UNIVERSITA' DEGLI STUDI DI VERONA

DIPARTIMENTO DI
BIOTECNOLOGIE

DOTTORATO DI RICERCA IN
BIOTECNOLOGIE MOLECOLARI, INDUSTRIALI ED AMBIENTALI

CICLO XXII

MOLECULAR, PHYLOGENETIC AND FUNCTIONAL CHARACTERIZATION OF *Burkholderia* sp. DBT1, A BACTERIAL STRAIN INVOLVED IN POLYCYCLIC AROMATIC HYDROCARBONS (PAHs) DEGRADATION

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ABBREVIATIONS

DBT. Dibenzotiofene; Dibenzothiophene

Bcc. *Burkholderia cepacia* complex

2-CBA. 2-carboxybenzaldehyde

CF. Fibrosi cistica; Cystic Fibrosis

EPA. Environmental Protection Agency

HDS. Hydrodesulfurization

HFBT. 3-hydroxy-2-formilbenzothiophene

cis-HTOB. cis-4- [2-(3-hydroxy)-tionaphthenil] 2-ossi-3-butenoic acid

trans-HTOB. trans-4- [2-(3-hydroxy)-tionaphthenil] 2-ossi-3-butenoic acid

ILs. Ionic Liquids

IPA. Idrocarburi Policiclici Aromatici

ISP. Iron Sulfur Protein Initial Dioxygenase

log K_{lip}. Lipid-water partition coefficients

log K_{ow}. Octanol-water partition coefficients

PAHs. Polycyclic Aromatic Hydrocarbons

TCA. Ciclo degli Acidi Tricarbossilici; Tricarboxylic Acid Cycle

“La gestione dell’imponderabile, il governo dell’imprevedibile, la comprensione dell’incertezza sono tensioni umane in atto da sempre, tanto forti e pesenti da rappresentare, nella loro stessa definizione, una specie di ossimoro, di eterodossia nella descrizione e nell’analisi dell’azione”

(M.L.)

Riassunto

Introduzione

La crescita dell'attività industriale avvenuta negli ultimi decenni, ed in particolar modo l'utilizzo di combustibili fossili come fonte d'energia, ha causato parallelamente la dispersione nell'ambiente di una larga varietà di composti non naturali, con bassa o assente biodegradabilità e spesso con dannose conseguenze per la salute umana. Tra gli inquinanti maggiormente presenti, gli idrocarburi policiclici aromatici (IPA) sono una vasta classe di composti principalmente originati in seguito alla parziale combustione di matrici fossili - petrolio e carbone -, o come emissione di numerosi processi industriali. Essi sono composti organici altamente idrofobici formati da due o più anelli benzenici o furanici condensati tra loro. Naftalene e fenantrene, - rispettivamente costituiti da due e tre anelli -, sono le due molecole a basso peso molecolare usate come modello per lo studio della degradazione degli IPA non sostituiti (Habe e Omori, 2003). Sono state identificate diverse vie che portano la degradazione del fenantrene. In condizione d'aerobiosi tutte le vie metaboliche iniziano tramite una diossigenasi. Generalmente, si ha un'ossidazione in posizione 3-4 generando, dopo diversi passaggi, l'acido 1-idrossi-2-naftoico. Tale composto può essere decarbossilato a naftalene-1,2-diolo, e quindi essere degradato a catecolo o a gentisato, attraverso l'acido salicidico, o a protocatecolo passando per l'acido ftalico (Fig. 1.5; Prabhu and Phale 2003; Stingley *et al.*, 2004). Alternativamente, l'acido 1-idrossi-2-naftoico può essere attaccato da un'ulteriore diossigenasi portando, come nel precedente caso, o alla formazione di catecolo attraverso l'acido salicidico, ovvero a protocatecolo passando per la 2-carbossibenzaldeide e acido ftalico (Fig. 1.6; Kiyohara *et al.*, 1976; Iwabuchi *et al*. 1997; 1998). Successivi studi hanno dimostrato com'esistono anche microrganismi che portano ad un'ossidazione del fenantrene in posizione 1-2, con la seguente formazione dell'acido 2-idrossi-1-naftoico. Il composto è quindi decarbossilato a naftalene-1,2-diolo, e quindi degradato a catecolo (Fig. 1.5; Pinyakong *et al.*, 2000). Anche la prima reazione di degradazione del naftalene avviene per opera di una diossigenasi, la quale incorpora due atomi d'ossigeno in posizione 1,2 generando il naftalene 1,2 diolo. Questo è in seguito convertito a catecolo o a gentisato passando per l'acido salicidico (Fig. 1.4; Davies ed Evans 1964). I composti a valle generati dalle vie sopra descritte - catecolo, protocatecolo e gentisato -, entrano quindi facilmente nel ciclo degli acidi tricarbossilici (TCA), portando a completa mineralizzazione il composto di partenza.

Gli IPA possono inoltre presentare degli eteroatomi d'azoto, ossigeno o zolfo. L'ultima classe di composti risulta essere la più presente delle tre all'interno dei combustibili fossili, ed è rappresentata, per più della metà, dalla classe dei tiofeni condensati. Essi sono costituiti da un anello furanico recante un atomo di zolfo unito ad uno o più anelli aromatici. Il dibenzotiofene (DBT) costituisce il modello di riferimento delle reazioni biodegradative a carico dei composti organici solforati, rappresentando il nucleo di molti composti solforati presenti nelle matrici combustibili (Kropp e Fedorak 1998). Ad oggi sono stati individuati due meccanismi di trasformazione microbica del DBT: il primo definito distruttivo, il secondo non distruttivo. La prima via, denominata anche "via di Kodama", inizia con l'attacco ad uno degli anelli omociclici da parte di una diossigenasi (ISP) in posizione 3,4, con la formazione del composto 1,2-diidrossi-1,2-diidrodibenzotiofene. Mediante una successiva azione rispettivamente di una deidrogenasi, un'extradiolo-diossigenasi ed un'idratasi-aldolasi, si ha una generazione del 3-idrossi-2-formilbenzotiofene (HFBT; Fig 1.7; Kodama *et al.*, 1973). La via non distruttiva, o "via delle 4 S", porta alla rimozione dell'atomo di zolfo senza andare ad alterare la struttura carboniosa della molecola. Tale processo procede per successive ossidazioni dell'atomo di zolfo con la formazione in successione di sulfossido, sulfone e, di seguito, la rottura dell'anello tiofenico con il rilascio dell'atomo di zolfo. Nel mezzo, quindi, si ha un accumulo di desolforato 2,2'-diidrossibefenile il quale non viene ulteriormente degradato dai microrganismi (Fig. 1.8; Gallagher *et al.*, 1993).

Molti IPA risultano essere tossici, cancerogeni e mutageni andando a costituire un serio problema per le popolazioni che risiedono in arie inquinate da tali sostanze. Piante da raccolto presenti su terreni contaminati possono assorbire gli inquinanti presenti che in questo modo possono raggiungere l'uomo e/o animali entrando

così nella catena alimentare. A fronte di tali considerazioni, negli ultimi anni sono stati svolti numerosi studi finalizzati alla bonifica di tali aree, e sempre più attenzione è stata rivolta verso una loro degradazione biologica tramite lo sfruttamento del metabolismo, o co-metabolismo microbico. Inoltre, è stato dimostrato che la rimozione e trasformazione dei composti organici dal suolo avviene in maniera più rapida nei suoli ricoperti da vegetazione rispetto a quelli in assenza di piante (Walton *et al.,* 2004). Questa osservazione ha spronato lo studio e l'uso di vegetali al fine di stimolare i processi di bonifica biologica in siti contaminati da IPA – *phytoremediation* –. Recentemente, si è inoltre provato come un protocollo di bonifica biologica basato sull'uso di piante possa essere utilizzato in associazione con l'attività metabolica ci ceppi microbici endofiti opportunamente selezionati (Barac *et al.,* 2004; Taghavi *et al.,* 2005). I batteri endofiti possono essere definiti come batteri in grado di colonizzare i tessuti interni d'organismi vegetali non determinando manifestazioni esterne d'infezione e non apportando effetti negativi all'ospite (Schulz and Boyle, 2006). Il passaggio fondamentale che va a differenziare la tradizionale *phytoremediation*, basata sulla fitoestrazione e il conseguente sequestro dei composti inquinati, dalla *phytoremediation* mediata da endofiti, è che quest'ultima prevede sia l'assorbimento della molecola contaminante dal terreno sia la sua successiva degradazione della stessa all'interno dei tessuti da parte del microrganismo. Infatti, l'inquinante è utilizzato come fonte di carbonio non dalla pianta, ma dall'endofita presente che lo degrada. Di contro, il microrganismo può sintetizzare molecole necessarie all'ospite, quali ormoni ACC e GABA (Taghavi *et al.,* 2008).

Nel 1992, grazie agli studi di Yabuuchi, fu descritto il nuovo genere *Burkholderia* (Yabuuchi *et al*., 1992) che, a differenza del genere *Pseudomonas*, appartiene alla suddivisione β dei proteobatteri. Ad oggi sono state identificate più di 60 specie appartenenti a questo genere, isolate sia da matrici ambientali, sia da uomo e animali (Coenye e Vandamme, 2003 ; http://www.bacterio.cict.fr/b/burkholderia.html). Questo genere si caratterizza per la per versatilità e diversità funzionale dovute principalmente alle caratteristiche del suo genoma: in primo luogo i microrganismi appartenenti al genere *Burkholderia* possiedono un genoma di grandi dimensioni, maggiore di 8 Mbp (Compant *et al.,* 2008). E' stato, infatti, dimostrato come le dimensioni dello stesso possono essere correlate alla capacità di sopravvivenza in condizioni avverse, come, per esempio, la presenza di contaminanti o semplicemente in ambienti come il suolo dove le risorse nutrizionali possono essere scarse (Konstantinidis and Tiedje, 2004). Gli studi si sono principalmente concentrati su *Burkholderia cepacia,* in quanto responsabile della putrefazione batterica delle cipolle, ma, in particolar modo in quanto patogeno opportunista per l'uomo in pazienti affetti da fibrosi cistica (CF) e, più in generale, in soggetti con il sistema immunitario compromesso (Parke e Gurian-Sherman, 2001). Attualmente, *B. cepacia* è conosciuto come un complesso batterico (Bcc) composto da quindici specie diverse che mostrano simili tratti fenotipici ma distinte caratteristiche genotipiche (Coenye *et al*., 2001c; Vermis *et al*., 2004; Vanlaere *et al.,* 2008). Inoltre, è importante ricordare che altri ceppi appartenenti a specie del genere *Burkholderia* sono risultati essere responsabili di patologie e disordini verso piante, uomo o animali: *B. gladioli* (Graves *et al.,* 1997; Shin *et al.,* 1997), *B. mallei* (Srinivasan *et al.,* 2001), *B. pseudomallei* (Dance, 1991) e *B. fungorum* (Gerrits *et al.,* 2005).

Nel nostro laboratorio è stato isolato da una matrice inquinata da IPA un ceppo batterico appartenente al genere *Burkholderia,* denominato *Burkholderia* sp. DBT1. Questo ceppo batterico è in grado di trasformare in maniera molto efficiente il dibenzotiofene a HFBT tramite la "via di Kodama" (Di Gregorio *et al.,* 2004). Mediante l'utilizzo di due ceppi mutati rispettivamente nella subunità alfa della diossigenasi iniziale - mutante mH1A -, e nell'enzima extradiolo-diossigenasi - mutante m51 -, sono stati individuati due operoni - p51 e pH1A -, che contengono sei degli otto geni coinvolti nella "via di Kodama" (Di Gregorio *et al.,* 2004). Nello specifico, le due sequenze mancanti risultano essere: il gene codificante per la subunità ferrodossina reduttasi dell'ISP; e il gene codificante l'idratasi-aldolasi coinvolto nell'ultimo passaggio della via di Kodama, da trans-HTOB a HFBT.

L'operone denominato pH1A risulta essere completo, in quanto sono stati identificati sia il sito d'inizio sia di fine trascrizione. Contrariamente, il sequenziamento dell'operone p51 termina a monte del sito di fine trascrizione, risultando quindi incompleto. Una peculiarità degli operoni pH1A e p51 risulta essere che essi non sono riconosciuti da primer in grado di identificare i *cluster* genici *nah* e *phn* (Takizawa *et al*., 1999; Laurie and Lloyd, 1999) largamente presenti in molti ceppi batterici in grado di degradare IPA.

Scopo

Lo scopo di questo progetto di tesi è la caratterizzazione molecolare, filogenetica e funzionale del ceppo *B.* sp. DBT1 al fine di valutare il suo utilizzo in protocolli di bonifica biologica per il recupero di suoli contaminati da IPA. Gli obiettivi generali della sperimentazione sono stati:

1. Identificare e sequenziare la regione a valle del frammento genico p51, al fine di valutare la presenza delle sequenze geniche mancanti nella via di degradazione di Kodama.

2. Condurre uno studio filogenetico del ceppo, al fine di collocare il ceppo DBT1 all'interno di una delle specie di *Burkholderia* ad oggi conosciute; o se, invece, esso andrà a costituire una nuova specie a se stante. Sarà inoltre fondamentale valutare se esso appartiene ad una specie potenzialmente patogena (e.g. Bcc).

3. Valutare il potenziale metabolico del ceppo DBT1 verso i composti IPA non solo di natura tiofenica, maggiormente rappresentati nei suoli contaminati da idrocarburi.

4. Condurre uno studio finalizzato ad una valutazione tossicologica del ceppo DBT1 verso cellule animali e umane.

5. Caratterizzare, mediante tecniche molecolari e biochimiche, ceppi endofiti isolati da pioppo ibrido - in relazione con il ceppo DBT1 -, potenzialmente utilizzabili in protocolli di bonifica biologica di suoli contaminati da IPA.

Risultati e discussione

Identificazione della regione a valle del frammento genico p51

L'analisi è stata condotta sui ceppi DBT1 e *B. fungorum* LMG 16225^T, quest'ultimo utilizzato come controllo negativo. La reazione di *nested-PCR*, eseguita con i set di primer WP-D74/SP-D74 e conseguentemente con i primer iWP-C47/iSP-C47, ha portato all'identificazione di un frammento genico di circa 2000 bp. L'analisi della relativa sequenza tramite il *software* "ORF – open reading frame – finder" (NCBI) ha permesso l'identificazione di due sequenze geniche. Le successive indagini d'omologia in banche dati hanno permesso di rilevare la funzione dell'enzima codificato. La prima sequenza genica, denominata *dbtAa*, codifica per la componente ferrodossina reduttasi dell'enzima ISP; mentre la seconda, denominata *dbtE,* codifica per l'enzima idratasi-aldolasi. Dai risultati ottenuti si può quindi affermare di aver identificato il completo set genico, e quindi enzimatico, coinvolto nella degradazione del DBT mediante il *pathway* di Kodama all'interno degli operoni p51 e pH1A nel ceppo DBT1.

Analisi tassonomica

La sequenza del gene 16S rDNA è la maggiormente utilizzata per effettuare analisi di identità a fine tassonomico. A questo scopo, il gene del ceppo *Burkholderia* sp. DBT1 è stato amplificato, clonato e sequenziato. L'albero filogenetico ottenuto con le sequenze omologhe di ceppi del genere *Burkholderia,* mostra che DBT1 s'inserisce all'interno di un *cluster* formato esclusivamente da ceppi appartenenti alla specie *fungorum* con un valore

d'identità che varia dal 99.5% al 100%. Tra questi, il ceppo N2P5 è stato isolato da sito contaminato da IPA (Mueller *et al*., 1997); SW6 e NW-2 sono stati identificati in assemblaggi deputati alla esplorazione dello spazio (Newcombe *et al.*, unpub. data; La Duc and Venkateswaran, unpub. data); mentre i ceppi W566 and Sw 120 sono stati isolati in seguito a infezioni del sistema nervoso centrale in maiali e cervi (Scholz and Vandamme, unpub. data). I risultati ottenuti mediante la sequenza genica 16S rDNA sono stati confermati anche dall'analisi di sequenza dei geni *recA* e *gyrB*. In particolar modo, l'albero filogenetico ottenuto con *recA* rivela sempre una correlazione tra DBT1 ed i ceppi W566 e Sw 120 (Scholz and Vandamme, unpub. data). E' importante in ogni modo sottolineare che la banca dati NCBI contiene, oltre al *type strain*, solo sequenze di *recA* di ceppi di *fungorum* derivate dal lavoro di Scholz and Vandamme (unpub. data). La prova definitiva al fine di confermare l'appartenenza del ceppo DBT1 alla specie *fungorum* giunge dall'analisi d'ibridazione DNA-DNA. La percentuale d'ibridazione tra il ceppo *B.* sp. DBT1 e il ceppo *B. fungorum* LMG 16225^T risulta essere del 78,2 ± 2,9%; una percentuale maggiore del valore soglia del 70%, sopra la quale due ceppi possano essere considerati della stessa specie (Wayne *et al.*, 1987).

Ceppi appartenenti alla specie *fungorum* sono stati isolati dal suolo, piante, a seguito d'infezioni del sistema nervoso centrale di maiali e cervi e in pazienti affetti da fibrosi cistica (CF; Coenye *et al.*, 2001; 2002; Scholz and Vandamme, unpub. data). Ad ogni modo non esistono dati di laboratorio riguardo questi pazienti, quindi il significato clinico degli isolati di *fungorum* è ancora tutto da chiarire. Solo recentemente è stata riportata la prima descrizione di setticemia causata da un ceppo di *B. fungorum* (Gerrits *et al.*, 2005). Di conseguenza, come in seguito descritto, è stata condotta una successiva analisi di patogenicità, ed in particolar modo sull'eventualità che il ceppo DBT1 sia in grado di produrre tossine verso cellule umane o animali.

Analisi fenotipiche e genotipiche

Le analisi fenotipiche condotte tramiti test API 20NE hanno rilevato solo poche differenze tra i ceppi *B.* sp. DBT1, *B. fungorum* LMG 16225^T and *B. cepacia* LMG 1222^T, provando come quest'analisi risulti poco discriminante verso queste specie. I risultati dei test biochimici hanno però evidenziato che DBT1 non è in grado di utilizzare il caprato come fonte di carbonio, contrariamente ad un ceppo di *B. fungorum* di cui si è dimostrato essere in grado di causare setticemia (Gerrits *et al.*, 2005).

Parallelamente ad un'analisi di tipo fenotipico, è stata compiuta un'indagine a livello genomico. A tal scopo è stato utilizzato un protocollo finalizzato all'estrazione e successiva visualizzazione tramite corsa elettroforetica, d'eventuali plasmidi all'interno del microrganismo (Kado and Liu, 1981). Il gel ha evidenziato due bande in corrispondeza del ceppo DBT1, mentre una sola relativamente a LMG 16225^T, un ceppo non in grado di utilizzare IPA come unica fonte di carbonio ed energia. Molti geni responsabili della degradazione di composti organici risiedono in operoni (Cho and Kim, 2001; Ma *et al.*, 2006). Di contro, i risultati fin qui ottenuti non permettono di affermare se la presenza della banda aggiuntiva mostrata dal gel elettroforetico relativa al ceppo DBT1, è dovuta alla presenza di un plasmide contenete gli operoni p51 e pH1A responsabili della degradazione del DBT. Per verificare questa ipotesi, il ceppo in esame è stato trattato con "agenti curanti" al fine di eliminare l'eventuale plasmide. Nessuno dei protocolli utilizzati - che prevedevano lo sviluppo microbico in presenza di concentrazioni di SDS, ovvero temperature sub-inibitorie della crescita batterica – sono stati in grado di annullare la capacità di formare intermedi della "via di Kodama" in presenza di DBT nel ceppo *B.* sp. DBT1. Da tali evidenze, quindi, non si può dedurre in maniera definitiva se la banda elettrofeoretica aggiuntiva relativa al ceppo DBT1 è dovuta alla presenza di un plasmide o di un cromosoma; e se essa è in qualche modo coinvolto nella degradazione degli IPA.

Studio del potenziale metabolico del ceppo DBT1 verso i composti IPA

La prima prova è stata la valutazione della crescita del ceppo utilizzando come mezzo colturale un terreno minimo (DM) contenente i singoli IPA come unica fonte di carbonio ed energia. I risultati ottenuti hanno evidenziato che *Burkholderia* sp. DBT1 è in grado di crescere utilizzando come substrati naftalene, fluorene, DBT e fenantrene. La maggior efficienza di crescita si è ottenuta impiegando il fenantrene, mentre lo sviluppo su naftalene e fluorene si ottiene solo previa induzione del ceppo con fenantrene o DBT. E' stata inoltre provata la trasformazione di questi composti anche in condizione di co-metabolismo. Il fenantrene risulta quindi il composto IPA che maggiormente supporta la crescita di DBT1. Analisi RT-PCR e successivi test di crescita dei ceppi mutati del ceppo DBT1 hanno evidenziato che i due operoni, responsabili della degradazione del DBT, sono indotti e coinvolti anche nella degradazione del fenantrene. Di seguito, sono stati testati diversi intermedi della via di degradazione di tale composto. Il ceppo in esame è risultato essere in grado di utilizzare la 2-carbossibenzaldeide, l'acido ftalico ed il protocatecolo. Diversamente non è in grado di crescere su catecolo e acido salicilico. I medesimi risultati sono stati ottenuti con il ceppo *B.* DBT1 mH1A – mutato nella subunità alfa dell'ISP -, mentre il mutante m51 – con la presenza della mutazione nel gene deputato alla sintesi dell'extradiolo diossigenasi - cresce su acido ftalico e protocatecolo ma non su 2-carbossibenzaldeide. L'acido 1-idrossi-2-naftoico può seguire due destini. Nel primo caso è decarbossilato a naftalene-1,2-diolo e quindi trasformato a potocatecolo passando per l'acido ftalico, ma non per la 2-carbossibenzaldeide. Nel secondo caso si ha un'ossigenazione dell'acido 1-idrossi-2-naftoico con la produzione di 2-carbossibenzaldeide poi, tramite una deidrogenasi, convertito ad acido ftalico, e quindi protocatecolo. Data la capacità del mutante di crescere su acido ftalico e non su 2-carbossibenzaldeide, e stabilito che l'unico enzima che separa questi due composti è la 2-carbossibenzaldeide deidrogenasi, sono stati condotti dei saggi enzimatici al fine di valutare il funzionamento dell'enzima nel ceppo m51. Nella fase di messa a punto sono state condotte delle prove preliminari sul ceppo *type strain* di *Burkholderia* sp. DBT1. Questi esperimenti avevano lo scopo di trovare le condizioni in cui il mutante sia in grado di crescere - utilizzando solo 2-carbossibenzaldeide come unica fonte di carbonio esso non si sviluppa - e, contemporaneamente, esprimere l'enzima. Le prove preliminari condotte con il ceppo *wild type* hanno rilevato come, sia alla presenza della sola 2-carbossibenzaldeide, sia con la co-presenza di mannitolo, si ha attività enzimatica della deidrogenasi. Al contrario, alla sola presenza dello zucchero non si è riscontrata attività. Trovate le condizioni opportune - utilizzare contemporaneamente il mannitolo e la 2-carbossibenzaldeide nel mezzo di coltura - si è quindi testato il mutante m51. Anche in questo caso, si è riscontrata attività enzimatica della 2-carbossibenzaldeide deidrogenasi. Constatato che l'enzima, sia nel ceppo *type strain,* sia nel ceppo mutante è presente e attivo, una possibile spiegazione della mancata crescita di tale ceppo su 2-carbossibenzaldeide può essere nel fatto che questo composto sia un intermedio di reazione non del fenantrene, ma di qualche altro composto IPA. Tale ipotesi è supportata anche dalla dimostrazione che nel caso *Burkholderia* sp. DBT1 utilizzi fenantrene come unica fonte di carbonio ed energia, non si riscontra alcun'attività enzimatica da parte della 2-carbossibenzaldeide deidrogenasi.

Analisi tossicologica e patogenetica

I risultati dell'analisi tassonomica sopra descritti, dimostrano che il ceppo *B.* sp. DBT1 afferisce alla specie *fungorum.* Tuttavia, all'interno di questa specie sono stati isolati ceppi con un non chiaro ruolo da soggetti affetti da fibrosi cistica e animali con infezioni a livello del sistema nervoso centrale (Coenye *et al.,* 2001; 2002; Scholz and Vandamme, unpub. data). Solo nel 2005 Gerrits *et al.,* ha identificato un ceppo di *B. fungorum* coinvolto in setticemia batterica. Quindi, è stata compiuta una successiva analisi tossicologica del ceppo DBT1. Diversi protocolli sono stati adottati. Il primo si basa sull'inibizione della mobilità delle cellule spermatiche, un fenomeno basato sulla fosforilazione ossidiativa dei mitocondri (Mann and Lutwak-Mann, 1982). E' stato dimostrato che concentrazioni anche nanomolari di tossine come la valomicina o cereulide sono in grado di bloccare il meccanismo di fosforilazione, andando quindi ad inibire la mobilità delle cellule (Hoornstra *et al.,* 2003). Il

protocollo si dimostra essere molto sensibile anche a basse concentrazioni di tossine. Inoltre, molti batteri patogeni ricorrono alla produzione di tossine capaci di lisare le membrane delle cellule ospiti (Alouf, 2001). Sono state, quindi, individuate due linee cellulari *target* umane. La linea cellulare Caco-2 al fine di monitorare le tossine in grado di intaccare cellule del tratto digestivo, e Paju con l'obiettivo di valutare l'eventuale neuro-tossicità del ceppo (Jääskeläinen *et al.,* 2003). I risultati ottenuti da *B.* sp. DBT1, *B. fungorum* LMG 16225^T ed il controllo negativo *Bacillus cereus* F-528, hanno mostrato che gli estratti cellulari derivanti da questi ceppi non sono in grado di alterare le membrane cellulari ed il potenziale di membrana mitocondriale delle cellule animali testate. Contrariamente, l'estratto cellulare derivante dal ceppo *B. cepacia* LMG 1222^T determina alterazioni del potenziale di membrana mitocondriale; mentre quello di *Bacillus cereus* 4810/72 - utilizzato come controllo positivo - determina sia sbilanciamenti del potenziale di membrana, sia danni alla membrana cellulare. Si può quindi affermare che *B.* sp. DBT1 non produce tossine in grado di danneggiare le cellule *target* utilizzate; malgrado ciò, non si può assicurare che il ceppo in studio non possa esplicare una forma di patogenicità non riscontrata da questo studio come, ad esempio meccanismi di inibizione della sintesi proteica.

Caratterizzazione molecolare e biochimica di ceppi endofiti isolati da pioppo ibrido

Una metodica promettente di bonifica biologica prevede l'utilizzo di ceppi endofiti dotati di capacità biodegradive al fine di migliorare la degradazione dei composti IPA *in planta* (Barac *et al.,* 2004). Inoltre, recenti pubblicazioni hanno identificato molte specie del genere *Burkholderia* come naturali endofiti di svariate piante (Mastretta *et al.,* 2006; Barac *et al*., 2004). E' stata quindi eseguita una caratterizzazione molecolare e biochimica di ceppi endofiti isolati da pioppo ibrido, al fine selezionare i ceppi potenzialmente utilizzabili in protocolli di bonifica biologica di suoli contaminati da IPA. Sono stati quindi selezionati 50 ceppi provenienti da una collezione d'endofiti isolati dal gruppo del Dott. Kim Yrjälä - Department of Biological and Environmental Sciences in Helsinki; Finlandia -.

Le prove di crescita effettuate su terreno minimo contenente IPA hanno evidenziato che dodici ceppi appartenenti al genere *Burkholderia* isolati dalle radici di pioppo sono in grado di utilizzare IPA come unica fonte di carbonio ed energia. Tra questi ceppi, dieci appartengono alla specie *fungorum* uno alla specie *sordidicola* e l'ultimo identificato con il nome di *Burkholderia* sp. R-701, un ceppo appartenente alla specie *sartisoli* (Vanlaere *et al.,* 2008a). Mentre l'identificazione di specie quali *fungorum* o *sordidicola* all'interno d'organismi superiori quali piante e/o funghi sono stati già documentati (Sun *et al.,* 2008; Lim *et al.,* 2003; Yrjälä *et al.,* in press), per la prima volta si è isolato un ceppo della specie *sartisoli* in tessuti vegetali. Le successive analisi di PCR hanno sorprendentemente evidenziato la presenza degli operoni pH1A e p51 nei dieci ceppi endofiti di *fungorum* e nel ceppo R-701. Analisi PCR-DGGE e analisi di restrizione non hanno mostrato variazioni nella sequenza di questi operoni tra i ceppi endofiti, rilevando, tuttavia, una notevole differenza con la sequenza di p51 e pH1A del ceppo DBT1. Le differenze riscontrate tra i ceppi di *fungorum* isolati dal pioppo ed il ceppo DBT1 non si sono rilevate essere solo a livello genico. Infatti, successive analisi biochimiche e microbiologiche hanno evidenziato che il ceppo DBT1 risulta essere più efficiente nella degradazione del fenantrene e del DBT rispetto ai ceppi endofiti *fungorum* sopra descritti. Ovviamente, con i dati ad oggi ricavati non si può correlare i diversi genotipi degli operoni p51 e pH1A con le diverse efficienze degradative degli IPA.

Conclusioni

Con il termine bonifica biologica si definiscono un insieme di metodiche in grado di usufruire delle capacità metaboliche di microrganismi e/o piante, al fine di recuperare siti contaminati da sostanze pericolose. In caso di forti contaminazioni, tuttavia, la sola stimolazione dei microrganismi autoctoni – mediante aggiunta d'ammendanti e/o tramite aerazione del suolo - risulterebbe troppo lenta. L'aggiunta di un inocula batterico direttamente in suolo

o, alternativamente, in piante selezionate al fine di aumentare il potenziale biodegradativo *in planta,* può aumentare l'efficienza nel recupero del sito contaminato. D'altra parte, la selezione di microrganismi opportuni a tal fine, riveste una fase critica per buon esito di un protocollo. In particolar modo, l'efficienza di degradazione verso i composti interessati, e la non pericolosità per uomo e/o animali, sono due aspetti da tenere in forte considerazione a monte di un loro eventuale sversamento *in situ*. A questo scopo, un'attenta caratterizzazione di ceppi microbici potenzialmente utilizzabili in protocolli di bonifica biologica appare di fondamentale importanza.

Il soggetto del presente studio, *B.* sp. DBT1, ha mostrato un'interessante capacità metabolica nei confronti di molecole IPA, sia se forniti come unica fonte di carbonio ed energia, sia in condizione di cometabolismo. In quest'ultimo processo un composto è degradato da un enzima prodotto da organismi impegnati in altre reazioni e la degradazione del composto inquinante è considerato un evento fortuito, da cui i microrganismi non traggono energia. Di conseguenza, non dovendo supportare la crescita microbica, possono essere degradate anche concentrazioni molto basse d'inquinante. Stabilite le potenzialità metaboliche del ceppo DBT1, è stato importante assicurare la sua non tossicità e pericolosità di un suo eventuale utilizzo. Gli studi tassonomici hanno dimostrato che il ceppo DBT1 appartiene alla specie *fungorum*, una specie in cui sono presenti alcuni membri che sono stati isolati da uomini e animali affetti da alcuni tipi di patologie (Coenye *et al.,* 2001; 2002; Scholz and Vandamme, unpub. data; Gerrits *et al.,* 2005). In quanto il ruolo di questi ceppi nel decorso della malattia, a parte per un singolo caso (Gerrits *et al.,* 2005), non è stato ancora determinato, è stato ritenuto importante effettuare uno studio più approfondito al fine di valutare le capacità di produrre tossine da parte del ceppo DBT1 verso sistemi animali e umani. I risultati hanno chiaramente evidenziato che il ceppo DBT1 non è in grado di danneggiare né il potenziale di membrana cellulare, né il gradiente protonico mitocondriale.

Da recenti studi, un protocollo di bonifica biologica che ha dimostrato una buon'efficienza nel recupero di siti contaminati è risultato essere l'utilizzo di un sistema in grado di sfruttare la capacità della pianta di assorbire i composti tossici, associata alle capacità degradative dei microrganismi presenti in essa (Barac *et al*., 2004; Taghavi *et al.,* 2005). La caratterizzazione effettuata sui ceppi endofiti isolati da pioppo ha evidenziato che *Burkholderia fungorum* è la specie maggiormente coinvolta nella bioderadazione di composti IPA, in particolar modo nei confronti di fenantrene e dibenzotiofene. Questi risultati incoraggiano un possibile utilizzo di DBT1 come ceppo endofita del pioppo, essendo anch'esso appartenente alla specie *fungorum*. D'altro canto, qualche dubbio può sorgere in quanto le molecole degradate – fenantrene e DBT – sia da DBT1, sia dai ceppi endofiti, non possano essere assorbiti dalle radici della pianta in quanto possiedono un coefficiente di ripartizione ottanolo-acqua (log K_{ow}) troppo elevato (Li *et al.,* 2005). Di conseguenza, tali composti non risultano essere disponibili per i microrganismi endofiti collocati all'interno dei tessuti vegetali. Tuttavia, recenti pubblicazioni hanno evidenziato che il modello basato sul coefficiente di ripartizione ottanolo-acqua sottostimava l'*up-take* di IPA da parte delle piante, in particolar modo riguardo i composti altamente idrofobici come fenantrene e DBT (Su and Zhu, 2007; Zhu *et al.,* 2007; Zhang and Zhu, 2009). Tuttavia, non esistono ad ora prove di un'effettiva funzionalità del modello proposto. Infatti, le prove d'inoculo in pianta non hanno portato ad alcun risultato per il motivo che l'eccessivo stress subito durante i vari espianti, hanno portato ad una forte disidratazione delle stesse, con conseguente morte. Un rimedio può essere l'utilizzo di talee di pioppo anziché della pianta in pieno sviluppo e di suoli sabbiosi, al fine di diminuire gli stress radicali. Concludendo, i risultati ottenuti da questo lavoro di tesi incentivano l'utilizzo del ceppo *Burkholderia fungorum* DBT1 in protocolli di bonifica biologica per il recupero di siti contaminati da composti IPA; e suggerisce una reale fattività di DBT1 come ceppo endofita in pioppo al fine di migliorare la biodegradazione *in planta*.

1. Introduction

1.1 Polycyclic Aromatic Hydrocarbons (PAHs)

Polycyclic aromatic compounds (PAHs) consist of fused benzene rings in linear, angular or clustered arrangements. They contain by definition only carbon and hydrogen atoms. However, sulfur, nitrogen, and oxygen atoms may readily substitute in the benzene ring to form heterocyclic aromatic compounds, which are commonly grouped with the PAHs. A wide variety of PAHs are found in the environment as a result of the incomplete combustion of organic matter, emission sources, automobile exhausts, stationary matter (e.g. coal-fired, electricity generating power plants), domestic matter (e.g. tobacco smoke and residential wood or coal combustion), area source matter (e.g. forest fires and agricultural burning) and also in food (Finlayson-Pitts and Pitts, 1997). PAHs have been studied due for their toxicity, persistency and environmental impact (Howsam and Jones, 1998; Blumer, 2003). Although there is no definitive legislation concerning PAH abatement, the Environmental Protection Agency (EPA) has listed 16 PAHs as priority pollutants, (Fig.1.1; EPA 1997). It is expected that air quality would be improved soon if the emission of these PAHs was controlled (Keith and Telliard, 1979). These 16 PAHs were chosen to be included in this profile because more information are available on these than on the others; they are suspected to be more harmful than some of the others and they exhibit harmful effects that are representative of the PAHs; there is a greater chance that humans will be exposed to these PAHs than to the others and of all the PAHs analyzed, these were the PAHs identified at the highest concentrations in the environment (Liu *et al.,* 2008).

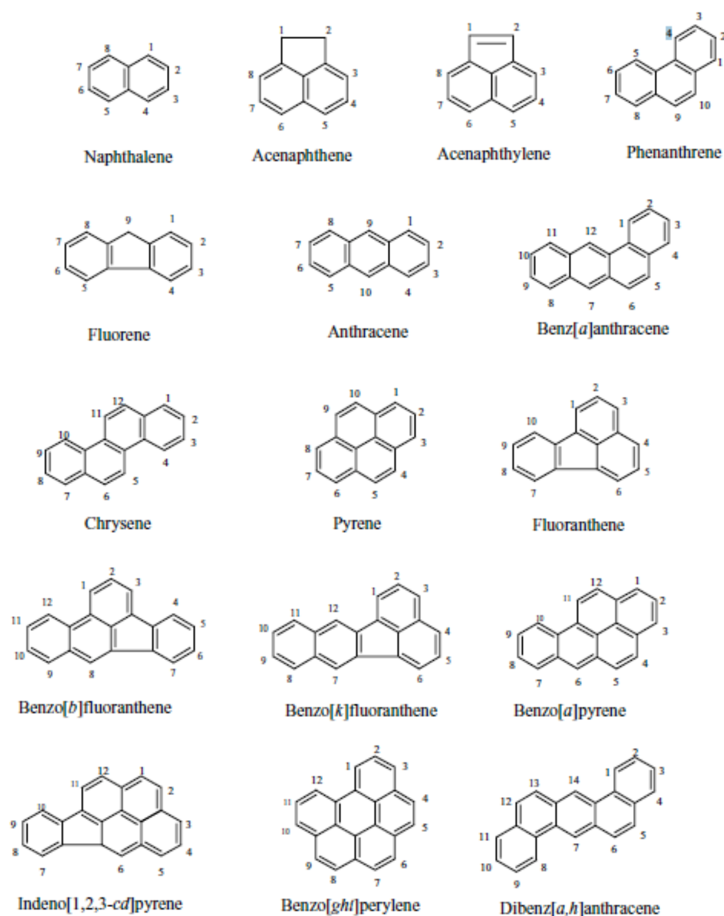

Fig. 1.1: Structures and nomenclatures of the 16 PAHs on the EPA priority pollutant list (Yan *et al.,* 2004).

1.1.1 Distribution and fate of PAHs in the environment

PAHs are found throughout the environment in the air, water, and soil. They can occur in the air, attached to dust particles or as solids in soil or sediment. PAHs generally occur as complex mixtures (e.g., as part of combustion products such as soot), and not as single compounds. As pure chemicals, PAHs generally exist as colorless, white, or pale yellow-green solids. A few PAHs are used in medicines and to make dyes, plastics, and pesticides. Others are contained in asphalt used in road construction. They can also be found in substances such as crude oil, coal, coal tar pitch, creosote, and roofing tar (Liu *et al.,* 2008). Many PAHs, especially carcinogenic PAHs, are found in all surface soils. Individual PAH concentration in soil produced by natural processes likes vegetation fires and volcanic exhalation have been estimated to be in the ranges of 1-10 µg kg^{-1} (Edwards, 1983). The PAHs concentrations in soils increase with increasing impact of industry, traffic and domestic heating (Jones *et al.,* 1989). Generally, an increase in the size and angularity of a PAH molecule results in a concomitant increase in hydrophobicity and electrochemical stability (Harvey, 1997; Zander, 1983). PAH molecule stability and hydrophobicity are two primary factors that contribute to the persistence of these compounds in the environment. The relationship between PAH environmental persistence and increasing numbers of benzene rings is consistent with the results of various studies correlating environmental biodegradation rates and PAH molecule size (Banerjee *et al.,* 1995; Bossert and Bartha, 1986). Moreover, most PAHs are strongly sorbed by organic matter in soil. Sorption generally increases with the number of benzene rings in PAH molecule (Wild and Jones, 1993; 1995). Thus, its biodegradability and extractability of organic compounds in soil decrease with the time they have been in contact with the soil: a phenomenon called "aging" or "weathering" (Hatzinger and Alexander, 1995; Loehr and Webster, 1996). On the other hand, these processes reduce the toxicity of the soil contaminants, by lowering the available fraction to living organism (Weissenfels *et al.,* 1992; Alexander, 1995).

1.1.2 PAHs toxicity

Many PAHs have toxic, mutagenic and/or carcinogenic properties (Goldman *et al.,* 2001; Mastrangelo *et al.,* 1997). PAHs are highly lipid soluble and thus readily absorbed from the gastro intestinal tract of mammals (Cerniglia, 1984). They are rapidly distributed in a wide variety of tissues with a marked tendency for localization in body fat. Metabolism of PAHs occurs via the cytochrome P450-mediated mixed function oxidase system with oxidation or hydroxylation as the first step (Stegeman *et al.,* 2001). The resultant epoxides or phenols might get detoxified in a reaction to produce glucoronides, sulfates or glutathione conjugates. Some of the epoxides might metabolize into dihydrodiols, which in turn, could undergo conjugation to form soluble detoxification products or be oxidized to diol-epoxides. Many PAHs contain a 'bay-region' as well as 'K-region', both of which allow metabolic formation of bay- and K-region epoxides, which are highly reactive. Carcinogenicity has been demonstrated by some of these epoxides (Goldman *et al.,* 2001). Therefore, many PAHs are considered to be environmental pollutants that can have a detrimental effect on the flora and fauna of affected habitats, resulting in the uptake and accumulation of toxic chemicals in food chains and, in some instances, in serious health problems and/or genetic defects in humans (Liu *et al.,* 2001).

Naphthalene, the first member of the PAH group, is a common micro pollutant in potable water. The toxicity of naphthalene has been well documented and cataractogenic activity has been reported in laboratory animals (Goldman *et al.,* 2001; Mastrangelo *et al.,* 1997). Naphthalene binds covalently to molecules in liver, kidney and lung tissues, thereby enhancing its toxicity; it is also an inhibitor of mitochondrial respiration (Falahatpisheh *et al.,* 2001). Acute naphthalene poisoning in humans can lead to haemolytic anaemia and nephrotoxicity. In addition, dermal and ophthalmological changes have been observed in workers occupationally exposed to naphthalene. Phenanthrene is known to be a photosensitizer of human skin, a mild allergen and mutagenic to bacterial systems

under specific conditions (Mastrangelo *et al.,* 1997). It is a weak inducer of sister chromatid exchanges and a potent inhibitor of gap junctional intercellular communication (Weis *et al.,* 1998). Equivocal results for tumour initiation have been obtained with skin-painting studies in mice. Interestingly, because phenanthrene is the smallest PAH to have a bay-region and a K-region, it is often used as a model substrate for studies on the metabolism of carcinogenic PAHs (Bücker *et al.,* 1979). Moreover, PAHs are rarely encountered alone in the environment and many interactions occur within a mixture of PAHs whereby the potency of known genotoxic and carcinogenic PAHs can be enhanced (Kaiser, 1997).

1.1.3 Condensed thiophenes

PAHs can show a backbone containing heteroatoms such as oxygen, nitrogen or sulphur. Condensed thiophenes consist of a furanic ring harboring a sulfur atom fused with one or more aromatic rings. Thiophenes can bear alkylic substituents or be condensed. After carbon and hydrogen, sulfur is typically the third most abundant element in petroleum, ranging from 0,05% to 5% w/w, depending upon the fossil fuel origin (Speight, 1980). Crude oils with higher density contain more sulfur compound, and distillation fraction with higher boiling point contains higher concentration of sulfur compounds (Kropp and Fedorak, 1998; Speight, 1980). About 30% of the sulfur in fuels is represented by inorganic compound (pyrite) and the remaining 70% by organic molecules. Thiophenes are the most recurrent sulphur heterocyclic compounds in the environment and they constitute about 50% of organic sulphur in fossil fuels (Nishioka *et al*., 1985; Nishioka, 1988; Thompson, 1981; Willey *et al*., 1981). Among these latter, dibenzothiophene (DBT) represents the prevailing compound, which is therefore taken as model chemical structure in biodegradation studies of organo-sulfur contaminants. DBT and its substituted forms resist to the cleavage in the conventional physico-chemical desulforization processes. Combustion of sulfur compound in fossil fuel produces sulfur oxides, which can cause adverse effects on health, environment and economy. SO_2 is the most abundant molecules among sulfur oxide, and can be cause of sulfate aerosol formation. The aerosol particles have an average diameter of 2,5 μm that can be transported into the lugs and cause respiratory illnesses (Dockery and Pope 1994). SO_2 can react with moisture in the air and cause acid rain or low pH fogs. The acid formed in this way can accelerate the erosion of historical buildings, depress the pH of the lakes with low buffer capacity and endanger the marine life (EPA, 2006).

Since 1979 an international cooperation has implemented more strict controls on sulfur dioxide emission. Most of these agreement targeted transport fuel because it was one of the most important sources of SO_2 emission. For instance, treatment procedures must be devised or improved to reach a sulfur level less than 10mg/kg (König *et al*., 2001; Song *et al*., 2000). Pyritic sulfur is effectively removed by various physical and chemical techniques such as heavy metal separation, magnetic separation, leaching, selective agglomeration and floatation (Kawatra and Eisele, 2001). Application of such methods to remove organic sulfur from coal is impossible, because is finely distributed among the matrix (Kawatra and Eisele, 2001; Kropp and Fedorak, 1998). Among conventional desulfurization process to remove organic sulfur from fossil fuel, hydrodesulfurization (HDS) and desulfurization by ionic liquid (ILs) are the most used. HDS operates at high temperature (>300°C) and high pressure (>100psi) in presence of an inorganic catalyst (Speight, 1980). In addition, this technique does not work well on certain sulfur molecules, particularly the polyaromatic sulfur heterocycles found in heavier fraction. Moreover, this technique also results in the release of carbon dioxide. Thus, HDS is energetically costly and highly polluting (Gupta *et al.,* 2005). ILs are a organic salts that are in liquid state at temperature below 100°C. Ionic liquid are predicted to take the place of organic solvent, because they have not measurable vapor pressure below their decomposition temperature and can be designed to have different properties depending on their structure. Desulfurization by ILs is based on extraction theories and it is a mild process (Bösmann *et al.,* 2001).

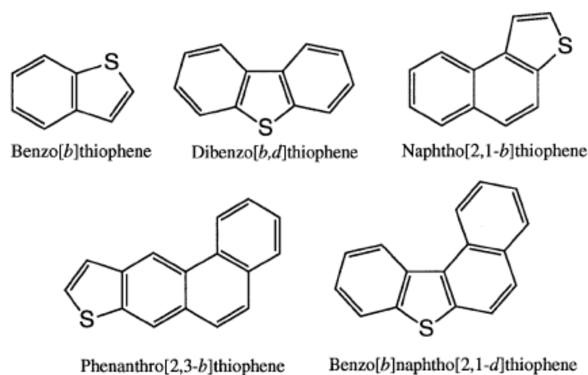

Fig. 1.2: Examples of un-substituted condensed thiophenes found in petroleum and coal derivatives (Bressler *et al.,* 1998).

1.1.3.1 Dibenzothiophene

ILs and HDS technique are expensive to build and operate. It is this limitation that has tempted researcher to venture into the inviting to find an alternative process of sulfur removal using microorganism, and DBT (Fig. 1.2) has been used for the last few decades as a polyaromatic sulfur model for the isolation and characterization of bacteria capable of transforming organosulfur compounds found in a variety of fossil fuels (Monticello and Finnerty 1985).

1.2 Bacterial degradation of PAHs

Numerous bacteria have been found able to degrade PAHs, and some of them can utilize PAHs as their sole carbon source. Moreover, the bacteria can use PAHs by co-metabolism process. Co-metabolism is defined as the oxidation of not growth of substrates during the growth of microorganism on another carbon or energy source. The common biochemical pathways for the bacterial degradation of PAHs such as naphthalene (Resnick *et al*., 1996; Annweiler *et al*., 2000), phenanthrene (Menn *et al*., 1993; Kiyohara *et al*., 1994; Pinyakong *et al*., 2000), anthracene and acenaphthene (Dean-Ross *et al*., 2001; Pinyakong *et al*., 2004) have been well investigated. Aerobic biodegradation mechanisms require the presence of molecular oxygen to initiate the enzymatic attack of PAH rings. In the initial step, dioxygenase catalyzed oxidation of arenes generally takes place in aerobic bacterial systems to yield vicinal cis-dihydrodiols as the early bioproducts by a multicomponent enzyme system (ISP). This dioxygenase system consists of four components, a ferredoxin reductase, a ferredodoxin, and of two not identical subunits α and ß. The electron transport is initiated by a single two-electron transfer from NAD(P)H to NAD in a ferredoxin recuctase. The reduced NAD provides one electron each to the [2Fe-2S] cluster in ferredoxin. These electrons are finally transferred to the ISP und used in its active site (subunits α and ß) to facilitate the addition of an oxygen molecule to the compounds (Fig 1.3; Habe and Omori; 2003). These dihydroxylated intermediates may then be cleaved by intradiol or extradiol ring-cleaving dioxygenases through either an ortho-cleavage pathway or a meta-cleavage pathway, leading to central intermediates such as protocatechuates and catechols that are further converted to tricarboxylic acid (TCA) cycle intermediates (Cerniglia, 1992; Gibson and Parales, 2000).

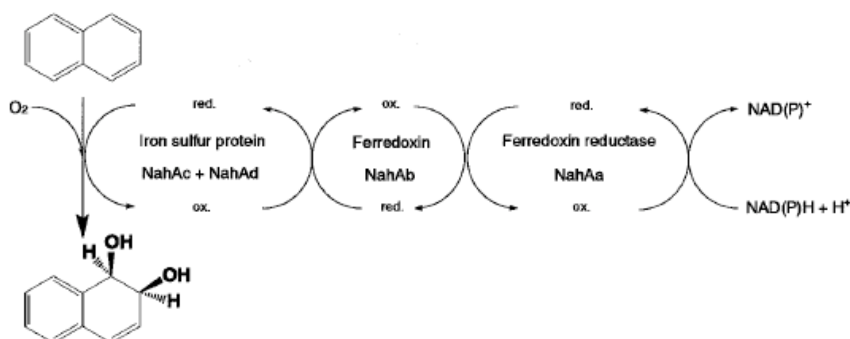

Fig 1.3: Naphthalene initial oxidation steps by relative subunits of ISP (Habe and Omori; 2003).

1.2.1 Naphthalene

Naphthalene is a bi-cyclic aromatic compound wide distributed in the environment. It has been used often as a model compound for PAHs degradation. The bacterial degradation of naphthalene is well understood. Therefore, information of bacterial degradation of naphthalene has been used to understand and predict pathways in the degradation of three- or more ring PAHs. Many bacteria that have been isolated and utilize naphthalene as a sole source of carbon and energy belong to different genera such as *Burkholderia, Pseudomonas, Ralstonia* and *Palaromonas* (Cerniglia, 1992; Kim *et al.,* 2003; Auger *et al.,* 1995; Denome *et al.,* 1993; Seo *et al.,* 2007; Zhou *et al.,* 2002; Pumphrey and Madsen, 2007).

Degradation of naphthalene starts through the multicomponent enzyme, naphthalene dioxygenase, attack on the aromatic ring to form *cis*-(1R, 2S)-dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol; Goyal and Zylstra, 1997; Simon *et al.,* 1993). The *cis*-naphthalene dihydrodiol formed by naphthalene dioxygenase is subsequently dehydrogenated to 1,2-dihydroxynaphthalene by a *cis*-dihydrodiol dehydrogenase (Goyal and Zylstra, 1997; Auger *et al.,* 1995). Subsequently, 1,2-dihydroxynaphthalene is metabolized to salicylate via 2-

hydroxy-2*H*-chromene-2-carboxylic acid, *cis*-*o*-hydroxybenzalpyruvate, and 2-hydroxy-benzaldehyde (Goyal and Zylstra, 1997; Denome *et al.*, 1993; Kiyohara *et al.*, 1993). Also, 1,2-dihydroxynaphthalene is not enzymatically oxidized to 1,2-naphthaquinone (Auger *et al.*, 1995). Salicylate is typically decarboxylated to catechol, which is further metabolized by ring fission in *meta*- and *ortho*-pathways. Fuenmayor *et al.* (1998) reported that salicylate is converted to gentisate by salicylate-5-hydroxylase (Fig. 1.4).

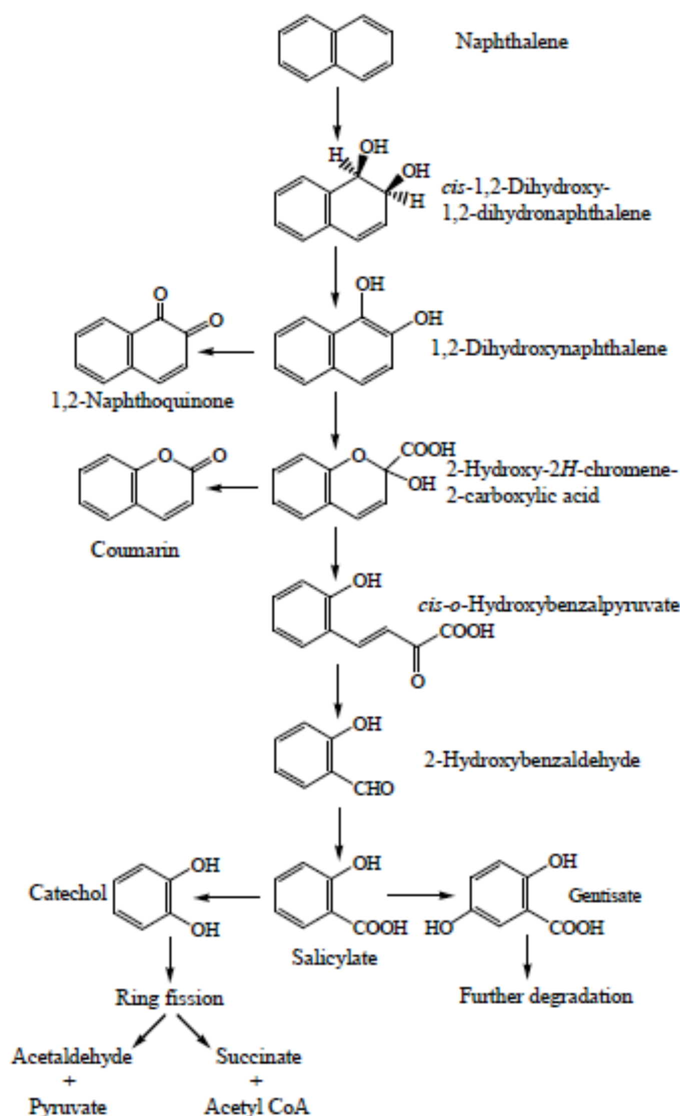

Fig. 1.4: Proposed catabolic pathways of naphthalene by bacteria (Seu *et al.*, 2009).

1.2.2 Phenanthrene

Phenanthrene is also used as a model compound in PAHs degradation pathway. Degradation of phenanthrene by many bacteria strains has been reported with different catabolic pathway (Balashova *et al*. 1999; Kang *et al*. 2003; Kim et al. 2005; Moody *et al*. 2001; Samanta *et al*. 1999). However, studies about degradation pathways of phenanthrene by *Burkholderia* are limited.

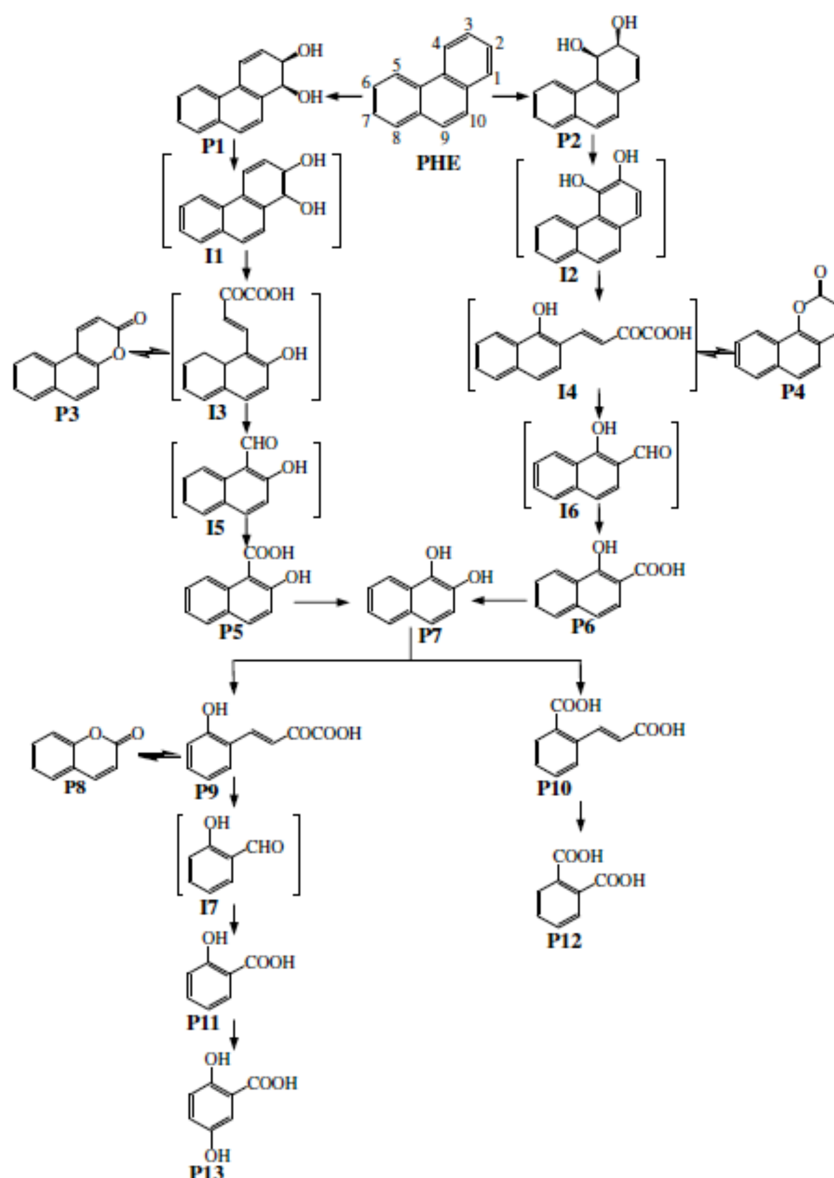

Fig. 1.5: Proposed catabolic pathways of phenanthrene. Metabolites in brackets are proposed structures, but not detected. P1. cis-Phenanthrene-1,2-dihydrodiol; P2. cis-Phenanthrene-3,4-dihydrodiol; P3. 5,6-Benzocoumarin; P4. 7,8-Benzocoumarin; P5. 2-Hydroxy-1-naphthoic acid; P6. 1-Hydroxy-2-naphthoic acid; P7. Naphthalene-1,2-diol; P8. Coumarin; P9. 2-Hydroxybenzalpyruvic acid; P10. 2-Carboxycinnamic acid; P11. Salicylic acid; P12. Phthalic acid; P13. Gentisic acid; I1. phenanthrene-1,2-diol; I2. phenanthrene-3,4-diol; I3. 4-(2-hydroxy-1-naphthyl)-2-oxobut-3-enoic acid; I4. 4-(1-hydroxy-2-naphthyl)-2-oxobut-3-enoic acid; I5. 2-hydroxy-1-naphthaldehyde; I6. 1-hydroxy-2-naphthaldehyde; and I7. salicylic aldehyde (Seo *et al.*, 2007).

In general, phenanthrene is metabolized from an initial 3,4-dioxygenation, and after several additional biochemical reactions, 1-hydroxy-2-naphthoic acid is formed. 1-hydroxy-2-naphthoic acid is further degraded via naphthalene-1,2-diol either through salicylic acid and gentisic acid or through phthalic acid and protocatechuic acid according to the bacterial species (Fig. 1.5; Prabhu and Phale 2003; Stingley *et al*. 2004). In another pathway, 1-hydroxy-2-naphthoic acid undergoes to ring-cleavage and is further metabolized via 2-carboxybenzaldehyde, *o*-phthalic acid and protocatechuic acid or via salicylic acid and catechol (Fig. 1.6; Kiyohara *et al.,* 1976; Iwabuchi *et al*. 1997;

1998) characterized the genes and enzymes involved in this pathway. 1-Hydroxy-2-naphthoate dioxygenase converts 1-hydroxy-2-naphthoate to *trans*-2'-carboxybenzalpyruvate and a *trans*-2'-carboxybenzalpyruvate aldolase transforms *trans*-2'-carboxybenzalpyruvate to 2-carboxybenzaldehyde. The next step is catalyzed by a 2-carboxybenzaldehyde dehydrogenase, which convert 2-carboxybenzaldehyde to *o*-phthalic acid.

There were few studies of initial dioxygenation at the 1,2-position of phenanthrene by Gram-negative bacteria. Although Pinyakong *et al*. (2000) suggested that *Sphingomonas* sp. strain P2 dioxygenated phenanthrene at 1,2-C positions to produce salicylic acid through 2-hydroxy-1-naphthoic acid, 2-hydroxy-1-naphthoic acid was not detected. Moody *et al*. (2001) reported that the dioxygenation and monooxygenation at 9,10-positions of phenanthrene yielded cis- and trans-9,10-dihydroxy-9,10-dihydrophenanthrene, respectively (Fig. 1.5).

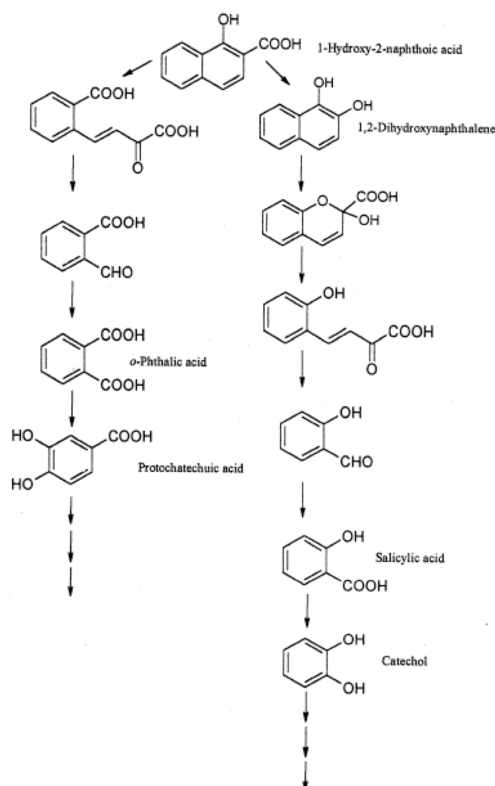

Fig. 1.6: Alternative phenanthrene pathway. 1-hydroxy-2-naphthoic acid is directly metabolized via *trans*-2'-carboxybenzalpyruvate, 2-carboxybenzaldehyde, *o*-phthalic acid and protocatechuic acid or via salicylic acid and catechol (Pinyakong *at al*., 2000).

1.2.3 Condensed thiophene

There are two type of pathways recognized in the metabolism of DBT. One is called ring destructive pathway or Kodama pathway (Kodama *et al*., 1973), while the other refers to hydrocarbon backbone conserving pathway (biodesulfurization) also known as the 4S pathway (McFarland *et al*., 1998). Thus, some bacteria assimilate the carbons of DBT as sole source of carbon and energy by Kodama pathway, degrading sulfur-containing molecules to water-soluble products that can be extracted from petroleum. In most of these systems, bacteria functioned in bioremediation protocol. On the other hand, sulfur removal from petroleum is important from the standpoint of the global environment because the combustion of sulfur compounds leads to the production of sulfur oxides, which are the source of acid rain.

1.2.3.1 Kodama pathway

The oxidation of DBT carbon skeleton and subsequence cleavage of aromatic ring can without remove the sulfur atom, act either as a source of carbon and energy or as a co-metabolic substrate during the degradation of others aromatic compounds. The oxidative pathway acts by a series of enzyme that attack the carbon atom in DBT phenyl ring is known as Kodama pathway and it consist of three main steps: hydroxylation, ring cleavage and hydrolysis. It results in degradation of one of the homocyclic rings of DBT to form 3-hydroxy-2 formylbenzothiophene (HFBT; Fig 1.7; Kodama *et al.,* 1970; 1973; Kropp and Fedorak, 1998). Briefly, the first step of Kodama pathway is a hydroxylation of one of the aromatic rings throughout a dioxygenase that leads to the formation of cis 1,2-dihydroxy-1, 2-dihydrodibenzothiophene. The second oxidation step, catalysed by a dihydrodiiol-dehydrogenase, produces 1,2 dihydroxydibenzothiophene. Afterwards, an extradiol-oxygenase activity determine a benzenic ring cleavage and produces a cis-4- [2-(3-hydroxy)-tionaphthenil] 2-ossi-3-butenoic acid (cis-HTOB). This compound stands in a chemical equilibrium with its hemiacetalic form trans-4- [2-(3-hydroxy)-tionaphthenil] 2-ossi-3-butenoic acid (trans-HTOB) that is the real forerunner of the following compound. The last step is catalysed by a hydratase-aldolase that forms pyruvate and 3-hydroxy-2-formilbenzothiophene (HFBT), the final compound that accumulates in the medium (Kodama *et al*., 1970; 1973; Monticello *et al*., 1985). HFBT accumulates in pure cultures, but there are few studies on its fate. Mormile and Atlas (1988) suggested that HFBT can be further biodegraded and Bressler and Fedorak (2001) reported some chemical properties of purified HFBT and described the abiotic condensation of HFBT to form *cis*- and *trans*-thioindigo. They also showed that a mixed bacterial community mineralized HFBT, and they identified benzothiophene 2,3-dione in the extracts of these cultures.

Monticello *et al*., (1985) showed that Kodama pathway for DBT degradation was plasmid associated in at least two *Pseudomonas* species: *P. alcaligenes* and *P. putida*. These two species harbored a single plasmid with an approximate molecular weight of 55 MDa. The same plasmid was also reported to mediate the biodegradation of others aromatic compound such as naphthalene and phenanthrene (Denome *et al*., 1993).

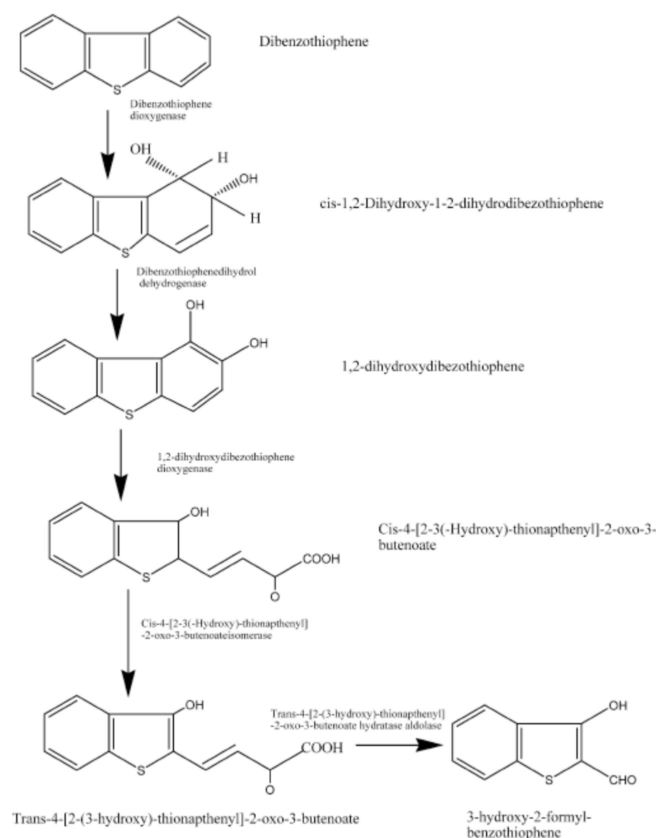

Fig 1.7: Kodama pathway of DBT oxidative degradation (Gupta *et al.,* 2005).

1.2.3.2 Bacterial desufurization

Bacterial desulfurization offers many advantages comparing traditional sulfur removal processes. They work under mild condition with no harmful reactions products (Monticello, 2000). Secondly, in biological activities, biocatalyst (enzymes) are involved. Therefore, the desulfurization would be highly selective. Bacteria are able to remove sulfur from a range of organo-sulfur compound and act as self-regenerating biocatalyst. Microorganism requires carbon and sulfur to growth. Under this condition, bacteria can use a carbon compound such as glucose and a sulfur-containing compound such as sulfur sources. In fact, the soils around oil wells are often lacking of such elements. Thus, in order to survive in these areas bacteria must degrade organo-sulfur compounds to obtain sulfur elements. From a industrial point of view, the biocatalytic activity that is needed for biodesulfurization is a specific cleave of the sulfur atom from thiophene molecules without breaking the hydrocarbon backbone of the molecule because this would decrease the energetic value of the desulfurized fuel (McFarland *et al.,* 1998).

“4S pathway”

This process removes sulfur and methyl DBT in a sulfur-specific manner without affecting the carbon skeleton thus preserving the fuel value. In fact, DBT serves only as sulfur sources and not as carbon sources. The metabolic pathway was first reported for *Rhodococcus rhodochrous* IGTS8 by Gallagher *et al.,* (1993). Other bacteria are subsequently reported, for instance: *R. erythropolis* D1 (Ohshiro *et al.,* 1997), *Gordona* CYKS1 (Rhee *et al.,* 1998), *Xantomonas* strain (Constanti *et al.,* 1994), *Nocardia globelula* (Wang and Krawiec, 1994), *Paenibacillus* strain (Ishii *et al.,* 2002) and *Mycobacterium* sp. X7B (Li *et al.,* 2003).

The “4S pathway” proceeds by sequential oxidation of sulfur atom with the formation of DBT sulfoxide (DBTO)

and then DBT sulfone ($DBTO_2$; Ohshiro and Izumi, 1999). The cleavage thiophenic ring transforms $DBTO_2$ to DBT sulfinate (HPBS), which is converted in 2-hydroxibiphenyl (2-HBP), and molecules of sulfuric acid, with the subsequence decrease of pH (Fig 1.8; Wang and Kraviec, 1996). The genes involved in "4S pathway" (*dsz*) have been cloned and well characterized. The *dsz* genes are arrange in a 4-KB single operon (Denome *et al.,* 1993a). It is a cluster of three genes (*dszA, dszB* and *dszC*) transcribed in the same direction, coding for three proteins DszA, DszB and DszC respectively (Piddington *et al.,* 1995). It was shown that the *dszC* gene encodes a sulfide/sulfoxide monooxygenase (Denome *et al.,* 1994; Lei and Tu, 1996), which catalyzes the step S oxidation of DBT via DBTO to $DBTO_2$, which is converted to HPBS by the *dszA* product gene. After which, HPBS is desulfunated by the aromatic sulfinic acid hydrolase encoded by *dszB* (Oldfield *et al.,* 1997).

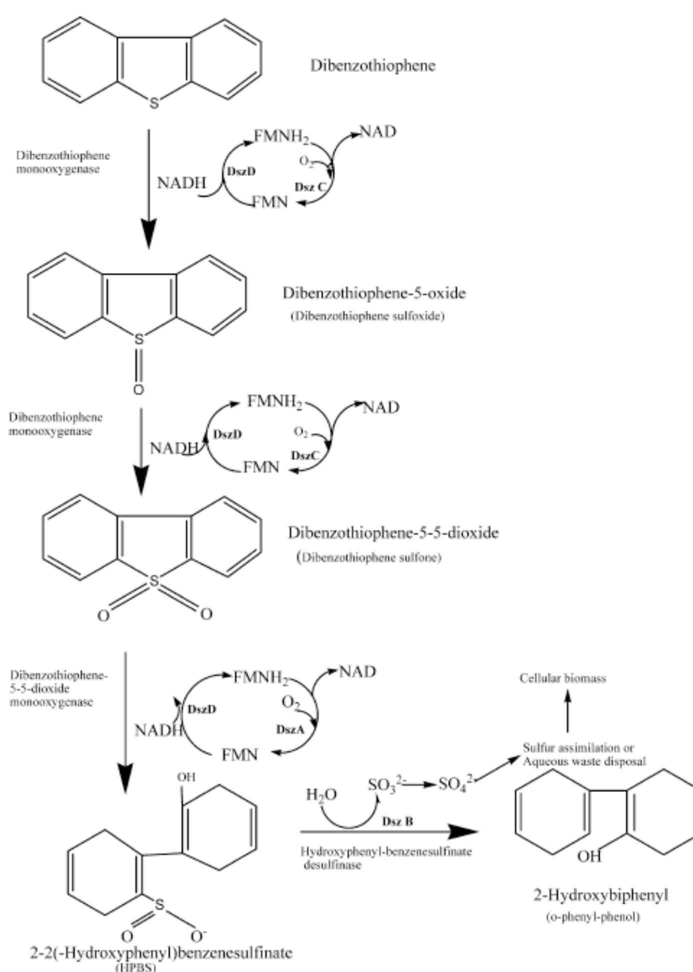

Fig. 1.8: Sulfur-specific DBT degradation via the 4S pathway (Gupta *et al.,* 2005).

1.3 Genetics in PAHs in diverse aerobic bacteria

Several genes involved in PAHs degradation have been characterized (Tab. 1.1). In the last two decades, genetic sudies of PAHs degradation have been focused on naphthalene such as *nah*-like of *Pseudomonas* strains (Dunn and Gunsalus, 1973; Simon *et al.,* 1993; Takizawa *et al.,* 1999) and phenanthrene catabolic genes.

Enzymes class	PAHs compound	Enzymes class	PAHs compound
arh	Acenaphtene	**nah**	Naphthalene
ben	Benzoate	**pah**	Naphthalene/Pheneanthrene
bph	Biphenyl	**pdo**	Phthalate
car	Carbazole	**phn**	Phenanthrene
dnt	2,4-dinitrotoluene	**pht**	Phthalate
etb	Ethylbenzene	**thn**	Tetralin
hca	3-phenylpropionate	**tou**	Toluene
nag	Naphthalene	**xil**	Xilene

Tab. 1.1: List of mains characterized genes involved in PAHs degradation and relative substrate. *arh*, (Kouzuma *et al.,* 2006); *ben*, (Kitagawa *et al*., 2001); *bph*, (Masai *et al*., 1995); *car*, (Nojiri *et al*., 2001); *dnt*, (Suen *et al*., 1996); *etb*, (Yamada *et al*., 1998); *hca* (Diaz *et al*., 1998); *nag*, (Jeon *et al*., 2006); *nah*, (Dunn and Gunsalus, 1973); *pah*, (Laurie and Lloyd, 1999); *pdo*, (Chauhan *et al*., 2008); *phd*, (Habe *et al*., 2003); *thn*, (Martinez-Perz *et al.,* 2004); *tou*, (Arenghi *et al*., 1999); *xil*, (Romine *et al*., 1999).

1.3.1 *nah*-like of *Pseudomonas* strains

The metabolism of naphthalene has been studied genetically in *Pseudomonas pudida* strain G7 (Dunn and Gunsalus, 1973). The catabolic genes are organized in three operons on the 83-kb plasmid NAH7: one encoding the "upper " pathway enzymes involved in the conversion of naphthalene to salicylate, the second encoding the lower pathway enzymes involved in conversion of salcylate to TCA cycle intermediate via meta-ring cleavage (Fig. 1.4), and the third encoding a regulatory protein (Yen and Gunsalus, 1985). Nucleic sequences of genes encoding the naphthalene catabolic enzymes from several *Pseudomonas* strains were reported, for instance: *ndo* genes from *Pseudomonas putida* NCIB 9816 (Kurkela *et al.,* 1988) and *dox* genes from *Pseudomonas* sp. strain C18 (Denome *et al.,* 1993). The gene organization and sequence homology (about 90%) among the upper catabolic genes of these strains were similar to those of the *nah* genes from the NAH7 plasmid of strain G7. These genes are usually called "classical *nah*-like genes"

1.3.2 *phn* genes and other phenanthrene-catabolic genes

The studies of phenanthrene-catabolic genes have been focused for many years on *phn* genes of *Burkholderia* sp. strain RP007 (Laurie and Lloyd, 1999). Strain RP007 was isolated from a PAHs-contaminated soil on the basis of its ability to degrade phenanthrene as sole carbon and energy source. This strain also utilizes naphthalene and anthracene as sole carbon source. The naphthalene and phenanthrene are degraded though a common pathway via salicylate and naphthalene-1,2-diol, respectively (Fig. 1.4; 1.5). Initially, the operon encode for iron–sulfur protein α and ß subunits of a PAH initial dioxygenase, but lack both ferredoxin and ferredoxin reductase components (Laurie and Lloyd, 1999).

Alcaligenes faecalis AFK2 can utilize phenanthrene as carbon sources though the 2-carboxybenzaldehyde and *o*-

phtalate pathway (Fig. 1.6), but not naphthalene (Kiyohara *et al.* 1982). Gene sequence information is available on database (Ac.No. AB024945), reporting a different stricture in comparison with classical operons; however, details have not been reported.

The *phd* genes of *Nocardioides* sp. strain KP7 are the most studied PAH-catabolic genes in Gram-positive bacteria, and belong to a new class of PAH-catabolic genes because of differences in gene organization and sequence similarity (Saito *et al.,* 1999). Strain KP7 was isolated on the basis of its ability to grow on phenanthrene at 40°C from marine samples, and it degrades phenanthrene *via* phtalate pathway (Fig. 1.6; Iwabuchi *et al.,* 1998). Saito *et al.,* (1999) reported the nucleotide sequence of the gene-cluster encoding enzyme responsible for the transformation of phenanthrene to 1-hydrixy-2-naphtoate (Fig. 1.5). Interestingly, the genes of this cluster, that encode α and ß subunits of ISP of phenanthrene dioxygenase, had less than 60% sequence identity to the corresponding ones of the other aromatic-ring dioxygenase. Phenanthrene ISP of *Nocardioides* sp. strain KP7 is encoded by *phdA*, *phdB*, *phdC*, and *phdD* genes, α and ß subunits of the dioxygenase component, a ferredoxin, and a ferredoxin reductase, respectively (Saito *et al.,* 1999). *E. coli* cells carrying *phdBCD* or *phdACD* exhibited no phenanthrene-degrading activity, and those carrying *phdABD* or *phdABC* exhibited phenanthrene-degrading activity which was significantly less than that in cells carrying the *phdABCD* genes. It was thus concluded that all of the *phdABCD* genes are necessary for the efficient expression of phenanthrene-degrading activity (Saito *et al.,* 2000).

Cycloclasticus sp. strain A5 is able to grow with petroleum polycyclic aromatic hydrocarbons (PAHs), including naphthalenes, dibenzothiophenes, phenanthrenes, and fluorenes. The set of genes responsible for the degradation such as PAHs was sequenced and found to contain 10 open reading frames (ORFs). Seven ORFs showed homology to previously characterized genes for PAH degradation. In particular, *phnA1*, *phnA2*, *phnA3*, and *phnA4* genes, which encode α and ß subunits of an iron-sulfur protein, a ferredoxin, and a ferredoxin reductase, respectively, were identified as the genes coding for PAH dioxygenase (Kasai *et al.,* 2003).

Recently, *Acidovorax* sp. strain NA3 was isolated from polycyclic aromatic hydrocarbon contaminated soil that had been treated in a bioreactor and enriched with phenanthrene (Singleton *et al.,* 2005). Further characterizations, have detected the presence of PAH degradation genes subsequently determined to be highly similar in both nucleotide sequence and gene organization to the uncharacterized *Alcaligenes faecalis* gene cluster (Singleton *et al.* 2009).

1.3.3 *dbt* genes of *Burkholderia* sp. DBT1

In our laboratory, two novel sets of genes for DBT transformation (*dbt* genes) were cloned and characterized from strain *Burkholderia* sp. DBT1 (Di Gregorio *et al.,* 2004). The description of these genes will be largely described in chapter 1.6.

1.4 Bioremediation of organic compound

The PAHs compound can be remove from the environment by the use of conventional methods, which involve removal, alteration, or isolation of the pollutant. Such techniques involve excavation of contaminated soil and its incineration or containment. These technologies are expensive, and in many cases transfer the pollutant from one phase to another. Therefore, alternative methods are required to restore polluted sites in a less expansive, safe and environmental friendly way. Such an alternative approach is consistent with bioremediation, a tool to transform the compounds to less hazardous/non-hazardous forms with less input of chemicals, energy, and time by living organism (Providenti *et al.,* 1993; Ward *et al.,* 2003; Bumpus, 1989; Yuan *et al.,* 2001). The PAH-degrading microorganism could be algae, bacteria, and fungi. It involves the breakdown of organic compounds through biotransformation into less complex metabolites, and through mineralization into inorganic minerals, H_2O, CO_2 – aerobic - or CH_4 – anaerobic -. The bioremediation of a pollutant and its rate depends on many factors including pH, temperature, oxygen, degree of acclimation, accessibility of nutrients, chemical structure of the compound, cellular transport properties (Singh and Ward, 2004). Microorganisms and/or plants play an essential role in a bioremediation protocol, and a carefully characterization of them is an essential prerequisite for development of efficient strategy. Thus, to plan a bioremediation system, several factors are to be counted for. In order to reclaim PAHs contaminated soil, different bioremediation protocol can be used. For instance, stimulation of indigenous bacterial community – landfarming - or inoculation with bacterial that were selected from PAHs contaminated matrix. The inoculation can be performed directly in polluted soil – bioaugmentation - (Barathi and Vasudevan, 2003) or alternatively, in selected plants in order to improve *in planta* degradation – phytoremediation – (Barac *et al.,* 2004). In particular, this study focused on phytoremediation and the relationship between plant and bacterial.

1.4.1 Phytoremediation

To be effective, remediation technique should enhance the rates of PAHs removal and degradation. Experimental evidence suggests that organic contaminants often disappear more quickly from planted soils than from soils without vegetation (Walton *et al.,* 1994). Such observation has led to propose the use of plants to trigger bioremediation of polluted soils. Most studies dealing with xenobiotic degradation have been carry out in the role of plants in enhancing of rhizosphere bacterial metabolism (Siciliano and Germida, 1998). However, little mechanism information regarding accelerated degradation in the rhizosphere is available.

Two different strategies for phytoremediation of organic compound are suggested: direct phytoremediation and phytoremediation *ex*-planta. Direct phytoremediation is primarily limited by the availability of the target compound and uptake mechanism (Salt *et al.,* 1998). The main factors that govern the uptake of pollutants are their physical-chemical features, and in particular their octanol-water partition coefficient (log K_{ow}). Organics that are most likely to be taken up by plants are moderately hydrophobic compounds with octanol-water partition coefficients ranging from 0.5 to 3.0 (Ryan *et al.,* 1988). Therefore, contaminants with a log $K_{ow} > 3.5$ show high sorption to the roots, but slow or not translocation to the stems and leaves (Trapp *et al.,* 2001).

Octanol-water partition coefficients (log K_{ow}) were assumed to be the same as the corresponding lipid-water partition coefficients (log K_{lip}) of contaminants for the reason that plant lipids were considered as the main storage sites for hydrophobic organic contaminants (Li *et al.,* 2005; Chiou et al., 2001). On the other hand, in a new model, plant organic components were categorized into both plant lipids and carbohydrates. Although the affinity of PAHs for lipids appears to be about 1.64 orders of magnitudes higher than that for carbohydrates, sorption of PAHs to carbohydrates could not be neglected because of its predominant weight fraction in plants - about 98 times of lipids for ryegrass root -. As a result, sorption of carbohydrates could not be ignored. Recent studies suggested that the extractable lipids were insufficient to represent the amounts of PAHs sorbed to plant samples

with the use of $K_{lip} = K_{ow}$. In fact, K_{ow} appears to underestimate K_{lip}, especially for the compounds with relative high hydrophobicity. As a result, the efficiency of K_{ow} as a substitute for K_{lip} was questioned, and the sorption of hydrophobic contaminants to plant carbohydrates may also have been highly underestimated (Fig. 1.9; Li *et al.,* 2005; Su and Zhu, 2007; Zhu *et al.,* 2007; Zhang *et al.,* 2009).

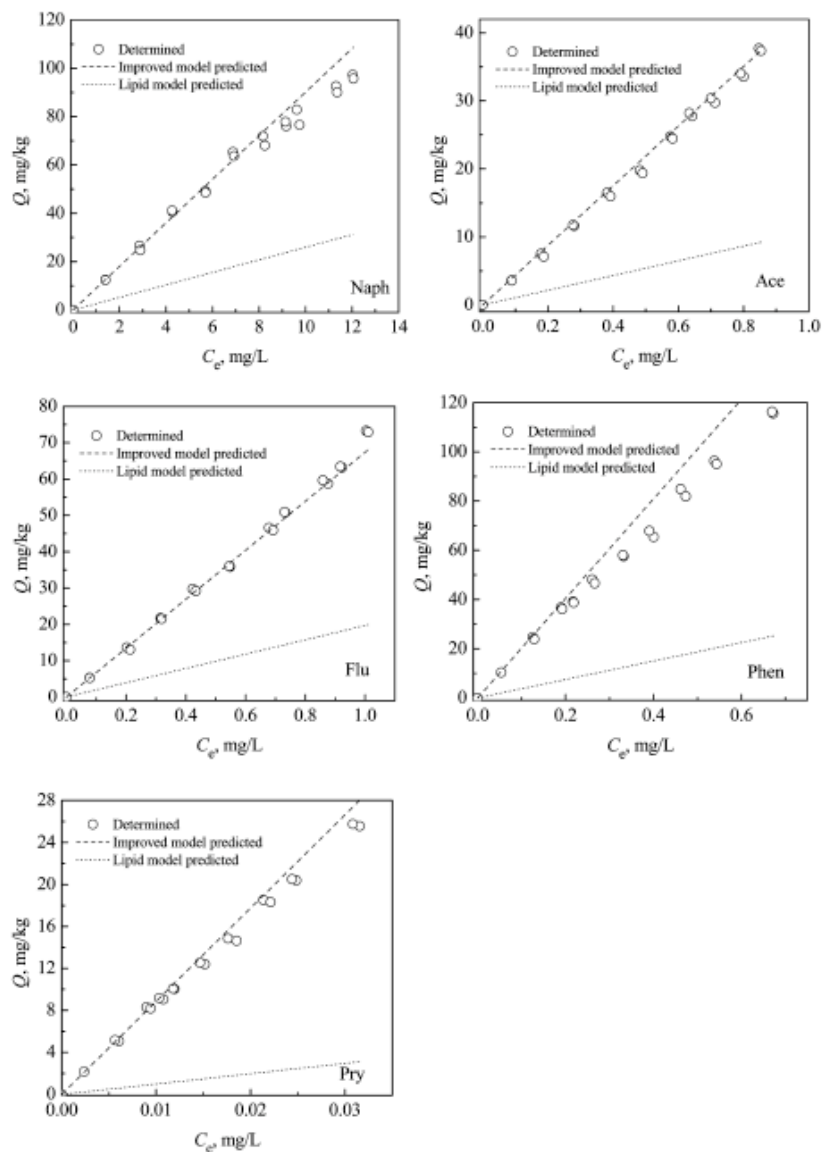

Fig. 1.9: Determined and predicted sorption isotherms of tested PAHs to ryegrass root. Naph, Ace, Flu, Phen, and Pyr stand for naphthalene, acenaphthene, fluorene, phenanthrene, and pyrene, respectively. (C_e) equilibrium concentrations of PAHs in water solution; (Q) sorption amount of PAHs on root (Zhang *et al.,* 2009).

However, PAHs phytoremediation seems to occur mainly by *ex*-planta strategy. This is based on the secretion by plants of their photosynthate in root exudates, which may support the overall growth and metabolic activities of bacterial communities in thee rhizosphere (Anderson *et al.,* 1994). Root exudates may also selectively enrich for specific degraders, or alternatively, secrete compounds that induce the necessary degradative pathway (Walton *et al.,* 1994). However, in most contaminated soils, the number of microorganisms is depressed so that, there are not enough bacteria either to facilitate contaminant degradation or to support plant growth (Glick, 1995; Siciliano and Germida, 1997). Despite the above-mentioned problem, it may be useful to facilitate phytoremediation through the massive inocula of specific contaminant degrading bacteria (Burd *et al.,* 1998; Siciliano and Germida, 1997).

However, the success of these processes of these processes is based on the ability of the bacteria to survive in the rhizosphere, compete for the exudates nutrients, sustain in sufficient numbers (Lugtemberg and Dekkers, 1999). Depending on their properties, organics may be degraded in the root zone of plants or taken up, followed by degradation, sequestration, or volatilization. In fact, after uptake in plant tissue, certain pollutants can leave the plant in volatile form; this is called phytovolatilization (Terry *et al.,* 1992). Phytovolatilization can be used for volatile organic compounds - VOCs – (Winnike-McMillan *et al.,* 2003). Poplar is also the most-used species for phytovolatilization of VOCs because of its high transpiration rate, which facilitates the movement of these compounds through the plant into the atmosphere (Burken and Schnoor, 1997; Schnoor *et al.,* 1995). It is important to underline, that if a toxic volatile pollutant is emitted by plants during phytoremediation, the fate of the gas in the atmosphere should be determined as part of risk assessment. For this reason, a previous bacterial degradation in plants could reduce the volatilization of dangerous compounds. In particular, degrader endophyte bacterial can play an important role in this mechanism (Barac *et al.,* 2004).

1.4.2 Endophyte bacterial used in phytoremediation

Endophytic bacteria can be defined as bacteria colonizing the internal tissues of plants without causing symptoms of infection or negative effects on their host (Schulz and Boyle, 2006). With the exception of seed endophytes, the primary site where endophytes can entry into plants is via the roots. (Pan *et al.,* 1997; Germaine *et al.,* 2004). Once inside the plant, endophytes either reside in specific plant tissues like the root cortex or the xylem, or colonize the plant systematically by transport through the vascular system or the apoplast (Mahaffee *et al.,* 1997; Quadt-Hallmann *et al.,* 1997). In comparison with rhizosphere and phyllosphere bacteria, endophytic bacteria are likely to interact more closely with their host. In these very close plants–endophyte interactions, plants provide nutrients and residency for bacteria, which in exchange can directly or indirectly improve plant growth and health (Mastretta *et al.,* 2006). On the other hand, endophytic bacteria can indirectly benefit plant growth by preventing the growth or activity of plant pathogens through competition for space and nutrients, production of hydrolytic enzymes, antibiosis, induction of plant defence mechanism and through inhibition of pathogen-produced enzymes or toxins (Mastretta *et al.,* 2006). In general, *Pseudomonaceae*, *Burkholderiaceae* and *Enterobacteriaceae* are among the most common genera of cultivable endophytic species found (Mastretta *et al.,* 2006). Moreover, to their beneficial effects on plant growth, endophytes have considerable biotechnological potential to improve the applicability and efficiency of phytoremediation. After plant uptake, the organic compound may be metabolized and/or released into the atmosphere via volatilization through the leaves. Plants, as photoautotrophic organism, have not developed the capacity to use organic compound as source of carbon and energy, but only a general transformations to more water-soluble forms, and sequestration processes (Burken, 2003). On the other hand, microbial metabolism often ends with the organics being converted into CO_2, water and cellular biomass. Thus, plants themselves have a positive effect on the microbial degradation of organic contaminants (Crowley *et al.,* 1997), increasing up take and degradation potential of the plant-endophyte system.

1.5 *Burkholderia* genus

The genus *Burkholderia* was proposed by Yabuuchi *et al.*, (1992) to accommodate the former rRNA group II *Pseudomonads*. The first isolated described was *Pseudomonas caryophylli* - formerly known as *Phytomonas caryophylli* - by Burkholder (1942) and in 1950, he reported the description of *Pseudomonas cepacia* as a plant pathogen causing sour skin of onion (Burkholder, 1950). Subsequently, in the early 1970s, rRNA-DNA hybridization analyses showed genetic diversity among the strains of this genus, thus, the *Pseudomonas* genus was divided into five rRNA homology groups (Palleroni *et al*., 1973). Therefore, the seven species belonging to rRNA homology group II (*Pseudomonas solanacearum, Pseudomonas pickettii, P. cepacia, Pseudomonas gladioli, Pseudomonas mallei* and *P. caryophylli*) were transferred to the novel genus *Burkholderia* (Yabuuchi *et al*., 1992). The members of this genera show the following general features: gram negative, aerobic, non-fermentative, non-spore-forming, mesophilic, straight rods, motile with a single flagellum or a tuft of polar flagella except *Burkholderia mallei* (this latter is characterized by being no motile); catalase-positive and often oxidase positive; able to accumulate poly-β-hydroxybutyrate as carbon reserve; with no production of fluorescent pigments and optimum growth temperature at 30-35°C. The *β*-proteobacteria class, that includes the *Burkholderia* genus, shows more than 86% of homology in the nucleotidic sequence of 16S rRNA. On the other hand, the homology with γ subclass members is between 81-83% (Govan *et al*., 1996). Up to this time, the genus comprises more than 60 species (see http://www.bacterio.cict.fr/b/burkholderia.html for a up-to-date) and is among the most abundant bacteria in the environment (Coeyne and Vandamme, 2003). As sequencing has become less expensive and sequencing facilities have become more available, the number of completely sequenced prokaryotic genomes publicly available is increasing (see http://www.genomesonline.org for a up-to-date). Examination of the completed *Burkholderia* genomes demonstrates that they harbor a large genome (Holden *et al*., 2004; Chain *et al*., 2006) with a minimum of two large chromosomal replicons, and both of them are essential for survival (Mahenthiralingam and Drevinek, 2007; Wigley and Burton, 2000). The large chromosome encodes many of the essential genes associated with central metabolism and cell growth, whereas the small chromosomes harbor accessory genes associated with adaptation and survival in different niches (Mahenthiralingam & Drevinek, 2007). Moreover, the *Burkholderia* genus harbors a genome rich in regulation and secondary metabolism genes (Konstantinidis and Tiedje, 2004), simple sequence repeat variation and genomic island (GIs) containing genes associated with mobile genetic element as insertion sequence (IS; Nierman *et al*., 2004). Lateral gene transfer, gene duplication and plasmid acquisition are also observed (Chain *et al*., 2006). These features may explain their ability to colonize environment where resources are scarce and different (Konstantinidis and Tiedje, 2004).

1.5.1 Ecology and physiology of *Burkholderia* genus

Members of the *Burkholderia* are versatile organisms that occupy a surprisingly wide range of ecological niches. Probably the members belonging to this genus are the most diverse and environmentally adaptable plant-associated bacteria.

Several species of the genus *Burkholderia* can induce plant diseases. As noted before, the well-known pathogenic bacterium *B. cepacia* is involved in onion rot mediated by the infection of onion leaves and bulbs (Burkholder, 1950), but other plants can also be infected by this strain (Saddler, 1994a). Moreover, *B. gladioli* induces bacterial soft rot in onions, leaf-sheath browning and grain rot in rice, and leaf and corm diseases in gladiolus and iris species (Palleroni, 1984a; Lee *et al*., 2005; Ura *et al*., 2006). *Burkholderia caryophylli*, induces formation of bacterial wilt in various plant species (Furuya *et al*., 2000) and *Burkholderia plantarii* provokes seedling blight on rice as well as on koyawarabi (Azegami *et al*., 1987; Tanaka and Katoh, 1999). *Burkholderia glumae* causes wilting symptoms in about 20 different plant species (Saddler, 1994b, Jeong *et al*., 2003) and *B. andropogonis* can

infect more than 52 species of 15 families of unrelated monocotyledonous and dicotyledonous plants (Li and De Boer, 2005; Takahashi *et al*., 2004).

Although some members of *Burkholderia* can be pathogenic for their hosts, the vast majority members of *Burkholderia* genus are nonpathogenic, and can be either neutral or beneficial to the host (De Costa & Erabadupitiya, 2005). It is well known that some *Burkholderia* sp. can inhabit the plant rhizosphere*,* and thus more beneficial plant–microbial associations can be formed with increase of plant health. *B. cepacia* complex has been isolated from the rhizosphere of maize in the US and Italy (Ramette *et al*., 2005; Bevivino *et al*., 2002), cotton (Parke & Gurian-Sherman, 2001), as well as of arum lily and wheat in the UK (Richardson *et al*., 2002). Other species of *Burkholderia* genus are isolated from plant-rhizosphere, for instance: *B. graminis* (Viallard *at al*., 1998), *B. unamae* (Caballero-Mellado *et al*., 2004), *B. caledonica* (Coenye *et al*., 2001a) and *B. vietnamiensis* (Gillis *et al*., 1995).

Some rhizosphere bacteria are able to enter the root tissue, cross from the root cortex to the vascular system, and subsequently establish endophytic populations in various organs such as leaves, stem and roots. *Burkholderia phytofirmans* (Sessitsch *et al*., 2005) has been isolated from onion roots (Nowak & Shulaev, 2003), and *B. cepacia* from branches of a *Citrus* (Araujo *et al*., 2002) and from roots of rice (Singh *et al*., 2006). Endophytic bacteria have several mechanisms by which they can promote plant growth and health. For instance, non-sterile poplar cuttings were inoculated with the endophyte *Burkholderia cepacia* VM1468, a derivative of *B. cepacia* Bu72 harboring the pTOM-Bu61 plasmid coding for toluene degradation pathway. It was observed a decreasing of the toluene phytotoxicity and a considerably improved of the growth of poplar trees in the absence of toluene (Barac *et al*., 2004; Taghavi *et al*., 2005). Recently, some *Burkholderia* strains isolated from hybrid poplar tree H11-11, are able to grow on phytohormone 1-aminocyclopropane-1-carboxylic acid (ACC) as sole nitrogen source (Taghavi *et al*., 2009).

Members of *Burkholderia* genus have also recently been isolated from nodules of some plant species and are now recognized as effective symbionts associated with roots, for instance, *B. cepacia* (Knowlton *et al*., 1980, 1983), *B. tuberum* (Moulin *et al*., 2001) and *B. phymatum* (Vandamme *et al*., 2002).

Most the species above cited, can be used for promotion of growth and health status of the plants. Their biological and metabolic properties have been exploited for biological control of fungal and abiotic diseases in plants but also for bioremediation and plant growth promotion. Many members of this genus have the ability to produce compound with antimicrobial activity and can be used against phytopathogenic fungi. For example, *B. ambifaria* LMG 19182 is very effective in controlling phytopathogenic *Pythium* species and *Aphanomyces euteiches* (Coenye *et al*., 2001c). Various strains of *Burkholderia* have been reported to produce a large variety of antifungals such as altericidins, pyrrolnitrin and xylocandins (Bisacchi *et al*., 1987; El-Banna and Winkelmann, 1998; Kirinuki *et al*., 1984). *Burkholderia* members can be used even for enhance resistance of the plant against abiotic stress. *B. phytofirmans* sp. PsJN enhance the heat stress tolerance in potato (Nowak & Shulaev 2003), and explant of *Vitis vinifera* inoculated with this strain increased grapevine growth and physiological activity at low temperature (Glick *et al*., 2007). *Burkholderia* strains have exhibited a beneficial effect on development of poplar trees (Taghavi *et al*., 2009) and inoculation of crops such as maize and sorghum have increased root and shoot biomass (Chiarini *et al*., 1998; Bevivino *et al*., 2000).

However, plants are not the only life habitation for *Burkholderia* genus. *Burkholderia* sp. has been found as fungal endosymbiont with *Suillus variegatus* and *Tomentellopsis submollis* (Izumi *et al*., 2007), *Rhizopagon luteolus* (Garbaye and Bowen, 1989). *Burkholderia* sp. have also been reported as inhabitants of several members of the Gigasporaceae (Bianciotto *et al*., 2000).

The capacity of certain bacterial groups to survive and grow in extreme or disturbed environments, such as under high levels of contamination, may be a useful feature for representing possible candidate to use in bioremediation protocol, and *Burkholderia* genera is often founded in adverse environment. Members of this genus have been

detected, for example, in agricultural or industrial soils contaminated with various heavy metals such as nickel (Hèry *et al*., 2003), lead (Templeton *et al*., 2001) and cadmium (Macnaughton *et al*., 1999; Sandrin *et al*., 2000; Lazzaro *et al*., 2008). Many strains members of *Burkholderia* genus are also isolated and identify from environment polluted with organic compounds. Some of them are capable to degrade these molecules and then be possible candidate in bioremediation protocol.

1.5.2 *Burkholderia* genus involved in bioremediation of organic compound

The exceptional metabolic versatility *Burkholderia* genus could be used for bioremediation purposes. Many members of *B. cepacia* complex are able to degrade xenobiotic compound as phthalates (Ribbons *et al*., 1984), herbicides (Sangodkar *et al*., 1988), and chlorinated hydrocarbons (Krumme *et al*., 1993). For instance, Bcc strain G4 can co-metabolically degrade toluene and tricholorethylene (TCE) via a pathway for aromatic compounds induced by phenol or tryptophan (Nelson *et al*., 1987; Shields and Reagin, 1992). Recently, pTOM toluene-degrading plasmid of *B. cepacia* G4 has been introduced in *B. cepacia* L.S.2.4, a natural endophyte of yellow lupine. Seeds were successfully inoculated with recombinant bacteria resulting in a marked decrease in toluene phytotoxicity (Barac *et al*., 2004). *Burkholderia cepacia* F297 grows on a wide variety of polycyclic aromatic compounds including fluorine - assimilating 40% of fluorene carbon - (methyl)naphthalene, phenanthrene, anchracene and dibenzothiophene. An analysis of the intermediates formed from these growth substrates has indicated that these compounds are degraded by catalytic reactions very similar to those for naphthalene degradation (Grifoli *et al*., 1995). *B. cepacia* AC 1100 is involved in the degradation of both pentachlorophenol and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T; Zaborina *et al*., 1998) a potent herbicide that is not easily biodegradable and persist for long time in the environment. Another herbicide widely used in agriculture is 2,4-dichlorophenoxyacetate (2,4-D). This compound can be degraded by *B. cepacia* CSV90 (Bhat *et al*., 1994). *B. cepacia* DBO1 was isolated in Florida for its ability to utilize phtalate as a sole source of carbon and energy for growth (Chang and Zylstra, 1998). Phthalates and phthalate esters are widely used in manufacture of plastics, textiles, papers, insect repellents, pesticides, munitions and cosmetics (Graham, 1973). These compounds are toxic for human and other organism (Autian, 1973, Peakall, 1975). Moreover, 1,2,4-trichlorobenzene (1,2,4-TCB) has various applications in industry as carrier for dyes in polyester materials, heat transfer medium and lubricant. It also functions as pesticide and aquatic herbicide. About 93% of 1,2,4-TCB enters the environment through industrial discharges. It has high acute and chronic toxicity to plants and aquatic life. The complete mineralization of 1,2,4-TCB as a sole carbon and energy source under aerobic condition has been observed by *Burkholderia* sp. PS12 (Lehning *et al.,* 1997).

B. xenovorans strain LB400 (Goris *et al*., 2004) is able to grow on biphenyl and co-oxidizes an exceptionally broad range of chlorobenzoate (CBs), the major intermediate products of aerobic degradation of polychlorinated biphenyls (PCBs). Many PCB-degrading microorganisms are not able to further metabolize clorobenzoates, therefore the degradation of these compounds is important for complete degradation of PCBs (Gibson *et al*., 1993; Erickson and Mondello, 1992; Hofer *et al*., 1993). *Burkholderia* sp. strain NK8, isolated from soil, even shows a broad specificity for chlorobenzoate degradation and it is able to grow on 3CB and 4CB (Ogawa *et al*., 2001).

Burkholderia sp. strains N3P2 and N2P5 were isolated from a creosote-contaminated Norwegian soil for their PAH degradation capacity. Creosote is a lumber preservative, constitued for 85% of PAH, found in more than 700 contaminated sites in United States; (Mueller *et al*., 1997). Strain N2P5 shows more than 99.5% 16S rDNA sequence similarity with *B. fungorum* strains (Coeyne *et al*., 2001).

Burkholderia RP007, originally isolated for its ability to degrade phenanthrene, contains *phn* locus a distinctive set of PAH catabolic genes (Laurie and Lloyd-Jones, 1999). *B. caryophylli* MCII-8 and *B.* sp. DhA54 are

phenanthrene degrading bacteria too (Friedrich *et al.,* 2000).

Burkholderia sp. DNT mineralized 2,4,5- trihydroxytoluene (THT) via an oxidative pathway (Haigler *at al.,* 1999). *Burkholderia* sp. strain NF100 was isolate from fenitrothion-treated soil (Hayatsu *et al.,* 2000), and is resulted to be capable of utilizing fenitrothion (*O,O*-mimethyl *O*-4-nitro-m-totyl phosphorothiate) as a sole carbon source. This compound is organophosphorus insecticide inhibitors of cholinesterase and can thus be hazardous in the areas of application.

A naphthalene degrading bacterium was obtained and identified as *Burkholderia* sp. (NCBI U37342; Sandrin *et al*., 2000). This bacterium is able to degrade naphthalene either in presence or in absence of cadmium by the biosurfactant rhamnolipid stimulative effect. This is an interesting feature. In fact, usually PAHs degradation of several bacteria is often inhibited by toxic metals, such as cadmium (Roane and Pepper, 1997).

Recently, *Burkholderia* sp. BS3702 was used for phenanthrene degradation in plant rhizosphere (Ovchinnikova *et al*., 2009) and *Burkholderia* sp. KR100 was characterized for 3,5,6-trichloro-2-pyridinol degradation (Kim and Ahn, 2009).

Finally, it is important to remember and underline that some species of *Burkholderia*, cause food poisoning (Zhao *et al*., 1995). They can also be plant and animal pathogens (Brett *et al*., 1998). Some Bcc strains cause life-threatening infections in humans such as cystic fibrosis (Govan *et al*., 1996). Few of them are true human pathogens. Thus, there are concerns about the use of potentially pathogenic strains of *Burkholderia* for biotechnological applications such as bioremediation of polluted sites (Vandamme *et al*., 1997; Parke and Gurian-Sherman, 2001).

1.5.3 *Burkholderia cepacia* complex (Bcc)

B. cepacia was identified over 50 years ago as a plant pathogen (Burkholder, 1950), and is widely isolated from the biosphere. The strain was differentiated from other closely related species by its ability to grow on more than 200 organic sources of carbon (Krieg and Holt, 1984), included *m*-hydroxy benzoate or tryptamine (Lessie and Gaffney, 1986). Actually, *B. cepacia* is knows as a bacterial complex (Bcc) with nine genomic species with similar phenotypical traits and different genomic ones, called genomovars. The complex is constituted by: *B. cepacia* (genomovar I), *Burkholderia multivorans* (genomovar II), *Burkholderia cenocepacia* (genomovar III), *Burkholderia stabilis* (genomovar IV), *Burkholderia vietnamiensis* (genomovar V), *Burkholderia dolosa* (genomovar VI), *Burkholderia ambifaria* (genomovar VII), *Burkholderia anthina* (genomovar VIII) and *Burkholderia pyrrocinia* (genomovar IX; Coenye *et al*., 2001c; Vermis *et al*., 2004). Recently, Vanlaere *et al.,* (2008) described new five Bcc species: *Burkholderia latens*, *Burkholderia diffusa*, *Burkholderia arboris*, *Burkholderia seminalis* and *Burkholderia metallica*. It is also demonstrate that a previous isolated, *Burkholderia ubonensis* (Yabuuchi *et al*., 2000) should be considered a member of the Bcc, increasing the number of Bcc species to 15 (Vanlaere *et al.,* 2008). In the last few years, severely unclassified Bcc were isolated from human and environment sources, and included in a distinct group know as taxon K (Baldwin *et al*., 2005; Dalmastri *et al*., 2005, 2007; Payne *et al*., 2005; Vermis *et al*., 2002). In 2009, this last group was re-examine by polyphasic taxonomic approach. It is propose to classify taxon K in two additional new species: *Burkholderia contaminans* and *Burkholderia lata* (Vanlaere *et al*., 2009).

The Bcc can live in several different environmental as an important component of the soil and plant microbial community (Butler *et al*., 1995; Bevivino *et al*., 1998; Salles *et al.,* 2004), and the prevalence of Bcc in the environment is underestimated (Fera *et al.*, 2007; Miller *et al.*, 2002; Payne *et al.*, 2005).

All species of the *B. cepacia*-complex share high degree of 16S rDNA sequence similarity (98-99%) and moderate levels of whole-genome DNA-DNA hybridisation (30-50%; Holmes *et al*., 1997; Mahenthiralingam *et al*., 2000a;

Rosello-Mora *et al*., 1995). The Bcc harbor a complex and plastic genome. Strains have from two to four (usually three) large (>500-kp) replicons (chromosomes) and one or more plasmids. The total genome size ranges from 4 to 9 Mb, more than twice the size of *Escherichia coli* (Lessie *et al*., 1996). Moreover, the microorganisms of the complex have a genome with a high mutation rate, being opportunistic pathogen to human and plants (Chaparro *et al*., 2001; Bernier *et al*., 2003). This is due mainly for the presences of numerous insertion sequences (IS; Cheng and Lessie, 1994; Lessie *et al*., 1996). IS elements are well known for causing mutations by insertion in or near genes, and IS-like elements are found flanking some "pathogenicity islands" (Baldwin *et al*., 2004).

The great attention and interest in this complex of many research groups is due for its notorious ability to be opportunistic pathogens in debilitated persons such as cystic fibrosis (CF) patients (Coenye *et al.,* 2001b). This aspect is of crucial importance since *B. cepacia* is a bacterium listed in the 2nd Risk Group of the Classification of Human Etiologic Agent. The Risk Group 2 (RG2) includes biological agents associated with human disease as CF. The main sources of infection are considered to be between patient-to-patient spread (Soni *et al.,* 2002). Moreover, acquisition from the environment it is also possible (Coenye and Vandamme, 2003; LiPuma *et al*., 2002). For these reasons, there is increasing concern about the risk of using this group of bacteria in beneficial processes such as biocontrol or bioremediation (Holmes *et al*., 1998; Parke and Gurian-Sherman, 2001).

1.5.3.1 Taxonomy of *Burkholderia cepacia* complex

One of the main aspects of microbiology, especially in ecological studies, is the identification and enumeration of species occurring in a given habitat. Moreover, there is a great interest in developing methods for rapid and efficient identification of bacterial species in environmental samples by molecular techniques. The attention in taxonomic study increase when *Burkholderia* members – and in particularly within Bcc - are involved. The exceptional metabolic diversity of this organism is a great advantage for them exploiting in the bioremediation of recalcitrant xenobiotics compound (Bhat *et al*., 1994; Nelson *et al*., 1987; Zaborina *et al*., 1998), as antagonists of soilborne plant pathogens (Holmes *et al.,* 1998) and as plant-growth-promoting agents (Hebbar *et al*., 1998). However, most strains used in biocontrol or bioremediation purposes are taxonomically poorly characterized, and their potential hazard to the CF community is unclear (Govan *et al*., 2000; Holmes *et al*., 1998). This aspect has become crucial since *B. cepacia* is a bacterium listed in the 2nd Risk Group of the Classification of Human Etiologic Agent. Thus, severely efforts have been done in order to investigate the taxonomic position of isolated strain within the *Burkholderia* genera. The diversity of environmental isolated in terms of both variation among strains and the number of different species which may be represented has made identification of environmental isolated very difficult. Since now, there is not a single technique capable to discriminate a member of Bcc, only a polyphasic approach can guarantee a reliable result (Vandamme *et al*., 1997; Coenye *et al.,* 2001b).

In diagnostic clinical laboratories identification of Bcc is performed using a combination of selective media, biochemical analysis and commercial test (Coenye *et al.*, 2001b; Henry *et al.*, 1999; Henry *et al.*, 1997; Henry *et al*., 2001; LiPuma, 1998). The most used selective media include: *P. cepacia* medium (PC medium; Gilligan *et al.*, 1985) and *B. cepacia* selective agar (BCSA; Henry *et al.*, 1997). For the recovery of environmental Bcc isolates, PCAT medium (Burbage and Sasser, 1982) or TB-T medium (Hagedorn *et al*., 1987), which are resulted the more efficient (Pirone *et al*., 2005).

Working on the single isolated, the Bcc identification can be achieved using different techniques. Whole-cell protein profiling has been used, although it has showed to have poor discriminatory efficiency between the genomovar (Vandamme *et al*., 1997). Analysis of *recA* gene is often used for taxonomy purpose. *RecA*-RFLP approach is resulted efficient for *B. multivorans* and *B. vietnamiensis* but not always sufficient to discriminate strains of *B. cepacia* genomovars I, III and *B. stabilis*. Moreover in this publication, there are no data for *B.*

anthina and *B. pyrrocinia* (Mahenthiralingam *et al*., 2000b). Specific primers for *recA,* and others gene, were also evaluated for the development of genomovar-specific PCR test (Mahenthiralingam *et al.*, 2000b; Whitby *et al.*, 2000a; Whitby *et al.*, 2000b). But, for instance, a publication showed that the assays for the identification was resulted specificity for *B. multivorans*, *B. cepacia* genomovar III and *B. ambifaria*, but showed low sensitivity towards *B. cepacia* genomovar I and cross-reacted with *B. pyrrocinia* (Vermis *et al.*, 2002). A nucleotide sequence-based identification approach based on this gene was then developed, providing a powerful means of both identification and classification of these bacteria, although more expansive (Payne *et al*., 2005). *16S* sequence analysis is less useful, but often permits the identification of Bcc-like bacteria (LiPuma *et al.*, 1999; Mahenthiralingam *et al*., 2000b). AFLP analysis was shown to generate specie specific profiles both within the genus *Burkholderia* and within the Bcc (Coenye *et al*., 1999). However, due to their complexity, this approach cannot be used for initial screening but rather only for confirmatory purpose.

Multilocus sequence analysis (MLST) allows to uncovers allelic variants in conserved genes for the purpose of bacterial identification and classification. In 2005, Baldwin *et al*., developed a MLST scheme sequencing of seven housekeeping gene - *ATP synthase β* chain, *atpD*; *glutamate synthase* large subunit, *gltB*; *DNA gyrase B*, *gyrB*; *recombinase A*, *recA*; *GTP binding protein*, *lepA*; *acetoacetyl-CoA reductase*, *phaC* and *tryptophan synthase*, *trpB* - distributed as much as possible across the chromosome (Baldwin *et al*., 2005). All known Bcc genovorans were distinguished with 100% bootstrap values, and no differences were found between clinical and environmental samples (Baldwin *et al*., 2005; 2007). Dalmastri *et al*., (2007) carried out MLST analysis for environmental sample – maize rhizosphere -, showing a massive diversity of Bcc.

Recently, Schönmann *et al*., (2009) developed an oligo-nucleotide microarray consisting of 131 hierarchically nested 16S rRNA gene-targed probes, for a culture – indipendent, parallel and high trough-put analysis of the members of *Burkholderia* genus. Eighty-eight per cent of the reference strains were correctly identified at the species level. Therefore, this technique can be useful only for an initial screening of Bcc in diagnostic purpose.

1.5.4 Other hazardous *Burkholderia* species

Bcc is the most studied and hazardous complex within the *Burkholderia* genus, but is not the only dangerous organism for human and animal.

B. gladioli is a plant pathogen typically recovered from *Gladiolus* sp., *Iris* sp. and rice (Palleroni, 1984b). *B. gladioli* are able of infecting certain vulnerable human hosts (Graves *et al.,* 1997; Shin *et al.,* 1997), chronic granulomatous disease (CGD) and again CF patients (Christenson *et al.,* 1989; Ross *et al.,* 1995; Khan *et al.,* 1996). The spectrum of infections caused by *B. gladioli* includes respiratory tract infections (Christenson *et al.,* 1989; Ross *et al.,* 1995), septicemia (Graves *et al.,* 1997; Ross *et al.,* 1995), abscesses (Jones *et al.,* 2001), osteomyelitis (Boyanton *et al.,* 2005) keratitis (Lestin *et al.,* 2009), and adenitis (Graves *et al.,* 1997). Although little is known about the epidemiology of human disease caused by this species. To date, person-to-person transmission of *B. gladioli* has not been reported and the presumed cross-infection of patients with the same strain described in New Zealand (Wilsher *et al.,* 1997) was later attributed to a strain of *B. cenocepacia* (Clode *et al.,* 1999a). Molecular identification of *B. gladioli* is mainly based on rDNA specific signatures and can be performed by means of species-specific PCR (Bauernfeind *et al.,* 1998; Clode *et al.,* 1999b), Amplified Ribosomal DNA Restriction Analysis (ARDRA; Segonds *et al.,* 1999) or 16S rDNA sequencing (Ferroni *et al.,* 2002). *B. gladioli* may also be involved in respiratory infections in non CF patients, especially in case of intubation (Segonds *et al.,* 2009).

B. mallei is primarily a pathogen in horses, in which it causes equine glanders, a disease characterized by fever, inflammation of the nasal mucosa, necrosis and obstruction of the oropharynx. In humans, infection can be limited

to subcutaneous tissues or can disseminate to cause sepsis. If inhaled *B. mallei* can cause pneumonia with necrosis of the tracheobronchial tree (Srinivasan *et al.,* 2001). *B. mallei* can be spread via contact with infected animals or through exposure in research laboratories (Srinivasan *et al.,* 2001). The highly contagious and potentially lethal nature of human infection with *B. mallei* makes this species well suited for use as an agent of bioterrorism. In fact, *B. mallei* was one of the first biologic weapons of the 20th century, being used by Germany during World War I (Wheelis, 1998).

B. pseudomallei is a saprophytic organism, broadly distributed in soil and water in Southeast Asia and northern Australia. It is an important cause of morbidity and mortality in Thailand, where the frequency of disease is most likely underestimated due to lack of access to adequate health care (Chaowagul *et al.,* 1989). The organism can also cause infections in a wide variety of animals, including livestock (Dance, 1991). It is thought that most infected humans and animals acquired the organism through inoculation on exposure to contaminated soil or water, although the possibility of inhalation or ingestion as modes of infection requires further investigation (Dance, 2000; Currie *et al.,* 2001).

Since the original report was made, *B. fungorum* has been identified in a range of soil and plant-associated samples, in infections of the central nervous system of a pig and a deer (H. Scholz and P. Vandamme, unpub. data), and in the respiratory secretions of people with cystic fibrosis (Coenye, *et al.,* 2001a; 2002). However, in these cases, the clinical significance of isolation of *B. fungorum* was also unclear. Only recently, the first case of *fungorum* septicemia was reported. The source of the *B. fungorum* bacteremia remained elusive, but, undoubtedly, it was a community-acquired infection manifested as a soft tissue infection of the leg (Gerrits *et al.,* 2005).

1.6 ***Burkholderia* sp. DBT1** (Di Gregorio *et al.*, 2004)

Burkholderia sp. DBT1, the object of this study, is a bacterial strain formerly isolated from the wastewater discharge pipeline of an oil refinery located in Tuscany. Since the first observation, it was clear the involvement of this strain in DBT transformation. In fact, both minimal and rich media supplied with DBT become red, suggesting the formation of intermediate of Kodama pathway. Spectroscopic data show as the DBT content of the culture decrease by 93% after 72h of incubation, and further chromatographic and mass spectroscopy analysis allowed identification of all the intermediates of Kodama pathway (Kodama *et al.*, 1970; 1973).
Molecular characterization of the strain DBT1 has shown unusual genetic features. The genes involved in dibenzothiophene transformation by the strain DBT1 are in fact harbored in two operons (p51 and pH1A; Fig. 1.10) and show low similarity to both *nah*-like and *phn*-like genes (Takizawa *et al.*, 1999; Laurie and Lloyd, 1999). The two genomic fragments were sequenced in *Burkholderia* sp. DBT1. The results shows that six of eight genes required for the conversation of DBT to HFBT by Kodama pathway are present in these two operons. The two genes missing are the ferredoxin reductase component of initial dioxygenase (ISP) and the aldolase, enzyme involved in the last step of Kodama pathway - from trans-HTOB to HFBT -.

pH1A genomic fragment

Eight putative ORFs were identified in the pH1A genomic fragment (GeneBank accession no. AF380367). Seven of these ORFs initiated with the expected ATG start codon followed by a putative ribosomal binding site, while the truncated ORF R (*dbt*R) started with a GTG start codon. ORF D, encoded for DbtD, a 2-hydroxychromene-2-carboxylate isomerase. ORFα and ß encoded DbtAc and DbtAd, respectively the large (α) and small (ß) subunit of the initial dioxygenase (ISP). ORF B encoded DbtB, a dihydrodiol dehydrogenase. All these genes are involved in Kodama pathway. On the other hand, ORF 5 encoded for a protein similar to a NADH:FMN oxidoreductase, a gene that are not involved in Kodama pathway. RT-PCR analysis has been showed that under inducing condition (minimal medium added with DBT) induces the transcription of *dbt*D,Ac,Ad,B,ORF5 in a single unit. No transcription was detected upstream *dbt*D and downstream ORF5. In un-inducing media (minimal medium supplied with citrate) no transcription of *dbt*D,Ac,Ad,B,ORF5 was observed. Downstream ORF 5 were detected ORF X, encoded for a protein homologous to PhnX cloned in *Burkholderia* sp. RP007 (Laurie & Lloyd-Jones, 1999) and the truncated ORF F encoded of a salicylaldehyde dehydrogenase. Neither protein is involved in the Kodama pathway. Upstream ORF D was detected the truncated ORF R encoded for a putative 54-dependent transcriptional regulator. ORF R was divergently transcribed with respect to *dbt*D,Ac,Ad,B,ORF5 unit.

p51 genetic fragment

Seven putative ORFs were detected in p51 genomic fragment (GeneBank accession AF404408) each initiated with an ATG start codon and a putative ribosomal binding site. ORF C and ORF b encoded, respectively, for DbtC, an extradiol dioxygenase, and for DbtAb, a ferrodoxin subunit of the initial dioxygenase (ISP) both genes involved in Kodama pathway. On the other hand, ORF 6 encoded for proteins homologous to an oxidoreductase and ORF 7 for the C-terminal of a hydrolase, which are not involved in the Kodama pathway. RT-PCR analysis, carry out as previously described, has been showed the co-transcription of *dbt*C,Ab,ORF6,ORF7 in a single replicative unit. No transcription was observed upstream *dbt*C. In un-inducing media no transcription of *dbt*C,Ab,ORF6,ORF7 was observed. The sequence of p51 genetic fragment stopped at the end of ORF 7 and the transcription terminator is not yet detected. Thus, this operon could harbor further gene sequences such as the aldolase and ferredoxin reductase, the two genes sequence missing in the degradation of DBT. On the other hand, upstream ORF C were

identified ORF 10 encoded the C-terminal of a protein homologous to 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 and ORF 9 encoded for a protein homologous to an oxidoreductase.

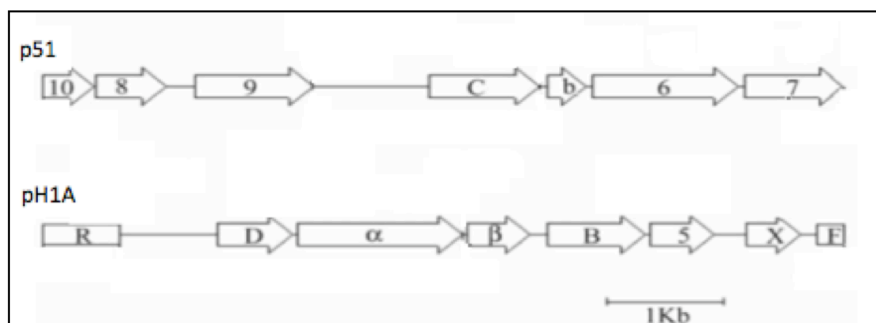

Fig. 1.10: Diagram of the p51 and pH1A replicative unit structures in *B.* sp. DBT1

pH1A genomic fragment			p51 genomic fragment		
ORF	**Gene**	**Protein feature**	**ORF**	**Gene**	**Protein feature**
R	*dbt*R	Transcriptional activator	10	ORF10	Aromatic oxygenase
D	*dbt*D	Isomerase	8	ORF8	Isomerase
α	*dbt*Ac	ISP α subunit	9	ORF9	Oxydoreductase
β	*dbt*Ad	ISP β subunit	C	*dbt*C	PAH extradiol dioxygenase
B	*dbt*B	Dihydrodiol dehydrogenase	b	*dbt*Ab	ISP ferrodoxin subunit
5	ORF5	Putative oxydoreductase	6	ORF6	Oxydoreductase
X	ORFX	Unknown protein	7	ORF7	Hydrolase
F	ORFF	Salicylaldeyde dehydrogenase			

Tab 1.2: *dbt* genes cloned on pH1A and p51 replicative units.

Recent publications identified nucleotide sequences of p51 and pH1A operons in PAHs contaminated sites (Chadhain *et al.*, 2006; Sipilä *et al.*, 2006) suggesting the involvement of DBT1 in degradation of PAHs other than condensed thiophenes frequently occurring in oil-contaminated sites. Since DBT, and generally PAHs, behaves as compounds persistent in the environment where it resist to the degradation, isolation and characterization of microbes able to use them as only source of carbon and energy is a great interest and, in particular, *Burkholderia* sp. DBT1 may become a useful candidate for bioremediation purposes.

Aims

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic compounds that have accumulated in the natural environment mainly as a result of anthropogenic activities. These compounds are largely suspected to act as potential mutagens, teratogens as well as carcinogens (Fujikawa *et al.,* 1993). Their molecular stability and hydrophobicity are among the prominent factors that contribute to the persistence of these pollutants in the environment. Moreover, their low aqueous solubility and, consequently, their low bioavailability are a great obstacle to microbial degradation (Cerniglia, 1992). Of the PAHs occurring in soils and groundwaters, about 0.04 - 5% (wt/wt) are sulfur heterocycles (Thompson, 1981), among which dibenzothiophene (DBT) represents the prevailing compound. This is therefore taken into account as model chemical structure in studies dealing with either biodegradation of organo-sulfur contaminants through the Kodama pathway (Kodama *et al.,* 1973) or petroleum biodesulfurisation through the 4-S pathway (Gallagher, 1993). Although there is no definitive legislation concerning PAH abatement, different treatment procedures have been investigated aimed to remove or degrade such compounds from the environment.

The word "bioremediation" has been used to describe the process of using microorganisms to degrade or remove hazardous compounds and wastes such as PAHs from the environment. Nevertheless, before developing an efficient protocol for bioremediation is necessary to perform an upstream study, in order to isolate, identify and characterize organisms suitable to this purpose. Moreover, important features are required in view of a possible exploitation of bacterial isolate in bioremediation protocols, such as the capability of the organism to transform or degrade toxic compounds in less or not hazardous metabolites, the exclusion of the organism to possible pathogenic species and ensure the eco-toxicological safety of the strain.

Burkholderia sp. DBT1 is a bacterial strain identified in an oil refinery wastewater which can degrade DBT nearly completely through the Kodama pathway within three days. Molecular characterization of the strain DBT1 has shown unusual genetic features. The genes involved in dibenzothiophene transformation are harbored in two operons - p51 and pH1A - and show low similarity to both *nah*-like and *phn*-like genes (Di Gregorio *et al.,* 2004).

These particular features have encouraged studies aimed at a functional, phylogenetic and toxicological characterization of *Burkholderia* sp. DBT1 for a possible explanation of such strain in protocol of bioremediation in PAHs contaminated soils. Aimed to clarify the effective potential and actual utilization of such strain in bioremediation protocol, different aspects of *B.* sp. DBT1 have been investigated.

Identification of DNA sequences flanking p51 operon: Molecular characterization of strain DBT1 has showed that six of eight genes required for DBT degradation by Kodama pathway are present in two operons – p51 and pH1A - (Di Gregorio *et al.,* 2004). pH1A genetic fragment harbor the single transcription unit *dbt*D,Ac,Ad,B,ORF5 and no transcription was detected upstream *dbt*D and downstream ORF5. On the other hand, p51 genetic fragment harbor *dbt*C,Ab,ORF6,ORF7 replicative unit. The sequence of p51 genetic fragment stopped at the end of ORF 7 and the transcription terminator was not yet detected. Thus, this operon could contain further gene sequence such as the hydratase-aldolase and ferredoxin reductase, the two genes sequence missing in the degradation of DBT by Kodama pathway. Therefore, identification of DNA sequence flanking p51 operon was carried out in order to detect possible gene sequence downstream ORF 7.

Taxonomic analysis: Taxonomic status of *Burkholderia* sp. DBT1 has never been resolved. It is important to remark that several species of *Burkholderia* can cause food poisoning (Zhao *et al.*, 1995) or can behave as plant and animal pathogens (Brett *et al*., 1998). Furthermore, different strains belonging to the *Burkholderia* cepacia complex (Bcc) are involved in life-threatening infections of humans such as cystic fibrosis (Govan *et al.*, 1996).

Thus, concerns exist about a misappropriate use of potentially pathogenic species of *Burkholderia* for biotechnological applications such as bioremediation of polluted sites (Vandamme *et al*., 1997; Parke and Gurian-Sherman, 2001). Therefore, the objective of this study is to investigate the true taxonomic position of DBT1 within the *Burkholderia* genera on the basis of a wide range of molecolar, phenotypic and genotypic tests.

Study of PAHs degrading activity: Since normally several organic pollutants contribute together to the contamination at different sites, isolation and characterization of microbes able to use a wide range of PAHs compounds as sole source of carbon and energy are of great interest in order to select useful candidates for applications in bioremediation. Therefore, the metabolic capability of strain DBT1 will be tested aimed at clarifying the possible role of *Burkholderia* DBT1 in the degradation of PAHs other than condensed thiophenes, frequently occurring in oil-contaminated sites.

Toxicology and pathogenic analysis: Several *Burkholderia* species are common soil inhabitants, nevertheless some species of this genus are known as toxin producer (Garcia *et al.,* 1999). Thus, before using isolates of this genus in bioremediation protocols, it appears of prominent importance to demonstrate that they are not hazardous organisms. Therefore, some tests will be carried out to even investigate the eco-toxicological safety of strain DBT1 for environmental use.

Study of endophytes bacterial strains: Once it is been clarified the metabolic, taxonomic aspect of strain DBT1, it results very important to set up an efficient protocol for a future application in open field (*in situ*). A new promise strategy is to use endophitic bacterial equipped with appropriate degradation pathway in order to improve *in planta* PAHs degradation. Moreover, a lot of published reports describe many species of *Burkholderia* as a natural endophyte (Barac *et al*., 2004), and *B.* sp. DBT1 presents all the potentiality for its use in a plant-endophytic bacteria protocol. The aim of this last year of *PhD* was to select and study bacterial strains able both to degrade PAHs and to colonize host plants (e.g. Aspen) in order to verify the bioremediation potential of plant-endophytic bacteria systems, using both molecular and physiologic analysis. Moreover, it is performed a taxonomic studies using different technique aimed to discover possible genetic polymorphism within endophyte *B. fungorum* species and DBT1 strain. The start point is a bacterial collection isolated from roots, stem and leaves of hybrid aspen (*Populus tremula* x *tremuloides*) grown on PAHs polluted soils (no published data) in Finland by group of Dott. Kim Yrjälä.

2. Materials and methods

2.1 Bacterial strains

Burkholderia sp. DBT1, *Burkholderia* sp. DBT1 mH1 mutant strain and *Burkholderia* sp. DBT1 m51 mutant strain were available at laboratories of Microbial Biotechnology & Environmental Microbiology - Department of Biotechnology, University of Verona - (Di Gregorio *et al*., 2004).

Burkholderia fungorum LMG 16225^T, *Burkholderia caledonica* LMG 19076^T, *Burkholderia graminis* LMG 18924^T, *Burkholderia cepacia* LMG 1222^T were purchased from DSM (German Collection of Microorganisms and Cell Cultures).

Escherichia coli strain Xl1Blu.

Burkholderia cepacia used as positive control in PCR detection of *esmR* was isolated from CF patient in hospital "Civile Maggiore" (Verona - Italy).

Collection of endophyte bacteria was obtained from group of Dott. Kim Yrjälä at Department of Biological and Environmental Sciences; Helsinki (Finland).

2.2 Growth media

- **Yeast Mannitol Broth (YMB):** 0.5 g l^{-1} K_2HPO_4; 0.1 g l^{-1} $MgSO_4$ H_2O; 0.1 g l^{-1} NaCl; 0.4 g l^{-1} yeast extract; 10 g l^{-1} mannitol.
- **Yeast Mannitol Agar (YMA)** is YMB solidified with 15 g l^{-1} of noble agar.
- **Tryptic Soy Broth (TSB)**: 15 g l^{-1} tryptone, 5 g l^{-1} soytone, 5 g l^{-1} NaCl.
- **Tryptic Soy Agar (TSA)** is TSB solidified with 15 g l^{-1} noble agar.
- **Defined Medium (DM)** is prepared as previously described in Frassinetti *et al*., (1998).
- **Defined Medium Agar** is DM solidified with 14 g l^{-1} of noble agar.
- **Luria Bertani (LB):** 10 g l^{-1} tryptone; 5 g l^{-1} yeast extract; 10 g l^{-1} NaCl.
- **Luria Bertani Agar** is LB solidified with 14 g l^{-1} of noble agar.
- **SOC broth:** 10 g/l^{-1} tryptone; 5 g/l^{-1} yeast extract; 0.5 g/l^{-1} NaCl; 10 ml/l^{-1} KCl [250 mM]; 5 ml/l^{-1}; $MgCl_2$ [2M]; 5 ml/l^{-1} glucose [1M].

All media were sterilized by heating at 121 °C under a pressure of 105 kPa for 15 minutes.

2.3 Identification of DNA sequences flanking p51 operon

-Targeted gene walking-

The identification of unknown sequence flanking adjacent to a known sequence can be performed by different technique: inverse PCR (Huang, 1994), ligation-mediated PCR (Liu *et al.,* 1995), or T-linker PCR (Yuanxin *et al*., 2003). However, these methods generally have been not applied because they are too inefficient and/or complicated. On the other hand, the "targeted gene walking" protocol by UP-PCR (Dominguez and Lopez-Larrea, 1994) offers several advantages: quality of products, simplicity, general applicability and a methodical approach to optimization.

2.3.1 Genomic DNA extraction with the bead-beater method

One anoxic colony was inoculate in a tube with 4 ml of liquid growth broth at 27°C in agitation over/night. 2 ml of liquid culture was drawn, centrifuged at 8000 rpm for 10 min and thus the supernatant was removed. 0.2 ml of extraction buffer (1% SDS, 0.2g/ml Yeast Extract, 0.02g/ml Triton X-100, 0.1M NaCl, 5mM EDTA), 0.3g of glass beads (diameter 0.4-0.5 mm sterilized in absolut ethanol) and 0.2ml of Phenol:Chloroform:Isoamyl Alcohol 24:25:1 (saturate with 10mM Tris, 1mM EDTA, pH 8.0) were added, and thus the microtubes were vortexed for 2

min. Therefore, the samples was centrifuged at 10000 rpm for 5 min and the watery phase transferred in a clean microtube. Isopropanol 1:1 was added and incubated for 5 min at room temperature. Thus, the samples were centrifuged at 10000 rpm for 5 min and the supernatant was removed. The pellet was cleaned with ethanol 70% and centrifuged at 10000 rpm for 5 min and the supernatant was removed. Finally, the pellet was air-dry and resuspend in 50µl of distillated water. To determinate the quality of DNA extracted, it was loaded 3µl of genomic in agarose gel 1% in TAE 1X buffer at 100V for 20 min.

2.3.2 Sequence amplification by "unpredictably primer (UP)-PCR" protocol

UP-PCR protocol employs two successive PCRs, for which four primers are needed: two sequence specific primers (SPs) and two universal walking primers (WPs). The WPs are oligonucleotides of defined and artificial sequence. Based on preliminary experimentation they have been given a similar basic design in order to fulfill the following requirements: promiscuous character for the first hybridization step and selectivity for subsequent PCR annealings. WPs differ from one another in their melting temperature, having therefore distinct working ranges, and at small changes at their 3' ends. The sequence of the short WP (iWP) is contained within that of the larger WP (Dominguez and Lopez-Larrea, 1994). The SPs are complementary to the known sequence from which one intends to walk. Both have the same orientation: 3' end towards the unknown region. They can be called outer specific primer (SP) and inner specific primer (iSP). The melting temperature (Tm) and consequently, the annealing temperature (Ta) of SP and iSP primers are the most critical parameter for UP-PCR. These parameters were calculated by BioEdit software (version. 7.0.9.0).

Reactions were performed after preparing the following mixes:

Sample mix (duplicate):

- 20 ng DNA
- 20 pmol WP-D74
- H_2O to 10 µl

Polymerase mix:

- 4 µl 5x buffer (Promega)
- 2 µl dNTP mix (2 mM dATP, 2 mM dCTP, 2 mM dGTP, 2 mM dTTP)
- 0.5 U Taq DNA pol GoTaq™ (Promega)

Replicas of sample mix were incubated for 30-60s at 90°C and suspended at 80°C. The next component were then added: 2 µl Mg^+ [25mM] in the first tube, 2 µl H_2O in the second tube and 1 µl Mg^+ [25mM] and 1 µl H_2O in the thirst and 6µl of polymerase mix in each tube. The final concentrations of Mg^+ result of 3mM, 1.5mM and 2.25mM respectively. Tubes were immediately chilled to 15 - 25°C for 15 minutes. This low stringency annealing and extension step will be referred to as first extension and it was considered complete after a final 1 minute incubation at 72°C. Samples were held at 90°C and 2 µl of 10 µM SP-D74 (20 pmol) were finally added. PCR reaction using DNA from *Burkholderia fungorum* LMG 16225^T was used as negative control for each concentrations of Mg^+.

The PCR reaction by WP-D74 and SP-D74 (Tab. 2.1) set of primers was performed as follows:

94°C for 30'' ⎤
56°C for 30'' ⎬ x 30
72°C for 1'30'' ⎦
72°C for 5'

Using as template the amplification previously described, it was amplified an internal fragment using iWP-C47 and iSP-C47 primers (Tab. 2.1). PCR reaction was carried out in 25µl volume containing 0.8 µM for each primer, 1 mM of deoxynucleotide triphosphate, 1 U of GoTaq™ DNA polymerase and 5 µl of 5x PCR-buffer (Promega). The PCR condition was as follows:

94°C for 5'
94°C for 30'' ⎤
46°C for 30'' ⎬ x 30
72°C for 2' ⎦
72°C for 7'

Primer sets	Sequence (5' to 3')	Reference
WP-D74	$T_{11}GT_3GT_2GTG_5TGT$	(Dominguez and Lopez-Larrea, 1994)
iWP-C47	$T_5GT_3GT_2GTG_3$	(Dominguez and Lopez-Larrea, 1994)
SP-D74	ACTGCGAGGTTGAAATAATTGACAACT	(This study)
iSP-C47	GAGAGATTCATTCTTACTTAA	(This study)
RT-Gw	TCGTCAAAACGTATACGCGC	(This study)

Tab. 2.1: Primers used in "gene targeted walking" protocol

All PCR are loaded in agarose gel with 0.5µg/ml of ethidium bromide in Joule Box™ Mini Gel Electrophoresis System (Stratagene).

2.3.3 Purification of PCR product, cloning and sequencing

Purification of PCR product: PCR products were electrophoresed on 0.8- 2% agarose gels containing ethidium bromide (10 mg ml−1), to ensure that a fragment of the correct size had been amplified. Then, the fragment was purified using QIAEX® II Gel Extraction Kit (Quiagen) following the manual instruction.

Ligation and cloning: PCR products were ligated and cloned in pGEM-T Easy (Promega). The ligation reaction was set up as described in manual protocol using 2-4:1 molar ratio of the PCR product to the vectors. The cloning was performed using *E. coli* XL1Blu $CaCl_2$ competent cells. Briefly, the cells were placed in ice bath until thawed. 10 µl of ligation reaction was transfer to the competent cells and incubated in ice for 20 min. The cells were heat-shocked for 35 sec in a water bath at 42°C. Immediately the tube was returned in ice for 2 min. 1ml SOC medium was added and subsequently incubated for 1 h at 37°C with shaking (200 rpm). 250 µl of the suspension and the pellet were plated on LB agar containing 100 µg/ml of ampicillin. 4 µl of IPTG (200 mg/ml) and 40 µl of X-Gal (20 mg/ml) were previously plated on the LB agar. The plates were incubated o/n at 37°C.

Transformation control: Selection of transformed cells was performed by screening of white-blue colonies. The white colonies were further verified by "colonies PCR" using M13for and M13rev primers (Tab. 2.2). Briefly, a single colony was added to reaction mix - 50µl volume containing 0.8 µM for each primer, 1 mM of deoxynucleotide triphosphate, 1 U of GoTaq^{TM} DNA polymerase and 10 µl of 5x PCR-buffer (Promega) - by picking the colony with a sterile loop, and twirling this in the liquid in the tube.
The PCR condition was as follows:

94°C for 7'
94°C for 30'' ⎤
42°C for 30'' ⎬ x 30
72°C for 1'30'' ⎦
72°C for 5'

The positive colonies were inoculated in LB media overnight at 37°C. Thus, the plasmid was extracted by kit Wizard® *Plus* SV Miniprebs (Promega) following the manual instruction.

Sequencing: The plasmids were sent to Primm (Milan, Italy) for sequencing.

Primer sets	**Sequence (5' to 3')**
M13for	GTAAAACGACGGCCAGT
M13rev	AACAGCTATGACCATG

Tab. 2.2: Primers used in "colony PCR"

All PCR are loaded in agarose gel with 0.5µg/ml of ethidium bromide in Joule Box^{TM} Mini Gel Electrophoresis System (Stratagene).

2.3.4 Targeted gene walking control

PCR performed in order to verify the concatenation of sequence detected by targeted gene walking protocol, was performed using RT-Gw/SP-D74 primers (Tab. 2.1). PCR reaction was carried out in 25µl volume containing 0.8 µM for each primer, 1 mM of deoxynucleotide triphosphate, 1 U of GoTaq^{TM} DNA polymerase and 5 µl of 5x PCR-buffer (Promega).
The PCR condition was as follows:

94°C for 5'
94°C for 30'' ⎤
56°C for 30'' ⎬ x 30
72°C for 1'30'' ⎦
72°C for 5'

The PCR has loaded in agarose gel with 0.5µg/ml of ethidium bromide in Joule Box^{TM} Mini Gel Electrophoresis System (Stratagene).

2.3.5 Bioinformatics tools

ORF finder: The sequence was analyzed by software Open Reading Frame (ORF) Finder – NCBI -.

BLAST: Putative gene identifications were assigned by nucleotide and amino acid sequence similarities to the resulting sequences obtained by BLAST – NCBI -.

Phylogenetic trees: ClustalW and phylogenetic trees were performed by Molecular Evolutionary Genetics Analysis (MEGA) software, version 4.

2.4 Taxonomic study

2.4.1 PCR amplification of *16S* rRNA, *recA* and *gyrB* sequences

All the PCR reaction was carried out in 25 µl volume containing 0.8 µM for each primers, 1 mM of deoxynucleotide triphosphate, 1 U of GoTaq™ DNA polymerase and 5 µl of 5x PCR-buffer (Promega).

PCR of *16S rRNA*: The gene encoding for 16S rRNA (1500-pb) was amplified using primers R11/F8 (Weisburg *at al*., 1991). The PCR condition was as follows:

96°C for 5'

96°C for 1' ⎤

50°C for 1' ⎬ x 30

72°C for 2' ⎦

72°C for 5'

recA: Specific *B. fungorum recA* PCR-amplification assay was performed using primers FunF and FunR (Chan *et al*., 2003) using the following condition:

96°C for 5'

96°C for 1' ⎤

66°C for 1' ⎬ x 30

72°C for 1' ⎦

72°C for 5'

PCR amplification of the *recA* (869-bp) was carried out using primers BUR1 and BUR2 (Payne *et al.,* 2005) as follows:

96°C for 5'

96°C for 1' ⎤

61°C for 1' ⎬ x 30

72°C for 1' ⎦

72°C for 5'

gyrB: For amplification of 432-bp *gyrB* fragment gene, were used primers gyrB1F/gyrB2R (Ait Tayeb *et al*., 2008). The PCR condition was as follows:

96°C for 5'

96°C for 1' ⎤

58-60°C for 1' ⎬ x 30

72°C for 1' ⎦

72°C for 5'

Primer sets	Sequence (5' to 3')	Reference
R11	ACGGCTACCTTGTTACGACT	(Weisburg *at al*., 1991)
F8	GAGTTTGATCCTGGCTCAG	(Weisburg *at al*., 1991)
FunF	GTCATGCGGCTCGGCGCAGGT	(Chan *et al*., 2003)
FunR	GAGTGCGTCGGCAATCTCGAGT	(Chan *et al*., 2003)
BUR1	GATCGA(AG)AAGCAGTTCGGCAA	(Payne *et al*., 2005)
BUR2	TTGTCCTTGCCCTG(AG)CCGAT	(Payne *et al*., 2005)
gyrB1F	GACAACGGCCGCGGSATTCC	(Ait Tayeb *et al*., 2008)
gyrB2R	CACGCCGTTGTTCAGGAASG	(Ait Tayeb *et al*., 2008)

Tab. 2.3: Primers used for amplification of *16S*, and the fragment of *recA* and *gyrB*.

All PCR are loaded in agarose gel with 0.5µg/ml of ethidium bromide in Joule Box™ Mini Gel Electrophoresis System (Stratagene).

2.4.2 Purification of PCR product, cloning and sequencing

Amplicons obtained were purified from agarose gel, cloned and sequenced such as reported in paragraph 2.3.3.

2.4.3 Bioinformatics tools

BLAST: BLAST of gene sequence were performed by nucleotide BLAST – NCBI -.

Phylogenetic trees: ClustalW and phylogenetic trees were performed by Molecular Evolutionary Genetics Analysis (MEGA) software, version 4.

2.4.4 DNA-DNA hybridization

The DNA-DNA hybridization was been performed by DSMZ (Braunschweig, Germany) by spectroscopic analysis in 2 X SSC + 5% formamide at 70°C. DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite (Cashion *et al*., 1997). DNA-DNA hybridization was carried out as described by De Ley *et al*., (1970) under consideration of the modification (Huss *et al*., 1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6x6 multi-cell changer and a temperature controller with *in-situ* temperature probe (Varian).

2.5 Phenotypical and genotypical analysis

2.5.1 API 20 NE test

Phenotypic characteristics of strains *Burkholderia* sp. DBT1, *Burkholderia fungorum* LMG 16225^T and *Burkholderia cepacia* LMG 1222^T were compared in duplicate by API 20 NE test strips according to the recommendation of the manufacturer (bioMèrieux, Marcy-L'Etoile, France). The results were recorded after 24 and 48 hours of incubation at 37 and 30°C.

2.5.2 Genomic organization

The presence of plasmids was test by a modified Kado and Liu (1981) extraction procedure. A pre-inoculum was growth until OD_{600}= 0.8 A. 4 ml of culture suspension was pelleted by centrifugation (6000 g x 10 min). The cells were suspended in 100 µl of Buffer E (40 mM Tris-acetato, 2 mM Na-EDTA at pH= 7.9). The cells were lysed by adding 200 µl Lysing Buffer (3% SDS, 50 mM Tris at pH= 12.6). The solution was heated at 60°C for 1 h. Two volumes of phenol-chloroform solution (1:1 vol/vol) were added. The emulsion was shaken and centrifuged (6000 g x 15 min) and the upper aqueous phase was transferred to a new tube. 50 µl of sample was electrophoresed. To avoid overheating and to resolve high-molecular-weight plasmids, a low- salt buffer system – Buffer E - was used. Electrophoresis was carried out at 12 V/cm and usually required about 2 h (~ 12 cm). The gel was stained with 0.5 µg/ml of ethidium bromide for 30 min.

2.5.3 Plasmid-Curing

A pre-inoculum in YMB of *B*. sp. DBT1 was grown in 5 ml of YMB medium for 48 h at 27°C.

SDS plasmid-curing agent: 10 µl of suspension cells was transferred in duplicate into a series of fresh tubes containing YMB supplied with several different concentration of SDS - 0.1%, 0.05%, 0.025%, 0.01% and 0.005% -. The cultures were incubated at 27°C for 48 h with shaking (200 rpm). Tube whose showed a just detectable increase of turbidity, were selected and plated at different serial dilutions on YMA. Therefore, individual colonies were streaked on YMA supplied with DBT powder on the bottom of the plate.

Elevated temperature plasmid-curing agent: Elevated temperature can also be used as another procedure for curing. Briefly, the pre-inoculum was diluited 1:20 into a fresh YMB medium and inoculated at several different temperatures from 30°C to 40°C for 48 h with shaking (200 rpm). Tubes whose showed a just detectable increase of turbidity was refreshed into a new tubes and allow to grow again at elevated temperatures. Repeat the step if necessary. Individual colonies were tested as previously described.

If DBT1 lost the ability to become red in presence of DBT, it means that the gene involved in PAHs degradation were harbored in a plasmid lost while the curing treatment.

2.6 Study of PAHs degrading activity

2.6.1 Chemicals

Dibenzothiophene (DBT), naphthalene, fluorene, phenanthrene, 2-carboxybenzaldehyde, phthalic acid, protocatechol, 2-hydroxy-1-naphthoic acid and 1-hydroxy-2-naphthoic acid were purchased from Sigma Aldrich. All the compounds were analytical grade. They were dissolved in *N*-*N*-dimethylformamide (Sigma Aldrich) before addition to the bacterial cultures.

2.6.2 Growth test in presence of different organic compounds as sole source of carbon and energy

Liquid medium: Carbon sources utilization was determined in DM medium by observing the bacterial growth and the appearance of colour intermediates.

Briefly, a pre-inoculum of the selected bacterial was grown in 5 ml of YMB medium for 48 h. Alternately, the pre-inoculum of selected strain was grown in 20 ml of DM supplied with DBT or phenanthrene for 72 h in order to induce the PAHs degrading genes. The cells were collected by centrifuge (5000 g for 5 min at 4°C) and washed twice with physiologic solution (NaCl 0.9%). The bacterial was supplied to 50 ml of DM supplemented with organic compound (100 ppm) to obtained a final concentration of OD_{600} = 0.01 A and growth at 27°C in constant agitation (250 rpm). A sample of this suspension was collected periodically throughout the whole incubation period. 100 µl of serial dilutions were spread in duplicate on YMA plates and incubated at 27°C for 4-5 days. The visible colonies were enumerated and calculated the colony-forming unit (CFU). Alternately, the bacterial growth was determined evaluating the absorption of the suspension at OD_{600} by spectrophotometer. A trial without bacterial inocula was performed as negative control.

Agarized media: Bacteria strains were streaked in minimal media (DM agar). An ethereal solution of single PAHs (10% wt/vol) was uniformly spayed over the surface of the agar plates. The PAHs were also supplied by spreading crystals on the agar surface or on the bottom of the plate. The plate was then incubated for 1 week. Bacteria streaked on DM media without PAHs are used as negative control.

2.6.3 Transformation of organic compounds by co-metabolism

The test was performed on YMA medium. The PAHs were supplied by spreading crystals on the bottom of the plate. The plates were then incubated at 27°C. The colonies were refreshed every week for a month. The subsequent development of pigmentation in the cells is due of the presence of PAHs transformation.

2.6.4 2-carboxybenzaldehyde dehydrogenase assay

Cell-free extraction: Bacterial strains were grown to exponential phase in DM or YMB supplemented with phenantrene or 2-carboxybenzaldehyde (2-CBA; 500 ppm). Cells were harvested by centrifugation (8000 g for 15 min at 4°C) and washed twice with phosphate buffer (50 mM, pH 7.8). Cells were then suspended in 1.5 ml ice-cold phosphate buffer and sonicated at 4°C for three cycles (8 min at 80 W) using an Ultrasonic Cleaner (model 1200M, Sonica). The homogenate abtained was centrifuged at 13000 rpm for 15 min at 4°C. The surnatant was used as cell-free extract for the enzymatic assay.

Bradford assay: Protein concentration was determinated through the Bradford assay (Bradford, 1976) by using bovine serum albumin as a standard.

2-CBA dehydrogenase assay: The activity of 2-carboxybenzaldehyde dehydrogenase was determinated spetrophotometrically by measuring the formation of NADH at 340 nm in phosphate buffer (50 mM, pH 7.8) containing 50 µg of protein, 10 mM NAD^+ and 75 mM 2-carboxybenzaldehyde.

2.6.5 Spectrophotometer characterization

Bacterial inocula – final concentration OD_{600nm}= 0.1 A – was performed in DM medium supplied with 2-carboxybenzaldehyde 100ppm. A sample of 2 ml was collected at begin of the trial and after 1 week. It was centrifuge (10000 g x 10 min), and 1 ml of sample was assayed by spectrophotometry (Unicam UV2) at a range of wavelengths from 200 nm to 600 nm. A trial without bacterial inocula was used as negative control.

2.6.6 RT-PCR analysis of p51 and pH1A operons in *Burkholderia* sp. DBT1

RNA extraction: *Burkholderia* sp. DBT1 was grown for three days in DM added with phenanthrene at final concentration of 500 ppm. Cells were then pelleted and RNA was obtained by TRIzol® reagent (GibcoBRL, Life Tecnology). Briefly, 1.5 ml of bacterial culture was pelletted by microcentrifuge. 1ml of TRIzol® was added, mixed well by pipetting up and down, and incubated at room temperature for 5 minutes. 0.2 ml of cold chloroform was added and mixed by shaking the tube vigorously by hand for 15 seconds. After a incubation at room temperature for 2-3 minutes the tube were centrifuged at 12,000 x g for 15 minutes at 4°C. The colorless, upper phase containing RNA was transfer to a fresh tube and 0.5 ml of cold isopropanol was added. After incubation at room temperature for 10 min the samples were centrifuged at 15,000 x g for 10 minutes at 4°C. The supernatant was carefully removed and the RNA pellet suspended in 1 ml 75% ethanol. After a centrifuge at 7,500 x g for 5 minutes at 4°C the pellet was suspended in 50 µl RNase-free water.

RT-PCR: RT-PCR was carried out with M-MLV Reverse Transcriptase (Promega), using 2µg of total RNA and, respectively, p11 primer for the p51 operon or p3 primer for the pH1A replicative unit (Tab. 2.4). The reaction conditions for the reverse transcripion were as follows: annealing at 42°C for 10 min and extension at 50°C for 1 h.

PCR reaction was carried out in 25µl volume containing 1-3µl of previous RT-PCR reaction, 0.8 µM for each primers, 1 mM of deoxynucleotide triphosphate, 1 U of GoTaq™ DNA polymerase and 5 µl of 5x PCR-buffer (Promega).

The PCR reaction using the p11 primer couple with p10 primer was as follows:

94°C for 1’ ⎤
69°C for 1’ ⎬ x 30
72°C for 1’ 30’’ ⎦
72°C for 5’

The PCR reaction utlizing the second set of primers, p3 and p2 was performed as follows:

94°C for 1’ ⎤
64°C for 1’ ⎬ x 30
72°C for 1’ ⎦
72°C for 5’

Positive and negative controls were included during the reactions.

Primer sets	Sequence (5' to 3')	Reference
p11	TTGTTCCGGTTTACATCGTAGCTC	(Di Gregorio *et al*., 2004)
p3	CGAATTTCCCGAAGTCCCAATT	(Di Gregorio *et al*., 2004)
p10	AGCGGCCAAGCGAATCAATCATTT	(Di Gregorio *et al*., 2004)
p2	GGTCGGAAACATGGGGTAATGGA	(Di Gregorio *et al*., 2004)

Tab. 2.4: Primers used for RT-PCR reaction.

All PCR are loaded in agarose gel with 0.5µg/ml of ethidium bromide in Joule Box^{TM} Mini Gel Electrophoresis System (Stratagene).

2.7 Toxicology and pathogenic analysis

2.7.1 Toxin extraction

Methanol extraction of toxins from pure bacterial culture was carried out in duplicate as described by the following protocol. After incubation on TSB at 28°C for three days, bacterial colonies were collected in a flask and weighted. Cells were frozen (-20 °C) and thawed for three times. Afterwards, 10 ml of methanol were added to the biomass and flasks were shaken at 170 rpm overnight. After that, biomasses were heated at 45 °C for 30' and centrifuged for 5' at 3800 rpm. Supernatant was transferred in a spherical flask and methanol was evaporated with rota-vapor. 2 ml of the same solvent were added and mixed with the residue and then transferred in a 4 ml screw cap glass vial. Methanol was further evaporated with nitrogen flow gas in a heat block. Finally the residue was weighted and leads to a final concentration of 10mg/ml with methanol.

2.7.2 Sperm cells motility essays

The test was performed on *Burkholderia* sp. DBT1, *Burkholderia fungorum*, and *Burkholderia cepacia*. *Bacillus cereus* F-528, and *Bacillus cereus* 4810/72 were respectively used as negative and positive controls (Andersson *et al.*, 1998). In this experiment was used extended boar semen (27x10^6cells/ml), composed by semen of five boars (AI Jalostuspavelu, Rauhalinna, Finland). Them can be kept at room temperature, and used until 2-4 days after collection. Two ml of extended boar semen for tube were exposed to 20 µl of the toxic extract. Diluted boar semen can tolerate 1% methanol. In the zero samples (sham exposure) the semen was exposed to 20 µl of methanol only. The tubes were turned upside down. The semen was exposed to the extracts for 1 day at room temperature. An aliquot of 200 µl was transferred in a new tube that was kept for 5 min in a thermo block at 37°C and mixed once. Finally the percentage of the spermatozoa that were mobile (flagellar movement) and progressive movement (percent of spermatozoa that change location), of a drop of sperm cells, was estimated on a pre-warmed microscope slide by means of a phase contrast microscopy with a stage heated at 37°C. The toxicity of extracts was tested also after 30 minutes of exposition of sperm cells. In this short test, 200 µl of extended semen was exposed to 5 µl of extract. The motility was checked as described above.

2.7.3 Staining of sperm cells

Plasma membrane integrity is determined considering the fluorescence emitted by propidium iodide (PI) (Molecular Probes Eugene, Oregon, USA). Mithocondrial depolarization is determined through change of fluorescence of 5,5',6,6'-tetrachloro-1,1',3-3'-tetraethylbenzimidazolycarbocyanine iodide (JC1) (Molecular Probes Eugene, Oregon, USA). 0.7 µl of JC-1 (1mg/ml in DMSO) and 1 µl of PI (1mg/ml in DMSO) were added, to 200 µl of sperm cells already exposed to toxic agents. The cells were kept for other 5 min at 37°C and viewed using a Leitz epifluorescence photomicroscope equipped with a 100-W mercury lamp, long pass filter system LP 520 nm (to visualize red and green fluorescence simultaneously) dichroic mirror RKP 510, and a 450-490 nm exciter.

2.7.4 Human cells essays

Caco-2 cells (colon adenocarcinoma cells) were obtained from Viikki Drug discovery center, Department of Pharmacy, University of Helsinki (Finland). They were cultivated in Dulbecco's modified eagle's medium (Gibko, UK) supplemented with 10% of fetal bovin serum (Gibko, UK), non-essential aminoacids, L-glutamine (2 mM) and penicilline-streptomycin (100 IU + 100 µg/ml). 120 000 cells for chamber were plated and incubated for 5 days. Paju cells (tumor cell line established from the pleural fluid of a girl with metastatic neural-crest derived tumor) were obtained from department of pathology Haartaman Institute, University of Helsinki (Helsinki,

Finland). These cells were grown in RPMI 1640 medium (Sigma, st. Louis, USA) supplemented with 10% FBS penicillin G (50 µg/ml) and 1mM L-glutammine. 80 000 cells for chamber were plated and incubated for three days. Human cell lines were grown at 37°C in water-saturated atmosphere with 5% CO_2 and 95% air in a four chambers Lab Tek II Chamber Slide™ System (Nalge Nunc International, Denmark). Afterwards the medium was removed from the corner of each well. 400 µl of it were mixed with 4 µl of toxin extract (for the negative control 4 µl of methanol were used) and immediately added to the well. After 20 min of incubation at 37°C, in water-saturated atmosphere with 5% CO_2 and 95% air, the toxin mixture was removed and cells were washed with 400 µl PBS. Subsequently PBS was removed and 200 µl dye solution (2 ml 1640 RPMI with 15 µl of P.I. and 10 µl of JC-1) were quickly added. The chamber was wrapped with foil to minimize light exposure and incubated for 20 min at 37°C in water-saturated atmosphere with 5% CO_2 and 95% air. Cells were washed with RPMI 1640 medium. Enough medium was left in the chambers to protect cells from drying. Chamber walls were removed and a cover slip was laid down on the slide. The fluorescence of human cells was checked by a Leitz epifluorescence photomicroscope equipped with a 100-W mercury lamp, long pass filter system LP 520 nm (to visualize red and green fluorescence simultaneously) dichroic mirror RKP 510, and a 450-490 nm exciter. Pictures to every well were made using Kodak Film 800 ISO.

2.7.5 Detection of *esmR* gene

PCR reaction: The presence of BCSEM was detected by PCR with primers BCESM1/BCESM2 as described in Mahenthiralinhgam *et al*., (1997). PCR reaction was carried out in 25µl volume containing 0.8 µM for each primer, 1 mM of deoxynucleotide triphosphate, 1 U of GoTaq™ DNA polymerase and 5 µl of 5x PCR-buffer (Promega).

The PCR condition was as follows:

96°C for 5'

96°C for 30'' ⎤

63°C for 30'' ⎬ x 30

72°C for 1'30'' ⎦

72°C for 5'

Primer sets	Sequence (5' to 3')	Reference
BCESM1	CCACGGACGTGACTAACA	(Mahenthiralinhgam *et al*., 1997)
BCESM2	CGTCCATCCGAACACGAT	(Mahenthiralinhgam *et al*., 1997)

Tab. 2.5: Primers used for amplification of *esmR gene*

***esmR* gene fragment digestion:** The PCR product was purified from the gel (Par. 2.3.3) and digested with *Pst*I and *Hae*III restriction enzymes (Promega). Briefly, ~500 ng of PCR product was added in 1 µl buffer 10x and 5 U of restriction enzyme. Deionized water was added to a final reaction volume of 10 µl. Afterwards, the digested products was loaded in 1.5% agarose gel in TBE 1X buffer at 100V for 60 minutes. The gel was analysed with the UV transilluminator (Eppendorf).

2.8 Study of endophytes bacterial strains

2.8.1 Screening of PAHs degrading bacteria

The screening was performed on agarized medium as previously described in paragraph 2.6.2.

2.8.2 Genomic DNA extraction with the bead-beater method

Genomic DNA extraction was performed as previously described in paragraph 2.3.1.

2.8.3 PCR primers-specific for p51 and pH1A operons

The PCR reactions were carried out in 25 µl volume containing 0.8 µM for each primers, 1 mM of deoxynucleotide triphosphate, 1 U of GoTaq™ DNA polymerase and 5 µl of 5x PCR-buffer (Promega).

Sets of primers p2/p3 and p1/p5 primers were used for the specific amplification of fragment of **pH1A** operon. The PCR reaction using the p2 primer couple with p3 primer was as follows:

94°C for 5’
94°C for 1’ ⎤
64°C for 1’ ⎬ x 30
72°C for 1’ ⎦
72°C for 5’

The PCR reaction utlizing the second set of primers, p1 and p5 was performed as follows:

94°C for 5’
94°C for 1’ ⎤
64°C for 1’ ⎬ x 30
72°C for 3’30” ⎦
72°C for 5’

Couples of primers p8/p11 and p12/p13 primers were used for the specific amplification of fragment of **p51** operon. The PCR reaction using the p8 primer couple with p11 primer was as follows:

94°C for 5’
94°C for 1’ ⎤
66°C for 1’ ⎬ x 30
72°C for 2’30’’ ⎦
72°C for 5’

The PCR reaction utlizing the second set of primers, p12 and p13 was performed as follows:

94°C for 5’
94°C for 1’ ⎤
68°C for 1’ ⎬ x 30
72°C for 1’30” ⎦
72°C for 5’

Primer sets	Sequence (5' to 3')	Reference
p1	GGGACGAGAAAACGTTATCAATTGCG	(Di Gregorio *et al*., 2004)
p5	GTCGGATCCCAATGCAGAAACTCC	(Di Gregorio *et al*., 2004)
p2	GGTCGGAAACATGGGGTAATGGA	(Di Gregorio *et al*., 2004)
p3	CGAATTTCCCGAAGTCCCAATT	(Di Gregorio *et al*., 2004)
p8	TAGGCTGGCCTATTCCACCTTCA	(Di Gregorio *et al*., 2004)
p11	TTGTTCCGGTTTACATCGTAGCTC	(Di Gregorio *et al*., 2004)
p12	GTCTTCCCGGTTATGTGGACATTA	(Di Gregorio *et al*., 2004)
p13	ATACTGCATGGGCGATATGTTTAAC	(Di Gregorio *et al*., 2004)

Tab. 2.6: Specific primers for p51 and pH1A operons.

2.8.4 PCR primers-specific for upper meta-pathway extradiol dioxygenases

The PCR reaction was carried out as described in Sipilä *et al*., (2006). DNA extracted from *Sphingomonas* sp. HV3 was used as positive control.

Primer sets	Sequence (5' to 3')	Reference
BP-r	TGVTSNCGNBCRTTGCARTGCATGAA	(Sipilä *et al*., 2006)
BP-f	TCTAYCTVCGNATGGAYHDBTGGCA	(Sipilä *et al*., 2006)

Tab. 2.7: Specific primers for upper meta-pathway extradiol dioxygenases.

All PCR are loaded in agarose gel with 0.5µg/ml of ethidium bromide in Joule Box^{TM} Mini Gel Electrophoresis System (Stratagene).

2.8.5 Restriction analysis

The digestion of PCR amplification product was performed with *HhaI* and *TaqI* restriction enzymes (Promega) at 37°C for 3 hours in a volume of 20µl. Afterwards, the digested products are loaded in 1.5% agarose gel in TBE 1X buffer at 100V for 60 minutes. The gel was analysed with the UV transilluminator (Eppendorf).

2.8.6 PCR-DGGE analysis

The theory behind DGGE is based upon the consideration that two strands of a DNA molecule separate, or melt, when heat or a chemical denaturant is applied. Melting temperature (Tm) of DNA molecule is determined by the nucleotide sequence. DGGE exploits the fact that otherwise identical DNA molecules, which differ by only one nucleotide within a low melting domain will have different melting temperatures.
DGGE was performed in the following way:

_V3_ and _V6-V8_ region of 16S rDNA gene sequence

All the PCR reaction was carried out in 25 µl volume containing 0.8 µM for each primers, 1 mM of deoxynucleotide triphosphate, 1 U of GoTaq^{TM} DNA polymerase and 5 µl of 5x PCR-buffer (Promega).

PCR of *16S rRNA*: The gene encoding for *16S rRNA* (1500-pb) was amplified using R11 and F8 primers (Tab. 2.3 Weisburg *at al.*, 1991). The PCR condition was as follows:

96°C for 5’

96°C for 1’ ⎤

50°C for 1’ ⎬ x 30

72°C for 2’ ⎦

72°C for 5’

Nested-PCR of *V3:* Using as template the amplification of 16S rDNA, it was amplified the hypervariable region *V3* using P3 and P2 universal primers (Tab. 2.8; Muyzer *et al.,* 1993).

The PCR condition was as follows:

95°C for 2’

95°C for 30’’ ⎤

53°C for 30’’ ⎬ x 30

72°C for 2’ ⎦

72°C for 5’

Nested-PCR of *V6-V8*: Using as template the amplification of 16S rDNA, it was amplified the hypervariable region *V6-V8* using F984GC and R1378 universal primers (Tab. 2.8; Heuer *et al.,* 1997).

The PCR condition was as follows:

95°C for 2’

95°C for 1’ ⎤

53°C for 1’ ⎬ x 30

72°C for 2’ ⎦

72°C for 5’

***dbtAc* fragment of p51 operon sequence**

All the PCR reaction was carried out in 25 µl volume containing 20 µM for each primers, 20 mM of deoxynucleotide triphosphate, 1,25 U of GoTaq^{TM} DNA polymerase and 5 µl of 5x PCR-buffer (Promega).

PCR of *dbtAc*: The gene encoding for *dbtAc* was amplified using p1 and p5 primers (Tab. 2.6 Di Gregorio *at al.*, 2004). The PCR condition was as follows:

94°C for 5’

94°C for 1’ ⎤

64°C for 1’ ⎬ x 30

72°C for 3’30” ⎦

72°C for 5’

Nested-PCR of *dbtAc*: Using as template the amplification of *dbtAc*, it was amplified an internal fragment of *dbtAc* using dbtAc forGC and dbtAbrev primers (Zocca, 2004). The PCR condition was as follows:

95°C for 5’

95°C for 45’’ ⎤

62°C for 45’’ ⎬ x 30

72°C for 45’’ ⎦

72°C for 5’

Primer sets	Sequence (5’ to 3’)	Reference
P3	(40bp-GCclamp)CCTACGGGAGGCAGCAG	(Muyzer *et al.*, 1993)
P2	ATTACCGCGGCTGCTGG	(Muyzer *et al.*, 1993)
F984GC	(40bp-GCclamp)ACGGGGGGACCGCGAAGAACCTTAC	(Heuer *et al.*, 1997)
R1378	CGGTGTGTACAAGGCCCGGGAACG	(Heuer *et al.*, 1997)
dbtAc forGC	(40bp-GCclamp)GGCAAGCTCTACCGAAAATTG	(Zocca, 2004)
dbtAbrev	GTGTTAGTACCCCAGAGATATGAGTTGT	(Zocca, 2004)

Tab. 2.8: Primers used for nested-PCR.

Poliacrilamide gel

The solution at 100% of denaturing was made by: 7M urea (BioRad), 40% (vol/vol) formamide (Sigma Aldrich), 8% acrilamide/bisacrilamide amd 0.5% TAE (pH 8) in distilled water.

	26% * 6% **	**58% * 9% ****	**40% * 10% ****	**60% * 10% ****	**40% * 8% ****	**55% * 8% ****
40% Bis-Acrilamide	2.73 ml	4.05 ml	4.55 ml	4.55 ml	3.6 ml	3.6 ml
TAE 50X	360 µl	360 µl	360 µl	360 µl	360 µl	360 µl
Formamide	1.872 ml	4.176 ml	2.88 ml	4.32 ml	2.88 ml	3.96 ml
Urea	1.963 ml	4.387 ml	3.02 ml	4.54 ml	3.02 ml	4.10 ml
H_2O	until 18 ml	until 18 ml	until 18 ml	until 18 ml	until 18 ml	until 18 ml

Tab. 2.9: Reagents and relative amounts used in DGGE solution; (*) Denaturing concentration; (**) Bis-Acrilamide concentration

Electrophoretic run in denaturing gel

The DGGE was performed using Dcode^{TM} Universal Detection System (BioRad). The electrophoretic run was made at 35V for 16h at 65°C (constant temperature) in 7 l of TAE 1X buffer. The poliacrilamide gel was colored in TAE 1X buffer supplemented with 1mg/l of ethidium bromide. The image was detected by the software AB.EL CAT with camera control version 2.0.0.

2.8.7 RAPD (Random Amplified Polymorphic DNA) analysis

RAPD analysis were performed by different primers.

Primer COC-1 (Cocconcelli *et al.*, 1995): The PCR reaction was carried out in 20 µl volume containing 2 µM of primer, 250 µM of deoxynucleotide triphosphate, 0,2 U of GoTaq^{TM} DNA polymerase and 1x PCR-buffer (Promega) supplied with Mg^{+} [25 mM] till reach the concentration of 3.75 mM. The PCR condition was as follows:

94°C for 15”

94°C for 1’ ⎤

29°C for 1’ ⎬ x 45

72°C for 2’ ⌋
72°C for 5’

Primer D1254 (Akopyanz *et al.,* 1992): The PCR reaction was carried out in 20 µl volume containing 2 µM of primer, 2.5 mM of deoxynucleotide triphosphate, 0,2 U of GoTaq^{TM} DNA polymerase and 1x PCR-buffer (Promega) supplied with Mg^{+} [25 mM] till reach the concentration of 3 mM. The PCR condition was as follows:

94°C for 5’ ⌉
36°C for 5’ } x 4
72°C for 5’ ⌋
94° for 1’ ⌉
36°C for 1’ } x 30
72°C for 2’ ⌋
72°C for 10”

Primer D8635 (Akopyanz *et al.,* 1992): The PCR reaction was carried out in 20 µl volume containing 0.8 µM of primer, 200 µM of deoxynucleotide triphosphate, 0,2 U of GoTaq^{TM} DNA polymerase and 1x PCR-buffer (Promega) supplied with Mg^{+} [25 mM] till reach the concentration of 3 mM. The PCR condition was as follows:

94°C for 5’ ⌉
40°C for 5’ } x 4
72°C for 5’ ⌋
94° for 1’ ⌉
55°C for 1’ } x 30
72°C for 2’ ⌋
72°C for 10”

Primer M-13 (Stenlid *et al.,* 1994): The PCR reaction was carried out in 20 µl volume containing 2.5 µM of primer, 250 µM of deoxynucleotide triphosphate, 0,2 U of GoTaq^{TM} DNA polymerase and 1x PCR-buffer (Promega) supplied with Mg^{+} [25 mM] till reach the concentration of 3 mM. The PCR condition was as follows:

94°C for 15”
94°C for 1’ ⌉
45°C for 20” } x 40
72°C for 2’ ⌋
72°C for 5’

Primer M-14 (Zapparoli *et al.,* 2000): The PCR reaction was carried out in 20 µl volume containing 2.5 µM of primer, 200 µM of deoxynucleotide triphosphate, 0,2 U of GoTaq^{TM} DNA polymerase and 1x PCR-buffer (Promega) supplied with Mg^{+} [25 mM] till reach the concentration of 4 mM. The PCR condition was as follows:

94°C for 5”
94°C for 1’ ⌉
45°C for 20” } x 40

72°C for 2' ⌋
72°C for 5'

Primer OPL-16 (Vincent *et al.,* 1998): The PCR reaction was carried out in 20 µl volume containing 0.2 µM of primer, 200 µM of deoxynucleotide triphosphate, 0,2 U of GoTaq™ DNA polymerase and 1x PCR-buffer (Promega) supplied with Mg^+ [25 mM] till reach the concentration of 2.5 mM. The PCR condition was as follows:

94°C for 5"
94°C for 1' ⌉
30°C for 1' } x 40
72°C for 2' ⌋
72°C for 5'

Primer sets	Sequence (5' to 3')	Reference
COC-1	AGCAGCGTGG	(Cocconcelli *et al.*, 1995)
D1254	CCGCAGCCAA	(Akopyanz *et al.*, 1992)
D8635	GAGCGGCCAAAGGGAGCAGAC	(Akopyanz *et al.*, 1992)
M-13	GAGGGTGGCGGTTCT	(Stenlin *et al.*, 1994)
M-14	GAGGGTGGGGCCGTT	(Zapparoli *et al.*, 2000)
OPL-16	AGGTTGCAGG	(Vincent *et al.*, 1998)

Tab. 2.10: Primers used for RAPD analysis.

All PCR are loaded in agarose gel with 0.5µg/ml of ethidium bromide in Joule Box™ Gel Electrophoresis System (Stratagene).

2.8.8 Growth test in presence of different organic compounds as sole source of carbon and energy

The tests were performed in liquid media as described in paragraph 2.6.2.

2.8.9 Spectrophotometer characterization

Bacterial inocula – final concentration OD= 0.01 A – was performed in DM medium supplied with PAH compound at 100ppm. A sample of 2 ml was collected at begin of the trial and for all bacterial growth curve. It was centrifuge (10000 g x 10 min), and 1 ml of sample was assayed by spectrophotometry (Unicam UV2) at a range of wavelengths from 200 nm to 600 nm. A trial without bacterial inocula was used as negative control.

2.8.10 Plant inoculation

Three selected strains (95, 316, 115) and DBT1 were grown on liquid medium till the end of log-phase (48h), then pelleted and resuspended in Hoagland's solution containing 5% of rich growth medium in order to obtain an inoculum with a cell concentration of 10^7 - 10^8 CFU/ml. Aspen roots were initially placed in bacterial suspension for 3 days. Control plants were placed in the same solution without bacteria for the same period of time. The plants were allowed to stabilize for two weeks in sands soils. Afterwards, the plants were sowed in non-sterile oil polluted (5%) sandy soil and in non-polluted sandy soil. Before sowing, root and sprout lengths as well as surface of three leaves were determined. Each test was performed in triplicate.

3. Results and Discussion

3.1 Identification of DNA sequences flanking p51 operon -Targeted gene walking-

Molecular characterization of *Burkholderia* sp. DBT1 showed the presence of two genetic fragments – p51 and pH1A - harboring the genes involved in Kodama pathway. The two genomic fragments were sequenced, and six of eight genes required for the conversation from DBT to HFBT were recovered. Thus, two genes have been missing: the hydratase-aldolase, which is involved in the last step of Kodama pathway and the ferredoxin reductase, a subunit of initial dioxygenase. Sequence analysis indicate the presence of either start or terminator sequences of transcription flanking to pH1A operon; otherwise, in p51 fragment only the start of transcription was identified, resulting truncate at 3' end (Di Gregorio *et al*., 2004). Therefore, the identification of DNA sequence flanking p51 operon was carried out in order to recover and identify the genes placed downstream the truncate sequence.

3.1.1 Sequence amplification by "unpredictably primer (UP)-PCR" protocol

Firstly, two specific primers were constructed on ORF7, the sequence placed at 3' end of p51 operon (Fig. 1.10; Di Gregorio *et al*., 2004). SP-D74 and iSP-C47 primers were constructed complementary to the known sequence from which one intends to walk, and having a Ta the most similar to WP-D74 (58°C) and iWP-C47 (45°C) respectively.

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                                                            5877 atgg
5881 aaaaaattca ggaaatcacc attaaaggat ccgagcgcac cgaggttgta aaggcttgga
5941 attcccagat cgatatcaag gtaaatattg cggggaatgg accggcaata gtctacttcc
6001 atccggctgc tggcctgttc tgggacccgt ttcttgatcg cttggctgag aaatttactg
6061 tctatgcgcc tgagatgccc ggcacaacgg ttggtgatcc ttatgccatc cacaaaattg
6121 acaccttttg ggatctattg cttatttatg aggaggttgt tcgaaagttg gagatcgaac
6181 cggtctgtgc gattggtcag tccctgggtg gcatggtgac ggcggatctc gccgcaaact
6241 ttactgaact gttcggcaag gtggtccttc tggatcccat cggtctatgg agcgacgacg
6301 cgccagtgct gacgaataag ttgctgatcg accccccctca aaatattccc ggatatcttt
6361 ttgtcgaccc cggcaatccg attgctgtga agatgttgac gccgccggcc gatccggagc
6421 aggccgttaa acatatcgcc catgcagtat ggtcgctcgg gtgttcagcc aaattcatct
6481 ggccattccc agatcaaggt ttggtaaagc gccttcaccg aatcgcggtc ccaactttgg
6541 tgatctgggg caaacaggac acccttgtgc cgtcggtgta tgccgaaagt ttcagaaagg
6601 atattgctga ctgcgaggtt gaaataattg acaactgcgg gcacataccg caggtggagc
  6661 agctcgagaa aactctggcg gcggtcgaga gattcattct tacttaatct gcag
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Seq. 3.1: Nucleotide sequence of ORF7 (GeneBank accession AF404408). Underline - bolt sequence are the pairing regions of primer SP-D74 (from 6609bp to 6637bp) and primer iSP-C47 (from 6689bp to 6697bp).

Therefore, UP-PCR protocol was performed as described in materials and methods. The first amplification was carried out by WP-D74/SP-D74 couple of primer. The electrophoresis resulting from the reaction with the higher concentration of Mg^{+} - 3mM - showed a smearing of DNA fragments, whereas the reaction performed with a 2.25mM Mg^{+} concentration revealed only one and weak band. On the other hand, no DNA fragments were detected from the reaction carried out with the lower concentration of the Mg^{+} - 1.5mM - and from both negative control: reactions performed by DNA of *B. fungorum* LMG 16225^{T} and without DNA (Fig. 3.1).

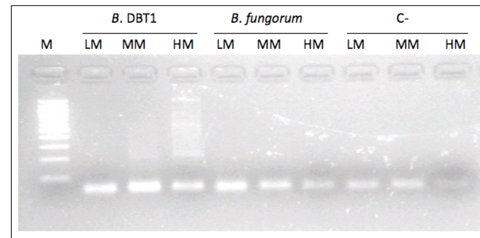


Fig. 3.1: Electrophoresis of PCR reaction performed by WP-D74/SP-D74 primers on *B*. sp. DBT1 and *B. fungorum* LMG 16225^T^. (M) 100 bp marker (BioRad). LM: [1.5mM], MM: [2.25mM], HM: [3mM] Mg^{+} concentration in the reaction buffer. C-: negative control.

Thus, 1:5 dilution obtained by WP-D74/SP-D74 amplification - 3mM Mg^{+} concentration - was re-amplified with iWP-C47/iSP-C47 primer set. A single amplicon was obtained with molecular size of about 2000 bp (data not show).

3.1.2 Cloning and functional analysis of the sequence

The band obtained by the previous reaction was purified, cloned and sequenced. The subsequently sequence analysis was performed by ORF Finder software – NCBI -, and showed the presence two open reading frame (Seq. 3.2).

CT**ATGACTTCACGAAGCCCTTTCAATATTTCGCTCAACGACAGTACTTTGAGCTTCGATGTCGATAACGGCGACACGATTCTCAGT**
GCCGCTTTGCGTAACGGTATTGGCCTCGCTTACGAGTGTAATTCTGGCGGTTGTGGAAATTGTAGCTTTGAGCTGAAAGATGGCGT
TGTCGAGGAGCTGTGGGCTGAAGCGCCGGGTCTTTCTGATGAGGCGAGAAAGAAAGGAAGATATCTCGCATGTCAGTGTGCTCCGG
CAAGCGATCTGAAGATATCTGCGAGGGTTAACAATCGGTTCCTGGCTCAATTTCCACCGATACGATTCAAGGCGCCTCTCATTGCC
AAACGCATGTTGACCAGCGATATGGCTGAATTCACGTTTCGAGTAAAAGAAGGAAGTGACTTTCTTCCGGGGCAGTTCGCCATTTT
TCGCTTGCCGGGGACGGACGGCTGCCGCGCCTACTCGATGGCGAATCTTACTGAAGACGGACAATTCTGGAGCTTCGTGATCAAGC
GCCTCCCGGCAGGAAGGACGACCAACTTCTTGTTCGAGAGGCTTGAAGTCGGCGCGGAAATTGAGTTCGACGGCCCGTACGGCTTA
TCTTATCTGCAACCGGCGATCGACCGAGACATTATCTGTATCGGAGGTGGGTCCGGTTTGGCGCCTTTACTTTCAATTACGCGAGC
GGCCATCCGGGCGCCGGAATTGAAACATAGGAAAATATTTTTCTTTTACGGTGCGCGGAGATCGGCGGATCATTGCTTGAAACATT
TTTTGGATCAGGATCCGTTAATCGCCGAGCGCGTGAAGATTATAGAAGTGGTATCCGACGAGGACCCTGAATGGCGTGGGGAGAAG
GGCCTCGTTCATGAGGTTTTAAAGCGACAGATGGCCGGAGATTTAGATAGATATGAATACTATGTCTGCGGACCCAAAGGAATGAC
TGATGCACTGCAGGTCTTCTTGTCTGTGGAAAACAAGGTGCCAACAAAGCAGATTCATTTCGATCGTTTTTATTAATCACTATTTT
GAGGCTGAAA**ATGAAGCGTCAAAAAAGACTACTCACCGTTGAAGACATCAAGGGCGCTTGGGCGATCATGCCGACGCCCGCTAAAG**
CAGGGGCCGACGATTGGCGGATGGCAGACACGGTTGATCTGGACGAGACCGCGCGCGTTGCCGAAGAGTTGGTGAAGGCCGGTGTA
GATGGAATTATTTCACTGGGTACGTTTGGCGAGTGCGCGGCCCTCACCTGGGACGAGAAGCGAGCTTTCATATCGACTGTGGTCGA
AACGGTTCGTGGTCGGATCCCCTTTTTTTGCGGTACAAGCAGCCTGAACACGCGCGAAACCATCCGGCAAACCAGAGAGGCGTTTG
ATATCGGCGCAGATGGAACGATGTTGGGCCTGCCAATGTGGCAGATTTGCGACGTGAAATCGGCTGTGCAATTTTTTAGCGATGTT
GCGGAAGCTGTGCCGGAAATGGCCATTGCGGTTTATGCGAATCCAGAGGCGTTCAAATTCGACTTTCCCCGCTCCTTTTGGGCTGG
AGTATCGGAGATTCCTCAGGTCGTGACCGCCAAATACATGGGCGTTGGCGCGCTGCACCTCGACATGATGCTGACAAAGCGCCGTA
TTCGCTTTTTGCCGATCGACATGGATTATGTGGCCGCTGCGCGGATTGATCCGGACTTTATCACTGGATTCTGGACCAGCGGAACG
AATTTAGGGCCTGCGCCGGCGATTCATTTGAGAGATGAAGTGTTAAAGGCGAAAAAGACCGGAGACTGGTCGAAAGCCCAGGAACT
GCAGAACGCGATTCTTGCTACTGCCCAAACTTTGTTCCCCAATGGATCTTTTAAGGAATTTTCGACTTACAACGTCCAGCTTGAAA
AGGCACGACTCAATGCTGGTGGCTGGATGAATGCAGGTCCCGCCCGGCCGCCCTATCGCTTCGATTCTGTTCCTGAAGCCTATCTC
GAAGGGGCGCGTATATCCGGAAGGAAGTGGGCAGAACTTCACGAGAAGTTAAGTCAGAATAGGTAAATTTGATTTTATGAGGACTG
CAGATGTTGTCGCGTCCGTAGCGCGATGAGACCGGAAATCCGCAGCTACCTGGGTTTCGTCCGTCTGTTTTGCAATGTAACTTCCG
GCGCGAGTCAAGCTAGTTGTCGTCTCGGTCATGCTGCCAATGGCTCTATATCGGTCGAGCGCGTATACGTTTTGACGA

Seq. 3.2: Nucleotide sequence identified by UP-PCR. In bolt is represented the sequence of the first ORF (*dbtAa;* 3 – 1022 bp), whereas in bolt-underline is represented the sequence of the second ORF (*dbtE:* 1043 – 2044 nt).

Based on nucleotide and amino acid sequence similarities, putative gene identifications were assigned to the resulting sequences. The information obtained from sequence data indicated the occurrence of two ORFs corresponding to genes encoding for enzymes involved in Kodama pathway. Both ORFs initiated with the expected ATG start codon and a putative ribosomal binding site preceded each start codon.

dbtAa encoded for DbtAa, the ferredoxin reductase component of initial dioxygenase. DbtAa amino acid sequence showed significant but moderate sequence identity to the subunits of known ferredoxin reductase component. It manifested similarity - spanning from 48.3% to 43.3% - to different class of isoenzymes: Phn, Thn, Arh and Tou (Fig. 3.2). The ferredoxin reductase component of initial PAH dioxygenase was one of the sequences missing in order to complete the enzymatic set of Kodama pathway in strain DBT1 (Di Gregorio *et al.,* 2004). The role of ferredoxin reductase is to initiate a single two-electron transfer from NAD(P)H to NAD in initial PAH dioxygenase multi-component enzyme (ISP; Habe and Omori; 2003).

dbtE encoded for DbtE, a hydratase-aldolase enzyme. DbtE manifested significant sequence identity to hydratase-aldolase enzyme of *Burkholderia* RP007, *Alcaligenes faecalis, Sphingobium xenophagum* and *Novosphingobium aromaticivorans* - 63.9%, 57.6%, 60.3% and 60.0% respectively - (Fig. 3.3). It was the second sequence missing in order to complete the enzymatic set of Kodama pathway in strain DBT1 (Di Gregorio *et al.,* 2004). It catalyzes the last step of Kodama pathway: from trans-HTOB to HFBT (Kodama *et al.,* 1970; 1973).

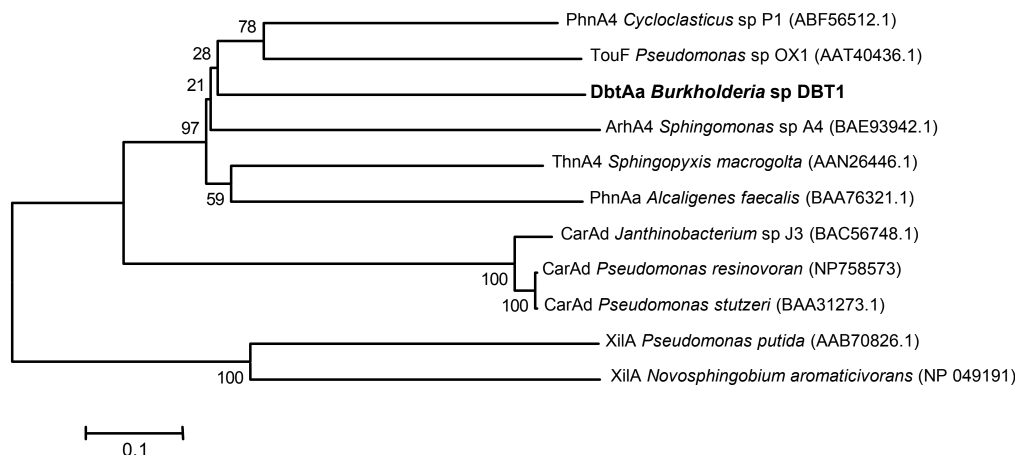


Fig. 3.2: Phylogenetic neighbour-joining tree obtained with ferredoxin reductase protein sequence (DbtAa) of strain DBT1 among ferredoxin reductase sequences of related bacterial strains. Numbers at nodes indicate levels of bootstrap support based on 500 resamplings. The scale represents the average numbers of nucleotide substitutions per site.

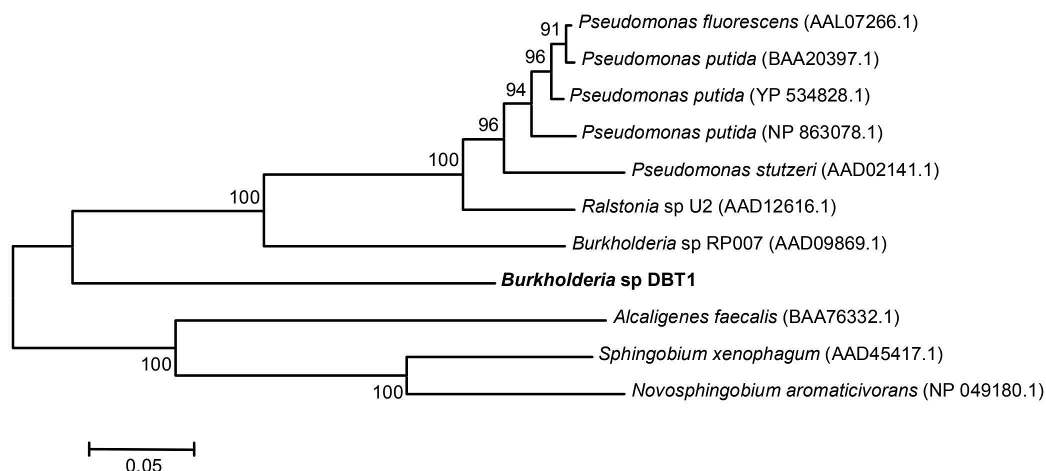


Fig. 3.3: Phylogenetic neighbour-joining tree obtained with hydratase-aldolase protein sequence (DbtE) of strain DBT1 and hydratase-aldolase protein sequences of related bacterial strains. Numbers at nodes indicate levels of bootstrap support based on 500 resamplings. The scale represents the average numbers of nucleotide substitutions per site.

Aimed to have a better overview of the functional and taxonomic collocation of initial PAH dioxygenase multi-component enzyme of strain DBT1, a multiple alignment and evolutionary distances were calculated for all the ISP subunits previously identified (Di Gregorio *et al*., 2004). The results showed that DbtAb, DbtAc and DbtAd formed a well-supported clade with Phn class of isoenzymes (Fig. 3.4; 3.5; 3.6), mainly for DbtAc and DbtAd. The phylogenies deduced from the three sequences were concordant to have a high similarity to Phn class of isoenzymes; otherwise, DbtAa showed similar identity to enzymes whose are involved towards different PAHs (Fig. 3.2). A possible explanation of these results could be the different role of ISP subunits. Subunits α and ß are the catalytic subunits of ISP and play an essential role in substrate recognition (Habe and Omori; 2003). Thus, amino acid sequence is highly conserved among isofunctional enzymes. Otherwise, ferredoxin and ferredoxin reductase subunits can share similar identity even among sequences derived from ISP implicated in different PAHs degradation, for the reason that are not involved in the recognition of substrate.

Eventually, the ISP of strain DBT1 shows high identity to ISP involved in degradation of phenanthrene. DBT and phenanthrene share similar chemical structure, consisting in tree aromatic rings. Thus, the activity of ISP towards both molecules is probable and in agreement with the literature (van Herwijnen *et al*., 2003).

100
ThnA3 *Rhodococcus* sp TFB (ACL31217.1)
77
EtbAc *Rhodococcus jostii* sp RHA1 (YP708496)
14
BphA3 *Novosphingobium aromaticivorans* (NP049211.1)
98
PhnA3 *Cycloclasticus* sp A5 (BAC81547.1)
DbtAb *Burkholderia* sp DBT1 (AF404408 4)
14
PhnAb *Alcaligenes faecalis* (BAA76320.1)
ThnA3 *Sphingopyxis macrogoltabida* (AAD52963.2)
58
ArhA3 *Sphingomonas* sp A4 (BAE93940.1)
100
CarAc *Pseudomonas stutzeri* (BAA31271.1)
53
CarAc *Pseudomonas* sp XLDN4-9 (AAY56343.1)
BphA3 *Marinobacter algicola* sp (ZP01894047.1)
100
HcaC *Shigella flexneri* (YP689991.1)
28
HcaC *Shigella boydii* sp Sb227 (YP408936.1)
NahAb *Pseudomonas stutzeri* (AAD02135.1)
89
NahAb *Pseudomonas putida* (YP 534821.1)
100
99
PahAb *Pseudomonas putida* (BAA20390.1)
0,1

Fig. 3.4: Phylogenetic neighbour-joining tree obtained with ferredoxin protein sequence (DbtAb) of strain DBT1 with ferredoxin protein sequences of related bacterial strains. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The scale represents the average numbers of nucleotide substitutions per site.

Fig. 3.5

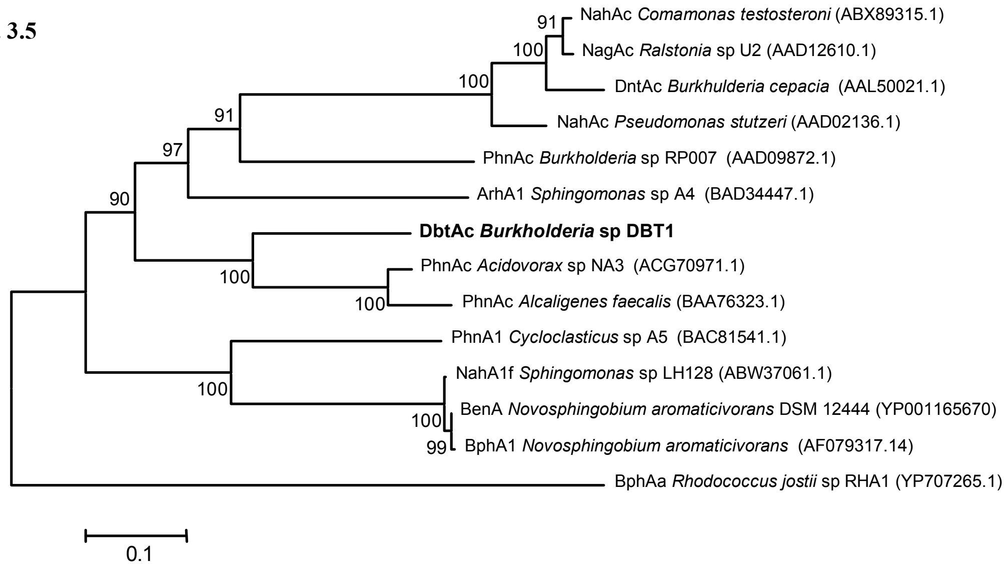


Fig. 3.6

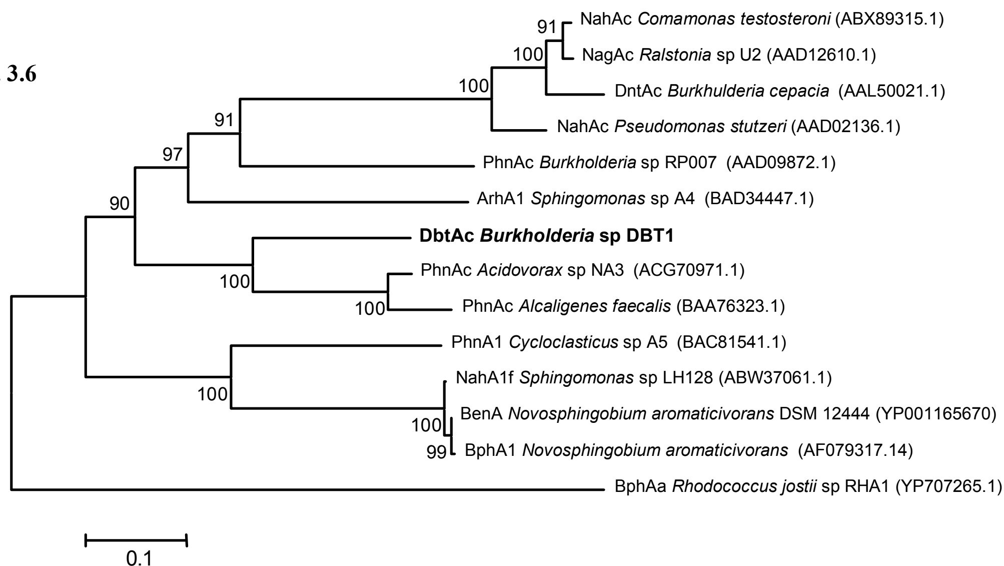


Fig. 3.5 and 3.6: Phylogenetic neighbour-joining tree obtained with beta (Fig. 3.5) and alfa (Fig. 3.6) subunits protein sequence of strain DBT1 and beta and alfa subunits protein sequences of related bacterial strains. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The scale represents the average numbers of nucleotide substitutions per site.

3.1.3 Targeted gene walking control

The effective concatenation of nucleotide sequence recovered by UP-PCR and p51 genetic fragment identified by Di Gregorio *et al.,* (2004) was verified by PCR. The 5' primer (RT-Gw) was designed in order to pair on p51 sequence, whereas the 3'primer (SP-D74) was constructed aimed to recognize the sequence previously identified. The presence of a single band at expected size confirmed the concatenation of the two sequences analyzed (data not show). Therefore, the results so far achieved can establish that the entire set of enzyme involved in Kodama pathway is detected in *B.* sp. DBT1 and are harbored by p51 and pH1A operons.

3.2 Taxonomic analysis

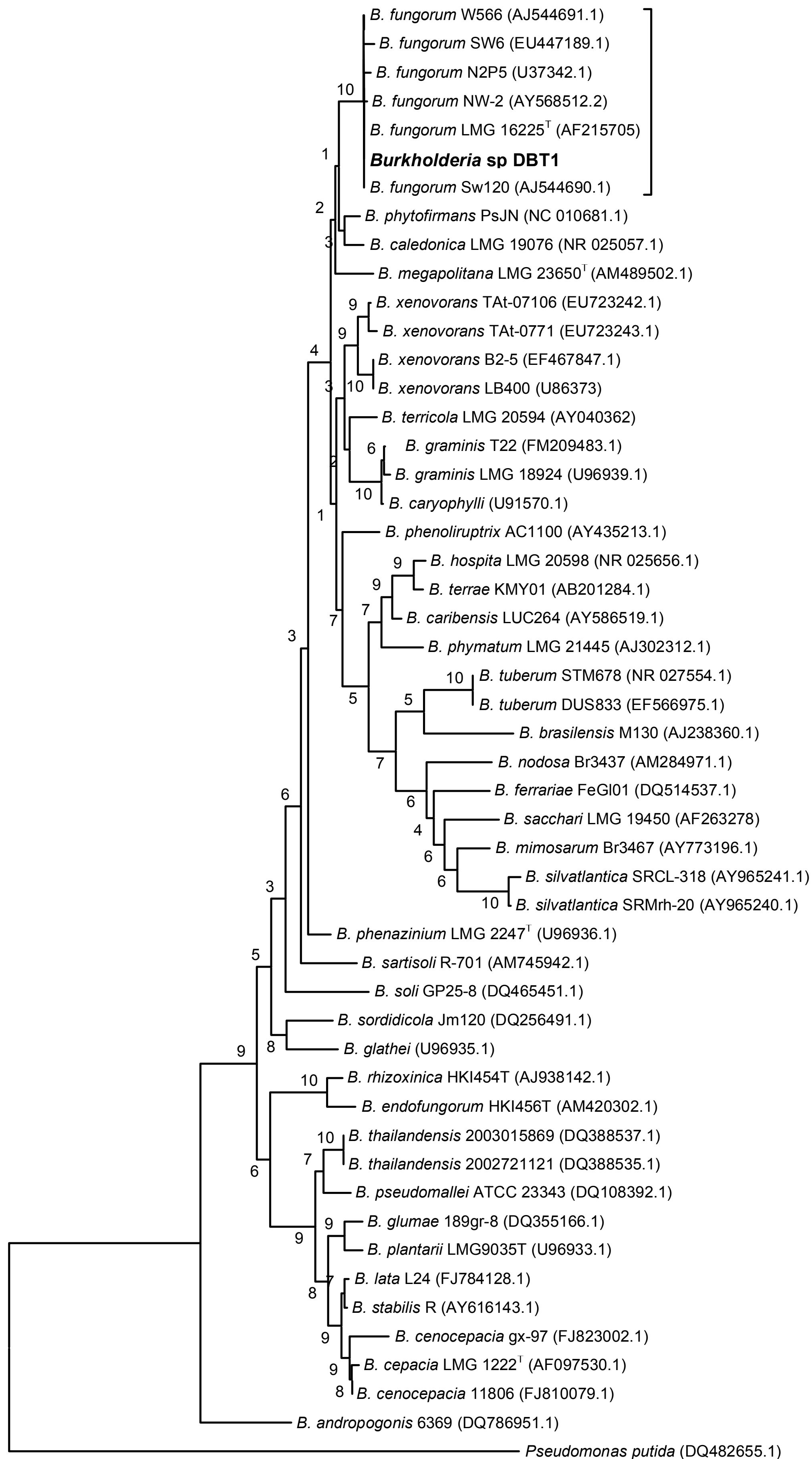
Taxonomic status of *Burkholderia* sp. DBT1 has never been resolved. Therefore, a study by polyphasic approach was employed in order to investigate which species belongs DBT1 strain. Moreover, the knowledge of taxonomic status of DBT1 is essential for its implication and its use in bioremediation protocol. In fact, it is important to remember and underline that some species of *Burkholderia*, cause food poisoning (Zhao *et al*., 1995), or be plant and animal pathogens (Brett *et al*., 1998). For instance, some Bcc strains cause life-threatening infections in humans such as cystic fibrosis (Govan *et al*., 1996). Thus, there are concerns about the use of potentially pathogenic strains of *Burkholderia* for biotechnological applications such as bioremediation of polluted sites (Vandamme *et al*., 1997; Parke and Gurian-Sherman, 2001). Therefore, for a safe exploitation of *Burkholderia* sp. DBT1 in environmental biotechnology, an accurate taxonomic study for the probative exclusion of this strain from *Burkholderia* dangerous species is an absolute pre-requisite.

3.2.1 *16S* rDNA sequence analysis

A 1493 bp fragment of DBT1 16S rDNA was sequenced and a nucleotide BLAST – NCBI - analysis was performed. Thereafter, multiple alignment and evolutionary distances were calculated with 16S rDNAs of related, in order to construct a phylogenetic tree based on the neighbour-joining algorithm (Fig. 3.7). In this way, a closing 16S rDNA similarity - spanning from 99.5% to 100% - of DBT1 to different strains of *Burkholderia fungorum* has been demonstrated. Strain N2P5 was isolated from an IPA contaminated soil (Mueller *et al*., 1997; Coenye *et al*., 2001) and might have similar useful xenobiotic compound-degrading properties to DBT1. Otherwise, strains SW6 and NW-2 were identified within structure involved in space exploration and no more information are available so far (Newcombe *et al.*, unpub. data; La Duc and Venkateswaran, unpub. data). On the other hand, concerns exist about strains W566 and Sw 120, owing to these strains were identified in infections of the central nervous system of a pig and a deer (Scholz and Vandamme, unpub. data).

B. phytofirmas strain PsJN is then resulted the second most related bacterial species with a 98.7% similarity to DBT1. Furthermore, DBT1 has shown good similarity of its 16S rRNA gene sequence even to *B. caledonica* LMG 19076 (98.4%), *B. megapolitana* (98.2%) and *B. xenovorans* (97,8%) respectively, as well as a still significant similarity - ranging from 97.5% to 97.1% - to different strains of *B. terricola, B. graminis* and *B. caryophylli.* On the other hand, the similarity of DBT1 to other *Burkholderia* species is resulted lower than 97.0%.

Next page. Fig. 3.7: Phylogenetic neighbour-joining tree obtained with *16S* gene sequence of strain DBT1 and *16S* sequence of related *Burkholderia* strains. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The scale represents the average numbers of nucleotide substitutions per site.


B. fungorum W566 (AJ544691.1)
B. fungorum SW6 (EU447189.1)
B. fungorum N2P5 (U37342.1)
B. fungorum NW-2 (AY568512.2)
B. fungorum LMG 16225T (AF215705)
Burkholderia sp DBT1
B. fungorum Sw120 (AJ544690.1)
B. phytofirmans PsJN (NC 010681.1)
B. caledonica LMG 19076 (NR 025057.1)
B. megapolitana LMG 23650T (AM489502.1)
B. xenovorans TAt-07106 (EU723242.1)
B. xenovorans TAt-0771 (EU723243.1)
B. xenovorans B2-5 (EF467847.1)
B. xenovorans LB400 (U86373)
B. terricola LMG 20594 (AY040362)
B. graminis T22 (FM209483.1)
B. graminis LMG 18924 (U96939.1)
B. caryophylli (U91570.1)
B. phenoliruptrix AC1100 (AY435213.1)
B. hospita LMG 20598 (NR 025656.1)
B. terrae KMY01 (AB201284.1)
B. caribensis LUC264 (AY586519.1)
B. phymatum LMG 21445 (AJ302312.1)
B. tuberum STM678 (NR 027554.1)
B. tuberum DUS833 (EF566975.1)
B. brasilensis M130 (AJ238360.1)
B. nodosa Br3437 (AM284971.1)
B. ferrariae FeGl01 (DQ514537.1)
B. sacchari LMG 19450 (AF263278)
B. mimosarum Br3467 (AY773196.1)
B. silvatlantica SRCL-318 (AY965241.1)
B. silvatlantica SRMrh-20 (AY965240.1)
B. phenazinium LMG 2247T (U96936.1)
B. sartisoli R-701 (AM745942.1)
B. soli GP25-8 (DQ465451.1)
B. sordidicola Jm120 (DQ256491.1)
B. glathei (U96935.1)
B. rhizoxinica HKI454T (AJ938142.1)
B. endofungorum HKI456T (AM420302.1)
B. thailandensis 2003015869 (DQ388537.1)
B. thailandensis 2002721121 (DQ388535.1)
B. pseudomallei ATCC 23343 (DQ108392.1)
B. glumae 189gr-8 (DQ355166.1)
B. plantarii LMG9035T (U96933.1)
B. lata L24 (FJ784128.1)
B. stabilis R (AY616143.1)
B. cenocepacia gx-97 (FJ823002.1)
B. cepacia LMG 1222T (AF097530.1)
B. cenocepacia 11806 (FJ810079.1)
B. andropogonis 6369 (DQ786951.1)
Pseudomonas putida (DQ482655.1)
0.02

3.2.2 *recA* and *gyrB* sequence analysis

Although the analysis of the 16S rRNA gene sequence represents a basic step in the taxonomic characterization of most bacterial genera (Vandamme *et al.,* 1996), its usefulness in comparing closely related species may result a non-resolving tool (Ash *et al.,* 1991; Fox *et al.,* 1992).

In order to overcome this limit, the *recA* gene sequence analysis has been introduced in taxonomic studies, demonstrating to be decisive even in phylogenetic comparisons within the *Burkholderia* genus (Vermis *et al.,* 2002; Payne *et al.,* 2005). In the present study, 869 pb portion of the *recA* gene sequence, from *Burkholderia* sp. DBT1 strain was amplified by PCR and subsequently cloned and sequenced. Related *recA* sequences were aligned and a phylogenetic tree was constructed (Fig. 3.8). While the *recA* gene sequence similarity of strain DBT1 to *B. fungorum* strains ranges from 99.7% to 99.1%, the similarity for the same sequence to *B. phytofirmas* PsJN, *B. xenovorans* LB400, *B. caledonica* and *B. graminis* drops to 95.5%, 93.5%, 92.0% and 91.4% respectively. Thus, DBT1 was more closely related phylogenetically to *fungorum* strains Sw 120, W566, LMG 16307, LMG 16225^T, DSM 15090 and LMG 15511. These strains were identified - except for LMG 16225^T – in infections of the central nervous system of a pig and a deer as previously reported for 16S analysis (Scholz and Vandamme, unpub. data). However, it is important to underline that NCBI database contain only *recA* sequences of *fungorum* strains obtained by Scholz and Vandamme reported in unpublished work, therefore it is not possible compare strain DBT1 to other *fungorum* strains.

Recently, species-specific primers, namely FunF and FunR, have been designed for *recA*-based PCR assays targeted for *B. fungorum* (Chan *et al.,* 2003). Therefore, in order to circumstantially ascribe *Burkholderia* sp. DBT1 to the *fungorum* species, a PCR-based analysis using FunF and FunR primers on DBT1 DNA was performed. PCR assays carried out with genomic DNA obtained from *B. cepacia* LMG 1222^T, *B. caledonica* LMG 19076 and *B. graminis* LMG 18924 were used as negative controls, while the test carried out with DNA from *B. fungorum* LMG 16225^T was taken as a positive control. An amplicon of 330 bp was obtained through PCR analysis of DNAs from either *B. fungorum* LMG 16225^T and strain DBT1. Afterwards, to confirm the identity of the fragments, the amplicons were purified and sequenced, resulting the proper sequence of *recA* gene. On the other hand, no amplification products were generated with DNA from the other *Burkholderia* strains tested (Fig. 3.9).

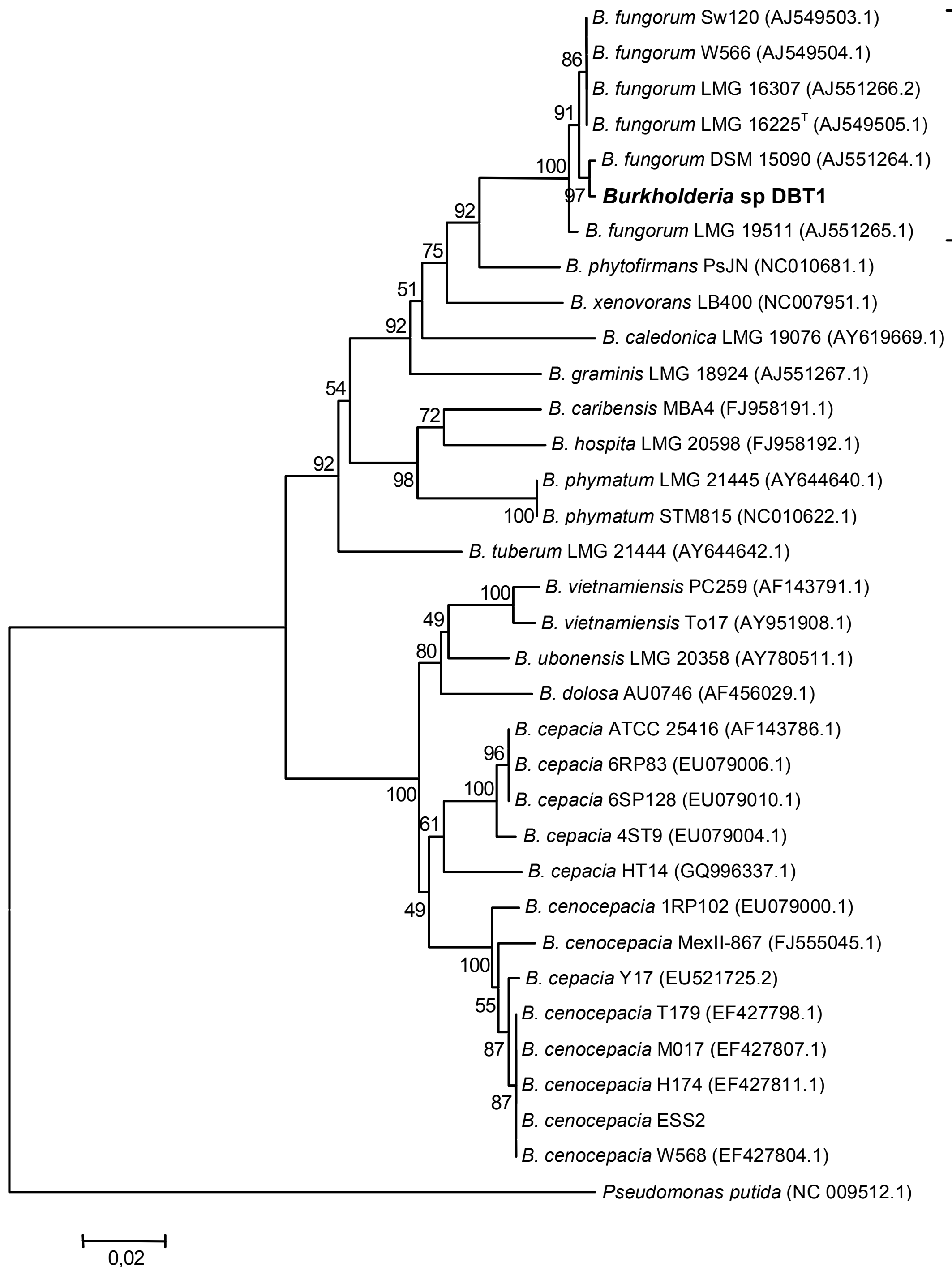


Fig. 3.8: Phylogenetic neighbour-joining tree obtained with *recA* gene sequence of strain DBT1 and *recA* sequences of related *Burkholderia* strains. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The scale represents the average numbers of nucleotide substitutions per site.

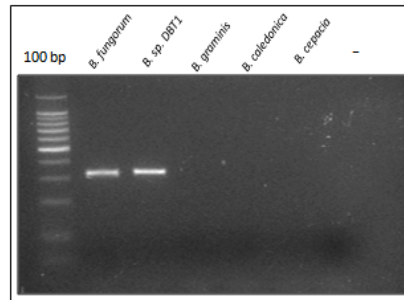


Fig 3.9: *RecA* PCR-amplification assay using species-specific primers for *B. fungorum*. (100 bp): 100bp molecolar marker (BioLabs)

Moreover, a 432 bp portion of *gyrB* gene was amplified by PCR starting from the genomic DNAs of *B. cepacia* LMG 1222^{T}, *B. fungorum* LMG 16225^{T} and *B.* DBT1. The amplicons were then cloned and sequenced. In this case, the degree of similarity of DBT1 to *B. fungorum* and *B. cepacia* resulted 98.2% and 86.5% respectively. The *gyrB* sequence of DBT1 was compared through the available DNA sequence databases by using the BLAST interface – NCBI -, giving similarities as follows: 95.0% to *B. fungorum* CIP 107096 (GeneBank accession EU024205.1), 94.0% to *B. xenovorans* LB 400 (GeneBank accession CP000270), 93.7% to *B. phytofirmans* PsJN (GeneBank accession CP001052), and 91.1% to *B. graminis* CIP 106649^{T} (GeneBank accession EU024212). Similarity to other species/strains of *Burkholderia* resulted lower than 90%.

Strain DBT1, within the phylogenetic trees based on the comparison of both 16S rRNA and *recA* gene sequences, forms a well-substantiated clade with *B. fungorum* strains. Moreover, also *gyrB* gene sequence similarity scoring indicates that DBT1 closely fits strains of the species *fungorum*, although databases are poor in bacterial *gyrB* sequence information. Thus, the results suggest that *B.* DBT1 can be ascribed to *B. fungorum* species. Nevertheless, the present study confirms how the 16S rRNA gene sequence characterization, widely used either in taxonomic or ecological investigations, suffers some intrinsic limitations. 16S rDNA sequences evolve so slowly that very closely related strains cannot always be distinguished each other (Ait Tayeb *et al*., 2008). For instance, strain DBT1 and *B. fungorum* LMG 16225^{T} show a similarity percentage of 100%. On the other hand, DBT1 and type strain of the species *fungorum*, were separated on the basis of a 98.4% similarity for the *gyrB* gene sequence- and a 99.5% similarity for the *recA* gene sequence, both evolving much faster than the 16S rDNA sequence, confirming as protein-coding gene are a powerful method for species identification.

3.2.3 DNA-DNA hybridization

Groups of bacteria sharing almost identical 16s rDNA sequences have been identified whose DNAs however hybridize at an extent significantly lower than 70%. This therefore indicates that these organisms represent distinct species (Fox *et al.,* 1992). Thus, to conclusively clarify the taxonomic affiliation of strain DBT1, DNA-DNA hybridization was performed against *B. fungorum* LMG 16225^{T}. A 78,2 ± 2,9% complementation demonstrated that *B.* DBT1 belongs to the species *fungorum* according to the definition of bacterial species by Wayne *et al.,* (1987). On this basis, *B.* DBT1 can be ascribed to *B. fungorum* species.

Since the original report, *B. fungorum* was identified in a wide range of environment such as soil, plant-associated samples, in infections of the central nervous system of a pig and a deer (Scholz and Vandamme, unpub. data), and in the respiratory secretions of people with cystic fibrosis (Coenye *et al.,* 2001; 2002). However, clinical data are not available from these patients, therefore the clinical significance of isolation of *B. fungorum* is not determined.

Only recently was reported the first description of bacteremia and invasive infection due to *B. fungorum* manifested as a soft tissue infection of the leg (Gerrits *eta al.,* 2005). Thus, further study of potential pathogenicity of DBT1 associated to production of toxin towards human and animal cell models is desirable before its intentional release into the environment for biotechnological applications.

3.3 Phenotypical and genotypical analysis

16S rRNA, *recA* and *gyrB* gene sequence analysis with support by DNA-DNA hybridization, indicate that *B.* sp. DBT1 can be consider a member of *fungorum* species. Hoverer, strains among the same species can own genetically unique features, but different phenotypic and biochemical traits such as members of *Burkholderia cepacia* complex (Bcc) or *Pseudomonas stutzeri* (Speert, 2002; Palleroni and Doudoroff, 1972; Wolterink *et al.,* 2002; Cladera *et al.,* 2006). Therefore, aimed to extend the characterization of strain DBT1, a further phenotypical analysis was performed.

3.3.1 API 20 NE test

API 20 NE tests procedure was performed on *B.* sp. DBT1. Results for strains *B. fungorum* LMG 16225^{T} and *B. cepacia* LMG 1222^{T} were also obtained for comparison as representative of the closely related phylogenetically to strain DBT1, and a representative strain of the Bcc, whose members are often responsible for opportunistic human infections (Govan *et al.,* 1993). Moreover, these two species were chosen because it has previously been shown that *B. fungorum* isolates can easily be misidentified as *B. cepacia* complex organisms (Coenye *et al.,* 2001a; 2002; Gerrits *et al.,* 2005). Results of gelatin hydrolysis and ß-galactosidase are not shown owing to weak positive reactions for one or more strains. Strains DBT1, LMG 16225^{T} and LMG 1222^{T} utilized D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, gluconate, malate, citrate and phenylacetate. Neither strain was positive for indole production, arginine dihydrolase, glucose acidification urease activity and ability to utilized maltose. Beside, strain DBT1 gave the same results as the type strains of *B. fungorum* as many of the tests shown in table 3.1 as it did with LMG 1222^{T}. Thus, the comparative analysis with the API 20 NE system revealed that little differences exist among strain DBT1, LMG 16225^{T} and LMG 1222^{T} reliving that biochemical test were not useful for distinguishing among these strains. The results for LMG 16225^{T} and LMG 1222^{T} are consistent with prior studies (Fain and Haddock, 2001; Coenye *et al.,* 2001). Biochemical test obtained by API 20NE test has showed that *fungorum* strain involved in bacterial septicemia and invasive infection utilized caprate as carbon source (Gerrits *et al.,* 2005), conversely to strain DBT1. Obviously, it would be unwise any speculation about this little metabolic difference.

Tests	**B. *cepacia***	**B. *fungorum***	**B. DBT1**
Nitrate reduction	-[a]	+	+
Aesculin hydrolysis	+[b]	-	+
Caprate assimilation	+	-	-
Adipate assimilation	+	-	+

([a]) negative

([b]) positive

Tab. 3.1: API 20 NE carbon source utilization and biochemical tests that differentiate strain DBT1 from *Burkholderia cepacia* LMG 1222^{T} and LMG 16225^{T}.

3.3.2 Genomic organization

PAHs catabolic genes, which encode different aromatic hydrocarbon degradation enzymes, are frequently located on plasmids, although degradative genes can be located on either chromosome or plasmid (Cho and Kim, 2001; Ma *et al.*, 2006; Sakai *et al.*, 2004). Thus, genomic organization of strain DBT1 was investigated by a protocol useful for extraction of chromosome and mega-plasmid, followed by agarose electrophoresis run (Kado and Liu, 1981). Results for strain *B. fungorum* LMG 16225^{T} was also obtained for comparison as representative of the closely related phylogenetically strain to DBT1, but unable to degrade PAHs (see below).

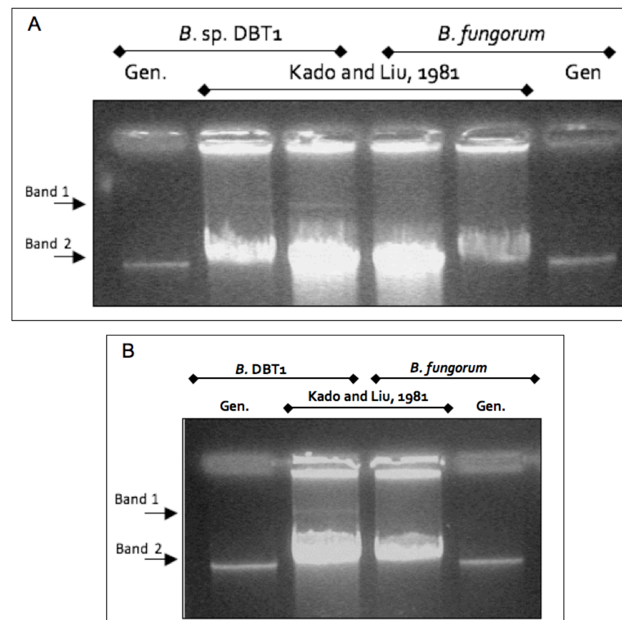


Fig. 3.10: Comparision between replicative units of *B*. sp. DBT1 and *B. fungorum* LMG 16225^{T}. (Kado and Liu, 1981) extraction performed by plasmids and mega-plasmid extraction protocol; (Gen.) standard genomic extraction protocol. (A) first replica and; (B) second replica of experiment.

The electrophoresis pattern showed the presence of two bands (Band 1 and 2) in *B*. sp. DBT1. On the other hand, *B. fungorum* LMG 16225^{T} does not shows the band 1 (Fig. 3.10). This result suggest the presence of a additional replicative unit in strain DBT1.

Characterization so far obtained, have demostrated a closely phylogenetic relationships, but different genomic organization between strain DBT1 and *B. fungoum* LMG 16225^{T}. On the other hand, these phenotypical differences are not enough to split strain DBT1 into new species.

3.3.3 Plasmid-Curing

Previous experiment has demostred a different genomic organization between *B*. sp. DBT1 and *B. fungorum* LMG 16225^{T}. In detail, genomic electrophoretic patterns showed two bands in *B*. sp. DBT1 (band 1 and 2) and a single band in LMG 16225^{T} (Band 2; Fig 3.10). However, the data are not sufficient to hypothesize if the band 1 is due to the presence of a plasmid or a chromosome, and we cannot assume if the metabolic capability of DBT1 to degrade PAHs is due to the additional replicative unit. Plasmids can be eliminated from bacterial strain by grown on rich

medium in the presence of sub-inhibitory concentrations of non-mutagenic compounds or by elevated growth temperature – plasmid-curing agent -. In this condition, bacterial strain is triggered to eliminate every not-essential structure for its replication, such as plasmids. In fact, plasmids are not essential for bacteria, rather provide a selective advantage under a given environmental state such as PAHs contaminated soils. On the other hand, chromosomes harbor essential genes for the microorganisms and will be maintained even after treatment by plasmid-curing agent.

SDS plasmid-curing agent: Initially, different concentrations of SDS were tested. *Burkholderia* sp. DBT1 was weakly able to grow on both 0.025% and 0.05% SDS concentration, although the growth on this latter proceeds less efficiently. On the other hand, no growth was attested with 0.1% concentration and no inhibitory effect was observed with concentrations of SDS lower than 0.01%. Thus, different concentration ranging between 0.025% and 0.05% were tested in order to find out the optimum sub-inhibitory concentrations. The minimal growth of DBT1 was attested around 0.035% - 0.04% of SDS. Thus, every two days the culture was refreshed in a new medium and serial dilutions were plated on YMA. Therefore, 60 individual colonies were streaked on YMA supplied with DBT powder on the bottom of the plate. No white colonies were observed even after 20 days from the beginning of the experiment.

Elevated temperature plasmid-curing agent: Different growth temperatures were tested on DBT1 strain, and the sub-inhibitory temperature was attested at 38.5°C. Every four days the culture was refreshed in a new medium and serial dilutions were plated on YMA. Therefore, 60 individual colonies were streaked on YMA supplied with DBT powder on the bottom of the plate. No white colonies were observed even after 20 days from the beginning of the experiment.

If DBT1 lost the ability to become red in presence of DBT, it means that the gene involved in PAHs degradation – p51 and pH1A operons - were harbored in a plasmid lost during the curing treatment. On the other hand, both protocols performed in order to eliminate possible plasmids present in strain DBT1 have been ineffective. This result could suggest the presence of a additional chromosome in strain DBT1 comparing to strain LMG 16225^T, but this statement may be only speculative. Therefore, further analysis would be performed in order to verify this hypothesis.

3.4 Study of PAHs degrading activity

Since normally several organic pollutants contribute together to the contamination at different sites (Liu *et al.*, 2008), microorganisms able to concomitantly degrade a wide range of substrates might play an important role in the bio-reclamation of polluted areas. *Burkholderia* sp. DBT1 is a bacterial strain isolated from an oil refinery wastewater which can degrade DBT nearly completely through the Kodama pathway within three days (Di Gregorio *et al.*, 2004). This particular features encouraged studies aimed at clarifying the possible role of *Burkholderia* DBT1 in the degradation of PAHs other than condensed thiophenes frequently occurring in oil-contaminated sites. Thus, the growth of strain DBT1 was tested in minimal medium supplied with different PAHs.

3.4.1 Growth test in presence of different organic compounds as sole source of carbon and energy

Growth test in presence of different organic compounds as sole source of carbon and energy were performed in presence of phenanthrene, dibenzothiophene, naphthalene and fluorene. Results for strains *B. fungorum* LMG 16225^T and *B. cepacia* LMG 1222^T were also obtained for comparison.

Results obtained with growth tests in presence of different PAHs showed that *Burkholderia* sp. DBT1 was able to grow in presence of phenanthrene and, although less efficiently, on DBT as sole source of carbon and energy (Fig. 3.11). Moreover, DBT1 was also able to utilize naphthalene and fluorene, even if only after an induction period of about tree days in presence of phenanthrene (Fig. 3.11) or DBT (data not shown). These evidences indicate that enzymes for the degradation of naphthalene and fluorene are induced by phenanthrene and DBT. This suggests that these compounds, particularly phenathrene, are the main substrates for *Burkholderia* sp. DBT1. Moreover, these results are agree with to the high similirity of ISP protein seqence of DBT1 to isoenzymes involved in phenanthtrene degradation (Fig. 3.4; 3.5; 3.6). Therefore, the results so far achieved suggest that the strain DBT1 presents a versatile metabolism towards PAHs. This behavior is quite interesting for the possible exploitation of *Burkholderia* DBT1 in bioremediation protocols of PAHs-contaminated sites.

On the other hand, results showed that *Burkholderia fungorum* LMG 16225^T and *Burkholderia cepacia* LMG 1222^T were not able to grow in presence of phenanthrene, DBT, naphthalene and fluorene as sole source of carbon and energy (data not shown).

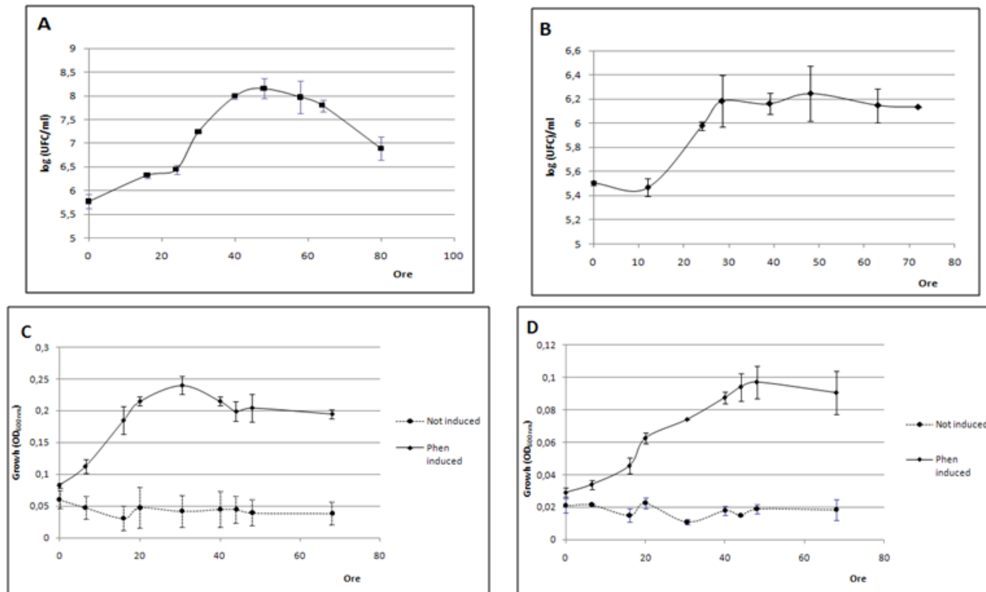


Fig. 3.11: Growth of *Burkholderia* sp. DBT1 in DM supplemented with (A), phenathrene, (B), DBT. (C), fluorene, (D), naphthalene. Concentrations of PAHs were provided at 100 ppm. (Phen induced), *Burkholderia* sp. DBT1 grows in DM after an induction period of about tree days in presence of phenanthrene. (Not induced), *Burkholderia* sp. DBT1 grows in DM without induction.

Phenanthrene and DBT are resulted the mains substrates of *B.* sp. DBT1. Moreover, DBT1 strain was proved to degrade 2-carboxybenzaldehyde, phthalic acid and protocatechol which are common intermediates in the phenanthrene degradation pathway (Fig. 1.5; 1.6). On the other hand, catechol and salicylic acid – both intermediates within the two upper pathways of phenanthrene degradation – did not support growth when provided even at concentrations ranging from 25 ppm to 1000 ppm (Tab. 3.2). These latter growth tests were performed by using either not induced cells or pre-induced cells with phenanthtrene.

The growth tests were performed even with mutant strains *B*. sp. DBT1 mH1 and *B*. sp. DBT1 m51 (Di Gregorio *et al.,* 2004). These results will be discusses in paragraphs 3.4.3 and 3.4.4.

	B. sp. DBT1 (wt)	**B. sp. DBT1 mH1**	**B. sp. DBT1 m51**
DBT	+	-	-
Phenanthrene	+	-	-
Salicylic acid	-	-	-
Catechol	-	-	-
2-carboxybenzaldehyde	+	+	-
Phthalic acid	+	+	+
Protocatechuate	+	+	+

(wt): Wild type

Tab. 3.2: Results of test growth in DM supplied with PAHs compounds. (wt): Wild type. (+) Bacterial growth. (-) No bacterial growth. Mutant strains mH1 and m51 were previously obtained to Di Gregorio *et al.,* (2004).

3.4.2 Transformation of organic compounds by co-metabolism

Co-metabolic aerobic oxidation is the microbial breakdown of a contaminant in which the contaminant is oxidized by an enzyme produced during the metabolism of another compound. This characteristic of bacteria provides an effective positive mechanism for *in situ* bioremediation of a wide range of organic contaminants. Co-metabolic bioremediation also has the advantage of being able to degrade contaminants to trace concentrations, since the biodegrader compound is not dependent on the contaminant for carbon or energy.

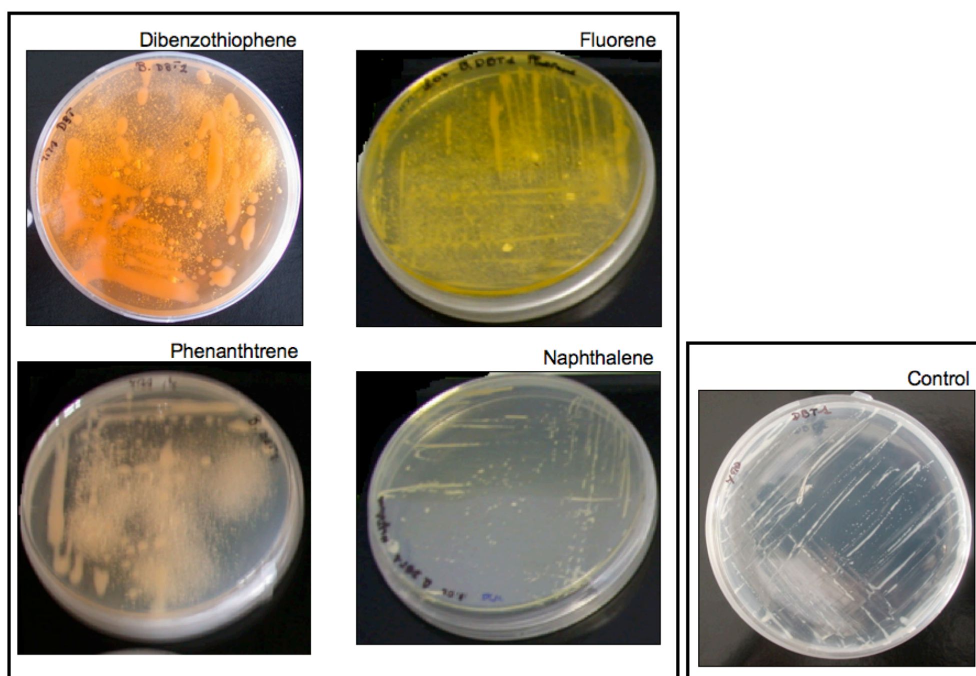


Fig. 3.12: Co-metabolism test of *B.* sp. DBT1 in presence of different organic compound. Control was strain DBT1 grown on YMB without PAHs compounds. The color change of the colony may be attributed to PAHs cleavage.

During growth of DBT1 on YMA plates with the presence of PAHs vapor, a change in color of the colonies was detected (Fig. 3.12). No attempt was made to identify the materials responsible for the color change, however this color change may be attributed to PAHs cleavage. In detail, the colonies of DBT1 became red in presence of DBT, owing to the presence of the Kodama pathway intermediate (Kodama *et al.,* 1970; 1973). In presence of fluorene, DBT1 accumulated yellow intermediates. This yellow compound is probably a *meta*-cleavage product resulting from transformation of 9-fluorenone. The fluorene degradation pathway via 9-fluorenone passes through phthalic acid and protocatechuate (Casellas *et al.,* 1997). In presence of phenanthrene the colonies of strain DBT1 produced an orange/pink pigment. The same phenotype was observed in *Alcaligenes faecalis* AFK2. This bacterial strain degrades phenanthrene by the ring-cleavage of 1-hydroxy-2-naphthoate via phthalic acid and protocatechuate (Fig. 1.5; Kiyohara *et al.,* 1982). In presence of naphthalene the colonies of DBT1 became weakly yellow. This pigmentation was originally identified in 1,2-naphthaquinone (Davies and Evans, 1963). The same phenotype of DBT1 was observed by Kiyohara and Nagao (1977) in *Pseudomonas* strain able to degrade either naphthalene or phenanthrene.

3.4.3 Study of possible involvement of the two operons in phenanthrene metabolism

Elsewhere and the present studies have shown that, in *Burkholderia* sp. DBT1 genes responsible for DBT degradation are clustered in two operons - p51 and pH1A - instead of in one as usual for the degradation of PAHs within most bacteria (Di Gregorio *et. al.,* 2004). This peculiarity has pushed to verify whether these transcriptional units are even involved in phenanthrene metabolism. Initially, the presence of specific transcripts (mRNAs) was verified during the growth of *Burkholderia* sp. DBT1 on phenanthrene as sole source of carbon and energy. RT-PCR analyses showed that both operons were strongly transcripted in presence of this compound (Fig. 3.13).

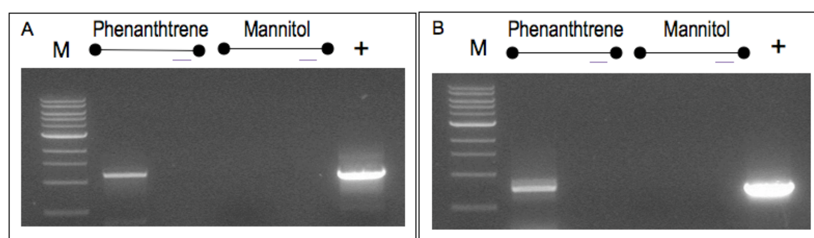


Fig. 3.13: RT-PCR analysis of the transcription of (A) p51 operon utilizing p10 and p11 sets of primers and (B) pH1A operon in DBT1 performed with p2 and p3 couple of primers. (M) 1Kb molecular marker (BioLabs). (–) It is the negative control and (+) is the positive control. The presence of specific transcripts was verified during the growth of *Burkholderia* sp. DBT1 on phenanthrene and mannitol as sole source of carbon and energy.

Moreover, the data is confirmed by previously evidences, in which two insertional mutants, m51 and mH1 of *Burkholderia* sp. DBT1, were grown in presence of different PAHs. Such experiments revealed that these mutated strains had lost the capacity to growth on phenanthrene as sole carbon and energy source (Tab. 3.2). In this way, the involvement of the operons in the abatement of phenanthrene was attested.

3.4.4 Identification of degradation pathway of phenanthrene

The results obtained by growth of strain DBT1 on minimal medium showed that phenanthrene and DBT are the main substrate among the PAHs tested. But, whereas the degradation pathway of DBT was previously identified in strain DBT1 (Di Gregorio *et al.,* 2004), data are not available for phenanthrene degradation so far. Previous growth test in minimal medium showed that DBT1 strain was proved to degrade 2-carboxybenzaldehyde, phthalic acid and protocatechol which are common intermediates in the phenanthrene degradation pathway. On the other hand, catechol and salicylic acid – both intermediates within two possible pathways of phenanthrene degradation – did not support bacterial growth (Tab. 3.2). The results obtained with strain DBT1 has suggested that this two possible phenanthrene degradation pathways via catechol and salicylic acid are not likely. On the other hand, other two pathways have been identified via phthalic acid and protocatechuate. In the first, phenanthrene is degraded via naphthalene-1,2-diol through 2-carboxycinnamic acid, phthalic acid and protocatechuic acid. In another pathway, 1-hydroxy-2-naphthoic acid undergoes to ring-cleavage and further metabolized via 2-carboxybenzaldehyde, phthalic acid and protocatechuate (Fig. 1.5, 1.6; Seo *et al.,* 2007; Pinyakong *at al.,* 2000).

Previous results showed that both mutants were also capable to degrade phthalic acid and protocatechol. On the other hand, only mH1A was proved to grow in presence of 2-carboxybenzaldehyde, while m51 strain whose mutation is inserted in PAH extradiol dioxygenase was not. It is worth noting that, 2-carboxybenzaldehyde is converted in phthalic acid by a 2-carboxybenzaldehyde dehydrogenase through a phenathrene lower pathway (Kiyohara *et al.,* 1982). Threfore, in the case of m51 strain, the decresase in biomass production when 2-

carboxybenzaldehyde was added to the growth medium might have a two explanation: (i) the PAH extradiol dioxygenase is involved downstream of 2-carboxybenzaldehyde in a not phenanthrene catabolic pathway or (ii) m51 mutant has lost 2-carboxybenzaldehyde dehydrogenase activity during the transposon mutagenesis protocol.

3.4.5 2-carboxybenzaldehyde dehydrogenase assay

A enzymatic assay was performed aimed to verify the 2-carboxybenzaldehyde dehydrogenase activity in p51 mutant. However, the protocol needs a proteins cell-free extraction from p51 strain induced by 2-carboxybenzaldehyde, but this strain does not grow on minimal medium supplied with this compound. Therefore, preliminary tests were performed on strain DBT1 in order to verify: (i) the releability of assay, (ii) the induction of 2-carboxybenzaldehyde dehydrogenase by co-metabolims.

Thus, under standard conditions, the activity of 2-carboxybenzaldehyde dehydrogenase was determined spectrophotometrically by measuring the formation of NADH at 340 nm. Results showed 2-carboxybenzaldehyde dehydrogenase activity in strain DBT1 either on DM or YMB supplied with 2-carboxybenzaldehyde. This observation allows to perform the enzymatic assay on m51 mutant in condition of co-metabolism. The cell-free extracts of m51 bacterial cultures grown in presence of YMB supplied with 2-carboxybenzaldehyde showed a high activity of this enzyme (Fig. 3.14).

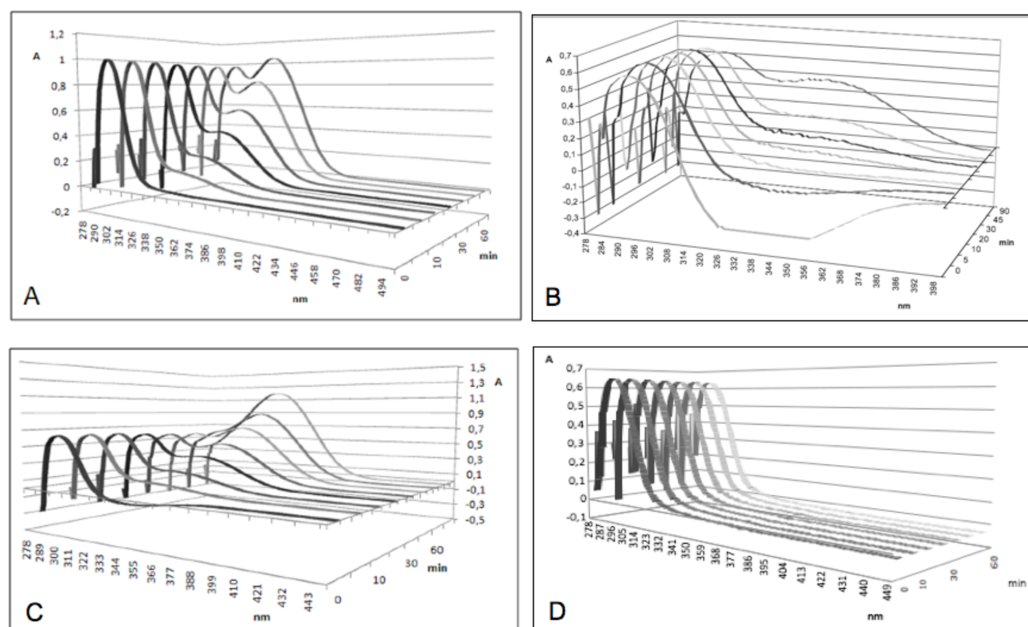


Fig. 3.14: UV-spectra chang during the 2-carboxybenzaldehyde dehydrogenase assay derived from (A) *Burkholderia* sp. DBT1 cultivated in DM, (B) DBT1 strain grown in YMB and (C) m51 strain grown in YMB. In these tests the medium was supplied with 2-carboxybenzaldehyde. (D) negative control, DBT1 strain grown in YMB without 2-carboxybenzaldehyde.

Enzymatic assays and growth tests have shown that *Burkholderia* sp. DBT1 harbors a 2-carboxybenzaldehyde dehydrogenase enzyme. However, strain DBT1 was not able to grow in presence of this molecule as sole source of carbon and energy without the PAH extradiol dioxygenase enzyme, clustered in the p51 operon. These results suggest that this bacterial strain is capable to degrade phenanthrene without 2-carboxybenzaldehyde as intermediate, through a metabolic pathway already founded in previus studies (Seo *et al.,* 2006). To verify this

hypothesis, an enzymatic assay to test the 2-carboxybenzaldehyde dehydrogenase activity has been performed on *Burkholderia* sp. DBT1 cells grown in presence of phenanthrene as sole source of carbon and energy. The results obtained showed no enzymatic activity (data not shown).

3.4.6 Spectrophotometer characterization

Spectrophotometer characterization of p51 mutant was performed in order to verify the 2-carboxybenzaldehyde degradation capability even without increase of biomass. The results showed that the peak at about 300 nm, corresponding to 2-carboxybenzaldehyde (Barnsley, 1983), was presents even after 1 week in the negative control. On the other hand, it disappeared in the medium supplied with strain p51mutant owing to bacterial degradation of 2-carboxybenzaldehyde, the only PAHs compound added to the medium (Fig. 3.15).
Thus, this data shows that the compound was efficient degrade by strain mutant m51, confirming that the PAH extradiol dioxygenase is involved downstream of 2-carboxybenzaldehyde in a not phenanthrene catabolic pathway.

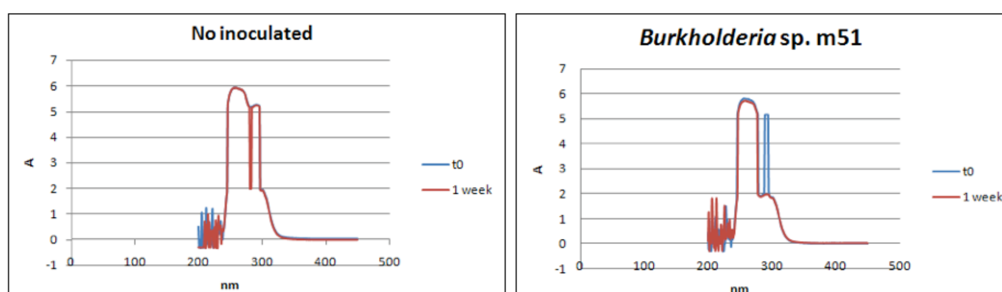


Fig. 3.15: Absorbent spectra of DM media supplied with 2-carboxybenzaldehyde.

Results so far achieved in order to detect the phenanthrene degradation pathway suggest the presence of phthalic acid and protocatechuate such as intermediates. On the other hand, has been demontrated that 2-carboxybenzaldehyde is not involved through phenathrene degradation. Thus, it is possible to hypothesize that phenanthrene is degraded via naphthalene-1,2-diol through 2-carboxycinnamic acid, phthalic acid and protocatechuic acid (Fig. 1.6; Pinyakong *at al.,* 2000). However, it is important to underline that further analysis by GC-MS will be performed in order to validate this hypothesize.

3.5 Toxicology and pathogenic analysis

The taxonomic data discussed in this work demonstrate that strain DBT1 belongs to the species *fungorum*, excluding any relationship of this isolate with the Bcc on the basis of a wide range of phenotypic, chemo-taxonomic and genotypic tests. On the other hand, as previously underline, concerns exist about strains belonging to *fungorum* species (Scholz and Vandamme, unpub. data; Gerrits *et al.,* 2005). Previous study was already conducted in order to investigate the safe utilizing of *Burkholderia* degrading strain LB400 for biotechnological applications such as bioremediation (Fain and Haddock, 2001). The report conclude with the exclusion of this strain from any *Burkholderia* pathogenic species, however it recommend further studies focused on potential pathogenicity with animal model before its intentional release into the environment. Therefore, the investigation about possible toxic effects by strain DBT1 to mammalian cells has been considered of prominent significance in the present study. The first assay is based on motility inhibition in boar spermatozoa. The motility of boar spermatozoa depends on oxidative phosphorylation in the mitochondria (Mann and Lutwak-Mann, 1982), responding with loss of motility when exposed to nanomolar concentrations of toxin such as valinomycin or of cereulide (Hoornstra *et al.,* 2003). These features make boar spermatozoan motility a sensitive indicator for detecting mitochondrial damage, a sublethal injury not easily detected by other types of cells. Moreover, many bacterial pathogens produce toxins that kill and lyse host cells by interacting with their plasma membrane. The majority of these cytolytic toxins are pore-forming proteins, and several of them have been shown to represent important virulence factors of the corresponding bacteria (Alouf, 2001). Thus, two human cells lines were used as model to investigate the contact of toxins with the human digestive tract, Caco-2 cells; and Paju cells to monitor the neurotoxicity activity (Jääskeläinen *et al.,* 2003).

3.5.1 Motility of sperm cells

In vitro toxicity bioassay is based on inhibition of motility of boar sperm cells. It has been widely used as an endpoint indicator of functional integrity of spermatozoa (Gao *et al.,* 1997). The motility of spermatozoa is exclusively depending on intact mitochondrial functions (Mann and Lutwak-Mann, 1982) and can be use as a tool for detecting toxins of microbial origin towards eukaryotic cells (Andresson *et al.,* 1997). The test is generally recorded as positive when treated sperm cells show a decrease in motility higher than 50% compared to the control (untreated spermatozoa).

Strains	EC_{50} [1]
Burkholderia sp. strain DBT1	>0.1
Burkholderia fungorum LMG 16225[T]	>0.1
Burkholderia cepacia LMG 1222[T]	0,05
Bacillus cereus F-528	>0,1
Bacillus cereus 4810/72	<0,05

[1]: mg of methanol soluble substance /ml

Tab. 3.3: Relative toxicity of bacterial cell extracts toward spermatozoa after 1 day.

The table 3.3 shows as cell extracts from *Bacillus cereus* F-528, *B. fungorum* and *B.* strain DBT1 do not negatively influence sperm cell motility. On the other hand, toxic substances from *B. cepacia* inhibited mobility of spermatozoa, after 1 day of exposure at 0.05 mg/ml bacterial cell extract, while *Bacillus cereus* 4810/72 was inhibitory at concentration lower than 0.05 mg/ml bacterial cell extract.

3.5.2 Staining of sperm cells

In order to check other viability parameters such as mitochondrial membrane potential ($\Delta\Psi_m$) and cell membrane integrity, sperm cells were stained with JC-1 and PI dyes respectively. The JC-1 dye accumulates in the mitochondria of healthy cells, which thus fluoresce red/orange. If the mitochondrial potential collapse, the JC-1 cannot accumulate any longer, remains in the cytoplasm as a monomeric form that fluoresces green (Reers *et al.*, 1995). On the other hand, plasma membrane integrity is determined considering the fluorescence emitted by PI which cannot penetrate living cells, but can only bind to and stain cellular DNA in damaged cells, giving them red fluorescence (Yeh *et al.*, 1981).

The dyes applied to sperm cells treated with methanol extracts of *Bacillus cereus* F-528 and *Burkholderia fungorum* LMG 16225^T showed mitochondria in the spermatozoan midpiece fluorescing bright orange. The same result was obtained with the extract from *Burkholderia* DBT1 (Fig. 3.16). This means that the mitochondrial potential was not dissipated as a consequence of the membrane integrity.

Not motile sperm cells exposed to the extract of *B. cepacia* (Fig. 3.16) evidenced the loss of red fluorescence of JC-1 dye in their spermatozoan midpiece related to a drop in $\Delta\Psi_m$. In this experiments, mitochondria of the spermatozoan midpiece lost about 50% of their orange fluorescence compared to perfectly viable sperm cells. Moreover, sperm cells negatively affected by toxic bacterial extracts show even head to head agglutination. With the exclusion of sperm cells treated with *Bacillus cereus* 4810/72 extract, spermatozoa did not revealed red fluorescence in all other PI stained specimens This means that membrane integrity was preserved after exposition to non-toxic bacterial extracts (data not show).

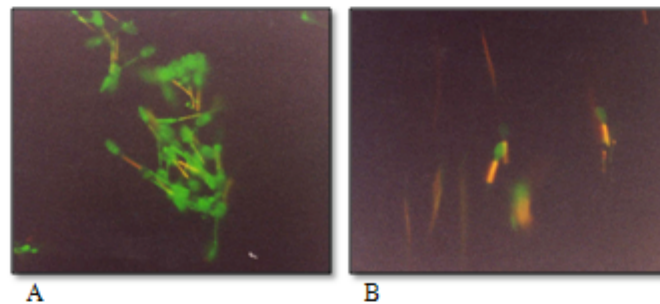


Fig. 3.16: Epifluorescence micrograph of sperm cells stained with JC-1 and PI. **Panel A** non motile sperm cells exposed to *Burkholderia cepacia* extract. **Panel B** motile sperm cells exposed to an extract of *Burkholderia* sp. DBT1.

The boar spermatozoa were sensitive indicator cells for mitochondrial toxins. The sperm cells differentiated between the mitochondria depolarising toxins: valinomycin, cereulide, gramicidin (A, B, C, D), nigericin, salinomycin, narasin, monensin, calcimycin, enniatin (A, A1, B, B1) and antimycin A. Moreover, were sensitive for other kinds of mitochondrial damage - oligomycin (A, B, C), ionomycin and staurosporine - not involving depolarization, but, for instance in inhibitor of the mitochondrial ATPase (Hoornstra *et al.*, 2003). These effects are not easy to detect with other type of assays. Thus, these toxins can be excluded to be producing by strain DBT1. On the other hand, spermatozoa are insensitive to substances affecting the synthesis of proteins or nucleic acids or their regulation.

3.5.3 Toxicity test on human cells

Human colon carcinoma cells (Caco-2 line) were exposed to extracts from bacterial cultures and then stained with JC-1 and PI dyes. Caco-2 cells were not sensitive to *B. fungorum, B.* sp. DBT1 and *Bacillus cereus* F-528 extract. On the other hand, bacterium cell extracts of *B. cepacia* caused mitochondria depolarization while did not compromise membrane integrity. Otherwise, *Bacillus cereus* 4810/72 extract induced both mitochondrial depolarization and membrane damage (Fig. 3.17).

Tests on Paju cells gave the same results as those with Caco-2 cells. Actually, excepted for the positive control with *Bacillus cereus* 4810/72 extract, none of the bacterial strains of interest negatively affected cells membrane integrity (Fig. 3.18).

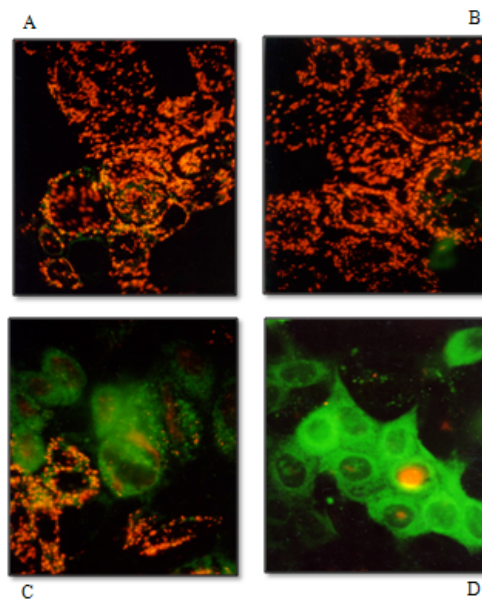


Fig. 3.17: Human colon carcinoma cells (Caco-2), stained with JC-1 and PI after exposure to extracts of: **Panel A:** methanol (control). **Panel B**: *Burkholderia* sp. strain DBT1. **Panel C:** *Burkholderia cepacia*. **Panel D:** *Bacillus cereus* 4810/72.

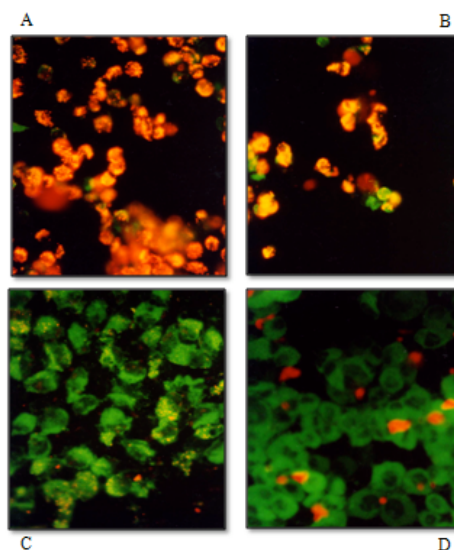


Fig. 3.18: Human neural cells (Paju), stained with JC-1 and PI after exposure to extracts of: **Panel A:** methanol (control). **Panel B**: *Burkholderia* sp. strain DBT1. **Panel C:** *Burkholderia cepacia*. **Panel D:** *Bacillus cereus* 4810/72.

In this study Paju cells were used to monitor for neurotoxicity of toxins such as cereulide and valinomycin – both potassium ionophores - (Mikkola *et al*., 1999; Teplova *et al*., 2004); Caco-2 cells (human colon adenocarcinoma cells) were used to model the contact of the sperm toxic bacterial metabolites with the human digestive tract such as amylosin (Apetroaie-Constantin *et al*., 2008). Thus, these toxins can be excluded to be producing by strain DBT1.

Mobility test of sperm cells and staining procedures with JC-1 and PI are sensible and rapid techniques for screening a wide range of mitochondrial plasma membrane toxin (Hoornstra *et al*., 2003). As far as the toxicity tests are concerned a role has been shown to be played by *B. cepacia* and *B. glumae* as toxin producers. Results concerning *B. cepacia* are in agreement with those reported by O'Quinn *et al*., (2001) and Hutchison *et al*., (1998). *B. glumae* and *B. cepacia* extracts de-polarize mitochondria preserving plasma membrane integrity of sperm cells and human cell lines (Caco2 and Paju cells). These symptoms have been shown to trigger apoptotic process in eukaryotic cells (Green and Reed, 1998; Inai *et al*., 1997). Thus, exposure to mitochondrial toxins produced by these strains may result in a severe health hazard. On the other hand, the tests used indicated that *B*. DBT1 and *B. fungorum*, type strain, resulted unable to damage mitochondrial membranes. However, it is important to underline that this strategy does not rule out the possibility that the strain investigated might be pathogenic under certain circumstances or on different targets not yet ascertained.

3.5.4 Detection of *esmR* gene

The potential pathogenesis of *B*. sp. DBT1 was also investigated by evaluating the presence of *esmR,* a molecular markers related to transmissibility which code for "*B. cepacia* epidemic strain marker" (BCESM; Mahenthiralingam *et al*., 1997). To verify the identity of 1,4-Kb BCESM marker obtained from positive control, the DNA fragment was purified from agarose gel and digested with *Pst*I and *Hae*III restriction enzymes. The profiles obtained were revealed identical to Mahenthiralingam *et al*., (1997). On the other hand, no amplicon was obtained with DNA from *B*. sp. DBT1 (Fig. 3.19).

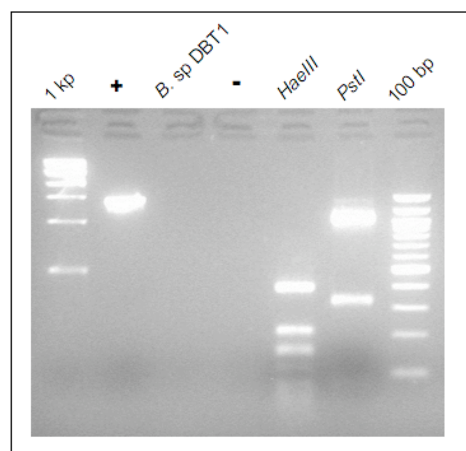


Fig. 3.19: PCR-amplification with BCSM1 and BCSM2 primers using DNA of *B*. sp. DBT1; **(+)** and DNA of epidemic *B. cepacia* strain as positive control; (-) negative control. (*HaeIII*) was loaded after *Hae*III digestion and (*PstI*) after *PstI* digestion of the BCESM band. (1 Kb): 1 Kb molecular marker (BioLabs).

Although the *esmR* genes, which are related to transmissibility, have been found mainly in clinical isolates of *B. cenocepacia* as well as in other species of the *B. cepacia* complex, the gene has also been identified from among environmental isolates of *B. cenocepacia* and *B. cepacia* (Bevivino *et al.,* 2002; Mahenthiralingam *et al.,* 1997). In the present study, *esmR* was not detected in *B.* sp. DBT1 which confirms their restricted presence in clinical and environmental isolates of opportunistic pathogens of the *B. cepacia* complex.

3.6 Study of endophytes bacterial strains

The results so far achieved suggest that the strain DBT1 presents a versatile metabolism towards PAHs. Afterwards, it is clarified and definitively assigned the taxonomic affiliation of *Burkholderia* sp. DBT1. The polyphasic taxonomic studies have showed the affiliation of DBT1 to *B. fungorum*. However, concern exists for possible application of DBT1 in environmental clean-up, although toxicology tests have demonstrated that strain DBT1 is not a mitochondrial toxin producer. Thus, while experiment are in progress by clinical microbiology laboratory in order to clarify the role of *fungorum* strains in bacterial septicemia, it could be anyway important to set up an efficient bioremediation protocol to test in laboratory scale, but with a future applicability in open field (*in situ*). A new promise strategy is to use endophitic bacterial equipped with appropriate degradation pathway in order to improve *in planta* PAHs degradation. Poplar trees (*Populus* spp.) are commonly used as phytoremediation tools because they are perennial, hardy, tolerant to high concentration of organic compounds, highly tolerant of flooding, fast growing, easily propagated and have a wide range adaptation. A key attribute of the poplar as related to bioremediation is the large quantity of contaminated water that it can contain that it can uptake from the soil (Schnoor *et al.,* 1995; Heilman *et al.,* 1990). Beside, a lot of published reports describe many species of *Burkholderia* as a natural endophyte (Mastretta *et al.,* 2006; Barac *et al*., 2004). Thus, *B.* sp. DBT1 could presents all the features for a bioremediation protocol such as described.

It has been recenly demonstrated that engineered endophytic *Burkholderia cepacia* strains improved phytoremediation and promoted plant tolerance to toluene (Barac *et al.* 2004) and there is an increasing interest on genetically modifying endophytes (Andreote *et al.* 2004). The advantages and obstacles to use bioengineered endophytes have been clearly discussed (Newman and Reynolds 2005). Neverthless, natural bacteria degrading recalcitrant compounds are more abundant among endophytic populations than in the rhizosphere of plants growth in contaminated sites (Siciliano *et al.* 2001), which could mean that endophytes have a role in metabolizing these substances. Therefore, selection and study of bacterial strains able both to degrade PAHs and to colonize host plants (e.g. Aspen) without use of molecular engineering can be an attractive approach. Thus, moleculars and physiologic analysis were carried out in order to verify the bioremediation potential of plant-endophytic bacteria systems.

The start point is a bacterial collection isolated and identified from roots, stem and leaves of hybrid aspen (*Populus tremula* x *tremuloides*) grown on PAHs polluted soils (submitted) in Finland by group of Dott. Kim Yrjälä - Department of Biological and Environmental Sciences in Helsinki; Finland -.

3.6.1 Screening of PAHs degrading bacteria

Bacterial strains able to PAHs degradation are important to develop an efficient bioremediation protocol. Therefore, fifty bacterial strains were tested aimed to detect the capability to growth on PAHs as sole source of carbon and energy for seven different compounds: naphtelene, phenanthrene, xylene, biphenyl, dibenzothiophene (DBT), fluorene and fluoranthrene (Tab. 3.4). The PAH molecules were chosen because more information are available on these than on the others and are largely presents in PAHs contaminated sites (see introduction). The bacterial strains have been selected in order to have a good representation of all the genera isolated from leaves, stems and roots.

No.	Bacteria Name	naph	phen	byphe	fluor	fluoranth	DBT	xylene
1	*Sphingomonas aerolata*	-	-	-	-	-	-	-
4	*Sphingomonas aerolata*	-	-	-	-	-	-	-
6	*Sphingomonas aerolata*	-	-	-	-	-	-	-
19	*Burkholderia sordidicola*	-	-	-	-	-	-	-
31	*Frigoribacterium veterifrondis*	-	-	-	-	-	-	-
32	*Arthrobacter rhombi*	-	-	-	-	-	-	-
49	*Rathayibacter tritici*	-	-	-	-	-	-	-
54	*Burkholderia fungorum*	-	-	-	-	-	-	-
55	*Burkholderia fungorum*	-	-	-	-	-	-	-
58	*Frigoribacterium veterifrondis*	-	-	-	-	-	-	-
67	*Rhodanobacter* sp.NP47	-	-	-	-	-	-	-
68	*Rathayibacter festuci*	-	-	-	-	-	-	-
88	*Burkholderia fungorum*	-	-	-	-	-	-	-
89	*Burkholderia fungorum*	+-	+	-	-	-	+ (red)	-
90	*Burkholderia fungorum*	+-	+	-	-	-	+ (red)	-
92	*Burkholderia fungorum*	+-	+	-	-	-	+ (red)	-
93	*Burkholderia fungorum*	+-	+	-	-	-	+ (red)	-
94	*Burkholderia fungorum*	-	-	-	-	-	-	-
95	*Burkholderia fungorum*	+-	+	-	-	-	+ (red)	-
96	*Burkholderia fungorum*	+-	+	-	-	-	+ (red)	-
97	*Burkholderia fungorum*	+-	+	-	-	-	+ (red)	-
98	*Burkholderia fungorum*	+-	+	-	-	-	+(red)	-
104	*Burkholderia fungorum*	+-	+	-	-	-	+(red)	-
105	*Burkholderia fungorum*	-	-	-	-	-	-	-
109	*Burkholderia fungorum*	+-	+	-	-	-	+ (red)	-
113	*Pseudomonas migulae*	-	-	-	-	-	-	-
115	*Burkholderia* sp. R-701	+-	+	-	-	-	-	-
123	*Microbacteriaceae* str.DB103	-	-	-	-	-	-	-
135	*Microbacteriaceae* str.DB103	-	-	-	-	-	-	-
152	*Pseudomonas migulae*	-	-	-	-	-	-	-
184	*Rahnella aquatilis*	-	-	-	-	-	-	-
191	*Rahnella aquatilis*	-	-	-	-	-	-	-
215	*Pseudomonas migulae*	-	-	-	-	-	-	-
270	*Pseudomonas migulae*	-	-	-	-	-	-	-
285	*Cupriavidus basilensis*	-	-	-	-	-	-	-
288	*Micrococcus luteus*	-	-	-	-	-	-	-
300	*Variovorax paradoxus*	-	-	-	-	-	-	-
314	*Cupriavidus basilensis*	-	-	-	-	-	-	-

316	*Burkholderia sordidicola*	++	-	-	-	-	-	-
331	*Wautersia basilensis*	-	-	-	-	-	-	-
350	*Micrococcus luteus*	-	-	-	-	-	-	-
361	*Agrobacterium rhizogenes*	-	-	-	-	-	-	-
369	*Dyella ginsengisoli*	-	-	-	-	-	-	-
382	*Pseudomonas migulae*	-	-	-	-	-	-	-
394	*Variovorax paradoxus*	-	-	-	-	-	-	-
410	*Burkholderia fungorum*	-	-	-	-	-	-	-
418	*Rathayibacter festuci*	-	-	-	-	-	-	-
463	*Burkholderia sordidicola*	-	-	-	-	-	-	-
466	*Methylobacterium fujisawaense*	-	-	-	-	-	-	-
507	*Plantibacter agrosticola*	-	-	-	-	-	-	-
DBT1	*Burkholderia* sp. DBT1	+-	++	-	+	-	++ (red)	-
HV3	*Sphingomonas* sp. HV3	++	++	++	-	+	-	++

Tab. 3.4: Bacterial strains tested on minimal media agar plate added with PAHs crystals and sprayed with ethereal solution. (naph), naphthalene; (phen), phenanthrene; (byphe),byphenyl; (fluor), fluorene; (fluoranth), fluoranthene; (DBT), dibenzothiophene; (xylene), xylene. From 1 to 32 the bacteria were isolated from leaves, from 49 to 68 from stem and from 88 to 507 from roots of aspen. (-) no growth; (+) growth; (++) very good growth; (red) the colony or the media become red.

Results showed that twelve strains were able to grown on some PAHs as sole source of carbon and energy. All of them were isolated from roots and belong to *Burkholderia* genera. It is not surprising that degrading strains are been found inside the roots. The root is the organ directly in contact to polluted soil containing the highest concentration of PAHs in the inner tissues (Gao and Zhu, 2004). This condition implies a selection towards strains able to tolerate and use PAHs as source of carbon and energy.

The isolation of endophyte *Burkholderia* strains is largely reported. For instance, McInroy and Kloepper (1995) found *Burkholderia pickettii* from sweet corn plants; Araujo *et al.,* (2002) and Barac *et al.,* (2004) isolated *B. cepacia* from yellow lupine, and citrus plants; and Weber *et al.,* (1999) and Engelhard *et al.,* (2000) detected different strains of *Burkholderia* sp. from banana, pineapple and rice. Beside, the role of these strains to PAHs degradation in plants was not detected.

Results obtained in this thesis show that among *Burkholderia* strains isolated from aspen able to grown on some PAHs, ten grouped with *B. fungorum* species, one with *B. sordidicola* and one with *Burkholderia* sp. R-701 - subsequently identified as a member of *sartisoli* species (Vanlaere *et al.,* 2008a) -. Among *fungorum* and *sartisoli* species, some strains were identified able to degrade PAH compounds (Vanlaere *et al.,* 2008a; Coenye *et al.,* 2001a). Moreover, Sun *et al.,* (2008) found an endophyte bacterial strain in rise roots belong to *fungorum*, confirming the versatility and adaptability of this species. On the other hand, this is the first case in which a strain belonging to *sartisoli* species is identified from vegetal tissue. *B. sordidicola* is a species formerly isolated and identified from a white-rot fungus (*Phanerochaete sordida*; Lim *et al.,* 2003) and subsequently detected in lichen (Cardinale *et al.,* 2005), *Lyophyllum* sp. (Warmink and van Elsas, 2009) and in birch rhizosphere planted in peat sandy soil amended with PAH compounds (Yrjälä *et al.,* in press). Therefore, the isolation of an endophyte strain from polar belonging to *sordidicola* species able to degrade xenobiotic compound is consistent with reported data. It is interesting to note the two species formerly isolated in association with fungi such as *fungorum* and

sordidicola (Lim *et al.,* 2003; Coenye *et al.,* 2001a) can be easily detected in association even in association plants, suggesting an inclination to these species to have a relationships with others organisms.

Burkholderia fungorum, R-701 and DBT1 strains seem to have similar characteristics in term of PAHs degradation, especially in the case of dibenzothiophene. These results suggest that *Burkholderia* - especially *fungorum* specie - is the most active genus involved in PAHs degradation in aspen plants. Therefore, these data encourages the applicability of strain DBT1 as endophyte in order to improve *in planta* PAHs degradation.

3.6.2 PCR primers-specific for: upper meta-pathway extradiol dioxygenases and p51 and pH1A operons

PCR analysis was carried out in order to detect the presence of specific PAHs catabolic genes. BP-f/BP-r primers were designed to amplify the extradiol ring-cleavage dioxygenase from *Sphyngomonas* sp. HV3 (Sipilä *et al.*, 2006) a strain that has diversified degradation capacities with a complex aromatic degradation pathway for degradation of biphenyls, polyaromatic hydrocarbons and BTEX compounds (Kilpi *et al*., 1980; 1983; 1988). Thus, the primer can detect catabolic genes involved in different PAHs degradation pathway (Sipilä *et al.*, 2006). On the other hand, primers designed on p51 and pH1A operon are more specific for the sequence identified in strain DBT1 (Di Gregorio *et al.,* 2004).

PCR analysis performed with BP-f/BP-r primers for extradiol ring-cleavage dioxygenase gives negative results except the positive control *Sphyngomonas* sp. HV3. Unexpectedly, an amplicon of correct size using the pairs of primers specific for either DBT1 p51 or pH1A operons was obtained for *B. fungorum* strains 89, 90, 92, 93, 95, 96, 97, 98, 104, 109 and *Burkholderia* R-701 (data not show). Siciliano *et al.* (2001) showed that the genes encoding catabolic pathways increased within the root endophyte population in response to the presence of a given pollutant. Thus, DBT and fenanthrene could be present at high concentration within the root tissues. Moreover, this result is very interesting because since now no bacteria - excepting strain DBT1 - containing p51 and pH1A was isolated. Publications present in literature report the identification of these operons in PAHs polluted sites (Chadhain *et al.,* 2006; Sipilä *et al.,* 2006) but only by molecular techniques and no bacteria isolated was obtained.

3.6.3 Study of genetic polymorphism of p51 and pH1A operons

Previous work showed by restriction analysis, that *nahAc* genes were found to be similar, but not identical, to the *nahAc* gene of the NAH7 plasmid carried by *Pseudomonas putida* G7, and a widely studied naphthalene-degrading bacterial strain, suggesting divergent homologs of *nahAc* are present in the PAH-degrading guilds at contaminated site (Ghiorse *et al.,* 1995). Thus, aimed to evaluate the sequence polymorphism on p51 and pH1A operons, PCR-DGGE and restriction analysis were performed from endophytes and DBT1 strains.

PCR-DGGE analysis

PCR-DGGE analysis was performed on α-subunits of dioxigenase in 89, 90, 92, 93, 95, 96, 97, 98, 104, 109, 115 and DBT1 strains. DGGE exploits the fact that otherwise identical DNA molecules, which differ by only one nucleotide within a low melting domain will have different melting temperatures. When separated by electrophoresis through a gradient of increasing chemical denaturant, the mobility of the molecule is retarded at the concentration at which the DNA strands of low melt domain dissociate.

A single band at the same height was obtained for each strains tested on α-subunits of dioxigenase (Fig. 3.20). Therefore, no polymorphisms were detected by PCR-DGGE analysis on α-subunits of dioxygenase. On the other

hand, the length of the segment amplified by dbtAcforGC and dbtAbrev primers (Zocca, 2004) is 140-bp, which is only a little fragment of the operon.

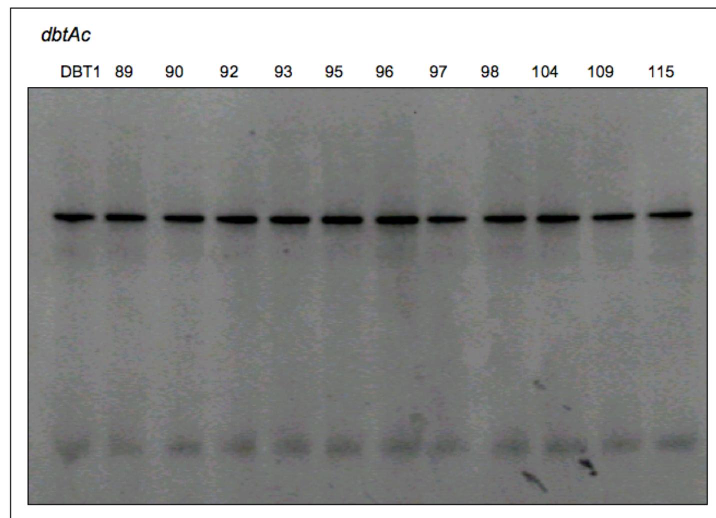


Fig. 3.20: DGGE profiles of a fragment of *dbt*Ac sequence – denaturing gradient and bis-acrilamide concentration of the gel: 40%-60 and 10% respectively- of endophytes able to degrade PAHs containing p51 and pH1A genetic fragment.

Restriction analysis of p51 and pH1A operons

Aimed to extend the polymorphism analysis to other regions of p51 and pH1A operons, genetic typing of fragments obtained from PCR analysis by primers p1/p5, p8/p11, p12/p13 and p2/p3 were performed through restriction analysis. Fragments amplified from strains 89, 90, 92, 93, 95, 96, 97, 98, 104, 109, 115 were analyzed and compared with DBT1. Interestingly the same pattern were obtained by the *TaqI* and *HhaI* enzymes for all the strain tested in the case of fragments generated by primers p2/p3 and p8/p11. Moreover, the same result was obtained by *TaqI* in the case of fragment generated with p1/p5. On the other hand, with p12/p13 primers using *TaqI* enzyme and p1/p5 primers using *HhaI* enzyme, the DBT1 pattern resulted different if compared with the endophytes strains (Fig. 3.21). In summary, specific profiles of DBT1 were detected compared to the other strains. These results prove the presence of two different sequences of p51 and pH1A operons: the first identified in *B*. sp. DBT1 and the second recognized in endophytes bacteria isolated in Finland.

Thus, no sequence polymorphism was observed in p51 and pH1A operons among endophyte strains, indicating that same homolog of the sequence was present in the native community. On the other hand, the genes of DBT1 were divergence homolog to the strain isolated in Finland.

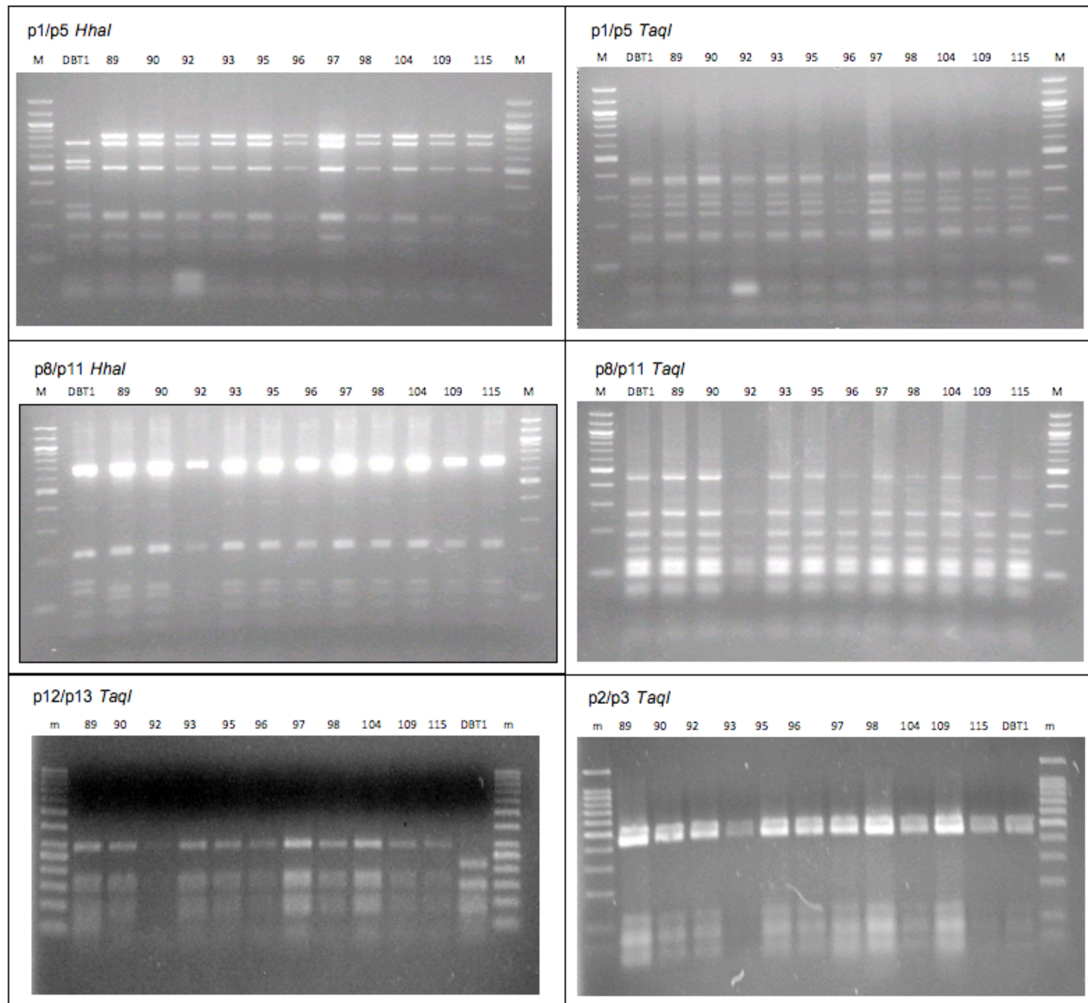


Fig. 3.21: Main restriction profiles of p51 (p8/p11; p12/13 couples of primers) and pH1A (p2/p3; p1/p5 couples of primers) operons obtained by the *Taq I* and *HhaI* enzymes. (m) 50-bp molecular marker (Promega); (M): 100 bp molecular marker (BioLabs).

3.6.4 Taxonomic study of *Burkholderia fungorum* endophytes

Previous results suggest a close relationship among *B. fungorum* and *Burkholderia* R-701 able to degrade PAHs isolated from root tissue. However, among the fifty bacterial strains tested on minimal medium supplied with different PAHs compounds are present additional six *B. fungorum* strains – 54, 55, 88, 94, 105 and 410 – not able to use PAHs as sole source of carbon and energy. Aimed to understand the taxonomic relationship among these strains in relation to *fungorum* degrading strains and *B.* sp. DBT1, preliminary taxonomic characterization by PCR-DGGE analysis on *V3* and *V6-V8* region of *16s* rDNA and RAPD analysis were performed.

PCR-DGGE analysis

PCR-DGGE analysis were performed on *V3* and *V6-V8* regions of *16s* rDNA. The results showed a band at the same height in strains 89, 90, 92, 93, 95, 96, 97, 98, 104, 109, 115 and a different height among strains 54, 55, 88, 94, 105, 410 and DBT1 (Fig. 3.22).

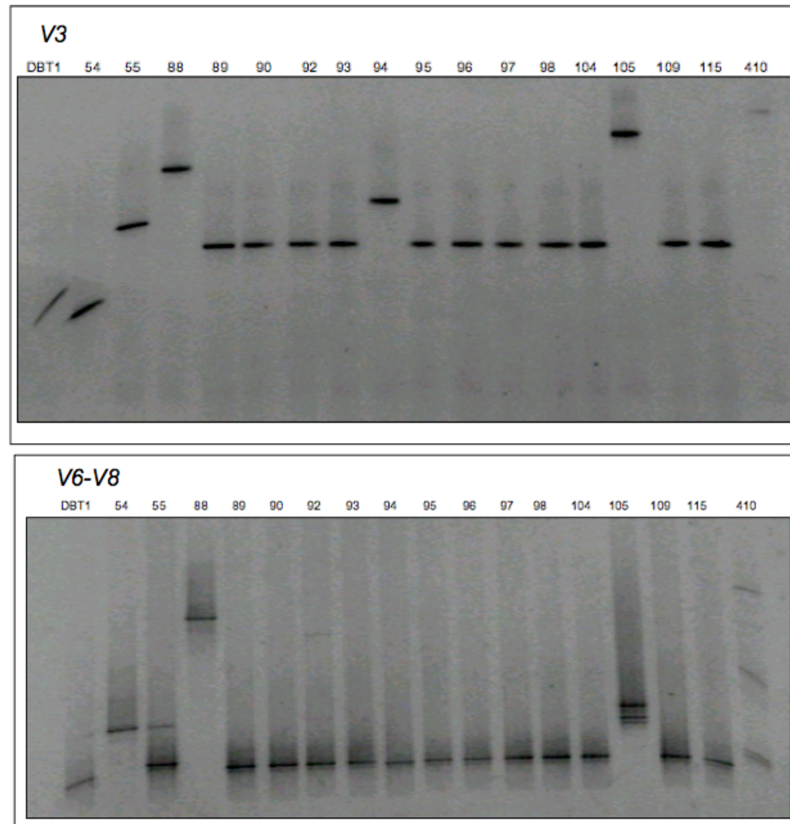


Fig. 3.22: DGGE profiles of *Burkholderia fungorum* endophytes strains, *Burkholderia* sp. R-701 and *B.* sp. DBT1. The gel used for *V3* sequence contains 40%-55% denaturing gradient and 8% bis-acrilamide concentration. The gel used for *V6-8* sequence contains 26%-58% denaturing gradient and 6%-9% bis-acrilamide concentration.

The profiles of PCR-DGGE analysis confirm the closely relationship among *fungorum* and R-701 degrading endophytes. On the other hand, DBT1 and bacterial strains 54, 55, 88, 94, 105 and 410 showed different profiles. This means that PAHs degrading strains share more similar 16S rDNA sequence comparing to bacteria not able to use xenobiotic compounds, whose own divergent 16S rDNA sequence.

From a methodological point of view it is possible to note that the V3 region of *16s* rDNA gene sequence presents more discriminatory powerful comparing to *V6-V8* region. In fact, 94 and 55 strains show a band at the same height to *fungorum* degrading strains by PCR-DGGE gel carried out using V6-V8 region, whereas in PCR-DGGE gel performed by V3 region the height of the bands are different. This observation is concordant with the literature where it was performed PCR-DGGE analysis on a mix of 16S rDNA sequence extract from an environmental matrix. In fact, it has been shown that though V3-derived DGGE gels on average contain more bands than V6–8-derived DGGE gels (Nielsen *et al.,* 2006).

RAPD (Random Amplified Polymorphic DNA) analysis

RAPD analysis was performed in order to discover possible genetic polymorphism within endophytes *B. fungorum* strains compared with DBT1 strain. The powerful and advantages of this technique is due to the fact that this is a whole genome fingerprinting. On the other hand, the technique shows low reproducibility among the replica. Different primers were tested such as OPL-16 (Vincent *et al*., 1998), M-14 (Zapparoli *et al.,* 2000), M-13 (Stenlid *et al.,* 1994), D8635 (Akopyanz *et al.,* 1992), D1254 (Akopyanz *et al.,* 1992) and COC-1 (Cocconcelli *et al.,* 1995;

data not show). OPL-16 has showed the better fingerprinting among the bacterial strains. The other primers were rejected for one of the following reasons: electrophoretic smear, the presence of too few amplicons or no amplification at all.

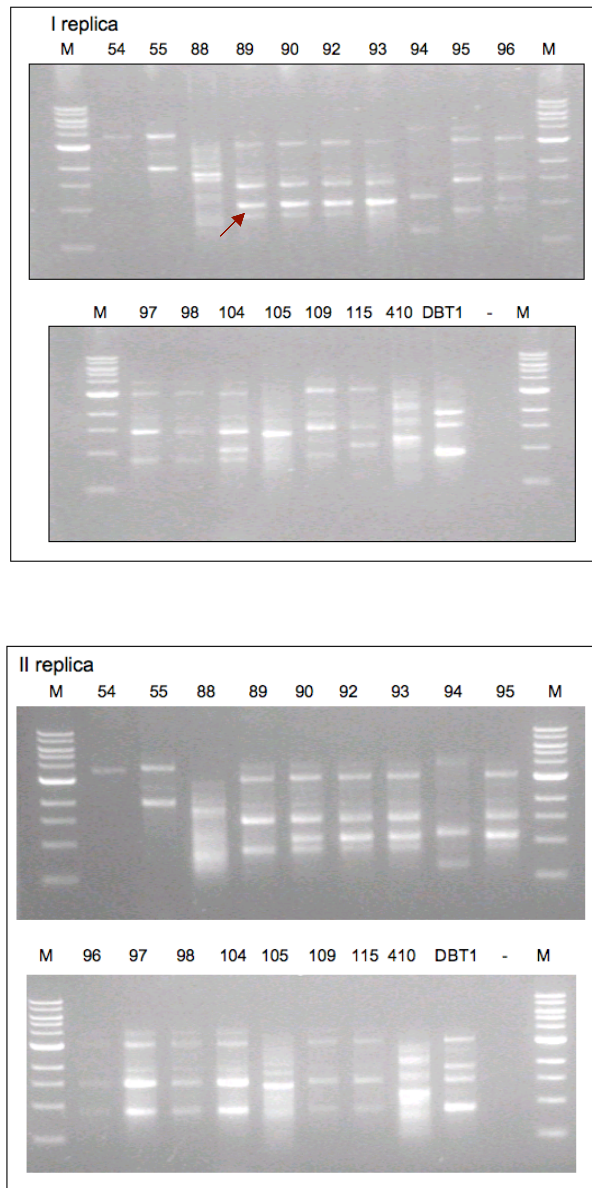


Fig. 3.23: First and second replica of RAPD profiles obtained by OPL-16 primer of *Burkholderia fungorum* endophytes strains, *Burkholderia* sp. R-701 and *B.* sp. DBT1. (M): 1 Kbp molecular marker (BioLabs). The red arrow indicates the band due to the low reproducibility of RAPD technique.

The first replica of RAPD analysis carried out by OPL-16 primer showed two different profiles among endophytes degrading strains tested. The first profile includes 89, 90, 92, 93, 96, 104, 109 and 115 strains, whereas the second group includes 95, 97 and 98 strains. The difference between profiles is due to the presence of an additional band at about 1 Kbp. The second replica showed, as the first, two different profiles among endophytes degrading strains tested. However, the members of the two groups are different. The first profile includes 90, 92, 93, 95 and 96 strains, whereas the second group includes 89, 97, 98, 104, 109 and 115 strains. The difference between profiles is

due to the presence of the same additional band at about 1 Kbp. Comparing results between the two replica is clear that the presence of two distinct group among the endophytes degrading strains is due to the low reproducibility of RAPD technique and not for genetic polymorphims among tested strains. Therefore, RAPD results show the same pattern in strains 89, 90, 92, 93, 95, 96, 97, 98, 104, 109 and 115. Even strain DBT1 shows a profile that can be included with these strains. On the other hand, a different pattern among strains 54, 55, 88, 94, 105 410 were detected (Fig. 3.23).

The results obtained by taxonomic study confirm the results achieved by the study of genetic polymorphism of p51 and pH1A operons: a closely relationships among 89, 90, 92, 93, 95, 96, 97, 98, 104, 109 and 115 strains. On the other hand, *fungorum* strains not able to degrade PAHs, show different genetic sequence both comparing degrading strains and among them. Moreover, *B.* sp. DBT1 strain shows distinctive sequence features. It presents a specific pattern of p51 and pH1A operons, and diverse V3 and V6-8 sequence of *16s* rDNA.

3.6.5 Growth test in presence of DBT and phenanthrene

Restriction analysis proves the presence of two genotypes of p51 and pH1A operons: the first harbored in *B.* sp. DBT1 and the second in endophytes degrading strains. Aiming to evaluate if beside the genomic difference subsist even a metabolic difference, growth tests on minimal medium supplied with DBT and phenanthrene –compounds degraded by both genotypes - were performed. Results for strains 95 and 109 were obtained for comparison with DBT1 as representative members of the new p51 and pH1A genotype, and strain 115 as representative of *Burkholderia sartisoli*, which harbor the new p51 and pH1A genotype as well.
Therefore, bacterial growth of strains 95, 109, 115 and DBT1 were compared using phenanthrene and DBT as sole source of carbon and energy. Moreover, UV-spectrophotometer analysis on the growth media was performed in order to evaluate the development of degradation intermediates during bacterial growth.

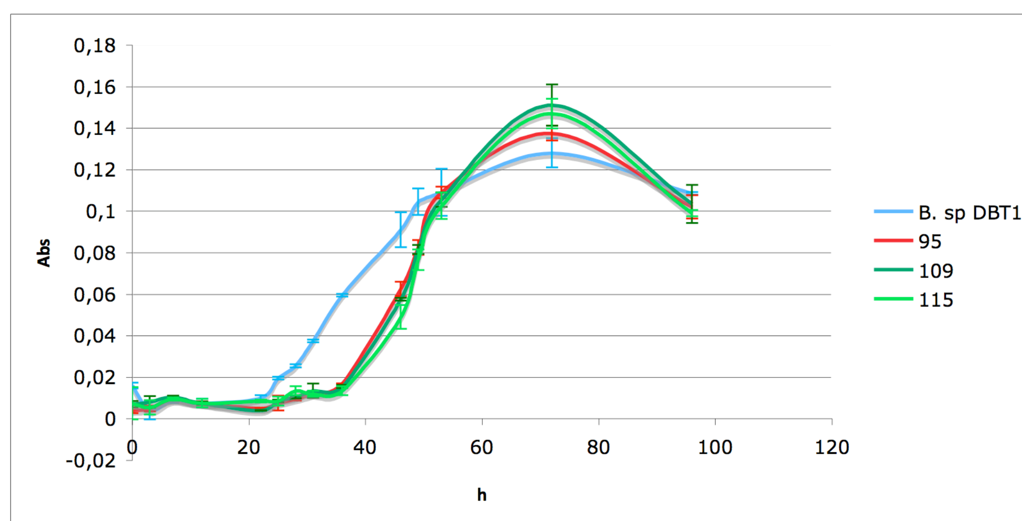


Fig 3.24. Growth of *B.* strain DBT1, *B.*strain 95, *B.*strain 109 and *B.* strain 115 in DM supplemented with phenanthrene.

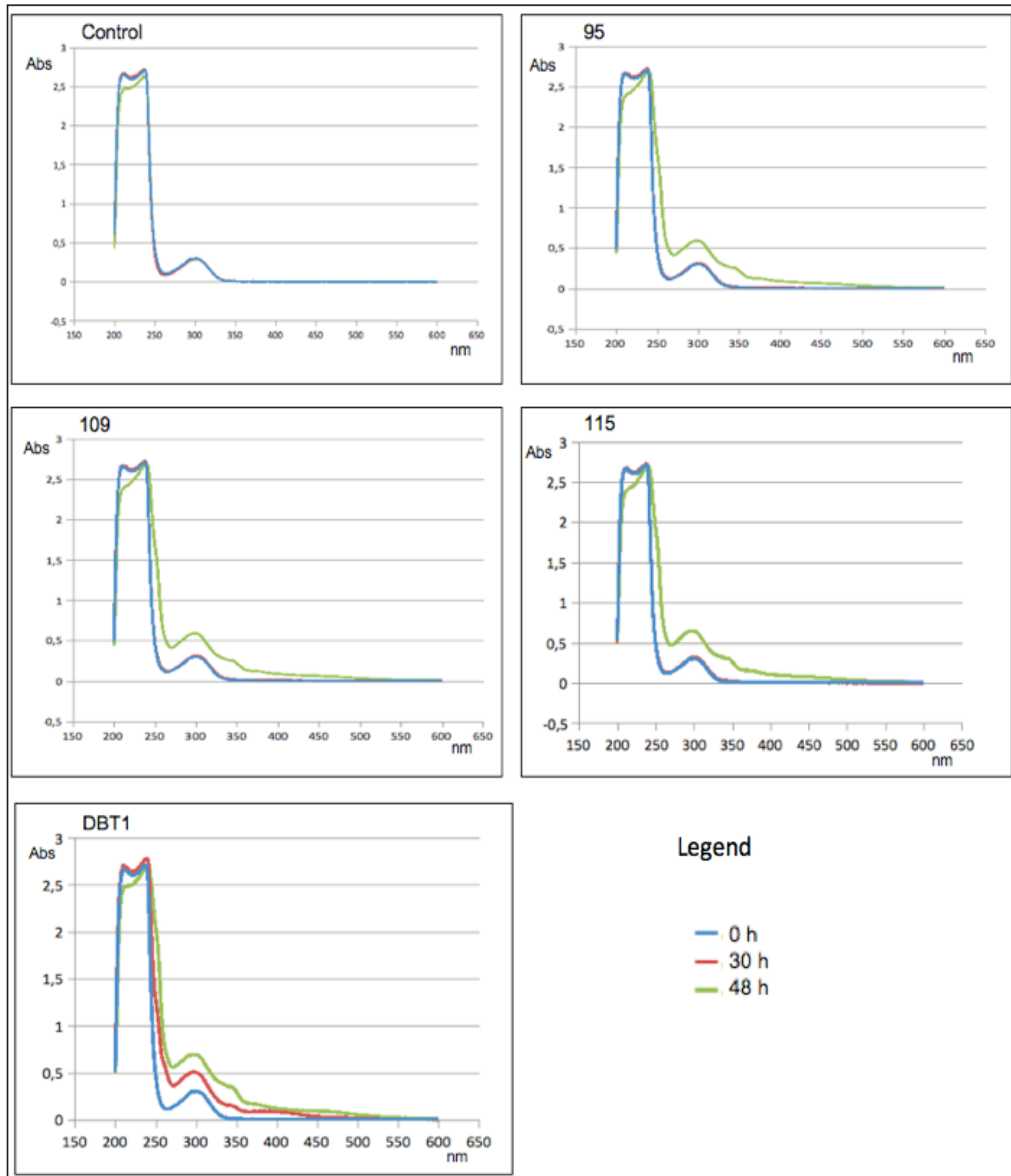


Fig. 3.25: UV-Vis spectra of DM media supplied with phenanthrene at 0, 30, and 48 hours. The media were respectively inoculated with strains 95, 109, 115 and DBT1.

The results obtained with phenanthrene showed a shorter lag phase in DBT1 strain compared with 95, 109 and 115 that means a major efficiency in phenanthrene degradation by DBT1 (Fig. 3.24). This data was subsequently confirmed by UV-Vis spectrophotometer analysis (Fig. 3.25). In fact, the strain DBT1 shows the development of intermediates after 30 hours, in coincidence with its mid-log growth phase. On the other hand, strains 95, 109, and 115 start to growth after 40 hours, with subsequently increase of amount of phenanthrene metabolites (Fig. 3.24; 3.25). The increase of the pick at 300 nm could be due to the development of a ring cleavage product of 1-hydroxy-2-naphthoate (Adachi *et al.,* 1999) in agreement with phenanthrene catabolic pathway previously suggested.

On the other hand, no difference was observed in the growth curve of the tested strain on DBT (data not show). However, it is important to underline that the growth resulted very limited for all the strain tested - included DBT1

– (Fig. 3.11). Otherwise, the development of Kodama pathway intermediates was more rapid in DBT1 compared with 95, 109 and 115 (Fig. 3.26). Strain DBT1 shows the presence of metabolites of DBT degradation approximately at 70 hours, about 40 hours later than phenanthrene degradation. The increasing of absorbance at 394 and 474 nm corresponding to hydroxy-2-formilbenzothiophene (HFBT) and trans-4- [2-(3-hydroxy)-tionaphthenil] 2-ossi-3-butenoic acid (trans-HTOB) respectively, both intermediates of Kodama pathway (Kodama *et al.,* 1973).

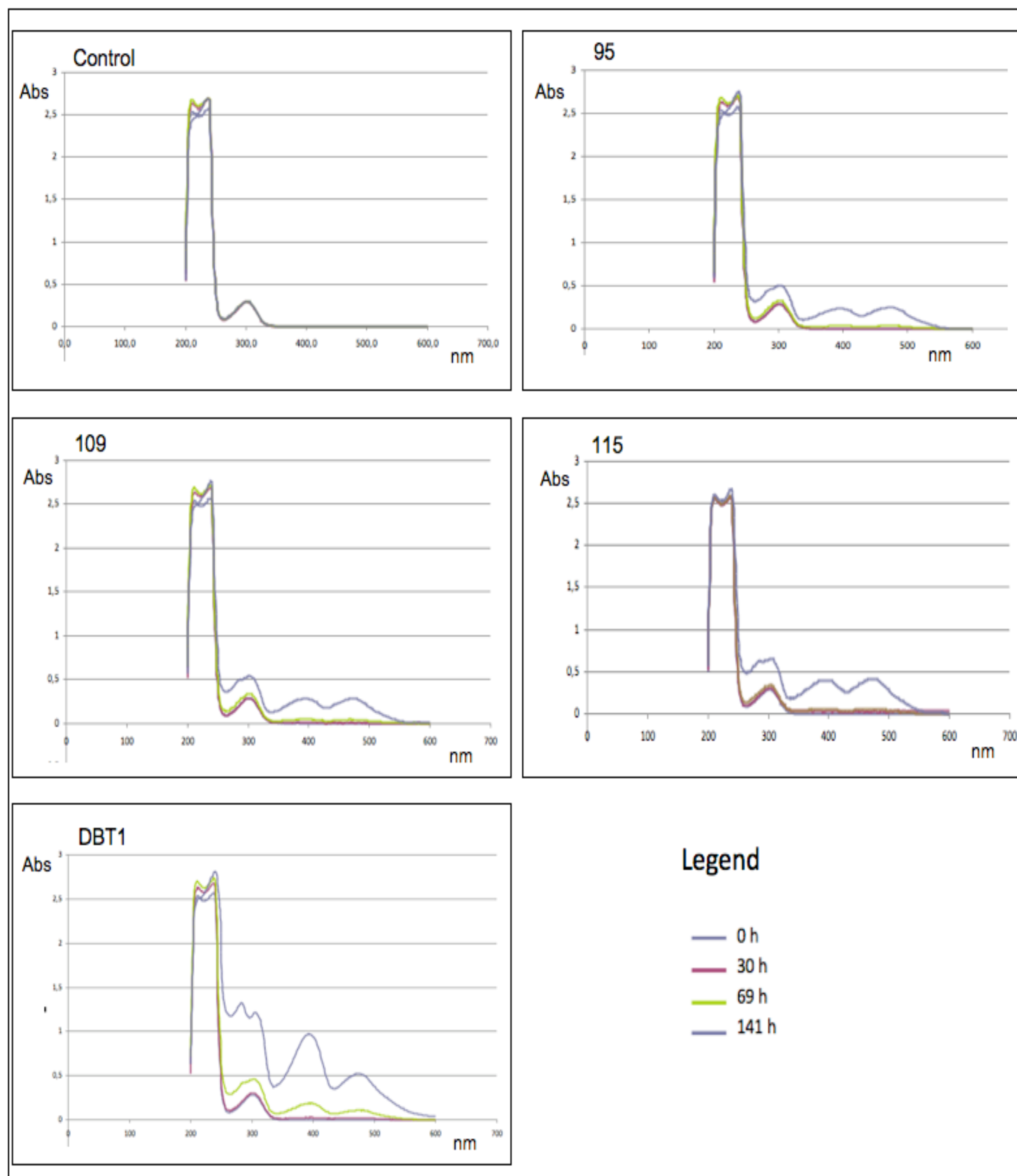


Fig. 3.26: UV-Vis spectra of DM media supplied with DBT at 0, 30, 69 and 141 hours. The media were respectively inoculated with strains 95, 109, 115 and DBT1.

These results suggest that *B.* sp. DBT1 is more efficient in degradation towards PAHs tested. Therefore, the difference between strains isolated in Finland and DBT1 is not only at genetic level. However, it is important to underline that it is not possible, with the results so far achieved, connect the diverse polymorphism of p51 and pH1A operons of DBT1 strain with its major efficiency degradation towards PAHs. However, the different habitat in which DBT1 was isolated - the wastewater dicharge pipeline of an oil refinery -, which contain higher concentration of PAHs than in the inner tissues of the plant could explain a selective pressure towards strain DBT1. Moreover, plants provide nutrients and residency for endophytes bacteria (Mastretta *et al.,* 2006), otherwise to strains such as DBT1 isolated from wastewater where the main carbon sources are represented by xenobiotic compounds. Therefore, this environmental condition implies a selection towards strains able to use PAHs as the only source of carbon and energy. In fact, it is interesting to note the big difference of efficiency between strains isolated in Finland and DBT1 on DBT, the molecule on which this strain has been formerly isolated (Di Gregorio *et al.,* 2004).

3.6.6 Plant inoculation

Results so far achieved suggest that the strain DBT1 presents a versatile metabolism towards PAHs especially towards phenanthrene and DBT. This behavior encourage the possible exploitation of *Burkholderia* DBT1 in bioremediation protocols. Morever, metabolic and genetic characterization of endophytes isolated in poplar plants in Finland has demonstrated the presence of *Burkholderia* strains able to degrade PAHs. In detail, most of degrading strains - 89, 90, 92, 93, 95, 96, 97, 98, 104, 109 and 115 - are able to degrade phenanthrene and dibenzothiophene, and a bacterial strain – 316 - was identified for its capability to degrade naphthalene. On the other hand, the efficiency of a bioremediation protocol, which uses plants infected with PAHs degrading-endophytic bacteria, is primarily affected by the degradation competence of the inoculated strains and the plant capability to uptake PAHs. PAHs sorption by plants is seriously affected either to physical-chemical proprieties of organic compound or plant species (Zhu *et al.,* 2007; Li *et al.,* 2005). It has been proved that the amounts of PAHs sorbed by plant predicted by octanol-water partition coefficient (log K_{ow}) model, appears to be highly underestimated, especially for the compounds with relative high hydrophobicity (Li *et al.,* 2005; Su and Zhu, 2007; Zhu *et al.,* 2007; Zhang and Zhu, 2009). Considering octanol-water partition model, the uptake of plant was possible to applied in phytoremediation protocol only towards PAHs with log K_{ow} between 0.5 and 3, excluding molecules such as phenanthrene and DBT (Basak *et al.,* 1996). Consequently, endophytes microorganism able to degrade these molecules would be not useful of this kind of bioremediation protocol owing to these compound are not available within plant tissues. On the other hand, the new and improved model containing integral roles of carbohydrates and lipids, showed that molecules such as phenanthrene may be sorbed by plants (Zhang and Zhu, 2009). Consequently, a bioremediation protocol based on endophytes bacteria able to degrade phenanthrene and/or DBT could be efficiently applied on polluted sites.

Thus, tree selected strains – 95, 115 and 316 - plus *B*. DBT1 were inoculated in aspen roots as described in in materials and methods. The plants were harvest after 10 weeks from the inoculation. At that time, root and sprout lengths as well as surface of three leaves will be measured. However, during the first week a rapid dehydration of the leaves for all the plants was observed. This was probably due to the stress that the explants have caused. In fact, the plants before inoculation were grown in soils - and not in vitro or perlite - and the removal from the ground probably caused the break of the roots tips and the following lost of water. Thus, no results are available for this experiment. In the future, it will be improve the protocol of plants infection in order to avoid the

dehydration of the plant tissues. The possible alternative is grow aspen in perlite in order to make easier the explants without break the roots tips, or using cutting of poplar instead of the whole plant (Taghavi *et al*., 2009).

4. Conclusions

Polycyclic aromatic hydrocarbons (PAHs) are a recalcitrant group of contaminants whose are known to be highly persistent in the environment (Cooke and Dennis, 1983). There are many sources for PAHs contamination in soils. These include creosote, petroleum and coke products (Cooke and Dennis, 1983). Remediation of persistent contaminants such as PAHs from soil is expensive and time consuming. Physical removal of contaminated soils and washing of these soils with solvent are expensive, and have met with mixed results (Dixon, 1996). Alternatively, microbial remediation – bioremediation - has been attempted. However, in most contaminated soils, the number of microorganisms is depressed so that there are not enough bacteria to facilitate contaminant degradation. Moreover, PAHs are very stable compounds and the initial oxidation step is biologically slow and metabolically expansive (Huang *et al.*, 2001). Thus, microbial degradation by only indigenous bacteria is probably too slow to be a realistic approach to this problem. More recently, there have been some improvements in the strategies for bacteria remediation of contaminated soils, including the stimulation of indigenous bacterial community – landfarming - or inoculation with bacterial previously selected from PAHs contaminated matrix. The inoculation can be performed directly in polluted soil – bioaugmentation - (Barathi and Vasudevan, 2003) or alternatively in selected plants in order to improve *in planta* degradation (Barac *et al.*, 2004). For instance, Barac *et al.*, (2004) infected lupine plants with genetically modified bacteria with the aim to degrade toluene. Plants grown in the presence of toluene had two important characteristics imparted by the new endophyte. First, the toxicity of toluene to the plants decreased significantly; and the second major change in the plants was the reduction in volume of toluene transpired. Nevertheless, the concepts of releasing engineered bacteria into the environment must be taken into account. Although these organisms have been transformed using naturally occurring bacterial traits for 'gene swapping', the public opinion is far from accepting the use of engineered bacteria in bioremediation protocols *in situ*. Therefore, the exploit of natural PAHs degrading bacteria could be an efficient and public accepted strategy. Thus, in order to better exploit and use the bioremediation potential of plant-endophytic bacteria systems, two possible ways can be followed. The first is based on using a well knows PAHs degrading bacteria and subsequently verifing its capability to colonize the host plant; the second is focused on isolating, identifying and characterizing the bacteria that act as bacterial endophytes in plants and that might be used for phytoremediation. In this thesis work, both these proposed strategies have been takes into account.

In our laboratory it had been previously isolated a bacteria strain called *Burkholderia* sp. DBT1 able to degrade dibenzothiophene (DBT) in liquid culture through the Kodama pathway within three days of incubation (Di Gregorio *et al.*, 2004). These features have encouraged to carry on news studies aimed at a functional, phylogenetic and toxicological characterization of *Burkholderia* sp. DBT1 for a possible explanation of such strain in protocol of bioremediation in PAHs contaminated soils.

The results so far achieved suggest that the strain DBT1 presents a versatile metabolism towards PAHs, and this is an important trait for the possible use of this strain in environmental clean up. This strain is able to use fluorene, naphtalene, DBT and phenanthrene for growing, although growth on the first two compounds needs a pre-induction. Therefore, it can be suggested that phenanthrene is metabolized through 2-carboxycinnamic acid, phthalic acid and further metabilized in protocatechol. On the other hand, further study with GC-MS is required to clarify the role of 2-carboxybenzaldehyde in PAHs degradation as well as to confirm the catabolic pathway of phenanthrene. The PAHs degradation can occur even by a co-metabolism process. Co-metabolism is the process by which a contaminant is fortuitously degraded by an enzyme or cofactor produced during microbial metabolism of another compound. Typically, there is no apparent benefit to the microorganism involved. Co-metabolic bioremediation enables remediation strategies to stimulate biodegradation of the contaminants to concentrations which are far below the concentration that could be of carbon or energy benefit to the biodegrader. Thus, co-metabolic bioremediation has the added advantage of allowing scrubbing of environmental contaminants down to undetectable concentrations. As cited before, studies of PAHs bioremediation can involve the inoculation of

degrading bacteria such as *B.* sp. DBT1 *in planta*. In this way, the addition of bacterial strain able to degrade PAHs by co-metabolic process is an advantage. In fact, plants provide nutrients – even carbon sources – (Mastretta *et al.*, 2006) and residency for bacteria that might be able to largely stimulate the degradation of PAHs compounds. The capability of DBT1 to degrade PAHs is due to *dbt* genes which show a novel genomic organization in comparison to previously described genes responsible for PAHs catabolism including DBT (Denome *et al.* 1993; Menn *et al.* 1993; Sanseverino *et al.* 1993; Kiyohara *et al.* 1994; Geiselbrecht *et al.* 1998). They constitute in fact two distinct operons - p51 and pH1A – instead of being clustered in a single transcript. The two genomic fragments were sequenced in *Burkholderia* sp. DBT1, and six of eight genes required for the conversation of DBT to HFBT were recovered. The work here presented has demonstrated that the hydratase-aldolase and ferredoxin reductase sequences, the two genes missing, are collocated downstream the putative sequence ORF 7 within p51 operon. Thus, the missing enzymes have been recovered, demonstrating that the whole set of enzyme involved in Kodama pathway degradation is harbored in p51 and pH1A operons.

The application of bacterial strains in environmental clean up is severely subject to another crucial pre-requisites: the ecotoxicological safety of the selected strain and its probative exclusion from pathogenic species such as Bcc. The data presented in this study indicate that strain DBT1 can be considered a member of the species *fungorum*, for which the name *Burkholderia fungorum* strain DBT1 is here proposed. Since the original report, *B. fungorum* was identified in a wide range of environment such as soil, plant-associated samples (Coeyne and Vandamme, 2003), in infections of the central nervous system of a pig and a deer (Scholz and Vandamme, unpub. data), and in the respiratory secretions of people with cystic fibrosis (Coenye *et al.*, 2001; 2002). However, clinical data were not available from these patients, therefore the significance of isolation of *B. fungorum* has not yet been determined. Just recently it was reported the first description of bacteria septicemia due to *B. fungorum* manifested as a soft tissue infection of the leg (Gerrits *et al.*, 2005). Therefore, further investigation may be performed by microbiology laboratory in order to clarify the role of *fungorum* strains in central nervous system of a pig and a deer and in human bacteria septicemia. On the other hand, biochemical test obtained by API 20NE test has showed that strain detected by Gerrits *et al.*, (2005) utilized caprate as carbon source, conversely to strain DBT1. Obviously, any speculation about this little metabolic difference would be unwise. Therefore, the investigation about possible toxic effects by strain DBT1 has been considered of prominent significance in the present study; and bioassay to detect mitochondrial toxicity in mammalian cells by JC-1 and PI staining of human cell lines would be useful to investigate toxic microbial metabolites. Results so far achieved show that *B.* DBT1 and *B. fungorum* type strain was unable to damage mitochondrial and human cells membranes. However, it is important to underline that this strategy does not rule out the possibility that the strain investigated might be pathogenic under specific circumstances or on different targets not yet ascertained, such as to substances affecting the synthesis of proteins or nucleic acids or their regulation. Besides, this is the first work in which an accurate study was performed in order to investigate the toxicological aspect of a bacteria strain in the perspective of an application in environment clean up. Thus, *B.* sp. DBT1 presents all the potentiality for its exploitation in plant-endophytic bacteria protocols.

Besides, as cited before, the second strategy for a better use the bioremediation potential of plant-endophytic bacteria systems is focused on isolating, identifying and characterizing the bacteria that already exist as bacterial endophytes in plants and which are applyable to phytoremediation protocols. Poplar trees (*Populus* spp.) are commonly used as phytoremediation tools because they are perennial, hardy, tolerant to high concentration of organic compounds, highly tolerant to flooding, fast growing, easily propagated and have a wide range adaptation. A key attribute of the poplar in relation to bioremediation is the large quantity of contaminated water that it can uptake from the soil (Schnoor *et al.*, 1995; Heilman *et al.*, 1990). Thus, a careful screening and characterization of endophytes from poplar plants growth on PAHs contaminated soils were carried out. The results obtained confirm

that natural bacteria degrading recalcitrant compounds are largely present among endophytic populations of plants growth in contaminated sites (Siciliano *et al.* 2001), this could mean that endophytes have a role in metabolizing these substances. Moreover, *Burkholderia* is resulted be the genera mainly involved in PAHs degradation, and *fungorum* the species more representative. Interestingly, the majorities of these bacteria are involved in phenanthrene and DBT degradation, and harbor p51 and pH1A, the operons formerly identify in DBT1 strain. Siciliano *et al.* (2001) noticed that the genes encoding catabolic pathways increased within the root endophyte population in response to the presence of a given pollutant. This increase was pollutant-concentration-dependent. This could mean that DBT and phenanthrene can be uptake from poplar roots, in agreement with the new PAHs plants uptake model proposed by (Zhang and Zhu, 2009). Moreover, it is interesting to point out the prevalence of p51 and pH1A operons in endophytes strains able to degrade PAHs. However, the subsequent DGGE and restriction analysis on these DNA fragments, showed the presence of a single genotype among the endophytic bacteria, but different from DBT1. The difference between strains isolated in Finland and DBT1 is not only at genetic level. In fact, *B*. sp. DBT1 resulted to be more efficient in the degradation towards PAHs tested, especially in the metabolism of DBT. At present, the explanation of the relation between high efficiency in PAHs transformation of strain DBT1 and different genotype from endophytes strains may be only speculative. However, the enlargement of the current information on endophytes strains is an important step in understanding the prevalence and distribution of bacterial species which are involved in PAHs degradation. Moreover, the results suggest the application of *B*. sp. DBT1 as poplar endophyte.

However, since now, there is not any demonstration of the endophyte nature of DBT1 for the reason that the plants infection experiment by DBT1 and selected endophytes strains was interrupted because the plants died. Thus, the protocol of plants infection will be improved in order to avoid the dehydration of the plant tissues. The possible alternative is growing aspen in perlite in order to allow an easier explants without breaking the roots tips, or using cutting of poplar instead of the whole plant (Taghavi *et al*., 2009).

Eventually, the results obtained from this thesis work incentives the use of the strain *Burkholderia fungorum* DBT1 for a possible exploitation in bioremediation protocols of PHA-contaminated sites; moreover it suggest an actual use of such strain as endophytes in poplar plant in order to improve *in planta* degradation.

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6. Acknowledgement

Innanzitutto vorrei ringraziare il prof. Giovanni Vallini per avermi coinvolto ormai più di tre anni fa in quest'avventura e per avermi messo a disposizione il laboratorio in cui è stata portata avanti la ricerca. Per laboratorio, tuttavia, non intendo strumentazioni, reagenti o vetreria d'ogni tipo, ma persone.

Silvia, vera anima del gruppo. Grazie per il tuo fondamentale aiuto, per i tuoi indispensabili consigli, per la tua allegra compagnia e, soprattutto, per la tua preziosa amicizia.

Grazie a tutti i dottorandi del gruppo per aver sopportato il mio incontenibile disordine e supportato in tutti i momenti: Nicola, Manuel, Anita, Chiara e se pur per un breve periodo, Alessia.

Grazie alla mia famiglia, sempre affianco, sempre vicina anche in città o Paesi diversi: mamma Raffaella, papà Giorgio e Francesca. Come poi dimenticare Roberto e i magnifici, meravigliosi stupendi etc etc etc nipotini Fabio e Teresa.

Grazie a tutto lo sterminato parentato di cugini, zii e, soprattutto, la nonna.

Grazie a tutti gli amici con cui ho condiviso serate e nottate a chiacchere davanti ad un boccone ed a bicchiere di vino, pomeriggi a zonzo e mattine a correre. Non voglio fare nomi, sarebbe impossibile non fare torto a qualcuno, ma penso che molti si possano riconoscere da soli.

Grazie a tutte le città, valli, pianure, montagne, mari e oceani in giro per il mondo in cui sono passato ed in cui passerò.

In una sola, semplice parola, grazie!

Conference abstract and proceeding

"4th European Bioremediation Conference" 3-6 September 2008, Chania, Crete (Greece)
"ORAL PRESENTATION"

BIODEGRADATIVE POTENTIAL OF *Burkholderia* sp. DBT1 IN THE ABATEMENT OF POLYCYCLIC AROMATIC HYDROCARBONS

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) represent a class of organic compounds that negatively affect human health. Due to combustion of fossil fuels and organic waste, PAHs are ubiquitous in the environment where they tend to accumulate. These compounds are of toxicological concern because some of them have been identified as carcinogenic, mutagenic, and teratogenic. *Burkholderia* sp. DBT1 is a bacterial strain, first isolated from a dibenzothiophene (DBT) contaminated oil refinery discharge, which can utlize dibenzothiophene, phenanthtrene, fluorene and napthalene as substrates for growth. This strain is capable of degrading DBT nearly completely through the "Kodama pathway" within three days. Molecular characterization of the strain DBT1 has shown the presence of an unusual genetic structure. Actually, strain DBT1 genes involved in dibenzothiophene transformation are harbored in two operons. Further analysis have shown the involvement of these two operons even in phenanthrene catabolism. For its capability to utilize a wide range of substrates including different PAHs, *Burkholderia* DBT1 might be interestingly exploited in bioremediation protocols of PHA-contaminated sites.

1. INTRODUCTION

In the last few decades, the interest for biodegradation mechanisms of polycyclic aromatic hydrocarbons (PAHs) has enormously risen due to the wide distribution of these pollutants in natural environments, with potentially dangerous effects on human health. These compounds are largely suspected to act as potential mutagens, teratogens as well as carcinogens [6]. PAHs are an extended class of organic molecules containing two or more fused aromatic rings. Their molecular stability and hydrophobicity are among the prominent factors that contribute to the persistence of these pollutants in the environment. Moreover, their low aqueous solubility and, consequently, their low bioavailability are a great obstacle to microbial degradation [2]. The presence of PAHs in polluted sites is due to both natural processes (biogenic and geochemical) and anthropogenic activities [10]. These latter account for the major release of PAHs into the environment through mainly either incomplete combustion of organic matrices including fossil fuels or transportation [11]. Of the PAHs occurring in soils and groundwaters, about 0.04 - 5% (wt/wt) are sulfur heterocycles [13], among which dibenzothiophene (DBT) represents the prevailing compound. This is therefore taken into account as model chemical structure in studies dealing with either biodegradation of organo-sulfur contaminants through the "Kodama pathway" [9] or petroleum biodesulfurisation through the "4-S pathway" [7]. *Burkholderia* sp. DBT1 is a bacterial strain identified in an oil refinery wastewater which can degrade DBT nearly completely through the "Kodama pathway" within three days. Molecular characterization of the strain DBT1 has shown unusual genetic features. The genes involved in dibenzothiophene transformation by the strain DBT1 are in fact harbored in two operons (p51 and pH1A) and show low similarity to both *nah*-like and *phn*-like genes [4]. Since normally several organic pollutants contribute together to the contamination at different sites, isolation and characterization of microorganisms able to concomitantly degrade a wide range of substrates might play an important role in the bioreclamation of polluted areas. This work aimed at clarifying the possible role of *Burkholderia* DBT1 in the degradation of PAHs other than condensed thiophenes, frequently occurring in oil-contaminated sites.

2. MATERIALS AND METHODS

2.1 Chemicals

Dibenzothiophene (DBT), naphthalene, fluorene, phenanthrene, 2-carboxybenzaldehyde, phthalic acid, protocatechol, 2-hydroxy-1-naphthoic acid and 1-hydroxy-2-naphthoic acid were purchased from Sigma Aldrich. All the compounds were analytical grade. They were dissolved in *N-N*-dimethylformamide (Sigma Aldrich) before addition to the bacterial cultures.

2.2 Cultivation media and bacterial growth conditions

Burkholderia sp. DBT1 was grown in Yeast Mannitol Broth (YMB): 0.5 g l^{-1} K_2HPO_4; 0.1 g l^{-1} $MgSO_4$ H_2O; 0.1 g l^{-1} NaCl; 0.4 g l^{-1} yeast extract; 10 g l^{-1} mannitol. Yeast Mannitol Agar (YMA) was YMB solidified with 15 g l^{-1} of noble agar. Enrichment cultures were set up in minimal defined medium DM [5] supplemented with one of the selected organic compounds as sole carbon source. *Burkholderia* sp. DBT1 was incubated at 27°C on a rotor shaker at 200 rpm.

2.3 RT-PCR

Burkholderia sp. DBT1 was grown for three days in DM added with phenanthrene at final concentration of 500 ppm. Cells were then pelleted and RNA was obtained by Tryzol reagent (GibcoBRL, Life Tecnology). RT-PCR was carried out with M-MLV Reverse Transcriptase (Promega), using 500 ng of total RNA and, respectively, RT2orf6 primer (TTGTTCCGGTTTACATCGTAGCTC) for the p51 operon or p1RT primer (CGAATTTCCCGAAGTCCCAATT) for the pH1A replicative unit [4]. The reaction conditions for the reverse transcripion were as follows: annealing at 42°C for 10 min and extension at 50°C for 1 h. The PCR reaction using the RT2orf6 primer couple with p1C primer (AGCGGCCAAGCGAATCAATCATTT) was as follows: 94°C for 1 min, 69°C for 1 min, 72°C for 1 min and 30 sec, followed by 25 cycles, 72°C for 5 min. The PCR reaction utlizing the second set of primers, p1RT and p1rev (GGTCGGAAACATGGGGTAATGGA), was performed for 30 cycles as follows: 94°C for 1 min, 64°C for 1 min , 72°C for 1 min and 1 cycle of 5 72°C for 5 min. Positive and negative controls were included during the reactions.

2.4 2-carboxybenzaldehyde dehydrogenase assay

Burkholderia sp. DBT1 was grown to exponential phase in DM or YMB supplemented with phenantrene or 2-carboxybenzaldehyde (500 ppm). Cells were harvested by centrifugation (8000 g for 15 min at 4°C) and washed twice with phosphate buffer (50 mM, pH 7.8). Cells were then suspended in 1.5 ml ice-cold phosphate buffer and sonicated at 4°C for three cycles (8 min at 80 W) using an Ultrasonic Cleaner (model 1200M, Sonica). The homogenate abtained was centrifuged at 13000 rpm for 15 min at 4°C. The surnatant was used as cell-free extract for the enzymatic assay. Protein concentration was determinated through the Bradford assay [1] by using bovine serum albumin as a standard. The activity of 2-carboxybenzaldehyde dehydrogenase was determinated spetrophotometrically by measuring the formation of NADH at 340 nm in phosphate buffer (50 mM, pH 7.8) containing 50 µg of protein, 10 mM NAD^+ and 75 mM 2-carboxybenzaldehyde.

3. RESULTS AND DISCUSSION

3.1 Growth in presence of different organic compounds

Results obtained with growth tests in presence of different PAHs showed that *Burkholderia* sp. DBT1 is able to grow in presence of phenanthrene and, although less efficiently, on DBT as sole source of carbon and energy (Fig. 1). Moreover, DBT1 was also able to utilize naphthalene and fluorene, even if only after an induction period of about 3 days in presence of phenantrene (Fig. 1) or DBT (data not shown).

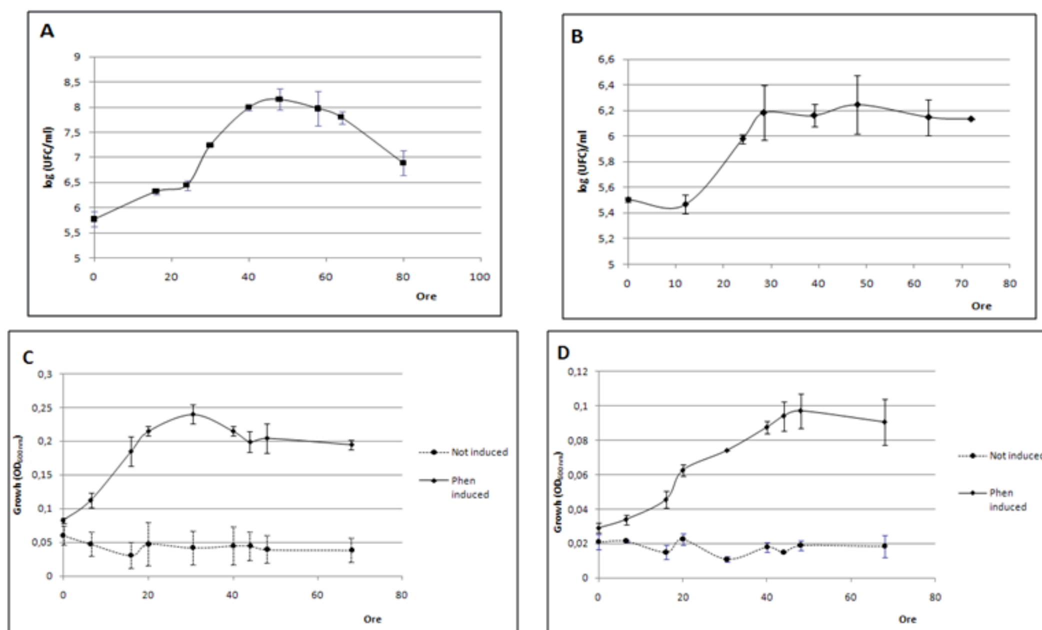

Figure 1: Growth of *Burkholderia* sp.DBT1 in DM supplemented with (A), phenathrene, (B), DBT. (C), fluorene, (D), naphthalene. Concentrations of PAHs were provided at 100 ppm.

These evidences indicate that enzymes for the degradation of naphthalene and fluorene are induced by phenanthrene and DBT. This suggests that these compounds, particularly phenathrene, are the main substrates for *Burkholderia* sp. DBT1. Actually, DBT1 strain was proved to degrade 2-carboxybenzaldehyde, phthalic acid and protocatechol which are common intermediates in the phenanthrene degradation pathway [8]. On the other hand, 2-hydroxy-1-naphthoic acid and 1-hydroxy-2-naphthoic acid – both key intermediates within the two possible upper pathways of phenanthrene degradation – did not support growth when provided even at concentrations ranging from 25 ppm to 1000 ppm. These latter growth tests were performed by using either not induced cells or pre-induced cells with phenanthtrene.

3.2 Study of possible involvement of the two operons in phenanthrene metabolism

Elsewhere studies have shown that, in *Burkholderia* sp. DBT1, genes responsible for DBT degradation are clustered in two operons (p51 and pH1A) instead of in one as usual for the degradation of PAHs within most bacteria [3]. This peculiarity has pushed the authors to verify whether these replicative units are even involved in phenanthrene metabolism. Initially, the presence of specific transcripts (mRNAs) was verified during the growth of *Burkholderia* sp. DBT1 on phenanthrene as sole source of carbon and energy. RT-PCR analyses showed that both operons were strongly transcripted in presence of this compound (Fig. 3).

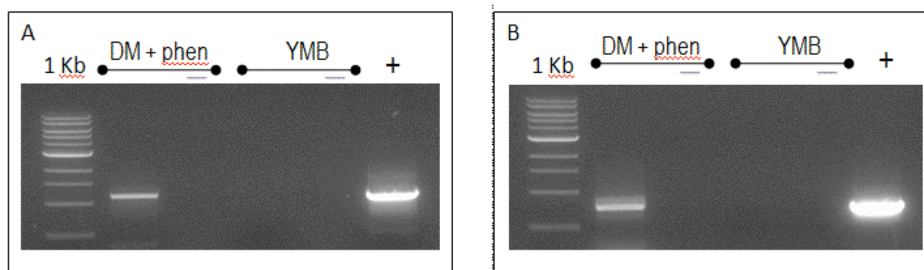

Figure 2: RT-PCR analysis of the transcription of (A) p51 operon utilizing RT2orf6 and p1C sets of primers and (B) pH1A operon performed with p1RT and p1rev couple of primers. (–) is the negative control and (+) is the positive control.

Afterwards, in order to confirm previous evidences,, two insertional mutants (one for each operon) of *Burkholderia* sp. DBT1 were grown in presence of different PAHs [4]. Such experiments revealed that these mutated strains had lost the capacity to growth on phenanthrene as sole carbon and energy source. In this way, the involvement of the operons mentioned above in the abatement of phenanthrene

and dibenzothiophene was attested. Furthermore, both mutants were also capable to degrade phthalic acid and protocatechol. However, only mH1A was proved to grow in presence of 2-carboxybenzaldehyde, while m51 strain whose mutation is inserted in PAH extradiol dioxygenase was not. It is worth noting that, 2-carboxybenzaldehyde is converted in phthalic acid by a 2-carboxybenzaldehyde dehydrogenase through a phenathrene lower pathway [8]. Threfore, in the case of m51 strain, the decresae in biomass production when 2-carboxybenzaldehyde was added to the growth medium might have a twofold explanation: (i) the PAH extradiol dioxygenase is involved downstream of 2-carboxybenzaldehyde in a not phenanthrene catabolic pathway or (ii) m51 mutant has lost 2-carboxybenzaldehyde dehydrogenase activity during the transposon mutagenesis protocol.

3.3 2-carboxybenzaldehyde dehydrogenase assay

An enzymatic assay has been performed to test the 2-carboxybenzaldehyde dehydrogenase activity in m51 strain. The cell-free extracts of bacterial cultures grown either in presence of YMB or DM supplied with 2-carboxybenzaldehyde showed a high activity of this enzyme (Fig. 3).

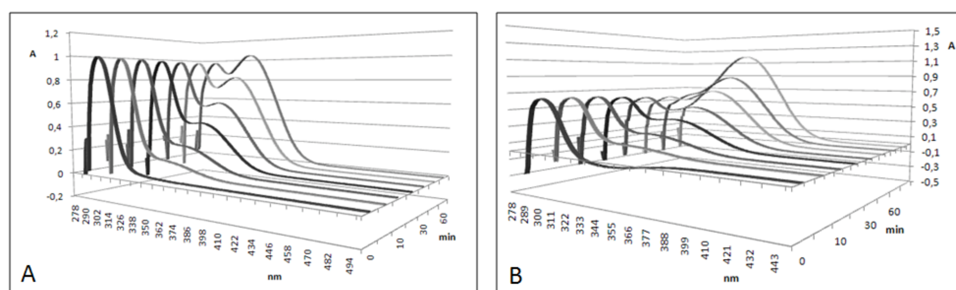

Figure 3: UV-spectra change during the 2-carboxybenzaldehyde dehydrogenase assay derived from (A) *Burkholderia* sp. DBT1cultivated in DM and (B) m51 strain grown in YMB. In both testes the medium was supplied with 2-carboxybenzaldehyde.

Both enzymatic assays and growth tests have shown that *Burkholderia* sp. DBT1 harbors a 2-carboxybenzaldehyde dehydrogenase enzyme. However, strain DBT1 was not able to grow in presence of this molecule as sole source of carbon and energy without the PAH extradiol dioxygenase enzyme, clustered in the p51 operon. These results suggest that this bacterial strain is capable to degrade phenanthrene without 2-carboxybenzaldehyde as intermediate, through a metabolic pathway already founded in previus studies [12] To verify this hypothesis, an enzymatic assay to test the 2-carboxybenzaldehyde dehydrogenase activity has been performed on *Burkholderia* sp. DBT1 cells grown in presence of phenanthrene as sole source of carbon and energy. The results obtained showed no enzymatic activity (data not shown).

CONCLUSIONS

The results so far achieved suggest that the strain DBT1 presents a versatile metabolism towards different PAHs. In fact, this strain is able to use fluorene, naphtalene, DBT and phenanthrene for growing, although growth on the first two compounds needs a pre-induction. Therefore, it can be suggested that phenanthrene is metabolized through 2-carboxycinnamic acid, phthalic acid and further metabilized in protocatechol. Further study with GC-MS are required to clarify the role of 2-carboxybenzaldehyde in PAHs degradation as well as to confirm the catabolic pathway of phenanthrene. Nevertheless, this behavior seems quite interesting for the possible exploitation of *Burkholderia* DBT1 in bioremediation protocols of PHA-contaminated sites.

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“The candidate Andreolli Marco planned and carried out the experiment. Moreover, he wrote the proceeding and presented the work at 4th European Bioremediation Conference by an oral presentation.”

"XXVIII Convegno nazionale SIMGBM" - *Società Italiana di Microbiologia Generale e Biotecnologie Microbiche* - 11-13 June, 2009, Spoleto (It).

"POSTER"

Burkholderia sp. DBT1 a promising bacterial strain for bioremediation protocols non-related to the *Burkholderia cepacia* complex (BCC)

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Burkholderia sp. DBT1 is a bacterial strain formerly isolated from the wastewater dicharge pipeline of an oil refinery located in Tuscuny. It is cable of biodegrading dibenzothiophene (DBT) in liquid culture through the "Kodama pathway" within three days of incubation. Molecular characterization of the strain DBT1 has shown the presence of an unusual genetic structure. Actually, the genes involved in dibenzothiophene transformation are harbored in two operons (namely, p51 and pH1A) and show low similarity to both *nah*-like and *phn*-like genes.

Since DBT results to be a recalcitrant compound and tends to bioaccumulate throughout the food chain, isolation and characterization of bacterial strains able to use it as sole source of carbon and energy is of great interest for bioremediation purposes. Nevertheless for a safe exploitation of *Burkholderia* sp. DBT1 in bioremediation protocols, the exclusion of such strain from the *Burkholderia cepacia* complex (BCC) is of prominent importance. Actually, members of this complex are responsible for opportunistic human infections.

Thus, the objective of this study was to investigate the taxonomic position of DBT1 within the genus *Burkholderia*, in order to demonstrate no affiliation of this strain to BCC. Both classical (API 20E, API 20 NE, fatty acid composition, carbon source utilization) and molecular (PCR protocols, DNA sequencing, DNA-DNA hybridization) analyses were carried out. *Burkholderia* DBT1 has been compared with strains belonging to its phylogenetic surrounding, (namely, *B. fungorum*, *B. graminis*, *B. cepacia* and *B. caledonica*). All results obtained indicate a strong relationship of DBT1 with *B. fungorum*. In particular, the sequencing of both 16S hypervariable regions (V3 and V6-V8) and housekeeping protein-coding genes (i.e. *RecA* and *GyrB*) evidenced such a high similarity with *B. fungorum* as well as a great divergence from *B. cepacia* strains. Moreover, the PCR reaction aimed to detect in DBT1 the presence of *esmR,* a molecular markers encoding for "*B. cepacia* epidemic strain marker" (BCESM), gave a negative result. Finally, the DNA-DNA hybridization analysis definitively revealed the affiliation of DBT1 strain to *Burkholderia fungorum* species, with a percentage of hybridization of 78,2 ± 2,9%.

"The candidate Andreolli Marco planned and carried out the experiment. Moreover, he compose the poster"

"The Final International Conference, COST Action 859" – Phytotechnologies to promote sustainable land use and improve food safety –11-16 October, 2009, Ascona (Ch).

"POSTER"

Birch (*Betula*) and hybrid aspen (*Populus*) for rhizoremediation: depicting the plant associated microbiome and the catabolic capacity

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The adequacy of birch and hybrid aspen for phytotechnology was studied in green house experiments using either polyaromatic hydrocarbons (PAHs) or petroleum hydrocarbons as pollutant. The tree-associated microbes from the different plant compartments were studied in order to take advantage of ecosystem services to benefit bioremediation. Below ground microbial communities were depicted by analysis of functional extradiol dioxygenase genes and structural 16S rRNA genes. The ectomycorrhizal (ECM) fungal diversity was analysed by PCR of the ITS region from the root tips. The PCR products were fingerprinted with PCR-RFLP and T-RFLP. Endophytic and epiphytic bacteria were isolated from all compartments of hybrid aspen and grouped in haplotypes by 16S rRNA analysis. PAH degradation with fifty endophytic bacterial strains was studied for evaluation of their biodegradative potential. In our rhizoremediation project the molecular tool box was successfully used together with bacterial isolation to describe the aspen microbiome.

Woody plant rhizoremediation was carried out and the degradation of PAHs was analysed (gas chromatography/masspectrometry). Biodegradation of PAHs was observed in planted as well as unplanted microcosms, showing differential degradation of individual PAHs. Genetic aromatic ring-cleavage potential was detected in rhizosphere soil demonstrated by higher diversity of extradiol dioxygenase genes than in bulk soil with no plant and *Burkholderia* were dominant in PAH-polluted aspen rhizosphere. The Wales birch clone tolerated high concentrations of PAHs (1200 ppm), whereas aspen was more sensitive to the pollutant, but still tolerated 900 ppm of PAHs. Tolerance tests with petroleum hydrocarbons showed that 10% oil was lethal, whereas 1% was tolerated by the aspen seedlings. ECM mycorrhizal diversity was moderate with 5-7 morphotypes per aspen family. They included *Thelephora*, *Cenococcum geophilum* and *Cortinarius*.

Thirteen aspen associated bacterial strains were able to grow on PAHs as sole source of carbon and energy. All of them were isolated from roots and belonged to the *Burkholderia* genera and same types were part of uncultured bacterial community in PAH-amended soil. Ten strains grouped with *B. fungorum* species, one with *B. sordidicola* and one with *Burkholderia* sp R-701. The behavior of these *Burkholderia* strains toward dibenzothiophene is similar to another and to a very closely related strain: *Burkholderia* sp DBT1. Unexpectedly an amplicon of correct size using two pairs of primers specific for DBT1 p51 and pH1A operons, was obtained for all ten *B. fungorum* and R-701 strains isolated from aspen plants growing on PAHs. The subsequent RFLP analysis of the amplicons generated by PCR from pH1A operon showed the same pattern in all the stains.

The findings improve our understanding of rhizoremediation associated microbes and may benefit bioremediation research by facilitating the development of molecular tools to detect and monitor populations involved in plant maintenance and degradative processes.

"The candidate Andreolli Marco planned and carried out part of the experiments"