slow growth suggesting *meta*-ring-cleavage and slow catabolism of accumulated products in the media. The HV3 strain grew less well on biphenyl when induced on naphthalene, but after successive transfers on biphenyl the growth was substantial. Toluene and *m*-xylene were also novel growth substrates when provided in vapour phase, but growth was completely inhibited when the organic solvent was added to the growth media. Growth experiments demonstrated that *Sphingobium* sp. HV3 is not just a herbicide degrader but degrades polyaromatics, BTEX and biphenyls as well.

## Discussion

*Sphingobium* sp. HV3 isolated from agricultural soil (Kilpi 1980) has a remarkable diverse degradation capacity of aromatics: in addition to chlorinated phenoxy herbicides and chlorobenzoates, it degrades BTEX compounds as well as PAHs and biphenyls. To the best of our knowledge no bacterial strain has been reported to have such wide degradation capacities of aromatics as *Sphingobium* sp. HV3. The *ortho*- and *meta*-pathways showed differential expression demonstrating how the intricate degradation machinery of the *Sphingobium* sp. HV3 is regulated on transcriptional level. The pSKY4 megaplasmid has an important role in the degradation of aromatics shown by Tn*5* mutagenesis.

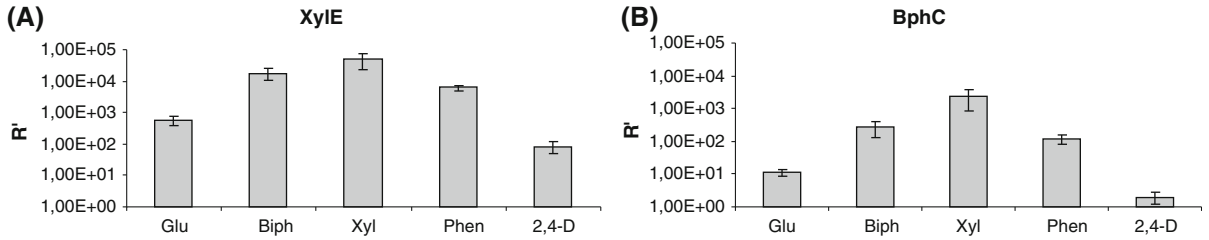


**Fig. 4** **a** qPCR of catechol 2,3-dioxygenase (*xylE*), and **b** 2,3-dihydroxybiphenyl 1,2 dioxygenase (*bphC*) operons in *Sphingobium* sp. HV3. R′ value in *y*-axis notes standardized mRNA equivalents. The expression of the operons were analysed from active growth stage cells (see "Materials and methods") using glucose (Glu), biphenyl (Biph), *m*-xylene (Xyl), phenanthrene (Phen) and 2,4-dichlorophenoxy acetic acid (2,4-D) as sole source of carbon

*Ortho*-pathway genes originally described in Beta- and Gammaproteobacteria are only known from two *Sphingomonas* strains (Kohler 1999; Thiel et al. 2005). We detected one *tfdC* gene from *Sphingomonas* sp. HV3 that was identical to the *tfdC* in *Sphingomonas* sp. TFD44. With newly constructed primers for the chlorocatechol pathway we were able to amplify two products that were of expected size, that is of similar size to described *ortho*-pathway operons (Thiel et al. 2005). This supports the presence of a functional chromosomal *ortho*-pathway like in the TFD44 strain. We show for the first time by qPCR the expression of *ortho*- and upper and lower *meta*-pathways in a single bacterium by study of the induction of transcription of genes encoding key enzymes of these pathways. The proper regulation of the pathways is essential to avoid bottlenecks and accumulation of toxic intermediates (Reineke 1998).

It was in our interest to reveal how coordinated regulation of transcription of the plasmid encoded *meta*-pathway and the evidently chromosomal *ortho*-pathway is accomplished. Expression of the intradiol dioxygenase (*tfdC*) and the extradiol dioxygenases (*xylE* and *bphC*) was studied in relation to different aromatic growth substrates. Upper and lower *meta*-pathway dioxygenases were induced by biphenyl, *m*-xylene and phenanthrene showing an aromatic compound-specific expression of pSKY4 plasmid genes. The highest induction of *meta*-pathways was achieved with *m*-xylene which might reflect the higher bioavailability and hence toxicity of this substituted benzene ring-containing compound in comparison to biphenyl and phenanthrene. The high toxicity of *m*-xylene to *Sphingomonas* sp. HV3 cells is counteracted by the fast induction of the catabolic pathway and subsequent degradation. *m*-xylene may also be considered more as a stressor than carbon source to the HV3 strain and must rapidly be degraded to prevent harmful effects to the cell membrane and macromolecules (Dominguez-Cuevas et al. 2006). The 2,4-D repressed the *meta*-pathway operons showing that the induction of *meta*-pathway genes is substrate specific to biphenyl *m*-xylene and phenanthrene. Minimal transcription of *tfdC* gene was also detected with cultures grown on glucose, *m*-xylene and phenanthrene. The repression of the *meta*-pathways by the 2,4-D reflect an ability of HV3 to avoid incomplete catabolism of substrates producing toxic intermediates by the relaxed specificity *meta*-pathway enzymes (Jouanneau and Meyer 2006).

Several degradation capacities of *Sphingobium* sp. HV3 were linked to the pSKY4 megaplasmid. The naphthalene$^-$ salicylate$^-$, *m*-toluate$^-$, phenanthrene$^-$ and biphenyl$^-$ phenotypes of Km187 and Km42 showed that the activity of reductase component of a dioxygenase in the pSKY4 megaplasmid is necessary in catabolism of these aromatics. It is thought that aromatic *meta*-pathways of *Sphingomonas* contain only one functional reductase (Keck et al. 2006; Romine et al. 1999). Disruption of the electron transfer chain of dioxygenases leads to an inactive *meta*-pathway in the HV3 strain shown by the mutant phenotypes. Km187 and Km42 mutants are still able to degrade herbicides (MCPA and 2,4-D) and benzoates evidently via a functional *ortho*-pathway. In mutant strain Km383 (Nah$^-$) the transposon disturbed the *bphB* gene supporting that BphB catalyses the second step in degradation of naphthalene and biphenyl (Pinyakong et al. 2003a; Romine et al. 1999). Very recently it was shown that *Sphingomonas* sp. CHY-1 dihydrodiol dehydrogenase

acts on various dihydrodiol derivates from biphenyl to benzo[a]pyrene (Jouanneau and Meyer 2006). Although the HV3 and CHY-1 strains are not phylogenetically closely related their partial BphB amino acid sequence is identical.

The Km24 strain with mutated large subunit gene of a ring hydroxylating dioxygenase could not grow on naphthalene or salicylate. The Km24 with disturbed *nah*-pathway could, however, grow on phenanthrene, although the degradation is suggested to proceed through naphthalene and salicylate derivates (Kiyohara et al. 1994). This suggests that naphthalene and phenanthrene are degraded independently in *Sphingobium* sp. HV3. In known *Sphingomonas* operons the *bphAc1* is followed by *nahD* (Kim and Zylstra 1999; Romine et al. 1999; Fig. 2). It is possible that the BphA1c subunit is not needed for phenanthrene hydroxylation. The mono-oxygenation of salicylate in *Sphingomonas yanoikuyae* B1 and *Sphingomonas* sp. CHY-1 (Demaneche et al. 2004; Cho et al. 2005) has been shown to be catalysed by BphA1/2c dioxygenase. The deficiency of the Km24 strain to grow on naphthalene and salicylate might be the lack of salicylate mono-oxygenation activity. The sequence of *bphA1/2c* was identical with the *phnA1b/A2b* from CHY-1 demonstrating that the HV3 strain also has a highly similar salicylate mono-oxygenase.

The HV3 strain is unique in its ability to degrade several aromatic, chloroaromatic and polyaromatic compounds. The *Sphingobium* sp. HV3 versatile degradation capacities are partly explained by the presence of upper and lower *meta*-pathway and the modified chlorocatechol degradation pathway. *Sphingomonas* are known to degrade BTEX compounds and PAHs (Kim and Zylstra 1999; Romine et al. 1999), various herbicides like 2,4-dichlorophenoxy acetic acid (2,4-D) and 2-(4-chloro-2-methylphenoxy) propanoic acid (Mecoprop; Kohler 1999) and even high molecular weight PAHs like four aromatic ring chrysene (Demaneche et al. 2004; Willison 2004), but these traits have so far not been reported to a single bacterial strain. The shown diverse catabolic features of the HV3 strain makes it promising for biotechnological applications like bioaugmentation of low concentration mixed pollution sites.

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