Biodegradation **15:** 111–123, 2004.

Identification of two new sets of genes for dibenzothiophene transformation in *Burkholderia* sp. DBT1

Simona Di Gregorio[1], Chiara Zocca[1], Stephan Sidler[2], Annita Toffanin[3], Daniela Lizzari[1] & Giovanni Vallini[1,*]

[1]*Department of Science and Technology, Laboratories of Microbial Biotechnology and Environmental Microbiology, University of Verona, Strada Le Grazie 15 - Ca' Vignal, I-37134 Verona, Italy;* [2]*Department of Biotechnology, Laboratories of Microbiology, E.T.S.I. - Agronomos, Technical University of Madrid (UPM), E-28040 Madrid, Spain;* [3]*Department of Agricultural Chemistry and Biotechnology, University of Pisa, Via del Borghetto 80, I-56124 Pisa, Italy (*author for correspondence: e-mail: giovanni.vallini@univr.it)*

Accepted 22 October 2003

Key words: dibenzothiophene transformation, gene organization, petroleum organosulfur heterocycles, transposon mutagenesis

Abstract

A novel genotype for the initial steps of the oxidative degradation of dibenzothiophene (DBT) is described in a *Burkholderia* sp. strain isolated from a drain receiving oil refinery wastewater. The strain is capable of transforming DBT with significant efficiency when compared to other microorganisms. Its genotype was discovered by investigating insertional mutants of genes involved in DBT degradation by the Kodama pathway. The cloned *dbt* genes show a novel genomic organization when compared to previously described genes capable of DBT catabolism in that they constitute two distinct operons and are not clustered in a single transcript. Sequence analysis suggests the presence of a σ^{54}-dependent positive transcriptional regulator that may be involved in the control of the transcription of the two operons, both activated by DBT. The achieved results suggest the possibility of novel features of DBT biotransformation in nature.

Introduction

Crude oils contain between 0.04 and 5% (wt/wt) sulfur (Speight 1980), which is mainly represented by organosulfurs including thiols, sulfides, and thiophenes. Condensed thiophenes such as dibenzothiophenes are among the most commonly found organosulfurs in fossil fuels (Bence et al. 1996) and represent the predominant portion of the so-called heavy fraction of crude oils. Moreover, they are more recalcitrant to biodegradation than the non-sulfur containing polycyclic aromatic hydrocarbons (PAH). Dibenzothiophene (DBT) serves as a model molecule for the description of the microbial capacity to transform organosulfurs (Kodama et al. 1970, 1973; Crawford & Gupta 1990; Kropp & Fedorak 1998). One of the described microbial DBT oxidative pathways, the Kodama pathway (Figure 1), transforms the molecule to the final product 3-hydroxy-2-formylbenzothiophene (HFBT). The intermediates of the Kodama pathway are colored compounds whose occurrence in suspension culture is an indication of the microbial activation of this specific oxidative process (Kodama et al. 1970, 1973). Denome et al. (1993) cloned the genes responsible for the Kodama pathway from *Pseudomonas* sp.. These genes are organized in a single operon, encode enzymes of the upper naphthalene catabolic pathway, and belong to a group of genes showing a high degree of homology to the *nah* genes from *Pseudomonas putida* G7 (Simon et al. 1993). The *nah*-like class of genes, cloned from different microorganisms, are highly conserved and are involved in the transformation of molecules that constitute the low molecular weight fraction of PAHs, including DBT (Denome et al. 1993; Menn et al. 1993; Sanseverino et al. 1993; Kiyohara et al. 1994; Geiselbrecht et al. 1998). They

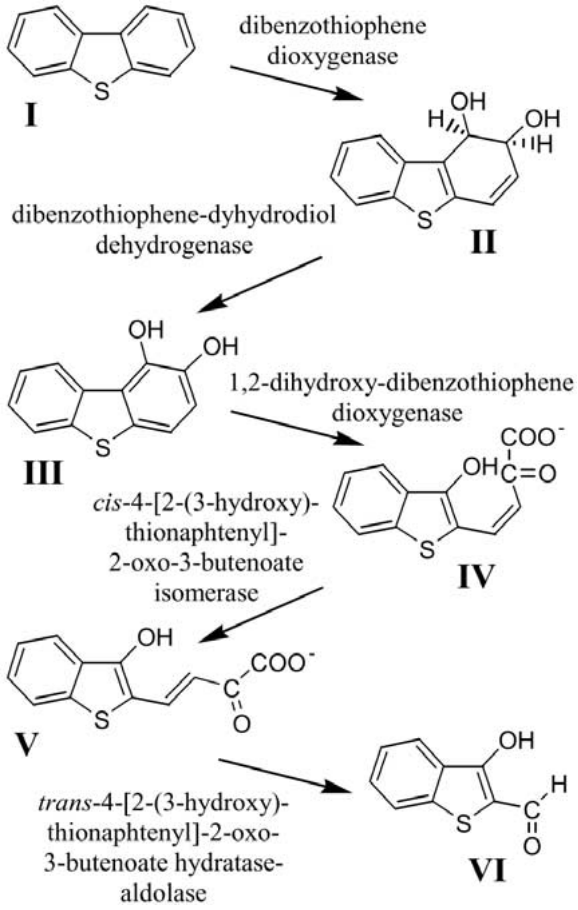

Figure 1. Kodama pathway showing the degradation of DBT to HFBT. (I) dibenzothiophene (DBT); (II) *cis*-1,2-dihydroxy-1,2-dihydrodibenzothiophene; (III) 1,2-dihydroxy-dibenzothiophene; (IV) *cis*-4[2-(3-hydroxy)-thionaphthenyl]-2-oxo-3-butenoate; (V) *trans*-4[2-(3-hydroxy)-thionaphthenyl]-2-oxo-3-butenoate; (VI) 3-hydroxy-2-formyl benzothiophene (HFBT).

are normally clustered in a single operon under the control of a single promoter.

The scope of the present work was the isolation and molecular characterization of microbial strains capable of transforming DBT. By analysis of enrichment cultures of an inoculum derived from a drain receiving oil refinery wastewater and grown in presence of DBT, we found a microbial candidate capable of transforming the organosulfur via the Kodama pathway. The microorganism belongs to the *Burkholderia* genus and transforms DBT with significant efficiency compared to other microorganisms. Detailed characterization of the isolate showed the presence of a novel set of genes responsible for the early part of the Kodama pathway. The genes are organized in two operons instead of one, and show only low similarity to the *nah*-like class of genes. The unusual gene organisation suggests the possibility of novel features of DBT transformation in natural context.

Materials and methods

Nucleotide sequences accession numbers

The nucleotide sequences of the DBT oxidizing genes (*dbts*) in *Burkholderia* sp. DBT1 are available in GenBank with accession numbers AF380367 and AF404408.

Chemicals

DBT and solvents were purchased from Sigma Aldrich. Bacteriological preparations were purchased from Difco. Other chemicals and solvents were analytical grade.

Cultivation media

Enrichment cultures were set up in minimal defined medium DM (Frassinetti et al. 1998) supplemented with DBT that was added in excess to DM medium: 500 mg l^{-1} DBT as *N-N*-dimethylformamide (Amersham Pharmacia Biotech) solutions. The solidified medium (noble agar) was supplemented with DBT spraying the surface of the plates with an ethanol free diethyl ether solution (5%) (Kiyohara et al. 1982).

Yeast Mannitol Broth (YMB): 0.5 g l^{-1} K_2HPO_4; 0.2 g l^{-1} $MgSO_4 \cdot 7H_2O$; 0.1 g l^{-1} NaCl; 1.0 g l^{-1} yeast extract; 10 g l^{-1} mannitol and Yeast Mannitol Agar (YMA): YMB solidified with 15 g l^{-1} of noble agar were utilized.

Molecular techniques

Standard procedures were used for plasmid DNA preparation, manipulation and agarose gel electrophoresis (Sambrook et al. 1989). Plasmid DNA was extracted using the Qiaprep Spin Plasmid Kit (Qiagen), bacterial genomic DNA using the Nucleospin Tissues Kit (Clontech), and total RNA using the TRIzol Reagent (Invitrogen), following the manufacturer's instructions. Genomic DNA was manipulated using enzymes purchased from Amersham Pharmacia Biotech. Cloned DNA was sequenced using a PRISM Ready Reaction DNA terminator cycle sequencing Kit (Perkin-Elmer) and run on an Applied Biosystems Inc. (ABI) 377 instrument. Nucleotide sequence data

were assembled using the ABI Fractura and Assembler computer packages and analyzed using ClustalW (Thompson et al. 1994) and Omiga (version 1.1) (Oxford Molecular Group, Oxford, UK).

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* DH5α was the host strain for replicative units pH1A, p46 and p51, derived from the insertional mutants, and for pMB393Ac and pMB393C plasmids used for the complementation of the insertional mutants. *E. coli* S17-1 (λ*pir*) was the host and mobilizing strain for the suicide vector pSS240, which harbors a self-cloning promoter-probe minitransposon m*Tn*5Ss*gus*AoriR (see below). *E. coli* harboring the recombinant constructs were routinely grown and maintained on either solidified or liquid Luria Bertani (LB). The antibiotics used were either 100 μg ml^{-1} ampicillin or 50 μg ml^{-1} streptomycin-spectinomycin (Sigma Aldrich). *Burkholderia* sp. DBT1 and its selected insertional mutants were routinely grown at 30 °C; *E. coli* strains at 37 °C.

Isolation of DBT1

Enrichment cultures were set up as described in Frassinetti et al. (1998). The enrichment inoculum was collected from a drain receiving oil refinery wastewater. Twenty-one isolates were recovered and clustered in different operational taxonomic units (OTUs) by ARDRA analysis using primers fD1 and rP2 (Weisburg et al. 1991) to amplify the 16S rRNA genes and digesting the amplification products with *Hae*III and *Hha*I. All the isolates were screened for their capacity to transform DBT based on the capacity to develop an orange color on agar plates supplemented with the molecule. One isolate showed the orange color indicating that the Kodama pathway was active. The corresponding microorganism was sub-cultured for further analysis and the 16S rRNA gene was amplified, sequenced on both strands, and aligned to the database sequences using BLAST (Altschul et al. 1997).

Testing of the isolate

The strain DBT1 was inoculated in tightly closed 125 ml flasks containing 25 ml of DM supplemented with DBT. The growth of the isolate was evaluated by means of CFU counts on YMA plates. The transformation of DBT was monitored spectrophotometrically at 325 nm analyzing the hexane extracts of microbial cultures filtrates, (pore size 0.2 μm), as described by Setti et al. (1995). The DBT concentration was measured using the content of the entire flask. The TLC analyses was performed as described in Frassinetti et al. (1998). The purification and the identification of HFBT were performed by ^{1}H-NMR and GC-MS analysis as previously described by Frassinetti et al. (1998).

Molecular characterization of DBT1

PCR reactions were set up in order to partially clone the genes involved in DBT transformation. The primers used were nahAcfor and nahAcrev, which amplify a 992 bp region encompassing nucleotides 63 to 1055 of *Pseudomonas putida* G7, *nah*Ac (GenBank accession no. M83949) (Simon et al. 1993) and P8073, P9047, which amplify a 993 bp region encompassing nucleotides 82 to 1075 of *Burkholderia* sp. RP007, *phn*Ac (GenBank accession no. AF061751) (Laurie & Lloyd-Jones 2000).

Transposon mutagenesis

Insertional mutants of the strain DBT1 were generated using the protocol by Ditta (1986). In order to obtain a self-cloning promoter-probe minitransposon, the origin of replication pMB1 (oriR), was amplified from plasmid pRL278 (GenBank accession no. L05083) with PCR primers GTCGACCAAAATCCCTTAACGTGA and GTCGACATACGCGTAAAACAGCCAGC. A 1.1 kb amplification product was obtained and cloned into plasmid pCAM140 (Wilson et al. 1995) partially digested with *Sma*I, to form plasmid pSS240. The resulting minitranposon construct harboured the pMB1 (oriR) origin of replication between the promoterless β-glucoronidase (*gus*A) gene and the antibiotic spectinomycin-streptomycin resistance cassette (Sm/sp). Putative insertional mutants (>10,000 total) selected on 50 μg ml^{-1} spectinomycin-streptomycin YMA plates, sprayed with DBT, were tested for the loss of the capacity to form orange colonies. Genomic DNA of putative transposon recipients was extracted and digested with the restriction endonuclease *Pst*I that does not cut inside the mT*n*5Ss*gus*AoriR minitransposon. The digested DNA was circularized using T4 ligase and electroporated into competent *E. coli* DH5α, successively streaked on solidified LB with 50 μg ml^{-1} spectinomycin-streptomycin. Different replicative units were obtained as plasmid

Table 1. Bacterial strains and plasmids

Strains and plasmids	Description	Sources
Strains		
Burkholderia sp. DBT1	Transforms DBT	Present study
E. coli DH5α	F-, *phi*80d *lac*Z Delta M15, Delta (*lac*ZYA-*arg*F), U169, *deo*R, *rec*A1, *had*R17 (rk−, mk+), *gal*-, *pho*A, *sup*E44, Lambda-, thi-1, *gyr*A96m *rel*A1	Invitrogen
E. coli S17-1 (λ-*pir*)	λ-pir lysogen of S17-1 [*thi pro hsd*R$^-$ *hsd*M$^+$ recA RP4 2-Tc::Mu-Km::*Tn*7(Tp^r/Sm^r)]	Victor de Lorenzo, Centro de Investigaciones Biologicas Madrid, Spain
Plasmids		
pMB393	Spectinomycin-resistant derivative of pBBR1MCS	(Gage et al. 1996)
pMB393Ac	Spectinomycin-resistant derivative of pMB393 complementing MH1A	Present study
pMB393C	Spectinomycin-resistant derivative of pMB393 complementing M51	Present study
pCAM140	Sm/sp, Ap, m*Tn*5S*gus*A40 in pUT/mini-*Tn*5 Sm/sp	(Wilson et al. 1995)
pSS240	Spectinomycin-resistant derivative of pCAM140 harbouring a pMB1 origin of replication	Present study

preparations from several insertional mutants and successively sequenced by inverse PCR on both strands to gain information on the nucleotide sequence of the transposon flanking regions. For this purpose, the primers gus55-TTTGATTTCACGGGTTGG and aadA1846-GCTGGCTTTTTCTTGTTATCG were designed, which anneal to the end regions of the m*Tn*5S*gus*AoriR minitransposon. Three replicative units, pH1A, p46 and p51, were completely sequenced by inverse PCR.

RT-PCR reactions

The strain DBT1 was grown to exponential phase in DM in the presence of DBT or 250 mg l^{-1} citrate (Sigma Aldrich). A total of 10^8 cells were pelleted from which RNA was isolated and successively treated with DNAse I. RNasin (Promega) was added to maintain the integrity of the mRNA. RT-PCR was carried out with M-MLV Reverse Transcriptase, RNA H Minus, Point Mutant (Promega), using respectively, primers p3-CGAATTTCCCGAAGTCCCAATT, p5-GTCGGATCCCAATGCAGAAACTCC, p7-CCGACAATGATAATATGGTCACCGC for the genes cloned on the pH1A replicative unit and p9-CTGATAATCGAGGCGCAAATAGA, p11-TTGTTCCGGTTTACATCGTAGCTC, p13-ATACTGCATGGGCGATATGTTTAAC for the genes cloned on the p51 replicative unit (Figure 2).

The reaction conditions were as follows: annealing at 42 °C for 10 min and extension at 50 °C for 1 h. The PCR reactions were carried out using respectively primers p1-GGGACGAGAAAACGTTATCAATT GCG, p2-GGTCGGAAACATGGGGTAATGGA, p4-GCTCCCATTTCAATGGCACCGTTCTT, p6-GGAATGAAAACGGAGCTGTCCGGA for the genes cloned on the pH1A replicative unit, (Figure 2) and p8-TAGGCTGGCCTATTCCACCTTCA, p10-AGCGGCCAAGCGAATCAATCATTT, p12-GTCTTCCCGGTTATGTGGACATTA or the genes cloned on the p51 replicative unit (Figure 2).

Appropriate positive and negative PCR controls were included. The cycling conditions for the RT-PCR amplification were as follows: 94 °C for 1 min, at 64 °C for 1 min, 72 °C for 2 min, followed by 25 cycles, 72 °C for 5 min. The amplification products were then sequenced.

Complementation of the characterized mutants

MH1A and M51 insertional mutants were complemented. The complementing genomic fragment was obtained by PCR reactions and cloning of the amplified fragments in the broad host range cloning vector pMB393 (Gage et al. 1996). The primers PAcF-CGG*CTCGAG*GCCGTCTCCTCTCGGGAATTTTG-C and PAcR-GGG*GGGCCC*TTATTTTCCGATGAG-CTCGCGGTTCC were designed for DBT1 genomic DNA (Figure 2) to complement the MH1A mutant.

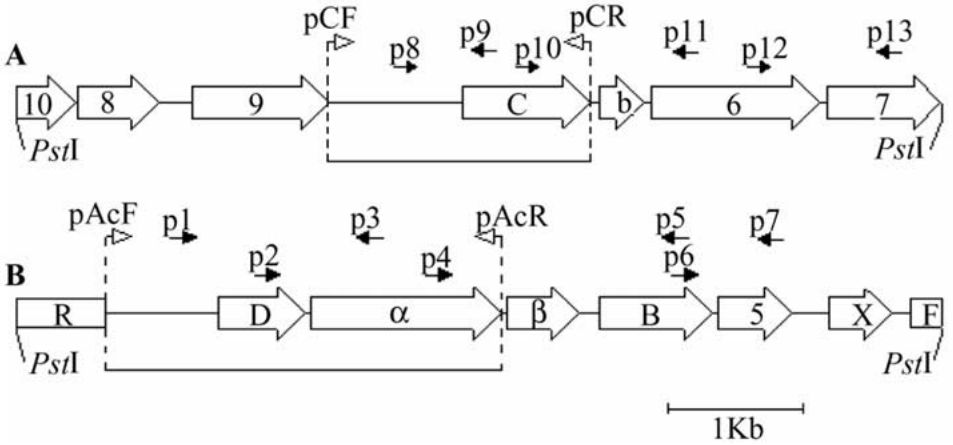

Figure 2. Map of the two DBT oxidizing operons in DBT1. (A) Map of the DBT1 *Pst*I genomic fragment mutagenized in MH1A and M46 insertional mutants. (B) Map of the DBT1 *Pst*I genomic fragment mutagenized in M51. The primers used for RT-PCR experiments (p1–p13) and complementation tests (pAcF/pAcR, pCF/pCR) are reported.

They amplified product from nucleotide 643 to 3486 on the genomic fragment deposited with the accession number AF380367. The cycling conditions were as follows: 94°C for 1 min, at 69 °C for 1 min, 72 °C for 2 min, followed by 25 cycles, 72 °C for 5 min. Both primers had a clamp: *Xho*I clamp was present in pAcF and *Sma*I clamp was present in the pAcR primer.

The primers pCF-CGG*GGTACC*CAATTGGAT TCCCGGATATCTGTCC and pCR-GGG*GGGCC C*TTATTCTTTCAATTTGACGTCAAGGCCG were designed for DBT1 genomic DNA (Figure 2) to complement the M51 mutant. They amplified from nucleotides 2351 to 4162 on the genomic fragment with the accession number AF404408. The cycling conditions were as described above. The pCF primer had a *Kpn*I clamp and the pCR primer had a *Sma*I clamp. The expected products were amplified using the Herculase DNA Polymerase (Stratagene). The PCR products were sequenced and successively cloned in pMB393 as *Xho*I *Sma*I and *Kpn*I *Sma*I fragments respectively, obtaining the pMB393Ac and the pMB393C plasmids complementing MH1A and M51 mutants. The two plasmids were transformed into competent MH1A and M51 mutant cells by electroporation (Dennis & Sokol 1995).

Fluorimetric GUS assay

The assay was performed growing *Burkholderia* sp. DBT1, MH1A, M46 and M51 mutants in YMB and YMB containing DBT. The bacterial cells were collected by centrifugation at different times, resuspended in 50 mM sodium phosphate buffer (pH 7.0), 10 mM β-mercaptoethanol, 10 mM disodium EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100, sonicated, and centrifuged at 16,000 g for 10 min at 4 °C. GUS activity was assayed in the supernatant. Fluorimetric GUS assays were performed as described by Jefferson (1987). Protein concentration in bacterial extracts was determined using the Bradford reagent (BioRad) according to the manufacturer's instructions.

Results

Isolation and biochemical characterization of DBT1

With the scope of isolating microorganisms capable of transforming organosulfurs, 21 candidates were recovered by enrichment cultures of a inoculum derived from a drain receiving oil refinery wastewater grown in the presence of DBT. A single microorganism capable of transforming DBT via the Kodama pathway was selected. The gene encoding for 16S rRNA was amplified, sequenced and analyzed, tentatively assigning the microorganism to the *Burkholderia* genus (99% homology to *Burkholderia* sp. isolate N2P5). The strain was designated *Burkholderia* sp. DBT1 due to its capacity to transform DBT.

The DBT transformation capacity of the strain, initially indicated by the release of colored compounds in the growth medium, was confirmed by the removal of DBT from the growth minimal medium when the molecule was used as a sole carbon and energy source (Figure 3). DBT removal was associated with corresponding microbial growth. The DBT content of the

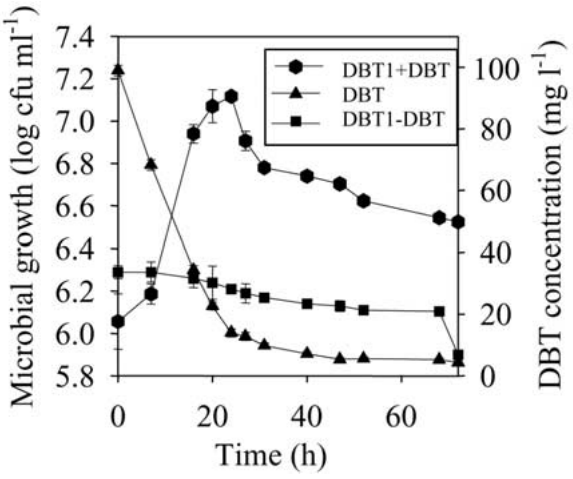

Figure 3. Growth of *Burkholderia* sp. DBT1 in DM supplemented with DBT. Growth of DBT1 cells is recorded only in presence of DBT and is concomitant with a decrease of DBT content in the growth medium. DBT1 does not growth in absence of DBT.

culture decrease by 93% after 72 h of incubation. Separation of the reaction intermediates at the end of the bacterial growth was performed by TLC and six colored spots characterizing the Kodama pathway were recovered (Kodama et al. 1973) (data not shown). A pale yellow compound with an absorption peak at 394 nm (Rf = 0.85) was purified by a column chromatography procedure (Frassinetti et al. 1998) and further analyzed by ^{1}H-NMR. The spectrum indicated four non-equivalent aromatic protons at δ 7.45 (triplet, J = 9 Hz, where J is the coupling constant value) that collapse to doublet upon irradiation at δ 7.96, 7.60 (triplet, J = 9 Hz), 7.80 (doublet, J = 9 Hz), and 8.05 (doublet, J = 9 Hz). The collapsing is ascribable to an aldehydic proton. The mass spectrum of the molecule showed the molecular ion at m/z 178 (100% relative intensity; $C_9H_6O_2S$ requires M^+178) and important fragment ions at m/z 177 (M^+–H), 121, 77 that are expected from an aldehyde. These data are consistent with the final product HFBT reported by Kodama et al. (1973) confirming that DBT1 transform DBT via the Kodama pathway (Figure 1), as expected.

Cloning of dbt genes

To clone the genes involved in the DBT transformation pathway, primers designed to amplify the *nah*-like class of genes were used. In the genus *Burkholderia*, an isofunctional cluster of genes (*phn* operon) has been cloned from a strain capable to transform phenanthrene (Laurie & Lloyd-Jones 1999). Primers designed from *phn* genotype were also used. However, using both the combination of primers, no amplification products were detected using the DBT1 genomic DNA as template.

To characterize the DBT1 genes involved in the transformation of DBT, insertional mutants of the isolate were generated and screened for the loss of the capacity to produce orange-colored intermediates on solidified medium sprayed with DBT. Three stable insertional mutants were obtained (Table 2). As expected, the selected insertional mutants lost the capacity to remove DBT from liquid culture (data not shown).

A set of inverse PCR reactions was programmed on the transposon flanking regions of the three insertional mutants. The results indicated that in two of these (MH1A, M46), the transposon was inserted in the iron sulfur protein (ISP) large (α) subunit of the initial dioxygenase with an opposite orientation. In the third mutant (M51) the transposon was inserted in the PAH extradiol dioxygenase (Table 2). Three replicative units, pH1A, p46 and p51 deriving, respectively, from the three insertional mutants (Figure 4), were completely sequenced.

Based on nucleotide and amino acid sequence similarities, putative gene identifications were assigned to the resulting sequences (Tables 3 and 4). The information obtained from sequence data indicated the occurrence of different ORFs corresponding to (i) genes encoding for enzymes catalysing the different steps of the Kodama pathway and (ii) genes encoding for protein not involved in the same pathway. Thus, the former ORFs were named *dbt* and the latter were not given names. Eight putative ORFs were recovered internal to the p46 and pH1A replicative units (GeneBank accession no. AF380367) (Figure 4). Seven of these ORFs initiated with the expected ATG start codon, while the truncated ORF R (*dbt*R) started with a GTG start codon. A putative ribosomal binding site preceded each start codon. ORF D, encoded for DbtD, a 2-hydroxychromene-2-carboxylate isomerase. ORF α and β encoded DbtAc and DbtAd, respectively the large (α) and small (β) subunit of the initial dioxygenase (ISP). ORF B encoded DbtB, a dihydrodiol dehydrogenase. Downstream the four genes, ORF 5 encoded for a protein similar to a NADH:FMN oxidoreductase, the first of these genes that are not involved in the Kodama pathway. The close arrangement of the described ORFs suggested that the related genes constitute a transcriptional unit (*dbt*D,Ac,Ad,B,ORF5). No ORF was detected 271 bp downstream ORF 5 and an inverted repeat was

Table 2. Burkholderia sp. DBT1 replicative units derived from insertional mutants

Replicative units – Insertional mutants	Sequencing results: primer *gus55*	Sequencing results: primer *aada1846*
pH1A–MH1A *dbt*Ac::m*Tn*5Ss*gus*AoriR	ISP α subunit IC	ISP α subunit
p46 - M46 *dbt*Ac::m*Tn*5Ss*gus*AoriR	ISP α subunit	ISP α subunit IC
p51–M51 *dbt*C::m*Tn*5Ss*gus*AoriR	PAH extradiol diox IC	PAH extradiol diox

ISP, iron sulfur protein; IC, inverse complement; diox, dioxygenase.

Table 3. dbt genes cloned on pH1A replicative unit

ORF	Gene	Nucleotide	Protein feature	% homology isofunctional genes	
R	*dbt*R	642-1	transcriptional activator	*phlR* 60% ***phnR*** 65%	(X91145) (AF061751)
D	*dbt*D	1443-2066	isomerase	***nahD*** 64% ***phnD*** 60%	(U09057) (AF061751)
α	*dbt*Ac	2119-3486	ISP α subunit	***ndoAc*** 68% ***phnAc*** 68%	(M23914) (AF061751)
β	*dbt*Ad	3516-4034	ISP β subunit	***pahAd*** 54% ***phnAd*** 59%	(AB004059) (AF061751)
B	*dbt*B	4182-5000	dihydrodiol dehydrogenase	***bphB*** 65% ***phnB*** 76%	(D17319) (AF061751)
5	ORF5	5029-5562	putative oxydoreductase	*ntaB* 62%	(I40751)
X	ORFX	5833-6282	unknown protein	***nahX*** 69% ***phnX*** 83%	(AF100302) (AF112137)
F	ORFF	6418-6643	salicylaldeyde dehydrogenase	***bphA1C*** 83%	(AF079317)

The cloned ORFs, where possible, were compared either to the highly conserved *nah*-like class of genes or to the *phn* sequences (indicated in boldface). The GeneBank accession numbers are reported.

detected 91 bp downstream the ORF 5 stop codon. The inverted repeat is capable of forming a stem-loop structure that may be a transcriptional terminator. Downstream the above-mentioned 271 bp, two putative ORFs, ORF X and the truncated ORF F, were detected. They encoded, respectively, for a protein homologous to PhnX cloned in *Burkholderia* sp. RP007 and for the N-terminal of a salicylaldehyde dehydrogenase. Neither protein is involved in the Kodama pathway.

Upstream all the described structural ORFs, separated by 800 bp, ORF R encoded for the N-terminal of DbtR, a putative σ^{54}-dependent transcriptional regulator. The above-mentioned regulative ORF R was divergently transcribed with respect to the structural ORFs described. The putative partial coding region showed 65% of homology to the amino acid sequences of the positive transcriptional regulators of the NtrC family (Buikema et al. 1995).

In summary, the described organization of the different ORFs cloned from the p46 and pH1A replicative units suggests that they contain two transcriptional units, the *dbt*D,Ac,Ab,B,ORF5 and the truncated ORFX,F both preceded by the regulative truncated gene *dbt*R.

With regards to p46 and pH1A, the complete nucleotide sequence of p51 replicative unit was determined, and seven putative ORFs were cloned (Figure 4) (GeneBank accession AF404408). The cloned ORFs initiated with a ATG start codon and a putative ribosomal binding site preceded each of the ORFs. ORF 10 encoded the C-terminal of a protein homologous to

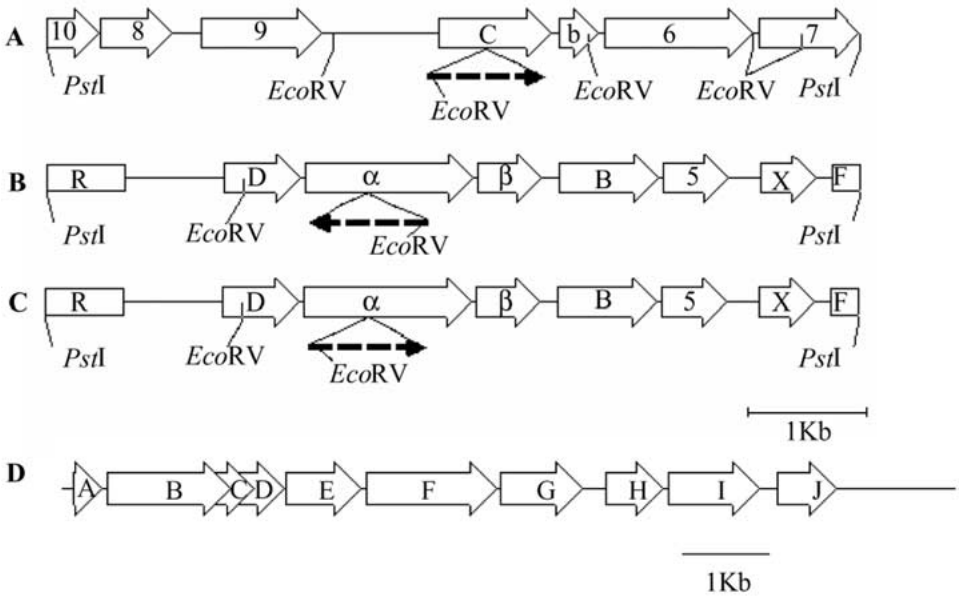

Figure 4. Diagram of the p51 (A), p46 (B), pH1A (C) replicative unit structures. The dotted arrow represents the transposon and the orientation of the reporter gene *gus*A with respect to the coding orientation of the mutagenized transcripts. The DBT1 genes responsible for oxidation of DBT via the Kodama pathway are designated as R for *dbt*R, D for *dbt*D, α for *dbt*Ac, β for *dbt*Ad, B for *dbt*B, C for *dbt*C, b for *dbt*Ab. The other ORFs were indicated as follows: 5, ORF5; 6, ORF6; 7, ORF7; 8, ORF8, 9, ORF9, X, ORFX; F, ORFF. (D) Diagram of the cluster of *dox* genes responsible for oxidation of DBT in *Pseudomonas* sp. (Denome et al. 1993). The genes are designated as A for *dox*A, B for *dox*B, C for *dox*C, D for *dox*D, E for *dox*E, F for *dox*F, G for *dox*G, H for *dox*H, I for *dox*I, J for *dox*J.

the large subunit of an aromatic oxygenase. ORF 8 encoded for a protein analogous to 2-hydroxychromene-2-carboxylate isomerase from the PAH catabolic pathway, showing 57% of homology to the isofunctional DbtD cloned on p46 replicative unit. ORF 9 encoded for a protein homologous to an oxidoreductase. No ORFs were observed in the 974 bp region downstream ORF 9. Downstream the untranscribed region, four ORF were recovered. ORF C and ORF b encoded, respectively, for DbtC, an extradiol dioxygenase, and for DbtAb, a ferrodoxin subunit of the initial dioxygenase (ISP). ORF 6 and ORF 7 encoded for proteins homologous to an oxidoreductase and for the C-terminal of an hydrolase, respectively, which are not involved in the Kodama pathway. The described organization of the different ORFs suggests that the p51 unit harboured two truncated transcriptional units, ORF10,9,8 and *dbt*C,Ab,ORF6,ORF7.

In summary, these results indicated that two independent insertional events in two genes encoding for dioxygenases determines the loss of the capacity of DBT1 to transform DBT via the Kodama pathway. The results of sequence analysis indicated that knocking out two genes putatively determines also the loss of two distinct transcriptional units, resulting from the close arrangement of the cloned genes on the flanking regions. Thus, the co-transcription of the genes constituting the putative transcriptional units involved in DBT transformation was verified by RT-PCR. RNA was extracted from DBT1 grown under inducing (DM, DBT) and uninducing (DM, citrate) conditions and used as target for RT-PCR amplification of partial gene sequences (Figure 2). The cDNAs derived from induced cells showed amplification products of the size expected by co-transcription (data not shown). No cDNAs were obtained from uninduced cells. All positive and negative PCR controls gave the expected results. The identity of the RT-PCR products was confirmed by sequencing the amplified fragments and indicated that DBT induces the transcription of the transcriptional units *dbt*D,Ac,Ad,B,ORF5 and *dbt*C,Ab,ORF6,ORF7. No transcription was observed upstream *dbt*D and *dbt*C and downstream ORF5.

The two transcriptional units might constitute two separate operons, and, by examination of the orientation of transcription, the expected transcription initiation sites and the promoter regions should be located upstream *dbt*D and *dbt*C. Thus, the untranscribed regions upstream the two clusters of oxidative genes, respectively 800 bp on pH1A and 974 bp

Table 4. *dbt* genes cloned on p51 replicative unit

ORF	Gene	Nucleotide	Protein feature	% homology isofunctional genes	
10	ORF10	1-435	aromatic oxygenase	*bphA1c* 83%	(AF079317)
8	ORF8	449-1039	isomerase	***pahD*** 60%	(AB004059)
				phnD 66%	(AF061751)
9	ORF9	1275-2264	oxydoreductase	*mll7162* 64%	(AP003011)
C	*dbt*C	3239-4162	PAH extradiol	***nahC*** 74%	(AF039533)
			dioxygenase	***phnC*** 21%	(AF061751)
b	*dbt*Ab	4236-4547	ISP ferrodoxin	***doxA*** 53%	(M60405)
			subunit	***phnAb*** 72%	(AF061751)
6	ORF6	4602-5822	oxydoreductase	*spr0246* 44%	(AE008406)
7	ORF7	5877-6707	hydrolase	*todF* 47%	(D90906)

The cloned ORFs, where possible, were compared either to the highly conserved *nah*-like class of genes or to the *phn* sequences (indicated in boldface). The GeneBank accession numbers are reported.

on p51 replicative unit, might have the consensus sequences necessary to function as promoters. In order to verify this hypothesis, the insertional mutants were tentatively complemented by constructing two expression vectors harboring the two untranscribed regions as inducible promoters (Figure 2). The complementing genomic fragments were obtained by PCR reactions on DBT1 genomic DNA using primers pAcF and pAcR to complement MH1A mutant, and pCF and pCR to complement M51 mutant (Figure 2). The two genomic regions amplified by PCR were cloned into pMB393, obtaining the pMB393Ac and the pMB393C plasmids complementing, respectively, the MH1A and M51 insertional mutants. The complemented insertional mutants recovered the capacity to produce orange-colored colonies on solid medium sprayed with DBT, indicating the occurrence of DBT transformation. The results indicated the presence of two distinct promoters upstream the two transcriptional units induced by DBT, confirming the hypothesis that these transcriptional units constitute two distinct operons.

GUS assay

In order to further analyze the induction of the two operons, a GUS activity fluorimetric assay was performed on MH1A, M51, M46 insertional mutants and on DBT1 as a control, in presence (inducing conditions) and absence (non-inducing conditions) of DBT (Figure 5). GUS activity was not detected in uninduced bacteria grown in YMB. Addition of DBT to the growth medium rapidly induced GUS activity in the MH1A and M51 mutants. GUS activity was not induced in either the M46 mutant (internal control) or in DBT1. The specific activity of GUS at the end of the exponential growth phases of the two mutants is 8 to 10 times higher than that observed at the two initial growth phases (Figure 5).

Discussion

The objective of the present study was the isolation and characterization of microorganisms capable of transforming DBT. By a culture-based approach, *Burkholderia* sp. DBT1, capable of transforming DBT via the Kodama pathway, was isolated from a drain receiving oil refinery wastewater. The assumption that DBT1 is able to transform DBT was initially based on the observation of the microbial release of colored intermediates in presence of DBT. This initial assumption was confirmed by the recovery, in the microbial growth medium, of the final product of the Kodama pathway (HFBT). Moreover, amending DBT as the sole carbon and energy source, DBT1 in liquid culture shows a clear growth, concomitantly to decreased levels of the compound in growth medium. In fact, after three days DBT is nearly completely removed. The DBT transformation time course may also be compared to that reported for other DBT transforming microorganisms. *A Rhizobium* strain, under similar growth conditions and following the same oxidative pathway, has been reported to remove the same DBT content in a significantly longer time (5 days) (Frassinetti et al. 1998). In our opinion, DBT1 may be

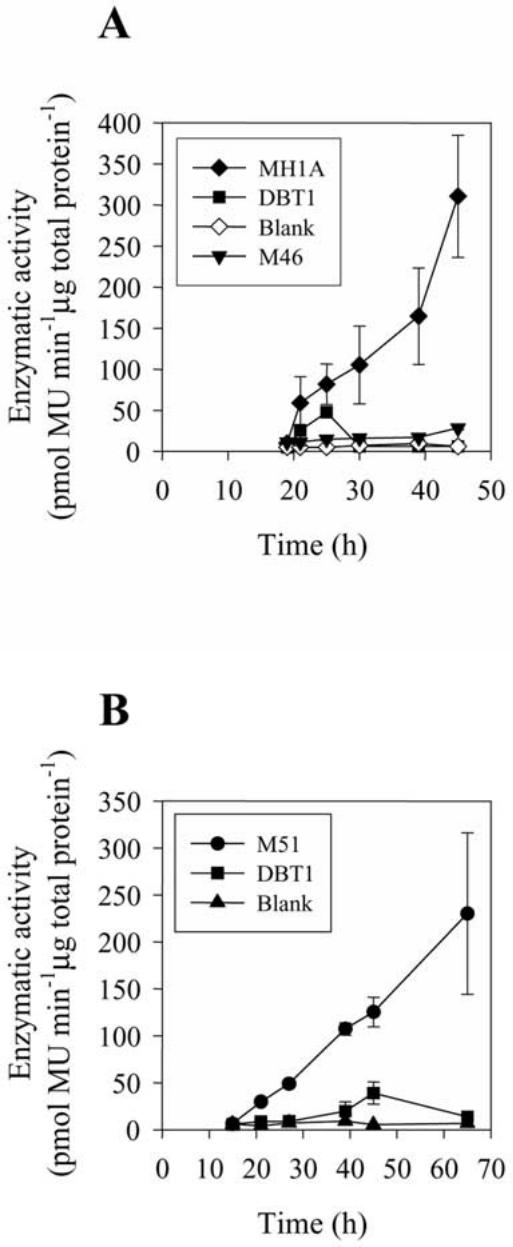

Figure 5. GUS activity in MH1A and M51 mutants harboring the reporter gene *gus*A with the same transcription orientation with respect to the one of the mutagenized operons. As controls, the GUS activity of DBT1 and M46 mutant (internal control because *gus*A in the reverse orientation with respect to the mutagenized operon) is reported.

considered an efficient bio-catalyst of DBT conversion to HFBT. Recently, mixed cultures capable to mineralize HFBT to CO_2 has been described (Bressler & Fedorak 2001). Thus, the isolation of a microorganism that rapidly transforms DBT to molecules that are easily mineralized, may prefigure new potentialities in the exploitation of microbial DBT biodegradation.

The transforming capacity of DBT1 is attributable to two novel sets of genes encoding for the initial steps of the oxidative Kodama pathway that were cloned by insertional mutagenesis.

The genes responsible for the Kodama pathway were initially cloned from *Pseudomonas* sp. by Denome et al. (1993) and were clustered into a single operon, responsible for the oxidation of naphthalene to catechol. They belong to the *nah*-like class of genes that, as previously mentioned, encode for enzymes catalyzing the transformation of the low molecular fraction of PAHs, and are usually involved in the oxidation of DBT via the Kodama pathway. These genes have been reported to be as highly conserved, clustered in one operon, and under the control of a single promoter (Zylstra et al. 1997).

The failure to recover an amplification product using primers designed to amplify the *nah*-like class of genes was the first evidence that DBT1 genes involved in DBT transformation were not highly similar to the conserved isofunctional oxidative genes. At the same time, the primers designed to amplify the isofunctional *phn* genes cloned from *Burkholderia* sp. RP007, also failed to yield a product, showing low similarity in the nucleotide sequences of isofunctional genes, even within the same microbial genus.

In order to clone the DBT1 genes involved in the Kodama pathway an insertional mutagenesis approach was adopted. The capacity of DBT1 to transform DBT was lost by knocking out the genes encoding for the initial dioxygenease (ISP) and the dihydrodiol dioxygenase (1,2-dihydroxy-dibenzothiophene dioxygenase). Sequence analysis of the replicative units derived from the insertional mutants suggested that in DBT1, the DBT transforming genes are organized in two separate transcriptional units. The results with RT-PCR corroborated this hypothesis, as co-transcription of the genes harbored by the two putative transcriptional units was observed and co-transcription of the latter with their upstream and downstream flanking regions was not recovered. As predicted, the cloned genes showed only 60% similarity to the highly conserved *nah*-like class of genes, instead of 90% reported

for the latter, as well as also 60% homology with the isofunctional *phn* genes.

The complementation of the insertional mutants indicated that the genomic regions cloned upstream of the two functional transcriptional units functioned as promoters, indicating that the knocking out of the genes encoding for the two dioxygenases also knocked out two separate operons. The two operons encode for the enzymes involved in the initial steps of the Kodama pathway (e.g. the large (α) and small (β) subunit of the initial dioxygenase (ISP), the corresponding ferrodoxin subunit, the dibenzothiophene-dihydrodiol dehydrogenase, the 1,2-dihydroxy-dibenzothiophene dioxygenase, and the isomerase). However, the enzyme catalyzing the last step of the HFBT formation (the *cis*-4-[2-(3-hydroxy)-thionaphtenyl]-2-oxo-3-butenoate hydratase-aldolase) (Figure1) has not been recovered by insertional mutagenesis, suggesting the presence of a third operon harboring the missing enzyme. In conclusion, even though as previously mentioned, isofunctional genes have been described as highly clustered, we conclude that DBT1 shows a scattered genomic organization of the genes involved in the catabolism of DBT.

An additional result of RT-PCR reaction consisted in the evidence that the two cloned operons were both transcribed in presence of DBT. Moreover, the GUS-assays showed that DBT strongly induces the translation of the two operons, as indicated by the significant increment in β-glucoronidase activity observed in presence of the molecule.

As a consequence, it can be suggested that the strong activation of the two operons by DBT observed may be responsible for the high efficiency of DBT transformation by DBT1. The presence of a gene (*dbt*R) encoding for a σ^{54}-dependent regulator indicates a possible involvement of the *dbt*R in the transcriptional activation of the two DBT1 operons. The location and direction of transcription of *dbt*R with respect to the structural genes harbored by one of the two operons has been reported in different aromatic catabolic operons (Inouye et al. 1988; Shingler et al. 1993; Ng et al. 1995). Therefore, despite the novel structural gene organization, DBT1 appears to have a conserved putative mechanism of transcription activation of catabolic genes by DBT.

The scattered genetic organization described in DBT1 resembles that one observed in *Sphingomonas* genus, where the catabolic genes involved in the oxidation of aromatic compounds to catechol intermediates are distributed on multiple gene clusters (Armengaud et al. 1998; Romine et al. 1999). It is also worth noting that the oxidative genes cloned in DBT1 are not organized in discrete pathway units. They are grouped on the same operon with oxidizing genes not associated with the Kodama pathway. Even though the DBT accumulating metabolite identified in the growth medium of DBT1 was HFBT, as expected, the involvement of genes not associated with the Kodama pathway in oxidative reactions on the DBT accumulating metabolites could not be excluded and must be further investigated. However, the involvement of these genes in the oxidative pathways of different classes of compounds may be also possible. This unusual organization of oxidizing genes in relation to pathway units has been described also in *Sphingomonas* sp. (Zylstra & Kim 1997), and, although speculative, it is possible to suggest that the activation of operons that encode for groups of genes of different oxidizing pathways may increase the capabilities of a microorganism in response to different environmental conditions.

Conclusions

A microbial strain, *Burkholderia* sp. DBT1, capable of efficiently transforming DBT to intermediates that can be mineralized has been isolated. The microorganism joins a high efficiency in DBT transformation to low similarity in nucleotide sequences and genomic organisation of *dbt* genes with isofunctional one. At present, the explanation of the relation between the two evidences may be only speculative. However, the cloning of the *dbt* genes expands current knowledge of the genetics of the microbial capacity to transform DBT. The enlargement of the current information on the genes governing the process is, in our opinion, an important step in understanding the prevalence and distribution of new groups of genes involved in the catabolism of organosulfur compounds. In fact, probes developed for specific transforming genes should eventually provide a useful tool for a detailed characterization of the ecology of organosulfur transforming microorganisms within a contaminated site.

Acknowledgements

Research in the Laboratories of Microbial Biotechnology and Environmental Microbiology at University of

Verona was supported by the Italian National Environmental Protection Agency (ANPA). Stephan Sidler was the recipient of a TMR Fellowship (83EU-53237) from the Swiss Federal Foundation. Research at Department of Biotechnology of the UPM was supported through the projects DGICyT PB95-0232 (to Thomás Ruiz-Argüeso) and MCyT BIO99-1159 (to Juan Imperial). Authors thank Leonardo Setti (University of Bologna, Italy) for ^{1}H-NMR and GC-MS analyses and helpful discussion, Juan Imperial (UPM) and Mirja Salkinoja-Salonen (University of Helsinki, Finland) for critically reading the manuscript.

References

Armengaud J, Happe B & Timmis KN (1998) Genetic analysis of dioxyn dioxygenase of *Sphingomonas* sp. strain RW1: catabolic genes dispersed on the genome. J. Bacteriol. 180: 3954–3966

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 17: 3389–3340

Bence AE, Kvenvolden KA & Kennicutt II MC (1996) Organic geochemistry applied to environmental assessments of Price William Sound, Alaska, after the Exxon Valdez oil spill – a review. Org. Geochem. 24: 7–42

Bressler DC & Fedorak PM (2001) Purification, stability, and mineralisation of 3-hydroxy-2-formylbenzothiophene, a metabolite of dibenzothiophene. Appl. Environ. Micribiol. 67: 821–826

Buikema WJ, Szeto WW, Lemley PV, Orme-Johnson WH & Ausubel FM (1995) Nitrogen fixation specific regulatory genes of *Klebsiella pneumoniae* and *Rhizobium meliloti* share homology with the general nitrogen regulatory gene *ntrC* of *K. pneumoniae*. Nucleic Acids Res. 13: 4539–4555

Crawford DL & Gupta R (1990) Oxidation of dibenzothiophene by *Cunninghamella elegans.* Curr. Microbiol. 21: 229–231

Dennis JJ & Sokol PA (1995) Elettrotransformation of *Pseudomonas.* In: Nickoloff JA (Ed) Elettroporation and Elettrofusion of Microorganism (pp 125–133). Humana press, Clifton, N.J.

Denome SA, Stanley DC, Olson ES & Young KD (1993) Metabolism of dibenzothiophene and naphthalene in *Pseudomonas* strains: complete DNA sequence of an upper naphthalene catabolic pathway. J. Bacteriol. 175: 6890–6901

Ditta G (1986) *Tn5* mapping of *Rhizobium* nitrogen fixation genes. In: Methods in Enzimology, Vol 118 (pp 529–536). Academic Press, New York

Frassinetti S, Setti L, Corti A, Farinelli P, Montevecchi P & Vallini G (1998) Biodegradation of dibenzothiophene by a nodulating isolate of *Rhizobium meliloti.* Can. J. Microbiol. 44: 289–297

Gage JD, Bobo T & Long SR (1996) Use of green fluorescent protein to visualize the early events of symbiosis between *Rhizobium meliloti* and Alfalfa (*Medicago sativa*). J. Bacteriol. 178: 7159–7166

Geiselbrecht AD, Hedlund BP, Tichi MA & Staley JT (1998) Isolation of marine polycyclic aromatic hydrocarbon (PAH)-degrading *Cycloclasticus* strains from the Gulf of Mexico and comparison of their PAH degradation ability with that of Puget Sound *Cycloclasticus* strains. Appl. Environ. Microbiol. 64: 4703–4710

Inouye S, Nakazawa A & Nakazawa T (1988) Nucleotide sequence of the regulatory gene *xylR* of the TOL plasmid from *Pseudomonas putida*. Gene 66: 301–306

Jefferson R (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. 5: 387–405

Kiyohara H, Nagao K & Yana K (1982) Rapid screen for bacteria degrading water insoluble, solid hydrocarbons on agar plates. Appl. Environ. Microbial. 43: 454–457

Kiyohara H, Torigoe S, Kaida N, Asaki T, Iida T, Hayashi H & Takizawa N (1994) Cloning and characterization of a chromosomal gene cluster, *pah*, that encodes the upper pathway for phenanthrene and naphthalene utilization by *Pseudomonas putida* OUS82. J. Bacteriol. 176: 2439–2443

Kodama K, Nakatami S, Umehara K, Shimizu K, Minoda Y & Yamada K (1970) Microbial conversion of petro-sulfur compounds Part III. Isolation and identification of products from dibenzothiophene. Agr. Biol. Chem. 34: 1320-1324

Kodama K, Umehara K, Shimizu K, Nakatami S, Minoda Y & Yamada K (1973) Identification of microbial products from dibenzothiophene and its proposed oxidation pathway. Agric. Biol. Chem. 37: 45–50

Kropp KG & Fedorak PM (1998) A review of occurrence, toxicity, and biodegradation of condensed thiophenes found in petroleum. Can. J. Microbiol. 44: 605–622

Laurie AD & Lloyd-Jones G (1999) The *phn* genes of *Burkholderia* sp. strain RP007 constitute a divergent gene cluster of polycyclic aromatic hydrocarbon catabolism. J. Bacteriol. 181: 531–540

Laurie AD & Lloyd-Jones G (2000) Quantification of *phnAc* and *nahAc* in contaminated New Zealand Soil by competitive PCR. Appl. Environ. Microbiol. 66: 1814–1817

Menn FM, Applegate BM & Sayler GS (1993) NAH plasmid-mediated catabolism of anthracene and phenanthrene to naphthoic acids. Appl. Environ. Microbiol. 59: 1938–1942

Ng LC, Poh CL & Shingler V (1995) Aromatic effector activation of NtrC-like transcriptional regulator PnhR limits the catabolic potential of the (methyl)phenol degradative pathway it controls. J. Bacteriol. 177: 1485–1490

Romine MF, Stillwell LC, Wong KK, Thurston SJ, Sisk EC, Sensen C, Gaasterland T, Fredrickson JK & Saffer JD (1999) Complete sequence of a 184-kilobase catabolic plasmid from *Sphingomonas aromaticivorans* strain F199. J. Bacteriol. 18: 1585–1602

Sambrook J, Fritsch EF & Maniatis T (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanseverino J, Applegate BM, King JM & Sayler GS (1993) Plasmid-mediated mineralization of naphthalene, phenanthrene, and anthracene. Appl. Environ. Microbiol. 59: 1931–1937

Setti L, Lanzarini G & Pifferi PG (1995) Dibenzothiophene biodegradation by a *Pseudomonas* sp. in model solutions. Process. Biochem. 30: 721–728

Shingler V, Bartilson M & Moore T (1993) Cloning and nucleotide sequence of the gene encoding the positive regulator (DmpR) of the phenol catabolic pathway encoded by pVI150 and identification of DmpR as a member of NtrC family of transcriptional activators. J. Bacteriol. 175: 1596–1604

Simon MJ, Osslund TD, Saunders R, Ensley BD, Suggs S, Harcourt A, Suen WC, Cruden DL, Gibson DT & Zylstra GJ (1993) Sequences of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains G7 and NCIB 9816-4. Gene 127: 31–37

Speight JG (1980) The Chemistry and Technology of Petroleum. Marcel Dekker Inc., New York, N.Y.

Thompson JD, Higgins DG & Gibson TJ (1994) CLUSTALW: improving the sensitivity of progressive multiple sequence align-

ment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic. Acids Res. 22: 4673–4680

Weisburg WG, Barns SM, Pelletier DA & Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173: 697–703

Wilson KJ, Sessitch A, Corbo JC, Giller K, Akkermans ADL & Jefferson RA (1995) β-glucoronidase (GUS) transposons for ecological and genetic studies of Rhizobia and Gram-negative bacteria. Microbiology 141: 1691–1705

Zylstra GJ & Kim E (1997) Aromatic hydrocarbon degradation by *Sphingomonas yanoikuyae* B1. J. Ind. Miocrobiol. Biotechnol. 19: 408–414

Zylstra GJ, Kim E & Goyal AK (1997) Comparative molecular analysis of genes for polycyclic aromatic hydrocarbons degradation. Gen. Eng. 19: 257–269