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ISOLAMENTO DI BATTERI DEGRADATORI DI ORGANOFOSFORICI DA SUOLI MEDITERRANEI

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Abstract

Organophosphorus pesticides are used for crop production and pest control. Although they are useful in agricultural management, they damage the living organisms because interfere with metabolic processes, furthermore the organophosphorus spread in environmental matrices such as the soil that is an important resource. The aim of this research was to study the biodiversity of organophosphorus degrader bacteria in mediterranean agricultural soils because the bacteria represent an useful source in the bioremediation of contaminated area. The bacteria were isolated from soils under different management systems, 47 strains were able to grow on parathion as carbon source. They were grouped into 20 Operational Taxonomic Units by analysing the Ribosomal Intergenic Spacer. This biodiversity was correlated to such physico-chemical properties of the mediterranean soils. In particular, a positive correlation was found between biodiversity, soil clay and carbon content. Representative isolates for each Operational Taxonomic Unit were randomly chosen for the partial sequencing of 16S rRNA gene. Strains phylogenetically related to *Sinorhizobium*, *Pseudoxanthomonas*, *Streptomyces iakyrus*, *Microbacterium takaoensis* and *Isoptericola dokdonensis* have never mentioned as organophosphorus degraders. The pesticide biodegradation ability was tested on identified strains. It was evaluated in liquid medium and in three standard soils. Solid Phase Micro-Extraction and solid liquid extraction methods were coupled with gas-chromatography and mass-spectrometry for the analysis of resulting organophosphorus compounds. Some of the analyzed strains showed a good ability in organophosphorus degradation.

Riassunto

I pesticidi organofosforici sono utilizzati per migliorare la produzione agricola e per il controllo dei parassiti. Nonostante i benefici effetti sull'agricoltura, i pesticidi danneggiano gli organismi viventi in quanto interferiscono con i processi metabolici e si diffondono nelle matrici ambientali, tra le quali il suolo, che rappresenta un'importante risorsa. Lo scopo del presente lavoro è stato quello di studiare la biodiversità di batteri degradatori di organofosforici, in suoli mediterranei, differenti per il tipo di coltura e le pratiche agronomiche. I batteri rappresentano infatti, un'utile strumento nella bonifica di aree contaminate. Dai suoli, sono stati isolati 47 ceppi, capaci di crescere in presenza di parathion, quale esclusiva fonte di carbonio. I batteri isolati sono stati raggruppati in 20 Unità Tassonomiche Operative, mediante analisi dello Spaziatore Intergenico Ribosomale. La biodiversità batterica è stata quindi correlata alle proprietà chimico-fisiche dei suoli considerati. In particolare si è evidenziata una correlazione positiva tra la biodiversità, il contenuto di argilla e di carbonio. Isolati rappresentativi, per ogni Unità Tassonomica Operativa, sono stati scelti casualmente, per il sequenziamento parziale del gene 16S rRNA. Alcuni ceppi sono stati affiliati a *Pseudoxanthomonas*, *Streptomyces iakyrus*, *Microbacterium takaoensis* e *Isoptericola dokdonensi*, mai citati in bibliografia tra i degradatori di organofosforici. Per alcuni dei ceppi identificati, è stata studiata la capacità di degradare gli organofosforici, in mezzo liquido ed in tre suoli standard. A tal fine, sono state utilizzate la microestrazione in fase solida e l'estrazione con solvente, unitamente alla gascromatografia e alla spettrometria di massa. Alcuni dei batteri esaminati, hanno mostrato una buona abilità degradativa.

Introduzione

L'ecosistema è costituito da un insieme di elementi biotici ed abiotici interagenti tra loro; il suo equilibrio è strettamente connesso all'interazione tra queste componenti che assumono un ruolo fondamentale all'interno dell'ecosistema.

Il suolo è parte integrante di questo ecosistema. Esso rappresenta un sistema naturale dinamico la cui storia è legata ad un insieme di fattori ecologici, climatici, geologici ed antropici. La formazione del suolo deriva, infatti, da una serie di trasformazioni fisiche, chimiche e biologiche della roccia madre che avvengono in milioni di anni. Il suolo può essere pertanto considerato una risorsa non rinnovabile che deve essere preservata nel tempo. Esso svolge una importante funzione in quanto concorre alla crescita delle colture erbacee ed arboree che forniscono cibo, fibre tessili e legname.

Negli ultimi anni si sta assistendo a cambiamenti climatici a livello globale che stanno influenzando l'intero ecosistema, compreso il suolo. Uno di fenomeni più evidenti riguarda l'innalzamento delle temperature che si erano mantenute intorno ad un valore costante nel periodo compreso tra l'anno 1000 e il 1850 per poi crescere all'aumentare della concentrazione di CO_2 (Figg. 1-2).

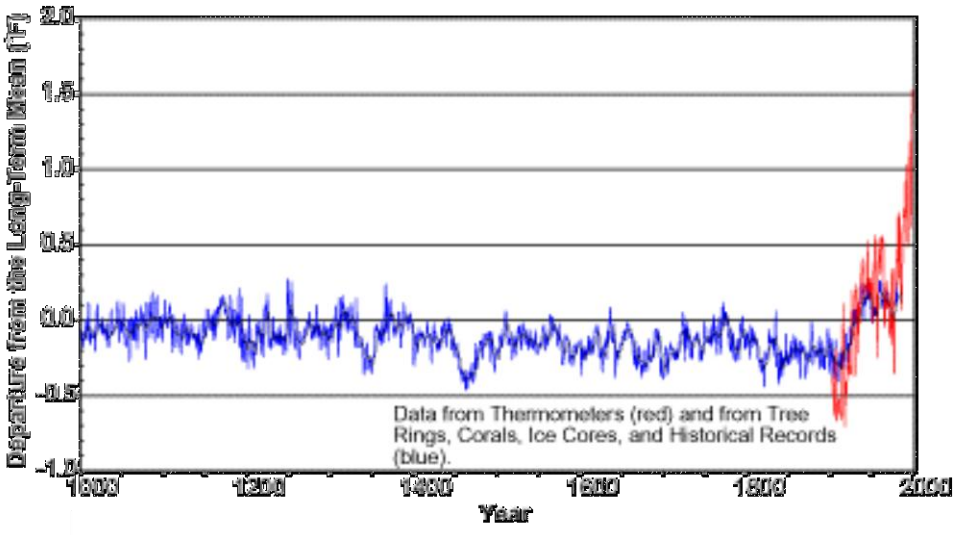

Figura 1 - Variazione della temperatura dell'emisfero nord tra gli anni 1000 e 2000 (fonte: IPCC, 2001)

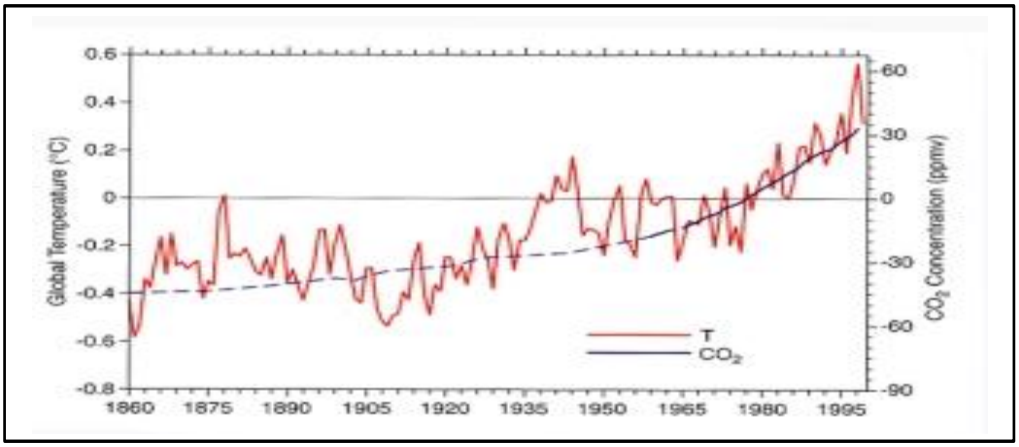

Figura 2 - Variazione della temperatura e della CO_2 tra il 1860 e il 1995 (fonte: IPCC, 2001)

L'innalzamento delle temperature è un fenomeno non indifferente; esso sta infatti causando una variazione del ciclo idrogeologico che avrà delle conseguenze alquanto negative su un'importante risorsa quale il suolo. Le precipitazioni più intense e meno frequenti e gli estremi di temperatura causeranno infatti, un progressivo aumento della salinità ed una graduale degradazione del suolo con un conseguente processo di desertificazione. Questo fenomeno si sta manifestando in buona parte dell'area mediterranea dove alla riduzione della piovosità e ad un innalzamento delle temperature, si aggiunge l'agricoltura intensiva che determina un intenso sfruttamento del suolo (Centritto e Loreto, 2005). Al degrado di tale risorsa sta inoltre contribuendo la deforestazione; alla quale fanno seguito frequenti fenomeni di erosione. In risposta ai citati fattori di disturbo il suolo possiede una capacità naturale di rigenerazione che è comunque limitata; essa può essere infatti compromessa in caso di uno sfruttamento eccessivo della risorsa che può influire sull'attività della microflora con conseguenze sulla fertilità.

Tra gli artefici del degrado ambientale rientra l'uso di fitofarmaci in agricoltura che, se da un lato apporta un contributo alla produzione agricola, dall'altro determina un inquinamento dell'ambiente in genere ed in particolare un inquinamento massiccio del suolo. I fitofarmaci comprendono: fungicidi, insetticidi, acaricidi e nematocidi impiegati per combattere microrganismi, virus, organismi animali e vegetali nocivi alle piante; fitoregolatori utilizzati per

incrementare la produzione e, per finire, diserbanti, repellenti, limacidi e rodenticidi (Conte e Imbroglini, 1994). Uno degli aspetti più tangibili, legato all'uso dei pesticidi, è rappresentato dalla contaminazione alimentare (Carvalho, 2006). In particolare, la frutta si classifica tra le categorie più inquinate con mele, agrumi e uva (Cabras e Angioni, 2000; Parveen *et al*., 2004). Non da meno la contaminazione di prodotti derivati quali il vino e l'olio (Cabras e Angioni, 2000; Amvrazi e Albanis, 2009). Per di più i pesticidi danneggiano gli ecosistemi alterandone l'equilibrio. Tali sostanze diffondono, infatti, nelle matrici ambientali quali l'aria (Kawahara *et al*., 2005), il suolo e l'acqua, per dilavamento dei terreni e attraverso le acque di ruscellamento e drenaggio. I pesticidi distribuendosi nell'ambiente hanno effetti tossici sugli organismi viventi influendo in modo negativo sulla natalità e sulla stessa sopravvivenza. Inoltre, tali composti, entrando a far parte della rete alimentare determinano fenomeni di bioconcentrazione e biomagnificazione (Pierzynski *et al*., 1994). Ad esempio la bioconcentrazione del pesticida nel fitoplancton e nello zooplancton, che sono alla base della catena alimentare, oltre ad incrementare la persistenza dello xenobiotico nell'ambiente, ha effetti negativi ai più alti livelli trofici (De Lorenzo *et al*., 2001).

Uno degli aspetti più importanti che caratterizza il pesticida consiste nella modalità di azione: ovvero il meccanismo mediante il quale esso interagisce con gli organismi viventi. L'assorbimento dei pesticidi da parte degli organismi bersaglio può avvenire per ingestione, contatto e inalazione. Una volta assorbiti, tali composti possono interferire con i processi fisiologici quali la divisione cellulare, la formazione di clorofilla nelle piante e lo sviluppo di tessuti o, con quelli metabolici, come la respirazione, l'attività enzimatica e la fotosintesi (Pierzynski *et al*., 1994).

I pesticidi più tossici appartengono alla classe degli organofosforici (OPs) e clororganici; questi ultimi non essendo biodegradabili hanno un tempo di persistenza ambientale superiore rispetto agli OPs (De Pasquale *et al*., 2005). Per tale motivo gli OPs hanno progressivamente sostituito i clororganici.

Gli OPs hanno effetti tossici sugli organismi viventi; espletando un'azione polivalente agiscono per contatto, ingestione ed inalazione. Una volta entrati nell'organismo gli OPs possono subire trasformazioni metaboliche ad opera di specifici enzimi che in molti casi riducono la loro tossicità ma, talvolta, al contrario la accrescono. I composti facenti parte degli organofosforici presentano proprietà fisico-chimiche differenti. Essi comprendono diversi principi attivi classificati in base ai radicali fosforici ma sono accomunati dallo stesso meccanismo d'azione ovvero l'inibizione irreversibile dell'enzima acetilcolinesterasi (Hurley *et al*., 2005). Questo enzima è deputato all'inattivazione dell'acetilcolina che agisce come neurotrasmettitore dell'impulso nervoso. L'inibizione dell'acetilcolinesterasi causa quindi un'azione protratta sui recettori rendendo difficoltosa la trasmissione degli impulsi nervosi (Ragnarsdottir, 2000). La specifica relazione tra OPs e acetilcolinesterasi è comunemente usata come biomarker nei casi di contaminazione da organofosforici (Yesilada *et al*., 2006).

Gli OPs inoltre danneggiano il sistema cardiovascolare, il sistema riproduttivo, il fegato e hanno effetti tossici sulle cellule, con conseguenti alterazioni sui mitocondri, anomalie cromosomiche, inibizione della sintesi proteica e del metabolismo del glucosio (Carlson *et al*., 2000; Kalipci *et al*., 2010).

Considerando che gli OPs esplicano un'azione non selettiva su tutti gli esseri viventi, diventa importante valutare i fattori ambientali e i processi di degradazione che concorrono a determinarne il destino nelle diverse matrici ambientali. Tra gli aspetti responsabili della distribuzione degli OPs nell'ambiente sono da citare gli effluenti di irrigazione (Müller *et al*., 2007), la volatilizzazione (Bedos *et al*., 2009), l'azione del vento, l'absorbimento (a livello cellulare) che consiste nella concentrazione del principio attivo da parte delle radici o degli animali e, per ultimo, l'adsorbimento (superficiale) da parte delle piante e del suolo. Quest'ultimo aspetto è particolarmente degno di nota in quanto l'interazione tra il suolo ed i contaminanti organici riduce la mobilità del pesticida nell'ambiente e influisce sull'attività biodegradativa (Pierzynski *et al*., 1994).

L’argilla e la matrice organica rappresentano i principali protagonisti di questa interazione (Pierzynski *et al*., 1994).

Tra i processi di degradazione rientrano la fotodecomposizione (Kiss e Virág, 2009), la degradazione chimica, la degradazione metabolica e, per finire, la biodegradazione, ovvero il processo tramite il quale gli organismi viventi degradano gli inquinanti organici pericolosi (Heinaru *et al*., 2005; Paul *et al*., 2005; Singh *et al*., 2006).

I principali responsabili della decomposizione sono i batteri e i funghi (Singh e Walker, 2006). Gli ambienti contaminati sono infatti, colonizzati da microrganismi che, sottoposti ad una pressione selettiva, metabolizzano alcuni inquinanti, tossici per la maggior parte degli organismi viventi.

Altri organismi sono comunque coinvolti nel determinare il destino dei pesticidi ovvero gli invertebrati mediante ingestione e le piante mediante enzimi; in seguito all’assorbimento da parte delle radici (Wild, 1993).

I processi di “bioremediation” ad opera di microrganismi apportano tuttavia il contributo maggiore alla decontaminazione o alla trasformazione dei fitofarmaci. A tal proposito i batteri rappresentano la frazione più consistente. Nel suolo essi costituiscono una ricchissima diversità biologica la cui distribuzione non è uniforme; la popolazione aumenta infatti, in presenza di substrato organico. Vicino le radici ad esempio la diversità può essere 100 volte più alta che nel resto del suolo (Wild, 1993). I batteri del suolo sono presenti con una densità molto elevata; compresa tra 106 e 109 batteri/grammo di suolo (Dommergues e Mangenot, 1970), a differenza dai funghi, presenti con una densità di 104-106 unità/grammo di suolo (Paszczynski e Crawford, 2000). I batteri, inoltre, mostrano una notevole capacità di occupare le più svariate nicchie ecologiche. Essi riescono, infatti, a sopravvivere in tutti i suoli, siano essi acidi o alcalini, ricchi di acqua o ben drenati, in zone molto calde o fredde, umide o secche (Wild, 1993). Alla luce di quanto detto, la potenziale eterogeneità e variabilità dei batteri è probabilmente più grande di ogni altro gruppo di organismi (Morris *et al*., 2002) sebbene una vasta gamma di batteri esistenti in natura sia ancora sconosciuta.

Considerando l'importante ruolo svolto dai ceppi batterici nella bonifica dei siti contaminati, la conservazione della biodiversità batterica si pone come valido mezzo per la salvaguardia dell'ambiente. La biodiversità è un elemento fondamentale per il funzionamento dell'ecosistema (Balvanera *et al*., 2006); variazioni anche minime nella composizione delle specie possono infatti alterarne il funzionamento con conseguenze negative sulle altre specie viventi e sull'ambiente in genere. Un più elevato numero di specie con differente capacità adattativa è quindi indice di maggiore stabilità per l'ecosistema (Clark e McLachlan, 2003).

L'accurata conservazione della comunità batterica diventa quindi un elemento basilare per lo sviluppo sostenibile ed in particolare per una corretta gestione del suolo che consenta di mantenerne la capacità produttiva mediante l'impiego di tecnologie di biorisanamento. Tali tecnologie permettono di bonificare il terreno senza comprometterne la fertilità garantendo in tal modo uno sviluppo che non compromette quello delle generazioni future. La comunità microbica svolge peraltro un ruolo di importanza fondamentale oltre che nel mantenimento degli equilibri dell'ecosistema, nei cicli biogeochimici e nei processi pedogenetici. Inoltre all'interno della comunità si ritrovano ceppi azotofissatori, molto utili per la crescita e lo sviluppo delle piante.

Il biorisanamento da fitofarmaci, ad opera dei microrganismi del suolo, può avvenire secondo processi di mineralizzazione, co-metabolismo, polimerizzazione e coniugazione, accumulo ed effetti secondari dell'attività microbica (Gianfreda, 1996). Nella mineralizzazione il pesticida viene utilizzato come substrato di crescita (Pierzynski *et al*., 1994). In questo caso la via metabolica seguita dipende strettamente dalla funzione che il composto svolge per lo specifico batterio, il quale può utilizzare il principio attivo per supplire uno o più elementi come carbonio, fosforo o zolfo (Singh e Walker, 2006). La biodegradazione è considerata il meccanismo principale che trasforma gli inquinanti organici in prodotti inorganici come CO_2, H_2O ed elementi minerali (Pierzynski *et al*., 1994). Nel co-metabolismo il fitofarmaco viene degradato mediante processi metabolici

ma non rappresenta una fonte di energia per il batterio (Serdar *et al*., 1982; Pierzynski *et al*., 1994; Horne *et al*., 2002). I principi attivi sono talvolta trasformati in intermedi che possono presentare una tossicità maggiore rispetto al composto di partenza, accrescendo in tal modo gli effetti negativi sull'ambiente in genere. I prodotti del co-metabolismo possono in altri casi diventare substrato di crescita per un altro batterio.

Per quanto riguarda gli altri processi coinvolti nella trasformazione del fitofarmaco, la polimerizzazione e la coniugazione mediate da batteri, consistono nella formazione di macromolecole per effetto di un legame tra il fitofarmaco ed altri pesticidi o composti naturali (Pierzynski *et al*., 1994). Nell'accumulazione il composto tossico viene invece incorporato all'interno delle cellule batteriche (Pierzynski *et al*., 1994). Per finire, l'attività microbica può causare la variazione di alcuni parametri, quali ad esempio il pH, con effetti secondari sul fitofarmaco (Pierzynski *et al*., 1994).

Considerando la tossicità dei pesticidi e le relative implicazioni sull'intero ecosistema, la conoscenza e l'impiego di microrganismi coinvolti nella loro biodegradazione, diventa un importante mezzo per la salvaguardia dell'ambiente e quindi dell'uomo.

La degradazione degli inquinanti ad opera di microrganismi, infatti, si pone come valida alternativa ai metodi convenzionali fisico-chimici di risanamento che possono risultare talvolta inefficaci e costosi.

Nel 1973 è stato isolato il primo microrganismo, appartenente al genere *Flavobacterium*, in grado di metabolizzare gli organofosforici (Somara e Siddavattam, 1995). Studi successivi hanno evidenziato la presenza di un notevole numero di specie tra Gram positivi e negativi, capaci di degradare un ampio insieme di pesticidi organofosforici (Singht e Walker, 2006).

La capacità di demolire composti organici del fosforo probabilmente si sviluppò con i primi procarioti, circa 3,5 miliardi di anni fa. Tali organismi, infatti, ricavano molecole di adenosina trifosfato (ATP) mediante la respirazione cellulare. L'ATP è una molecola ad alto contenuto energetico che funge da anello

di congiunzione tra il catabolismo e l'anabolismo. Tale composto, infatti, mette in gioco energia mediante l'idrolisi, una reazione catalizzata dall'enzima ATPasi che coinvolge il trasferimento di un gruppo fosfato. L'ADP che si genera da questo processo viene successivamente riconvertito in ATP.

La formula generale degli organofosforici è mostrata in figura 3. I gruppi R_1 e R_2, generalmente appartenenti ad un gruppo arilico o alchilico, sono legati ad un atomo di fosforo direttamente o attraverso un atomo di zolfo o di ossigeno. Il gruppo X può appartenere alla categoria dei composti alifatici, aromatici o eterociclici .

Il principale passo nel processo di biodegradazione degli organofosforici è rappresentato dall'idrolisi del legame estereo con rilascio del gruppo X (Fig. 3) (Singht & Walker, 2006).

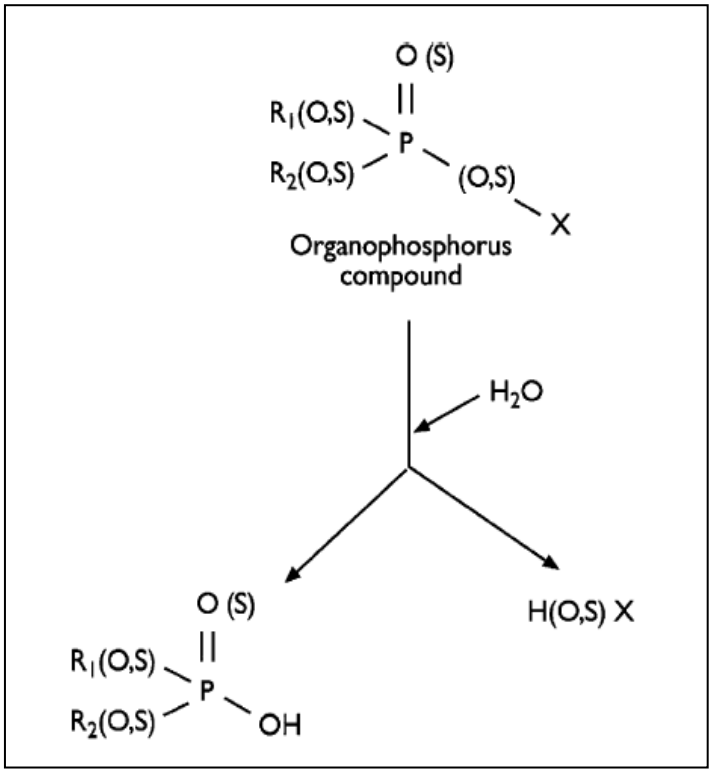

Figura 3 - Formula generale degli organofosforici e principale via di degradazione (fonte: Singh e Walker, 2006)

Tra i pesticidi organofosforici rientra il parathion (Fig. 4). Quest'ultimo ampiamente impiegato in passato su diverse colture: agrumi, drupacee, pomacee, fragola, castagno, noce, vite, olivo e ortaggi (Conte e Imbroglini, 1994) per la sua scarsa selettività è stato classificato dall' US Environmental Protection Agency (EPA) tra i pesticidi più tossici (Singht e Walker, 2006). L'estrema nocività di tale principio attivo è stata dimostrata dagli effetti negativi sull'uomo e su diverse specie animali (Puga e Rodrigues, 1996; McConnell *et al*., 1999).

Il parathion oltre ad inibire l'acetilcolinesterasi, interagisce con il DNA o con l'RNA (Rodriguez *et al*., 2005). Inoltre mediante reazioni ossidative tale composto è trasformato in un prodotto più reattivo ovvero il paraoxon (Rodriguez *et al*., 2005).

La principale via di degradazione del parathion consiste nell'idrolisi con produzione di un intermedio di degradazione ovvero il para-nitro-fenolo (Singh e Walker, 2006). Tra i degradatori di parathion sono noti ceppi appartenenti ai generi *Flavobacterium*, *Pseudomonas*, *Bacillus*, *Arthrobacter*, *Xanthomonas*, *Agrobacterium* (Singht e Walker, 2006). Alcuni batteri mineralizzano il parathion utilizzandolo come fonte di carbonio (Munnecke e Hsieh, 1976; Rani e Lalithakumari, 1994) o di fosforo (Rosenberg e Alexander, 1979).

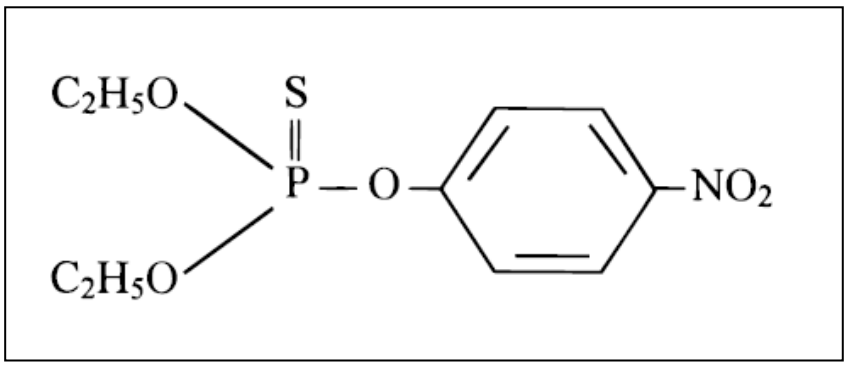

Figura 4 - Formula del parathion

Considerando la tossicità del parathion, il diffuso impiego in passato di tale pesticida e l'importanza nel preservare la biodiversità di un territorio ai fini ambientali, lo scopo del presente lavoro è stato quello di valutare la biodiversità di batteri degradatori dell'organofosforico parathion in suoli mediterranei. Inoltre dai riferimenti bibliografici si evince che il parathion rappresenta un modello per valutare l'abilità dei batteri nella degradazione dei pesticidi organofosforici (Choi *et al*., 2009). Per l'isolamento batterico si è scelto di mettere a confronto campioni di suolo prelevati da diverse località della Sicilia, differenti per il tipo di coltura e le pratiche agronomiche. I suddetti suoli sono stati studiati da un punto di vista chimico-fisico per conoscere l'ambiente di crescita dei ceppi isolati, dal momento che la qualità e la fertilità del suolo dipendono dall'interazione tra le componenti chimico-fisiche e biologiche (Kennedy e Smith, 1995). In particolare alle proprietà fisiche come ad esempio la tessitura sono collegate alcune importanti caratteristiche dei suoli quali l'aerazione, la permeabilità, la capacità di ritenzione idrica e la disponibilità di elementi nutritivi mentre le proprietà chimiche ovvero la concentrazione dei costituenti organici ed inorganici, influiscono sulla fertilità del suolo, l'attività biologica, il grado di inquinamento e la salinità (Pierzynski *et al*., 1994). La componente biologica a sua volta assume un'importanza fondamentale nella decomposizione di residui animali e vegetali contribuendo alla costituzione della frazione organica del suolo. La matrice organica determina la formazione (Lundström *et al*. 1995) e la struttura del suolo (Stevenson, 1994) giocando un importante ruolo nella chimica del terreno. Alcune proprietà del suolo come la presenza di macro e micronutrienti, la capacità di scambio cationico e il pH sono determinate dalla componente organica (Pierzynski *et al*., 1994). La matrice organica inoltre svolge una importante funzione nel trasporto dei nutrienti e dei contaminanti (Marschner, 1998; Kalbitz e Popp, 1999; Zsolnay, 2003). Essa è capace di assorbire alcuni elementi potenzialmente tossici per gli organismi viventi quali piombo, cadmio e rame, riducendone in tal modo la diffusione in ambiente acquatico. Anche i pesticidi ed altri composti organici possono essere assorbiti dalla frazione organica con un duplice vantaggio: ridurre la diffusione

degli inquinanti nell'ambiente e rendere i pesticidi più disponibili per la degradazione batterica (Pierzynski *et al*., 1994). Tale processo richiede comunque la presenza di condizioni idonee per la crescita della popolazione batterica. I fattori più importanti sono l'ossigeno, la temperatura, l'umidità, l'acidità e i nutrienti organici e inorganici. Per quanto riguarda il fabbisogno di ossigeno, i batteri si distinguono in aerobi, anaerobi ed anaerobi facoltativi che possono crescere anche in condizioni di scarsa aerazione. L'ottimo di temperatura e di umidità per molti batteri oscillano rispettivamente tra 20-40^{o}C e 50-75%. Estremi valori di pH non sono generalmente adatti per la crescita dei batteri (Pierzynski *et al*., 1994). Per finire, la disponibilità dei nutrienti è un elemento determinate per la crescita batterica.

La capacità degradativa dei ceppi isolati nel corso del presente lavoro è stata valutata in condizioni controllate di laboratorio. I batteri sono stati isolati in coltura pura, secondo la tecnica classica di analisi delle comunità microbiche. Ogni colonia è stata quindi piastrata su MSM (Mineral Salt Medium) agar, contenete parathion come esclusiva fonte di carbonio. I ceppi sono stati studiati mediante analisi del gene 16S rRNA e dello spaziatore intergenico tra i geni 16S e 23S (Internal Transcribed Spacer, ITS). Il primo, presente in tutti i procarioti nell'operone ribosomale, codifica per l'RNA ribosomale 16S, costituente della subunità minore del ribosoma dei procarioti. Poiché tale molecola è coinvolta nel processo di sintesi proteica, è indispensabile alla vita, non è influenzata dall'ambiente esterno, né soggetta a trasferimento laterale. La sequenza del gene 16S pertanto varia esclusivamente in funzione del tempo di divergenza tra le specie; motivo per cui il gene 16S rappresenta un utile orologio molecolare. Inoltre, considerando l'abbondanza di informazioni in banca dati, tale gene può essere impiegato nell'identificazione dei ceppi batterici. Lo spaziatore intergenico 16S-23S ITS è una regione capace di registrare mutazioni senza compromettere la vita degli organismi in quanto non codifica per alcun prodotto finale. Questa regione dell'operone ribosomale presenta sia polimorfismi di sequenza che di lunghezza e la sua analisi permette di discriminare i batteri sia a livello inter-

specifico che intra-specifico. Alla luce di quanto detto, per valutare la diversità dei ceppi batterici isolati, è stata utilizzata l'analisi dei polimorfismi di lunghezza dello spaziatore intergenico ribosomale (Fisher e Triplett, 1999). In seguito all'amplificazione del frammento di DNA si ottiene, infatti, un profilo diverso per ciascun batterio che viene quindi assegnato ad una OTU (Unità Tassonomica Operativa). Si tratta quindi di una tecnica utilmente sfruttata per la valutazione della biodiversità (Hugo *et al*., 2003) e per la classificazione dei ceppi batterici (De Angelis *et al*., 2007; Cardinale *et al*., 2008). La biodiversità dei batteri isolati, evidenziata mediante tale tecnica, è stata correlata alle proprietà chimico-fisiche del suolo. Per ciascun ceppo è stata in seguito analizzata l'abilità nell'utilizzare il parathion quale fonte di carbonio in mezzo liquido e in suoli standard. La capacità degradativa, in entrambi i mezzi, è stata osservata mediante analisi in gas-cromatografia e spettrometria di massa del parathion residuo, estratto dopo un periodo di incubazione. Per l'estrazione del pesticida dai microcosmi è stata utilizzata la tecnica classica di estrazione mediante solvente mentre in mezzo liquido il parathion è stato estratto con la microestrazione in fase solida (SPME).

Il vantaggio della microestrazione in fase solida consiste nel combinare la preparazione, l'estrazione e il campionamento degli analiti in una singola fase; caratteristica che rende questa procedura veloce rispetto alle classiche e dispendiose fasi che caratterizzano l'estrazione con solvente. Inoltre si tratta di una tecnica facilmente accoppiabile alle analisi cromatografiche. Per l'estrazione in SPME viene utilizzata una fibra di silice rivestita da una appropriata fase adsorbente, rappresentata principalmente dal polidimetilsilossano (PDMS, 100 mm) per analiti non polari, dal poliacrilato (PA, 85 mm) o dalla carbowax (CW-DVB, 65 mm) per composti polari. L'estrazione avviene per immersione diretta della fibra nel campione da analizzare o in spazio di testa. Durante il campionamento gli analiti vengono trasportati dalla matrice alla fibra. Si tratta quindi di un processo di equilibrio nel quale la quantità di analiti estratti dalla fibra dipende dalla costante di distribuzione fibra/matrice, dalla superficie della fibra, dal volume del campione e dalla concentrazione iniziale di analiti. Inoltre,

trattandosi di una tecnica non esaustiva il metodo di campionamento, il tipo di fibra e la temperatura di estrazione, che influisce sulla pressione di vapore degli analiti, sono determinanti per la cinetica di estrazione.

L'SPME è stata applicata con successo per lo studio di varie sostanze organiche quali pesticidi in acqua, suoli e altre matrici più complesse come per esempio gli alimenti. Infine tale tecnica oltre ad essere più veloce consente di ridurre gli errori dell'operatore legati alle fasi di purificazione.

Materiali e Metodi

Campionamento dei suoli e analisi chimico-fisiche

Per l'isolamento batterico sono stati scelti campioni di suolo provenienti da differenti località della Sicilia occidentale: Partinico, Misilmeri e Ribera e diversi tra loro per il tipo di coltura e le pratiche agronomiche (Tab. 1).

Codice identificativo	Origine	Coltura	Pratica agronomica
VCH	Partinico	vigna	convenzionale
APCH	Misilmeri	agrumi	convenzionale
POV	Partinico	pomodoro	convenzionale
KAD	Misilmeri	cachi	convenzionale
RIBCON	Ribera	agrumi	organica
RIBBIO	Ribera	agrumi	organica
RIBFAN	Ribera	agrumi	convenzionale
RIBINO	Ribera	agrumi	convenzionale

Tabella 1 - Codice identificativo dei suoli, provenienza, coltura e pratiche agronomiche

Il campionamento è avvenuto nei primi 3 cm di profondità. I suoli utilizzati come fonte di inoculo sono stati analizzati da un punto di vista chimico-fisico per evidenziare una eventuale relazione tra la biodiversità batterica e le caratteristiche di ciascun suolo. Un'aliquota dei campioni è stata quindi essiccata all'aria e filtrata per ottenere una frazione di suolo di dimensione inferiore ai 2 mm. La terra fine è stata infine utilizzata per lo studio delle proprietà chimico-fisiche. Per la determinazione del pH è stata preparata una soluzione di suolo e acqua distillata (1:2.5 *w*/*v*). Il pH è stato quindi misurato mediante un elettrodo a vetro (Mettler-

Toledo). La conduttività elettrica (EC) è stata determinata su una soluzione di suolo e acqua distillata in rapporto 1:5 (*w*/*v*). Per valutare la capacità di scambio cationico (CEC) è stata utilizzata una soluzione di cloruro di bario e trietanolammina a pH 8.2. La percentuale di carbonio è stata misurata utilizzando il metodo di Walkley–Black basato sull'ossidazione con dicromato (Ministero delle Politiche Agricole e Forestali, 1999).

Isolamento batterico

L'allestimento delle colture di arricchimento è avvenuto dopo 24 h dalla raccolta dei suoli, in modo da lavorare in condizioni biologiche inalterate. Ciascun campione (2 g) è stato aggiunto a 20 ml di una soluzione di MSM (Cullington e Walker, 1998) pH 7, contenente 400 µl di una soluzione di parathion in esano/acetato di etile (1:1) come fonte di carbonio. Quest'ultima è stata aggiunta all'MSM previa sterilizzazione, utilizzando appositi filtri da 0.2 µm (Filtro Siringa PTFE, mdi), fino a raggiungere una concentrazione finale di 20 µg ml^{-1}. Le colture di arricchimento sono state incubate a 30 °C su un agitatore orizzontale a 200 r.p.m. per quattro giorni. Successivamente 2 ml di ogni coltura sono stati re-inoculati in MSM fresco contenente una concentrazione doppia di parathion. Le colture di arricchimento sono state quindi incubate nelle stesse condizioni. Aliquote delle colture sono state diluite serialmente e piastrate su MSM agar, contenente parathion come fonte di carbonio (40 µg ml^{-1}). Le piastre sono state incubate at 30 °C per quattro giorni. Le colonie cresciute in ogni piastra sono state distinte in base ad alcuni caratteri fenotipici quali il colore, la morfologia e la colorazione di Gram. Ogni colonia è stata quindi piastrata sullo stesso mezzo fino ad ottenere colonie pure. Ogni ceppo isolato è stato infine conservato in glicerolo al 20% a -80°C.

Preparazione del mezzo liquido di crescita MSM

La soluzione di MSM è stata preparata a partire da tre soluzioni secondo la procedura seguente :

Soluzione 1

KH_2PO_4 (g)	11.35
$Na_2HPO_4x12H_2O$ (g)	29.85
NaCl (g)	5

Portare a 500 ml con acqua distillata e autoclavare

Soluzione 2

$MgSO_4x7H_2O$ (g)	2.5
$CaCl_2x2H_2O$ (g)	0.05
$MnSO_4x4H_2O$ (g)	0.1

Portare a 500 ml con acqua distillata e autoclavare

Soluzione 3

$FeSO_4$ (g)	0.25

Portare a 100 ml con acqua distillata e sterilizzare per filtrazione (filtri ALBET da 0,20 μm).

In una bottiglia contente 790 ml acqua distillata sterile aggiungere le soluzione 1 e 2 in ragione di 100 ml ciascuna. Aggiungere infine 10 ml di soluzione di ferro.

Colorazione di Gram

Reagenti utilizzati:

- Cristal violetto
- Lugol
- Alcol al 95%
- Safranina

Procedura:

Allestire i vetrini

Colorare con cristal violetto e lasciare agire per circa un minuto

Lavare con acqua

Coprire il preparato con lugol per un minuto

Sciacquare con acqua

Decolorare con alcol al 95%

Lavare con acqua

Applicare safranina per 45 s

Sciacquare con acqua

Amplificazione dello spaziatore ribosomale tra i geni 16S e 23S dell'operone ribosomale

Il 16S-23S ITS dei ceppi isolati è stato amplificato mediante colony-PCR (Polymerase Chain Reaction). Tale metodica consente di amplificare frammenti di DNA direttamente dal lisato cellulare. Il processo di lisi si articola nelle seguenti fasi:

- Prelevare un frammento di colonia con un'ansa sterile e porlo in un eppendorf
- Aggiungere 25 µl di TE (tampone Tris-EDTA, pH 8)
- Mettere l'eppendorf in acqua bollente per circa 3 min
- Porre l'eppenorf in ghiaccio per 5 min
- Centrifugare a 14000 rpm per 5 min
- Recuperare 1 µl come templato per la PCR

Per l'amplificazione sono stati utilizzati i primers ITSF/ITSReub (Cardinale *et al*., 2004). La miscela di reazione per la PCR comprendeva i seguenti componenti in un volume finale di 20 µl:

1 µl di lisato cellulare di ogni colonia singola come templato

0.3 µM di ogni primer

0.2 mM di deossinucleotidi trifosfati

Buffer 1X

1.5 mM di $MgCl_2$

0.05 u/µl di HotStarTaq® DNA Polimerasi (QIAGEN)

Il profilo di temperatura è stato il seguente:

95 ^{o}C for 20 min

30 cycles of 1 min at 95 ^{o}C

1 min at 55 ^{o}C

1.5 min at 72 ^{o}C

72 ^{o}C for 10 min

I prodotti di PCR sono stati visualizzati su un gel di agarosio all'1,5% in TBE 1X, mediante colorazione con Etidio Bromuro 0,0015%.

I frammenti sono stati confrontati con un DNA marker (DNA Ladder Mix, MBI Fermentas) di peso molecolare noto. Gli isolati con lo stesso profilo ITS sono stati raggruppati nelle stesse OTUs. In base al numero di OTUs è stato possibile valutare la biodiversità dei ceppi isolati ed in particolare la biodiversità per ciascun suolo.

Amplificazione del gene 16S rRNA

Uno o più ceppi per ogni OTU sono stati scelti casualmente per il sequenziamento parziale del gene 16S rRNA (Janssen, 2006) e l'identificazione filogenetica.

Il gene 16S rRNA è stato amplificato utilizzando i primers fD1/rD1 (Weisburg *et al*., 1991) e secondo il protocollo suddetto. I prodotti di amplificazione sono stati purificati utilizzando il QIAquick Gel Extraction Kit (QIAGEN) e sequenziati presso il BMR genomics. Le sequenze ottenute sono state confrontate con quelle note presenti in banca dati mediante il programma BLAST (Altschul *et al*., 1997; http://www.ncbi.nlm.nih.gov/BLAST/). Le sequenze identificate nell'ambito del presente lavoro sono state infine inserite nel GenBank/EMPL/DDBJ database, ciascuna con uno specifico numero di accessione compreso tra GU902282 e GU902303.

Prova di degradazione del parathion in MSM

La capacità degradativa del parathion in MSM liquido è stata verificata su 9 differenti ceppi batterici. L'inoculo è stato aggiunto ad una soluzione di MSM sterile in seguito a contaminazione con parathion.

Fasi di preparazione dell'inoculo

- Prelevare una parte della colonia batterica con un'ansa sterile
- Sospendere la colonia in 3 ml di LB sterile (Luria-Bertani broth-Difco)
- Incubare a 30^{o}C al buio, su un agitatore orizzontale per 24 ore a 150 rpm
- Risospendere 100µl di pre-inoculo in 5 ml di LB sterile
- Incubare l'inoculo per 24h a 30^{o}C al buio su un agitatore orizzontale
- Centrifugare l'inoculo a 4000 rpm per 20 min
- Eliminare il supernatante
- Aggiungere 5 ml di MSM sterile
- Agitare in vortex
- Centrifugare a 4000 rpm per 20 min
- Eliminare il supernatante
- Ripetere la procedura altre due volte
- Risospendere la coltura in MSM sterile fino ad una OD pari a 590 nm

Contaminazione del mezzo di crescita con parathion e inoculazione con i ceppi batterici

Parallelamente alla preparazione dell'inoculo, 20 ml di MSM sterile, per ciascun ceppo, sono stati contaminati con una soluzione 1000 µg ml^{-1} di parathion (1:1 esano/acetato di etile), fino a raggiungere una concentrazione finale di 15 µg ml^{-1}. La soluzione di pesticida è stata aggiunta previa sterilizzazione con filtri da 0.2 µm (Filtro Siringa PTFE, mdi). L'MSM contaminato è stato posto per 24h su un agitatore orizzontale; si è quindi proceduto all'evaporazione del solvente sotto flusso di azoto. Alla soluzione contaminata sono stati aggiunti 0.5 ml di inoculo. L'esperimento è stato ripetuto in triplicato per ciascun ceppo. Sono stati inoltre

allestiti due controlli, rispettivamente senza ceppo batterico (abiotico) e senza pesticida. I campioni sono stati incubati al buio, per 72 h, a 30 ^{o}C su un agitatore orizzontale a 150 r.p.m.

Studio della procedura estrattiva in SPME

La procedura analitica precedentemente sviluppata da De Pasquale *et. al.*, 2005 è stata investigata in MSM liquido per valutare l'efficienza di tale metodica nei sistemi analizzati. Sono state testate tre fibre: PDMS, PA e CW-DVB, tutte provviste di holder (Supelco UK) per l'iniezione manuale. Prima dell'uso le fibre sono state condizionate; la PDMS a 250 ^{o}C per 30 min, la PA a 300 ^{o}C per 2 h e la CW-DVB a 220 ^{o}C per 30 min. Ognuna è stata inserita nella porta di iniezione del GC in un liner avente un diametro di 0.75 mm.

Gli studi sono stati condotti in triplicato in sistemi non inoculati a pH 5 con il 10 % di NaCl (*w*/*v*). Il coefficiente di partizione (*Kd*) per il parathion è stato esaminato mediante valutazione della quantità di analita absorbito o adsorbito dalla fibra di SPME rispetto al contenuto iniziale di pesticida nel sistema.

Estrazione del parathion residuo dal mezzo liquido

Il parathion residuo, dopo incubazione, nelle colture di MSM è stato estratto, purificato e concentrato simultaneamente mediante SPME come precedentemente affermato. Il pH di ogni campione è stato portato a 5 mediante aggiunta di soluzioni di NaOH o HCl (0.1, 0.5, 1 M). In seguito, 20 ml della coltura sono stati trasferiti in vials da 40 ml. L'estrazione del parathion residuo è avvenuta per immersione della fibra nella coltura, a temperatura ambiente (20 °C) per un tempo di 15 min. Durante questo intervallo il campione è stato posto in agitazione.

Prova di degradazione del parathion nei microcosmi

L'abilità degradativa in suoli standard è stata valutata su due isolati batterici. Per il suddetto esperimento sono stati presi in considerazione tre differenti suoli denominati 2.1, 6S e 3A, rispettivamente sabbioso, sabbioso-argilloso e limoso (De Pasquale *et al*., 2005). I suoli standard sono stati acquistati da Landwirtschaftiche Untersuchungs und Forschhungsanstalt (LUFA) Speyer (Germania). Ogni suolo (2g) è stato autoclavato e contaminato con una soluzione di parathion (esano/acetato di etile 1:1) previa sterilizzazione con filtri da 0.2 µm (Filtro Siringa PTFE, mdi) fino a raggiungere una concentrazione finale di 100 µg ml^{-1}. Dopo 24h di agitazione, il solvente è stato fatto evaporare sotto flusso di azoto. I microcosmi sono stati quindi inoculati con 0.5 ml di inoculo, preparato secondo il protocollo descritto precedentemente, ed incubati su un agitatore orizzontale al buio, per 72 h, a 30^{o}C. Sono stati inoltre allestiti due controlli rispettivamente abiotico e non contaminato.

Procedura seguita per l'estrazione del parathion residuo dai microcosmi

Il parathion residuo presente nei microcosmi è stato estratto in base al seguente protocollo: a ciascun campione sono stati aggiunti 3 ml di acqua distillata, la coltura è stata quindi miscelata in ultra-turrax (IKA T18 Basic). Sono stati aggiunti 2g di Na_2SO_4 e 3g di $NaHCO_3$. Sono stati infine addizionati 30 ml di acetato di etile. Il campione è stato quindi posto in agitazione per 1h su agitatore orizzontale. Il supernatante recuperato è stato filtrato mediante un filtro Millipore e filtri in PTFE da 0.45 µm (ALBET). L'estratto è stato concentrato mediante evaporazione in rotavapor (HEIDOLPH LABOROTA 4000 EFFICIENT, WB ECO) a 40 ^{o}C. Infine il campione è stato portato ad un volume finale di 5 ml mediante aggiunta di acetato di etile.

Analisi della capacità degradativa mediante gas-cromatografia e spettrometria di massa

L'abilità degradativa nel mezzo liquido e nei suoli standard è stata valutata mediante uno studio della percentuale di parathion residuo dopo incubazione. Tale parametro è stato confrontato rispettivamente con i controlli abiotico e non contaminato per una appropriata valutazione della capacità degradativa.

L'analita residuo è stato valutato mediante gas-cromatografia e spettrometria di massa (Hewlett-Packard 5890 e 5973 MS). È stata utilizzata una colonna HP5–MS (5% difenile-95% dimetil polisiloxano 30 m x 0.2 mm, 0.25 μm film, J&W Scientific, Folsom CA, USA). Per le analisi sono stati fissati i seguenti parametri:

Programmata di temperatura del forno: 80 ^{o}C per 1 min con un incremento di 5 ^{o}C min^{-1} fino a raggiungere una temperatura di 220 ^{o}C, mantenuta per 5 min.

Flusso di elio 1 ml min^{-1}

Modalità di iniezione: "split" 1/10 per l'iniezione dei campioni estratti con solvente e "splitless" per i campioni estratti con SPME.

Temperatura della porta di iniezione: 280^{o}C

Relativamente al campione estratto con solvente è stato iniettato un volume di 1 μl.

Per i campioni, estratti rispettivamente mediante SPME e solvente, sono stati utilizzati due differenti "liners" aventi diametro di 0.75 e 2 mm.

Le analisi sono state fatte in full-scan con una ionizzazione a impatto di elettroni di 70 eV ed una conseguente registrazione di spettri di massa corrispondenti ad un rapporto massa-carica (m/z) compreso tra 42 e 550 UMA. Lo spettro di massa del parathion è stato comparato con quelli presenti nella banca dati NIST 05, inoltre l'identificazione del pesticida è stata confermata utilizzando uno standard (99% purezza – Fluka, Sigma-Aldrich Chemie GmbH, Switzerland).

Analisi statistica

Tutte le analisi statistiche sono state eseguite utilizzando il programma Statistica 6.0 di Windows (StatSoft Inc., Tulsa, OK, USA).

È stata condotta l'analisi delle componenti principali (PCA) considerando le variabili chimico-fisiche dei suoli quali tessitura, percentuale di carbonio, pH, conduttività elettrica e capacità di scambio cationico in funzione degli isolati batterici. La significatività delle differenze quantitative di parathion residuo in seguito alle degradazioni batteriche è stata analizzata mediante *t* tests.

Risultati e Discussione

Proprietà chimico-fisiche dei suoli

Diversi fattori possono influire sulla biodiversità batterica dei suoli tra questi la vegetazione presente, il tipo di suolo, le pratiche agronomiche i composti chimici applicati al suolo e alle piante e i costituenti delle radici (Balandreau, 1986; Van Diepeningen *et al*., 2006).

I batteri in esame sono stati isolati da 8 suoli, differenti tra loro per alcuni aspetti quali le pratiche agronomiche, il tipo di coltura e le proprietà chimico-fisiche, tra queste la tessitura (Tab. 2). In particolare nei suoli VCH e POV prevale nettamente la frazione sabbiosa; nei suoli RIBCON, RIBBIO, RIBFAN, RIBINO, APCH e KAD sono presenti in buona parte sia la componente argillosa che quella sabbiosa, sebbene in proporzioni differenti. Nel suolo APCH la frazione sabbiosa si presenta in proporzioni maggiori rispetto a quella argillosa, a differenza dai suoli provenienti da Ribera mentre nel suolo KAD le componenti argillosa e sabbiosa si presentano in proporzioni pressoché simili. La percentuale di carbonio è chiaramente inferiore nei suoli VCH e POV come la capacità di scambio cationico.

Suolo	VCH	APCH	POV	KAD	RIBCON	RIBBIO	RIBFAN	RIBINO
Argilla (%)	6.3	33.3	9.7	37.5	40.8	40.2	43.3	38.9
Limo (%)	3.7	23.3	3.5	24.9	24.8	27.2	23.7	27.3
Sabbia (%)	90.0	43.4	86.8	37.6	34.4	32.6	33.0	33.7
Tipo di suolo	sabbioso	argilloso-limoso	limoso-sabbioso	argilloso-limoso	argilloso	argilloso	argilloso	argilloso-limoso
Carbonio organico (%)	0.54	1.58	0.71	1.66	2.60	2.82	3.33	2.48
pH (H_2O)	7.2	8	6.4	8.1	7.8	7.8	8	7.9
Conduttività elettrica (1:5) dS/m	0.2	0.4	0.3	0.5	0.3	0.4	0.4	0.4
Capacità di scambio cationico ($cmol^+/kg$)	13.1	27.5	13.7	30.6	27.4	32.6	33.8	32.4

Tabella 2 - Proprietà chimico-fisiche dei suoli

Biodiversità fenotipica di batteri isolati da suoli mediterranei

Dai suoli mediterranei sono state isolate 47 colonie fenotipicamente differenti (Figg. 5-6) e capaci di crescere su piastre contenenti il parathion come esclusiva fonte di carbonio.

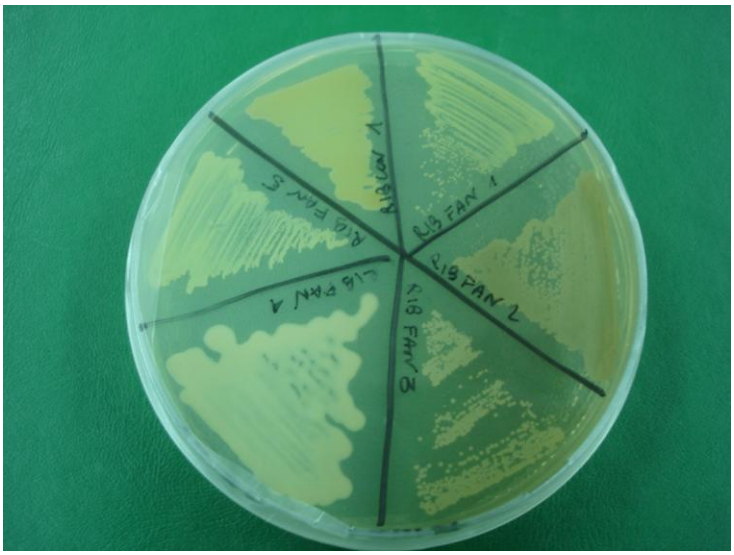

Figura 5 - Colture di batteri isolati da suoli mediterranei

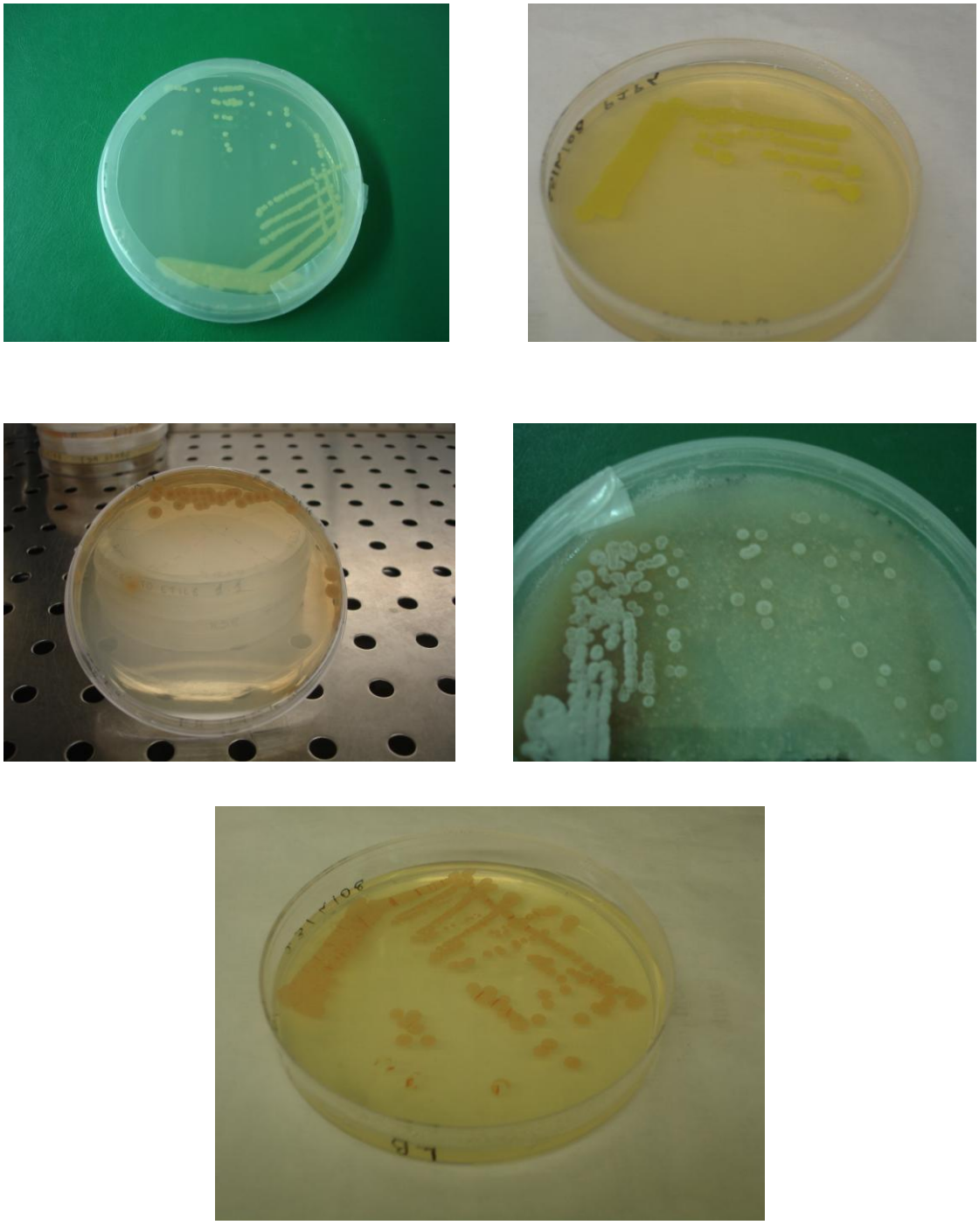

Figura 6 - Esempio di batteri isolati in coltura pura capaci di utilizzare il parathion come unica fonte di carbonio

I ceppi sono stati in seguito classificati in Gram positivi e negativi mediante colorazione di Gram. Tale tecnica è ampiamente utilizzata nella classificazione dei batteri in quanto consente di evidenziare alcune proprietà distintive della parete cellulare. Quest'ultima, infatti, reagisce diversamente ai coloranti utilizzati in funzione della sua struttura. Dalla colorazione dei ceppi isolati si è evidenziata una netta prevalenza dei Gram negativi rispetto ai Gram positivi.

Raggruppamento degli isolati in OTUs e biodiversità batterica

L'amplificazione dello spaziatore ribosomale 16S-23S mediante colony-PCR ha permesso di caratterizzare genotipicamente gli isolati e valutare la biodiversità batterica. In base ad un confronto, su gel di agarosio, tra i prodotti di PCR, i ceppi isolati sono stati raggruppati in 20 OTUs denominate con le lettere comprese nell'intervallo A–T .

Considerando la biodiversità presente in ogni suolo, si è evidenziato che ciascuno di essi comprende da 2 a 8 OTUs (Tab. 3). In particolare nei suoli APCH, RIBCON, RIBBIO e RIBFAN è presente il più alto numero di OTUs (da 5 a 8); si tratta di suoli coltivati con agrumi mentre la più bassa biodiversità batterica si è riscontrata nei suoli VCH e POV caratterizzati rispettivamente dalla presenza di un vigneto e di una serra di pomodoro.

Suolo	Numero batteri isolati	OTU
VCH	2	C-K
APCH	6	B-D-F-N-P-Q
POV	4	F-K-T
KAD	4	A-B-J-M
RIBCON	8	C-D-F-G-Q-R
RIBBIO	9	D-I-J-M-O
RIBFAN	9	B-D-E-G-H-L-O-S
RIBINO	5	B-C-D-K

Tabella 3 - Numero di batteri isolati da ciascun suolo e assegnazione alle Unità Tassonomiche Operative mediante analisi dell'ITS

Analisi delle componenti principali

La PCA ha permesso di evidenziare, tra le variabili studiate, quelle che detengono una maggiore correlazione alla biodiversità batterica. Il numero di variabili è stato ridotto mediante una PC1 ed una PC2 che detengono l'80.4% della varianza totale (Fig. 7). Come si osserva in figura è presente una correlazione positiva tra la biodiversità batterica e i suoli che presentano una più elevata percentuale di argilla e carbonio. Diversamente da questi ultimi, i suoli sabbiosi si collocano in posizione opposta rispetto alla biodiversità.

Il pH e la conduttività elettrica non influiscono in alcun modo sulla biodiversità batterica.

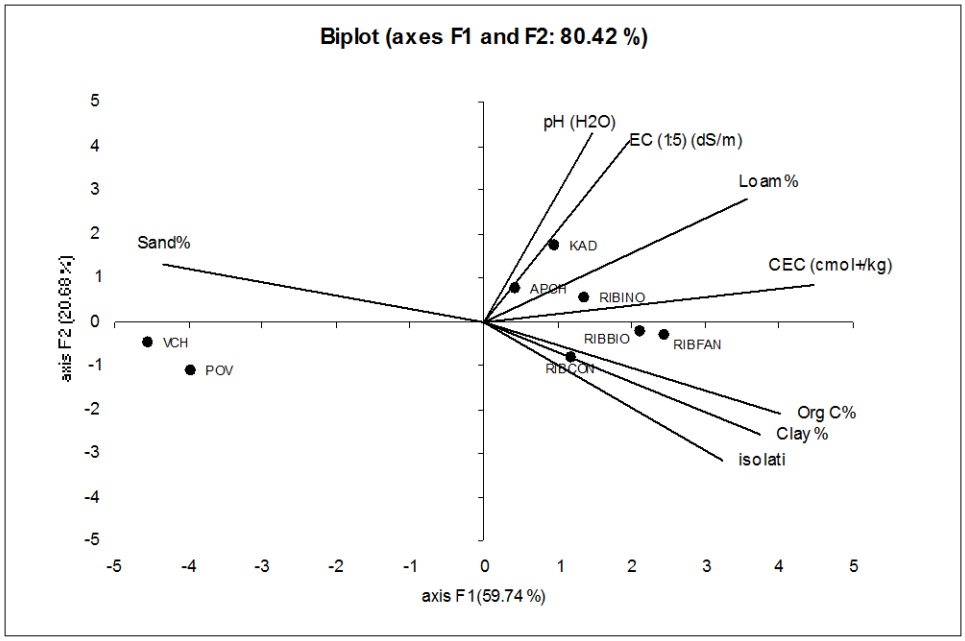

Figura 7 - Analisi delle componenti principali che mette in relazione le proprietà chimico-fisiche dei suoli e la biodiversità batterica

Identificazione dei ceppi isolati

Uno o più rappresentanti per ciascuna OTU sono stati scelti per il sequenziamento del gene 16S rRNA e identificati mediante confronto delle sequenze con i data base presenti in BLAST. I risultati dell'allineamento sono mostrati in tabella 4. Delle 20 OTU, 8 sono affiliate alla famiglia delle Rhizobiaceae e sono presenti in quasi tutti i suoli analizzati (Tab. 3), aspetto interessante considerando l'importante ruolo di azotofissatori svolto dai rizobi. Come si osserva in tabella 4 ogni OTU comprende da 1 a 9 isolati.

Gli isolati sono stati affiliati a 13 differenti generi che comprendono ceppi ben noti nella degradazione di OPs e taxa mai citati tra i degradatori.

OTU	Numero di isolati per OTU	Gene 16S rRNA (nucleotidi allineati)	Isolati rappresentativi	Sequenza più vicina numero di accessione (% identità)	Suolo di origine
A	1	732	M1P2	*Sinorhizobium medicae* EU445262 (97%)	KAD
B	4	955	M1D3	*Arthrobacter* sp. EF612307 (99%)	KAD
C	3	1109	RCP1	*Rhizobium* sp. EU529842 (98%)	RIBCON
D	9	1094	RFP2	*Xanthomonas translucens* AY572961 (97%)	RIBFAN
		1088	ROP3	*Stenotrophomonas* sp. AB461831 (98%)	RIBINO
E	1	1056	RFD1	*Isoptericola dokdonensis* DQ387860 (99%)	RIBFAN
F	4	1146	P2P1	*Pseudoxanthomonas* sp.EU025131 (98%)	POV
G	2	1027	RCP3	*Stenotrophomonas maltophilia* AJ131117 (99%)	RIBCON
H	1	1017	RFD4	*Paracoccus* sp.AM403616 (99%)	RIBFAN
I	1	1015	RBP2	*Rhizobiales* EF219048 (98%)	RIBBIO
J	2	1046	M1P1	*Streptomyces iakyrus* AB184877 (99%)	KAD
K	5	988	P1P4	*Microbacterium takaoensis* AB201047 (98%)	VCH
L	1	1083	RFP4	*Agrobacterium* sp.GU085230 (98%)	RIBFAN
M	2	995	RBD1	*Sinorhizobium meliloti* AY196963 (100%)	RIBBIO
		1026	M1D4	*Sinorhizobium* sp.EU399910 (99%)	KAD
N	1	1143	M2D3	*Sinorhizobium* sp.DQ196475 (98%)	APCH
O	3	1033	RBD4	*Streptomyces peucetius* AB249907 (96%)	RIBBIO
P	1	613	M2P3	*Pseudomonas putida* AY395005 (98%)	APCH
Q	2	1073	RCD2	*Azospirillum brasilense* EF634031 (99%)	RIBCON
R	1	1087	RCD1	*Rhizobium* sp.EF437254 (97%)	RIBCON
S	2	1119	RFP1	*Agrobacterium tumefaciens* EU697966(97%)	RIBFAN
T	1	1151	P2P3	*Roseomonas fauriae* AY150046 (97%)	POV

Tabella 4 - Identificazione filogenetica degli isolati per ogni Unità Tassonomica Operativa (A – T)

Tra i ceppi identificati, 4 sono stati affiliati al genere *Sinorhizobium*, noto per la capacità di degradare gli OPs (Parker *et al*., 1999); altri ceppi affiliati al genere *Agrobacterium* e ad altre rhizobiaceae sono citati in bibliografia come degradatori di glyphosate, diazinon, coumaphos e parathion (Liu *et al*., 1991; Ghassempour *et al*., 2002; Singh e Walker, 2006);

Diversi ceppi isolati appartenenti ai Gram negativi fanno parte di generi noti in bibliografia come degradatori di OPs: *Stenotrophomonas*, *Pseudomonas* (Rosenberg e Alexander, 1979; Kim *et al*., 2009; Choi, *et al*., 2009), *Xanthomonas*, *Roseomonas* (syn. *Azospirillum brasilense*) (Jiang *et al*., 2006) e *Paracoccus* (Jia *et al*., 2006). Tuttavia, per alcuni di essi, sono disponibili poche informazioni in merito alla capacità degradativa in quanto in questi ultimi anni sono stati isolati pochi batteri degradatori inclusi nei generi suddetti. Per esempio un unico ceppo di *Stenotrophomonas* (YC-1) è stato recentemente isolato e per quest'ultimo è stata saggiata la capacità degradativa di chlorpyrifos, parathion, methyl parathion e fenitrothion (Yang *et al*., 2006).

Nell'ambito del presente lavoro, è stato inoltre isolato un ceppo di *Pseudoxanthomonas*, correlato filogeneticamente a *Xanthomonas* e mai citato in bibliografia come degradatore di organofosforici.

Tra i ceppi isolati appartenenti ai Gram negativi, degni di nota sono quelli correlati alle rhizobiaceae e ad *Azospirillium brasilense* grazie all'importante funzione che assolvono per la vita vegetale. Tali batteri, infatti, insediandosi nelle radici di alcune piante instaurano con esse un rapporto di simbiosi, nel quale il batterio fissa l'azoto molecolare in forme utilizzabili dalla pianta e usa i metaboliti prodotti da quest'ultima come fonte di energia. Inoltre, *Azospirillium* potrebbe incrementare lo sviluppo delle piante fornendo loro componenti utili alla crescita come auxine, citochinine, giberelline e vitamine; ragione per cui i batteri appartenti al genere *Azospirillium* sono definiti PGPR (Plant Growth Promoting Rhizobacteria) (Glick, 1995).

Tra i Gram positivi, isolati nel corso della presente attività di ricerca, membri del genere *Arthrobacter* degradano un ampio numero di composti organofosforici per

esempio glifosato (Pipke' *et al*., 1987), monocrotophos (Bhadbhade *et al*., 2002), chlorpyrifos, diazinon, EPN, fenitrothion, isofenphos, parathion ed ethoprophos (Ohshiro *et al*., 1996; Racke e Coats, 1988; Choi *et al*., 2009). Inoltre alcuni ceppi di *Arthrobacter* possono degradare il p-nitrofenolo, che rappresenta il prodotto di idrolisi del parathion (Paul *et al*., 2006; Singh e Walker, 2006).

I ceppi isolati affiliati alle specie *Streptomyces iakyrus*, *Microbacterium takaoensis* e *Isoptericola dokdonensis* non sono noti in bibliografia per la capacità degradativa. Sono peraltro disponibili poche informazioni riguardo la capacità degradativa ad opera di altri ceppi appartenenti ai generi suddetti. Ad esempio il ceppo StC facente parte del genere *Streptomycetes* è capace di degradare il glifosato mediante rottura del legame C-P (Obojska *et al*., 1999) mentre un ceppo identificato come *Microbacterium esteraromaticum* è capace di idrolizzare il fenamiphos (Cáceres *et al*., 2009).

Sebbene tutti i batteri isolati nel corso del presente lavoro sono stati capaci di crescere in presenza di parathion come esclusiva fonte di carbonio, si è ritenuto opportuno approfondire le conoscenze in merito alla capacità degradativa di alcuni isolati particolarmente interessanti per l'ambiente. A tal proposito l'abilità degradativa è stata verificata in mezzo liquido e in suoli standard quindi in condizioni più vicine a quelle naturali.

Capacità degradativa dei batteri

Analisi SPME

Da un'attenta analisi dell'efficienza estrattiva in MSM liquido, in relazione alle tre fibre esaminate, è emerso che la PDMS è la più idonea per l'estrazione in mezzo liquido. Infatti, il coefficiente di partizione fibra/matrice per il parathion, relativamente alle fibre PDMS, PA e CW-DVB è stato rispettivamente 808, 388.7, 404.

Capacità degradativa in MSM

L'abilità degradativa in mezzo liquido è stata saggiata su 3 ceppi non annoverati in bibliografia tra i degradatori di organofosforici ovvero M1P1, P1P4, RFD1 e su batteri affiliati a generi o specie già noti per la loro abilità degradativa: M2D3, RCP3, P2P3, RFP1, RBP2, RCD2. La figura 8 mostra l'abilità degradativa di ogni ceppo. I ceppi P2P3, M2D3, RFP1, RBP2 ed RCD2 mostrano una buona capacità degradativa. Infatti, la percentuale di parathion residua dopo 72 h di incubazione è inferiore al 30% per ognuno di essi. Tra i ceppi suddetti P2P3 ed M2D3 si distinguono per una maggiore abilità nel degradare il parathion (~ 90-95 %) entro 72h.

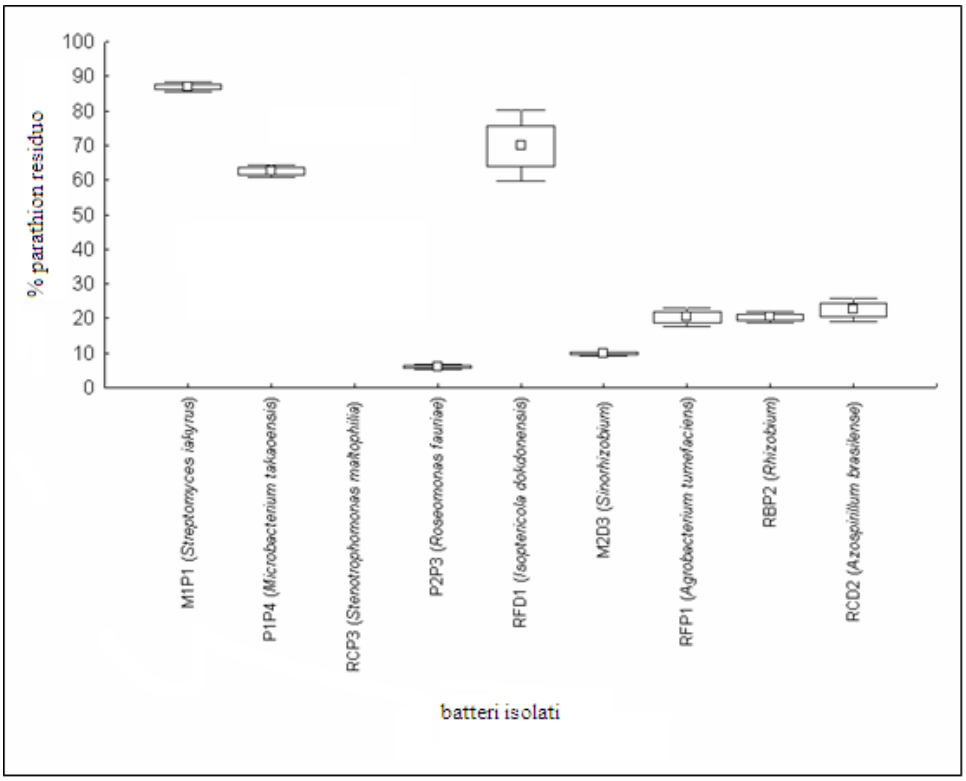

Figura 8 - Abilità degradativa dei ceppi isolati, espressa in % di parathion residuo rispetto alla concentrazione iniziale. Il quadrato piccolo rappresenta la media, il quadrato grande rappresenta la media ± l'errore standard, la barra di errore rappresenta la media ± la deviazione standard

Capacità degradativa nei microcosmi

Le prove di degradazione nei microcosmi sono state condotte sui ceppi P2P3 ed RFD1. Il primo è stato selezionato per l'ottima capacità degradativa in mezzo liquido, oltre che per gli effetti positivi esercitati sulle piante, come precedentemente descritto. Il ceppo RFD1 è stato scelto in quanto batterio annoverato per la prima volta tra i degradatori. Dai risultati è emerso che entrambi i ceppi sono capaci di degradare il parathion nei microcosmi, sebbene con una diversa efficienza per ogni suolo (Fig. 9). In particolare si è osservato che

il ceppo RFD1 è più efficiente di P2P3 in tutti i suoli considerati, a differenza da quanto osservato in mezzo liquido (Fig. 8).

Considerando che la biodegradazione è un processo che dipende dall'interazione tra la comunità microbiologica e l'ambiente (Davis e Madsen, 1996) in cui essa vive, i risultati hanno evidenziato un'influenza della tessitura del suolo sulla biodegradazione del parathion. Mettendo a confronto i tre suoli standard si osserva, infatti, che la percentuale di pesticida residuo è considerevolmente inferiore nei suoli 2.1 e 6S rispetto al suolo 3A per entrambi i ceppi. In particolare, il parathion residuo nei suoli 2.1, 6S e 3A è rispettivamente inferiore al 10%, tra il 10% e il 20% e attorno al 40%, con una maggiore efficienza degradativa nel suolo sabbioso 2.1.

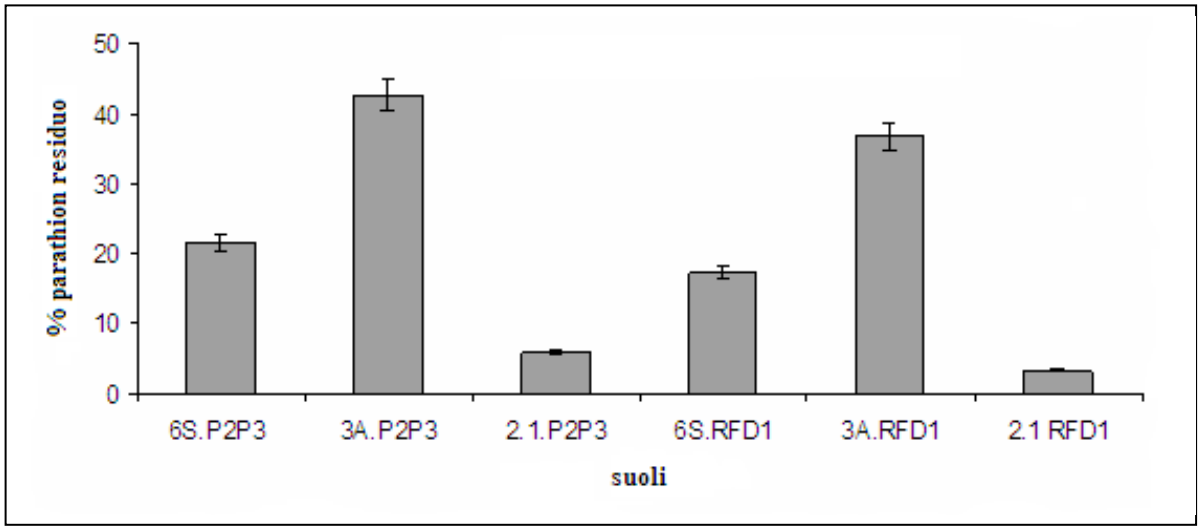

Figura 9 - Capacità degradativa dei ceppi P2P3 ed RFD1 in tre suoli standard (6S, 3A, 2.1), espressa in % di parathion residuo rispetto alla concentrazione iniziale

Conclusioni

La questione ambientale ha assunto, negli ultimi anni, una rilevanza sempre maggiore, interessando tutte le matrici, tra le quali anche il suolo. Il presente lavoro ha voluto apportare un contributo alla decontaminazione di questa importante risorsa, ponendo l'attenzione su alcuni territori della regione mediterranea.

Dagli studi effettuati, è emerso che i suoli esaminati, distinti per il tipo di coltura e di pratiche agronomiche, ospitano un considerevole numero di batteri differenti, che sono capaci di crescere in presenza del pesticida parathion, quale esclusiva fonte di carbonio. Questa abilità si può estendere con buona probabilità agli altri organofosforici. In bibliografia, infatti, il parathion è spesso citato come modello, per valutare la capacità biodegradativa degli organofosforici in generale. La biodiversità batterica presente nei suoli mediterranei, oggetto di indagine, è correlata positivamente ad alcune proprietà chimico-fisiche del suolo, ovvero al contenuto di argilla e carbonio. Inoltre, il tipo di coltura sembra influire sulla biodiversità, che risulta maggiore nei suoli coltivati ad agrumi, indipendentemente dai trattamenti organici o convenzionali. La biodiversità degli agrumeti è infatti frutto della selezione naturale, avvenuta nel corso del tempo, in un ambiente che ospita, una tra le colture più antiche in Sicilia ed esprime chiaramente l'attitudine del territorio, nel sostenere una vasta varietà di specie batteriche.

La collezione dei batteri, isolati durante la presente attività di ricerca, è rappresentata principalmente da Gram negativi, tra i quali i ceppi di *Sinorhizobium* sono predominanti. Questi batteri sono capaci di fissare l'azoto, per cui svolgono un ruolo molto importante sia da un punto di vista economico, favorendo la crescita di piante facenti parte della dieta alimentare, che da un punto di vista ecologico, perchè l'azoto fissato dai batteri simbionti, entra nelle reti alimentari attraverso i livelli trofici.

Tra i Gram negativi, sono stati inoltre isolati due ceppi di *Roseomonas*, un genere che oltre a fissare l'azoto atmosferico, promuove la crescita delle piante. È quindi

opportuno sottolineare, che uno degli isolati, appartenente a tale genere si è distinto per una maggiore abilità degradativa, rispetto agli altri ceppi.

Tra i Gram positivi, cresciuti in presenza di parathion, come fonte di carbonio, alcuni sono degni di nota, in quanto mai citati in bibliografia tra i degradatori di organofosforici.

Nel corso del presente lavoro si è evidenziata infine, una notevole influenza della tessitura sull'abilità degradativa. In particolare, una maggiore percentuale di sabbia sembra favorire la biodegradazione.

Alla luce degli studi effettuati, il biorisamento si conferma come un processo molto complesso, il cui successo dipende, oltre che dalla natura e dalla quantità di eventuali microrganismi utilizzati, dal tipo di suolo. Tale metodo, presenta comunque diversi vantaggi rispetto ai più diffusi trattamenti chimico-fisici; si tratta, infatti, di una tecnologia che non richiede costi elevati, si realizza con una spesa energetica ridotta e minimizza i rischi di inquinamento ambientale. A tal fine, la biodiversità di batteri, capaci di crescere in presenza di parathion, come esclusiva fonte di carbonio, costituisce un'importante ricchezza naturale; una risorsa, per il risanamento ambientale di alcuni siti contaminati, nel territorio siciliano.

L'impiego di batteri autoctoni, infatti, consente interventi di bonifica *in situ*, senza compromettere, gli equilibri naturali del territorio interessato. Alcuni studi hanno rivelato che la biodegradazione, insieme ad altri processi naturali, può consentire una graduale decontaminazione da pesticidi, in tempi relativamente brevi, sebbene in ambiente naturale l'efficienza della biodegradazione dipenda da diversi fattori quali: la disponibilità di pesticidi per i microrganismi, il loro stato fisiologico, la sopravvivenza e la proliferazione di batteri degradatori di pesticidi e la dinamica di popolazione in relazione agli altri microrganismi, presenti nello stesso habitat.

Tra i batteri isolati, i ceppi *Sinorhizobium* e *Roseomonas* si potrebbero impiegare per il biorisanamento, in quanto, tali batteri, oltre a decontaminare il suolo, hanno effetti positivi sulle piante, quindi sull'intero ecosistema.

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Appendice A

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LOCUS         GU902282 747 bp    DNA     linear   BCT 14-APR-2010
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VERSION
KEYWORDS    .
SOURCE      Sinorhizobium medicae (Ensifer medicae)
ORGANISM  Sinorhizobium medicae
Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
Rhizobiaceae; Sinorhizobium/Ensifer group; Sinorhizobium.
REFERENCE   1  (bases 1 to 747)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini, P.
TITLE     ISOLATION OF ORGANOPHOSPHORUS-DEGRADING BACTERIA FROM AGRICULTURAL MEDITERRANEAN
SOILS
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 747)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     Direct Submission
JOURNAL   Submitted (18-FEB-2010) Dpt. Biologia Cellulare e dello Sviluppo,University of
Palermo, Viale delle Scienze Edif 16, Palermo, PA
90128, ITALY
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      661 tcgggcagtt tactgttcgg tggcccactt aacacattta aacattccgc ctggggagaa
      721 cggacgcaag attaaactca aaggaat

LOCUS       GU902283            958 bp    DNA     linear   BCT 14-APR-2010
DEFINITION  Arthrobacter sp. strain M1D3 16S ribosomal RNA gene, partial sequence.
ACCESSION GU902283
VERSION
KEYWORDS    .
SOURCE      Arthrobacter sp.
ORGANISM  Arthrobacter sp.
Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;
Micrococcineae; Micrococcaceae; Arthrobacter.
REFERENCE   1  (bases 1 to 958)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     ISOLATION OF ORGANOPHOSPHORUS-DEGRADING BACTERIA FROM AGRICULTURAL MEDITERRANEAN
SOILS
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 958)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     Direct Submission
JOURNAL   Submitted (18-FEB-2010) Dpt. Biologia Cellulare e dello Sviluppo,University of
Palermo, Viale delle Scienze Edif 16, Palermo, PA
90128, ITALY
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18-FEB-2010
DEFINITION  Rhizobium sp. strain RCP1 16S ribosomal RNA gene, partial sequence.
ACCESSION GU902284
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SOURCE      Rhizobium sp.
ORGANISM  Rhizobium sp.
Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
Rhizobiaceae; Rhizobium/Agrobacterium group; Rhizobium.
REFERENCE   1  (bases 1 to 1119)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     ISOLATION OF ORGANOPHOSPHORUS-DEGRADING BACTERIA FROM AGRICULTURAL MEDITERRANEAN
SOILS
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1119)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     Direct Submission
JOURNAL   Submitted (18-FEB-2010) Dpt. Biologia Cellulare e dello Sviluppo,University of
Palermo, Viale delle Scienze Edif 16, Palermo, PA
90128, ITALY
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ACCESSION GU902285
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SOURCE    Xanthomonas translucens
ORGANISM  Xanthomonas translucens
Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales;
Xanthomonadaceae; Xanthomonas.
REFERENCE   1  (bases 1 to 1117)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E.,Alonzo,G. and Quatrini,P.
TITLE     ISOLATION OF ORGANOPHOSPHORUS-DEGRADING BACTERIA FROM AGRICULTURAL MEDITERRANEAN
SOILS
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1117)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
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JOURNAL   Submitted (18-FEB-2010) Dpt. Biologia Cellulare e dello Sviluppo,University of
Palermo, Viale delle Scienze Edif 16, Palermo, PA
90128, ITALY
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ACCESSION GU902286
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ORGANISM  Stenotrophomonas sp.
Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales;
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REFERENCE   1  (bases 1 to 1095)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     ISOLATION OF ORGANOPHOSPHORUS-DEGRADING BACTERIA FROM AGRICULTURAL MEDITERRANEAN
SOILS
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1095)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     Direct Submission
JOURNAL   Submitted (18-FEB-2010) Dpt. Biologia Cellulare e dello Sviluppo,University of
Palermo, Viale delle Scienze Edif 16, Palermo, PA
90128, ITALY
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      541 gggcgtaaag cgtgcgtagg tggtcgttta agtctgttgt gaaagccctg ggctcaacct
      601 gggaactgca gtggaaactg gacgactaga gtgtggtaga gggtagcgga attcctggtg
      661 tagcagtgaa atgcgtagag atcaggagga acatccatgg cgaaggcagc tacctggacc
      721 aacactgaca ctgaggcacg aaagcgtggg gagcaaacag gattagatac cctggtagtc
      781 cacgccctaa acgatgcgaa ctggatgttg ggtgcaattt ggcacgcagt atcgaagcta
      841 acgcgttaag ttcgccgcct ggggagtacg gtcgcaagac tgaaactcaa aggaattgac
      901 gggggcccgc acaagcggtg gagtatgtgg tttaattcga tgcaacgcga gaaccttacc
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      961 tgggccttga catgtcgaga actttccaga gatggattgt gcttcgggac tcgaacacag
     1021 gtgctgcatg gctgtcgtca gctcgtgtcg tgagatgttg ggtttaagtt cccgcaacga
     1081 gcgcaacctt ggtcc

LOCUS       GU902287 1060 bp    DNA     linear   BCT 14-APR-2010
DEFINITION  Isoptericola dokdonensis strain RFD1 16S ribosomal RNA gene, partial sequence.
ACCESSION GU902287
VERSION
KEYWORDS    .
SOURCE      Isoptericola dokdonensis
ORGANISM  Isoptericola dokdonensis
Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;
Micrococcineae; Promicromonosporaceae; Isoptericola.
REFERENCE   1  (bases 1 to 1060)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     ISOLATION OF ORGANOPHOSPHORUS-DEGRADING BACTERIA FROM AGRICULTURAL MEDITERRANEAN
SOILS
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1060)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     Direct Submission
JOURNAL   Submitted (18-FEB-2010) Dpt. Biologia Cellulare e dello Sviluppo,University of
Palermo, Viale delle Scienze Edif 16, Palermo, PA
90128, ITALY
FEATURES             Location/Qualifiers
source          1..1060
                     /organism="Isoptericola dokdonensis"
                     /mol_type="genomic DNA"
                     /strain="RFD1"
                     /db_xref="taxon:372663"
                     /note="PCR_primers=fwd_name: fD1"
 BASE COUNT      235 a    255 c    375 g    195 t
ORIGIN
        1 tgctgggggg atcagtggcg aacgggtgag taacacgtga gcaacctgcc ccccacttcg
       61 ggataagcct tggaaacggg gtctaatacc ggatatgagt gcctgctgca tggtgggtgc
      121 tggaaagttt ttcggtgggg gatgggctcg cggcctatca gcttgttggt ggggtaatgg
      181 cctaccaagg cgtcgacggg tagccggcct gagagggcga ccggccacac tgggactgag
      241 acacggccca gactcctacg ggaggcagca gtggggaata ttgcacaatg ggcgaaagcc
      301 tgatgcagcg acgccgcgtg agggatgacg gccttcgggt tgtaaacctc tttcagcagg
      361 gaagaagcgc aagtgacggt acctgcagaa gaagcgccgg ctaactacgt gccagcagcc
      421 gcggtaatac gtagggcgca agcgttgtcc ggaattattg ggcgtaaaga gctcgtaggc
      481 ggtttgtcgc gtctggtgtg aaaacctcag gctcaacctg gggcgtgcat cgggtacggg
      541 cagactagag tgcggtaggg gagactggaa ttcctggtgt agcggtggaa tgcgcagata
      601 tcaggaggaa caccgatggc gaaggcaggt ctctgggccg caactgacgc tgaggagcga
      661 aagcatgggg agcgaacagg attagatacc ctggtagtcc atgccgtaaa cgttgggcac
      721 taggtgtggg gctcattcca cgagttccgt gccgcagcta acgcattaag tgccccgcct
      781 ggggagtacg gccgcaaggc taaaactcaa aggaattgac gggggcccgc acaagcggcg
      841 gagcatgcgg attaattcga tgcaacgcga agaaccttac caaggcttga catgcaccgg
      901 aaacactcag agatgggtgc ccgcaaggtc ggtgcacagg tggtgcatgg ttgtcgtcag
      961 ctcgtgtcgt gagatgttgg gtagtcccgc acgagcgcac cctcgtccta tgttgccagc
     1021 gggttatgcc gggactcatg ggagaactgc cggggtcaac

LOCUS       GU902288          1153 bp    DNA     linear            14-APR-2010
DEFINITION  Pseudoxanthomonas sp. strain P2P1 16S ribosomal RNA gene, partial sequence.
ACCESSION GU902288
VERSION
KEYWORDS
SOURCE      Pseudoxanthomonas sp.
ORGANISM  Pseudoxanthomonas sp.
Unclassified.
REFERENCE   1  (bases 1 to 1153)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     ISOLATION OF ORGANOPHOSPHORUS-DEGRADING BACTERIA FROM AGRICULTURAL MEDITERRANEAN
SOILS
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1153)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E.,Alonzo,G. and Quatrini,P.
TITLE     Direct Submission
JOURNAL   Submitted (18-FEB-2010) Dpt. Biologia Cellulare e dello Sviluppo,University of
Palermo, Viale delle Scienze Edif 16, Palermo, PA
90128, ITALY
FEATURES             Location/Qualifiers
     source          1..1153
                     /organism="Pseudoxanthomonas sp."
                     /mol_type="genomic DNA"
                     /strain="P2P1"
                     /note="PCR_primers=fwd_name: fD1"
BASE COUNT      284 a    256 c    385 g    228 t
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ORIGIN
        1 agtgacgctg gcggtaggcc taacacatgc aagtcgaacg gcagcacagg agagcttgct
       61 ctctgggtgg cgagtggcgg acgggtgagg aatacatcgg aatctacctt gtcgtggggg
      121 ataacgtagg gaaacttacg ctaataccgc atacgacctt cgggtgaaag tgggggaccg
      181 caaggcctca cgcgattaga tgagccgatg tcggattagc tagttggcgg ggtaaaagcc
      241 caccaaggcg acgatccgta gctggtctga gaggatgatc agccacactg gaactgagac
      301 acggtccaga ctcctacggg aggcagcagt ggggaatatt ggacaatggg cgcaagcctg
      361 atccagccat accgcgtggg tgaagaaggc cttcgggttg taaagccctt ttgttgggaa
      421 agaaatcttg ccggttaata cctggcgagg atgacggtac ccaaagaata agcaccggct
      481 aacttcgtgc cagcagccgc ggtaatacga agggtgcaag cgttactcgg aattactggg
      541 cgtaaagcgt gcgtaggtgg tggtttaagt ctgctgtgaa agccctgggc tcaacctggg
      601 aattgcagtg gatactgggt cactagagtg tggtagaggg atgcggaatt tccggtgtag
      661 cagtgaaatg cgtagagatc ggaaggaaca tccgtggcga aggcggcatc ctgggccaac
      721 actgacactg aggcacgaaa gcgtggggag caaacaggat tagataccct ggtagtccac
      781 gccctaaacg atgcgaactg gatgttgggt gcaacttggc acccagtatc gaagctaacg
      841 cgttaagttc gccgcctggg gagtacggtc gcaagactga aactcaaagg aattgacggg
      901 ggcccgcaca agcggtggag tatgtggttt aattcgatgc aacgcgaaga accttacctg
      961 gccttgacat gtcgcgaact ttccagagat ggattggtgc cttcgggacg cgaacacagg
     1021 tgctgcatgg ctgtcgtcag ctcgtgtcgt gagatgttgg ggtagtcccg cacgagcgca
     1081 tcattgttct tagtgcagca cgtaatggtg gactctaagg agaccgccgg tgacaaaccg
     1141 gaggagggtg ggg

LOCUS       GU902289               1029 bp    DNA     linear   BCT 14-APR-2010
DEFINITION  Stenotrophomonas maltophilia strain RCP3 16S ribosomal RNA gene, partial sequence.
ACCESSION   GU902289
VERSION
KEYWORDS
SOURCE      Stenotrophomonas maltophilia
ORGANISM    Stenotrophomonas maltophilia
Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales;
Xanthomonadaceae; Stenotrophomonas.
REFERENCE   1  (bases 1 to 1029)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     Isolation of organophosphorus-degrading bacteria from agricultural
mediterranean soils
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1029)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     Direct Submission
JOURNAL   Submitted (22-FEB-2010) Dpt. Biologia Cellulare e Dello Sviluppo,University of
Palermo, Viale Delle Scienze Edif 16, Palermo, PA
            90128, Italy
FEATURES             Location/Qualifiers
source          1..1029
                     /organism="Stenotrophomonas maltophilia"
                     /mol_type="genomic DNA"
                     /strain="RCP3"
                     /db_xref="taxon:40324"
                     <1..>1029
                     /product="16S ribosomal RNA"
ORIGIN
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       61 atgggtggcg agtggcggac gggtgaggaa tacatcggaa tctacctttt cgtgggggat
      121 aacgtaggga aacttacgct aataccgcat acgaccttcg ggtgaaagca ggggaccttc
      181 gggccttgcg cggatagatg agccgatgtc ggattagcta gttggcgggg taaaggccca
      241 ccaaggcgac gatccgtagc tggtctgaga ggatgatcag ccacactgga actgagacac
      301 ggtccagact cctacgggag gcagcagtgg ggaatattgg acaatgggcg caagcctgat
      361 ccagccatac cgcgtgggtg aagaaggcct tcgggttgta aagccctttt gttgggaaag
      421 aaaagcagtc ggctaatacc cggttgttct gacggtaccc aaagaataag caccggctaa
      481 cttcgtgcca gcagccgcgg taatacgaag ggtgcaagcg ttactcggaa ttactgggcg
      541 taaagcgtgc gtaggtggtt gtttaagtct gttgtgaaag ccctgggctc aacctgggaa
      601 ttgcagtgga tactgggcga ctagagtgtg gtagagggta gtggaattcc cggtgtagca
      661 gtgaaatgcg tagagatcgg gaggaacatc catggcgaag gcagctacct ggaccaacac
      721 tgacactgag gcacgaaagc gtggggagca aacaggatta gataccctgg tagtccacgc
      781 cctaaacgat gcgaactgga tgttgggtgc aatttggcac gcagtatcga agctaacgcg
      841 tttaagttcg ccgcctgggg agtacggtcg caagactgaa actcaaagga attgacgggg
      901 gcccgcacaa gcggtggagt atgtggttta attcgatgca acgcgagaac cttacctggt
      961 cttgacatgt cgagaacttt ccagagatgg attgttgcct tcgggaactc gaacacaggt
     1021 gctgcatgg

LOCUS          GU902290        1018 bp    DNA     linear   BCT 14-APR-2010
DEFINITION  Paracoccus sp. strain RFD4 16S ribosomal RNA gene, partial sequence.
ACCESSION GU902290
VERSION
KEYWORDS    .
SOURCE      Paracoccus sp.
ORGANISM  Paracoccus sp.
Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales;
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Rhodobacteraceae; Paracoccus.
REFERENCE   1  (bases 1 to 1018)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     ISOLATION OF ORGANOPHOSPHORUS-DEGRADING BACTERIA FROM AGRICULTURAL MEDITERRANEAN
SOILS
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1018)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     Direct Submission
JOURNAL   Submitted (18-FEB-2010) Dpt. Biologia Cellulare e dello Sviluppo,University of
Palermo, Viale delle Scienze Edif 16, Palermo, PA
            90128, ITALY
FEATURES             Location/Qualifiers
     source          1..1018
                     /organism="Paracoccus sp."
                     /mol_type="genomic DNA"
                     /strain="RFD4"
                     /db_xref="taxon:267"
                     /note="PCR_primers=fwd_name: fD1"
 BASE COUNT      257 a    230 c    327 g    204 t
 ORIGIN
        1 aacgctggcg gcaggcctaa cacatgcaag tcgagcggat ccttcgggat tagcggcgga
       61 cgggtgagta acgcgtggga atatgccctt ctctacggaa tagcctcggg aaactgggag
      121 taataccgta tacgccctac gggggaaaga tttatcggag aaggattagc ccgcgttgga
      181 ttaggtagtt ggtggggtaa tggcctacca agcctacgat ccatagctgg tttgagagga
      241 tgatcagcca cactgggact gagacacggc ccagactcct acgggaggca gcagtgggga
      301 atcttagaca atgggggaaa ccctgatcta gccatgccgc gtgagtgatg aaggccttag
      361 ggttgtaaag ctctttcagc tgggaagata atgacggtac cagcagaaga agccccggct
      421 aactccgtgc cagcagccgc ggtaatacgg agggggctag cgttgttcgg aattactggg
      481 cgtaaagcgc acgtaggcgg accggaaagt tggaggtgaa atcccagggc tcaaccttgg
      541 aactgccttc aaaactatcg gtctggagtt cgagagaggt gagtggaatt ccgagtgtag
      601 aggtgaaatt cgtagatatt cggaggaaca ccagtggcga aggcggctca ctggctcgat
      661 actgacgctg aggtgcgaaa gcgtggggag caaacaggat tagataccct ggtagtccac
      721 gccgtaaacg atgaatgcca gtcgtcgggt agcatgctat tcggtgacac acctaacgga
      781 ttaagcattc cgcctgggga gtacggtcgc aagattaaaa ctcaaaggaa ttgacggggg
      841 cccgcacaag cggtggagca tgtggtttaa ttcgaagcaa cgcgcagaac cttaccaacc
      901 cttgacatta caggacatcc ccagagatgg ggctttcact tcggtgacct gtggacaggt
      961 gctgcatggc tgtcgtcagc ctcgtgtcgt gagatgttcg gttaagtccg gcaacgag

LOCUS       GU902291           1028 bp    DNA     linear   BCT 14-APR-2010
DEFINITION  Rhizobiales strain RBP2 16S ribosomal RNA gene, partial sequence.
ACCESSION GU902291
VERSION
KEYWORDS    .
SOURCE      Rhizobiales
ORGANISM  Rhizobiales
Bacteria; Proteobacteria; Alphaproteobacteria.
REFERENCE   1  (bases 1 to 1028)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G. and Quatrini,P.
TITLE     ISOLATION OF ORGANOPHOSPHORUS-DEGRADING BACTERIA FROM AGRICULTURAL MEDITERRANEAN
SOILS
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1028)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     Direct Submission
JOURNAL   Submitted (18-FEB-2010) Dpt. Biologia Cellulare e dello Sviluppo,University of
Palermo, Viale delle Scienze Edif 16, Palermo, PA
90128, ITALY
FEATURES             Location/Qualifiers
source          1..1028
                     /organism="Rhizobiales"
                     /mol_type="genomic DNA"
                     /strain="RBP2"
                     /db_xref="taxon:356"
                     /note="PCR_primers=fwd_name: fD1"
 BASE COUNT      261 a    230 c    321 g    216 t
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        1 gggtgagtaa cgcgtgggaa tctacccatc tctacggaat aactcaggga aacttgtgct
       61 aataccgtat acgcccttcg ggggaaagat ttatcggaga tggatgagcc cgcgttggat
      121 tagctagttg gtggggtaaa ggcctaccaa ggcgacgatc catagctggt ctgagaggat
      181 gatcagccac attgggactg agacacggcc caaactccta cgggaggcag cagtggggaa
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      541 ggtgaaattc gtagatattc ggaggaacac cagtggcgaa ggcggcttac tggtccatta
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      661 ccgtaaacga tgaatgttag ccgtcggcat gcatgcatgt cggtggcgca gctaacgcat
      721 taaacattcc gcctggggag tacggtcgca agattaaaac tcaaaggaat tgacgggggc
      781 ccgcacaagc ggtggagcat gtggtttaat tcgaagcaac gcgcagaacc ttaccagccc
      841 ttgacatccc gatcgcggtt agtggagaca ctttccttca gttaggctgg atcggagaac
      901 aggtgctgca tggctgtcgt cagctcgtgt cgtgagatgt gggtaagtcc cgcaacgagc
      961 gcaaccctcg cccttagttg ccagcattca gttgggcact ctaaagggac tgccggtgaa
     1021 taagccga

LOCUS       GU902292               1052 bp    DNA     linear   BCT 14-APR-2010
DEFINITION  Streptomyces iakyrus strain M1P1 16S ribosomal RNA gene, partial sequence.
ACCESSION   GU902292
VERSION
KEYWORDS
SOURCE      Streptomyces iakyrus
ORGANISM    Streptomyces iakyrus
Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;
Streptomycineae; Streptomycetaceae; Streptomyces.
REFERENCE   1  (bases 1 to 1052)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     Isolation of organophosphorus-degrading bacteria from agricultural
mediterranean soils
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1052)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     Direct Submission
JOURNAL   Submitted (22-FEB-2010) Dpt. Biologia Cellulare e Dello Sviluppo,University of
Palermo, Viale Delle Scienze Edif 16, Palermo, PA
          90128, Italy
FEATURES             Location/Qualifiers
source               1..1052
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                     /mol_type="genomic DNA"
                     /strain="M1P1"
                     /db_xref="taxon:68219"
                     <1..>1052
                     /product="16S ribosomal RNA"
ORIGIN
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       61 agtggcgaac gggtgagtaa cacgtgggca atctgccctg cactctggga caagccctgg
      121 aaacggggtc taataccgga tactgatccg tctgggcatc cagatggttc gaaagctccg
      181 gcggtgcagg atgagcccgc ggcctatcag cttgttggtg aggtagtggc tcaccaaggc
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      541 tcggttgtga aagcccgggg cttaaccccg ggtctgcagt cgatacgggc aggctagagt
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      721 gcgaacagga ttagataccc tggtagtcca cgccgtaaac ggtgggcact aggtgtgggc
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      841 ccgcaaggct aaaactcaaa ggaattgacg gggcccgca caagcggcgg agcatgtggc
      901 ttaattcgac gcaacgcgaa gaatctttac caaggcttga catacaccgg gaacgttcag
      961 agatgggcgc ccccttgtg gtcggtgtac aggtggtgca tggctgtcgt cagctcgtgt
     1021 cgtgagatgt tgggttaagt cccgcaacga gc

LOCUS       GU902293           997 bp    DNA     linear   BCT 14-APR-2010
DEFINITION  Microbacterium takaoensis strain P1P4 16S ribosomal RNA gene,partial sequence.
ACCESSION GU902293
VERSION
KEYWORDS    .
SOURCE      Microbacterium takaoensis
ORGANISM  Microbacterium takaoensis
Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;
Micrococcineae; Microbacteriaceae; Microbacterium.
REFERENCE   1  (bases 1 to 997)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     ISOLATION OF ORGANOPHOSPHORUS-DEGRADING BACTERIA FROM AGRICULTURAL MEDITERRANEAN
SOILS
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 997)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     Direct Submission
JOURNAL   Submitted (18-FEB-2010) Dpt. Biologia Cellulare e dello Sviluppo,University of
Palermo, Viale delle Scienze Edif 16, Palermo, PA
90128, ITALY
FEATURES             Location/Qualifiers
     source          1..997
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                     /mol_type="genomic DNA"
                     /strain="P1P4"
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                     /note="PCR_primers=fwd_name: fD1"
 BASE COUNT      240 a    232 c    336 g    189 t
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       61 gatcagtggc gaacgggtga gtaacacgtg agcaacctgc cctggactct gggataagcg
      121 ctggaaacgg cgtctaatac tggatatgag ctctcatcgc atggtggggg ttggaaagat
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      301 cagactccta cgggaggcag cagtggggaa tattgcacaa tgggcggaag cctgatgcag
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      601 agtgcggtag gggagattgg aattcctggt gtagcggtgg aatgcgcaga tatcaggagg
      661 aacaccgatg gcgaaggcag atctctgggc cgtaactgac gctgaggagc gaaagggtgg
      721 ggagcaaaca ggcttagata ccctggtagt ccaccccgta aacgttggga actagttgtg
      781 gggtcctttc cacggattcc gtgacgcagc taacgcatta agttccccgc ctggggagta
      841 cggccgcaag gctaaaactc aaaggaattg acggggaccc gcacaagcgg cggagcatgc
      901 ggattaattc gatgcaacgc gaagaacctt accaaggctt gacatacacg agacacccta
      961 gaaatagggg actctttgga cactcgtgaa caggtgg

LOCUS       GU902294      1092 bp    DNA     linear   BCT 14-APR-2010
DEFINITION  Agrobacterium sp. strain RFP4 16S ribosomal RNA gene, partial sequence.
ACCESSION GU902294
VERSION
KEYWORDS    .
SOURCE      Agrobacterium sp.
ORGANISM  Agrobacterium sp.
Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
Rhizobiaceae; Rhizobium/Agrobacterium group; Agrobacterium.
REFERENCE   1  (bases 1 to 1092)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E.,Alonzo,G. and Quatrini,P.
TITLE     ISOLATION OF ORGANOPHOSPHORUS-DEGRADING BACTERIA FROM AGRICULTURAL MEDITERRANEAN
SOILS
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1092)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G. and Quatrini,P.
TITLE     Direct Submission
JOURNAL   Submitted (18-FEB-2010) Dpt. Biologia Cellulare e dello Sviluppo,University of
Palermo, Viale delle Scienze Edif 16, Palermo, PA
90128, ITALY
FEATURES             Location/Qualifiers
     source          1..1092
                     /organism="Agrobacterium sp."
                     /mol_type="genomic DNA"
                     /strain="RFP4"
                     /db_xref="taxon:361"
                     /note="PCR_primers=fwd_name: fD1"
 BASE COUNT      274 a    239 c    348 g    231 t
 ORIGIN
        1 gggtgagtaa cgcgtgggaa catacccttt cctgcggaat agctccggga aactggaatt
       61 aataccgcat acgccctacg ggggaaagat ttatcgggga aggattggcc cgcgttggat
      121 tagctagttg gtggggtaaa ggcctaccaa ggcgacgatc catagctggt ctgagaggat
      181 gatcagccac attgggactg agacacggcc caaactccta cgggaggcag cagtggggaa
      241 tattggacaa tgggcgcaag cctgatccag ccatgccgcg tgagtgatga aggccttagg
      301 gttgtaaagc tctttcaccg atgaagataa tgacggtagt cggagaagaa gccccggcta
      361 acttcgtgcc agcagccgcg gtaatacgaa gggggctagc gttgttcgga attactgggc
      421 gtaaagcgca cgtaggcgga tatttaagtc aggggtgaaa tcccgcagct caactgcgga
      481 actgcctttg atactgggta tcttgagtat ggaagaggta agtggaattc cgagtgtaga
      541 ggtgaaattc gtagatattc ggaggaacac cagtggcgaa ggcggcttac tggtccatta
      601 ctgacgctga ggtgcgaaag cgtggggagc aaacaggatt agataccctg gtagtccacg
      661 ccgtaaacga tgaatgttag ccgtcgggca gtatactgtt cggtggcgca gctaacgcat
      721 taaacattcc gcctggggag tacggtcgca agattaaaac tcaaaggaat tgacgggggc
      781 ccgcacaagc ggtggagcat gtggtttaat tcgaagcaac gcgcagaacc ttaccagctc
      841 ttgacattcg gggtatgggc attggagacg atgtccttca gttaggctgg gccccagaaa
      901 caggtgctgc atggctgtcg tcagctcgtg tcgtgagatg ttgggttaag tcccgcaacg
      961 agcgcaaccc tcgcccttag tgcagcattt agttgggcac tctaagggga ctgcgtgata
     1021 agcgaaagaa ggtggggaat gaacgtcagt ctcatggccc ttacgggctg gcttaccaca
     1081 cgtgctacaa tg

LOCUS       GU902295            995 bp    DNA     linear   BCT 14-APR-2010
DEFINITION  Sinorhizobium meliloti strain RBD1 16S ribosomal RNA gene,partial sequence.
ACCESSION GU902295
VERSION
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KEYWORDS    .
SOURCE      Sinorhizobium meliloti (Rhizobium meliloti)
ORGANISM  Sinorhizobium meliloti
Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
Rhizobiaceae; Sinorhizobium/Ensifer group; Sinorhizobium.
REFERENCE   1  (bases 1 to 995)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     ISOLATION OF ORGANOPHOSPHORUS-DEGRADING BACTERIA FROM AGRICULTURAL MEDITERRANEAN
SOILS
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 995)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G. and Quatrini,P.
TITLE     Direct Submission
JOURNAL   Submitted (18-FEB-2010) Dpt. Biologia Cellulare e dello Sviluppo,University of
Palermo, Viale delle Scienze Edif 16, Palermo, PA
90128, ITALY
FEATURES             Location/Qualifiers
     source          1..995
                     /organism="Sinorhizobium meliloti"
                     /mol_type="genomic DNA"
                     /strain="RBD1"
                     /db_xref="taxon:382"
                     /note="PCR_primers=fwd_name: fD1"
 BASE COUNT      253 a    223 c    321 g    198 t
 ORIGIN
        1 aacgctggcg gcaggcttaa cacatgcaag tcgagcgccc cgcaagggga gcggcagacg
       61 ggtgagtaac gcgtgggaat ctacccтttt ctacggaata acgcagggaa acttgtgcta
      121 ataccgtatg agcccttcgg gggaaagatt tatcgggaaa ggatgagccc gcgttggatt
      181 agctagttgg tggggtaaag gcctaccaag gcgacgatcc atagctggtc tgagaggatg
      241 atcagccaca ttgggactga gacacggccc aaactcctac gggaggcagc agtggggaat
      301 attggacaat gggcgcaagc ctgatccagc catgccgcgt gagtgatgaa ggccctaggg
      361 ttgtaaagct ctttcaccgg tgaagataat gacggtaacc ggagaagaag ccccggctaa
      421 cttcgtgcca gcagccgcgg taatacgaag ggggctagcg ttgttcggaa ttactgggcg
      481 taaagcgcac gtaggcggat tgttaagtga ggggtgaaat cccagggctc aaccctggaa
      541 ctgcctttca tactggcaat ctagagtcca gaagaggtga gtggaattcc gagtgtagag
      601 gtgaaattcg tagatattcg gaggaacacc agtggcgaag gcggctcact ggtctggaac
      661 tgacgctgag gtgcgaaagc gtggggagca aacaggatta gataccctgg tagtccacgc
      721 cgtaaacgat gaatgttagc cgtcgggcag tttactgttc ggtggcgcag ctaacgcatt
      781 aaacattccg cctggggagt acggtcgcaa gattaaaact caaaggaatt gacgggggcc
      841 cgcacaagcg gtggagcatg tggtttaatt cgaagcaacg cgcagaacct taccagccct
      901 tgacatcccg atcgcggata cgagagatcg tattcttcag ttcggctgga tcggagacag
      961 gtgctgcatg gctgtcgtca gctcgtgtcg tgaga

LOCUS       GU902296       1027 bp    DNA     linear   BCT 14-APR-2010
DEFINITION  Sinorhizobium sp. strain M1D4 16S ribosomal RNA gene, partial sequence.
ACCESSION GU902296
VERSION
KEYWORDS    .
SOURCE      Sinorhizobium sp.
ORGANISM  Sinorhizobium sp.
Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
Rhizobiaceae; Sinorhizobium/Ensifer group; Sinorhizobium.
REFERENCE   1  (bases 1 to 1027)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     ISOLATION OF ORGANOPHOSPHORUS-DEGRADING BACTERIA FROM AGRICULTURAL MEDITERRANEAN
SOILS
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1027)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     Direct Submission
JOURNAL   Submitted (18-FEB-2010) Dpt. Biologia Cellulare e dello Sviluppo, University of
Palermo, Viale delle Scienze Edif 16, Palermo, PA
90128, ITALY
FEATURES             Location/Qualifiers
     source          1..1027
                     /organism="Sinorhizobium sp."
                     /mol_type="genomic DNA"
                     /strain="M1D4"
                     /db_xref="taxon:42445"
                     /note="PCR_primers=fwd_name: fD1"
 BASE COUNT      253 a    237 c    329 g    208 t
 ORIGIN
        1 gcggcaggct taacacatgc aagtcgagcg ccccgcaagg ggagcggcag acgggtgagt
       61 aacgcgtggg aatctaccct tttctacgga ataacgcagg gaaacttgtg ctaataccgt
      121 atacgccctt cgggggaaag atttatcggg aaaggatgag cccgcgttgg attagctagt
      181 tggtggggta aaggcctacc aaggcgacga tccatagctg gtctgagagg atgatcagcc
      241 acattgggac tgagacacgg cccaaactcc tacgggaggc agcagtgggg aatattggac
      301 aatgggcgca agcctgatcc agccatgccg cgtgagtgat gaaggcccta gggttgtaaa
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      361 gctctttcac cggtgaagat aatgacggta accggagaag aagccccggc taacttcgtg
      421 ccagcagccg cggtaatacg aaggggggcta gcgttgttcg gaattactgg gcgtaaagcg
      481 cacgtaggcg gacatttaag tcaggggtga aatcccgggg ctcaaccccg gaactgcctt
      541 tgatactggg tgtctagagt atggaagagg tgagtggaat tccgagtgta gaggtgaaat
      601 tcgtagatat tcggaggaac accagtggcg aaggcggctc actggtccat tactgacgct
      661 gaggtgcgaa agcgtgggga gcaaacagga ttagataccc tggtagtcca cgccgtaaac
      721 gatgaatgtt agccgtcggg cagtttactg ttcggtggcg cagctaacgc attaaacatt
      781 ccgcctgggg agtacggtcg caagattaaa actcaaagga attgacgggg gcccgcacaa
      841 gcggtggagc atgtggttta attcgaagca acgcgcagaa ccttaccagc ccttgacatc
      901 ccgatcgcgg attacggaga cgttttcctt cagttcggct ggatcggaga caggtgctgc
      961 atggctgtcg tcagctcgtg tcgtgagatg ttgggtttag tcccgcaacg agcgcaaccc
     1021 tcgccct

LOCUS       GU902297     1150 bp    DNA     linear   BCT    14-APR-2010
DEFINITION  Sinorhizobium sp. strain M2D3 16S ribosomal RNA gene, partialsequence.
ACCESSION GU902297
VERSION
KEYWORDS    .
SOURCE      Sinorhizobium sp.
ORGANISM  Sinorhizobium sp.
Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
Rhizobiaceae; Sinorhizobium/Ensifer group; Sinorhizobium.
REFERENCE   1  (bases 1 to 1150)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     ISOLATION OF ORGANOPHOSPHORUS-DEGRADING BACTERIA FROM AGRICULTURAL MEDITERRANEAN
SOILS
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1150)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     Direct Submission
JOURNAL   Submitted (18-FEB-2010) Dpt. Biologia Cellulare e dello Sviluppo, University of
Palermo, Viale delle Scienze Edif 16, Palermo, PA
90128, ITALY
FEATURES             Location/Qualifiers
     source          1..1150
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                     /mol_type="genomic DNA"
                     /strain="M2D3"
                     /db_xref="taxon:42445"
                     /note="PCR_primers=fwd_name: fD1"
 BASE COUNT      286 a    260 c    368 g    236 t
 ORIGIN
       1 gcggcaggct taacacatgg aagtcgagcg ccccgcaagg ggagcggcag acgggtgagt
      61 aacgcgtggg aatctaccct tttctacgga ataacgcagg gaaacttgtg ctaataccgt
     121 atacgccctt cgggggaaag atttatcggg aaaggatgag cccgcgttgg attagctagt
     181 tggtggggta aaggcctacc aaggcgacga tccatagctg gtctgagagg atgatcagcc
     241 acattgggac tgagacacgg cccaaactcc tacgggaggc agcagtgggg aatattggac
     301 aatgggcgca agcctgatcc agccatgccg cgtgagtgat gaaggcccta gggttgtaaa
     361 gctctttcac cggtgaagat aatgacggta accggagaag aagccccggc taacttcgtg
     421 ccagcagccg cggtaatacg aaggggggcta gcgttgttcg gaattactgg gcgtaaagcg
     481 cacgtaggcg gacatttaag tcaggggtga aatcccgggg ctcaaccccg gaactgcctt
     541 tgatactggg tgtctagagt atggaagagg tgagtggaat tccgagtgta gaggtgaaat
     601 tcgtagatat tcggaggaac accagtggcg aaggcggctc actggtccat tactgacgct
     661 gaggtgcgaa agcgtgggga gcaaacagga ttagataccc tggtagtcca cgccgtaaac
     721 gatgaatgtt agccgtcggg cagtttactg ttcggtggcg cagctaacgc attaaacatt
     781 ccgcctgggg agtacggtcg caagattaaa actcaaagga attgacgggg gcccgcacaa
     841 gcggtggagc atgtggttta attcgaagca acgcgcagaa ccttaccagc ccttgacatc
     901 ccgatcgcgg attacggaga cgttttcctt cagttcggct ggatcggaga caggtgctgc
     961 atggctgtcg tcagctcgtg tcgtgagatg ttggggttaa gtcccgcaac gagcgcaacc
    1021 ctcgccttag ttgccagcat ttagttgggc actctaagga ctgcgtgaat aagcgagaag
    1081 gaaggtggga tgacgtcaag tctcatgcct tacgctggct acccacgtgc taacaatggt
    1141 ggtgaacagt

LOCUS      GU902298          1071 bp    DNA     linear   BCT 14-APR-2010
DEFINITION  Streptomyces peucetius strain RBD4 16S ribosomal RNA gene,partial  sequence.
ACCESSION GU902298
VERSION
KEYWORDS    .
SOURCE      Streptomyces peucetius
ORGANISM  Streptomyces peucetius
Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;
Streptomycineae; Streptomycetaceae; Streptomyces.
REFERENCE   1  (bases 1 to 1071)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     ISOLATION OF ORGANOPHOSPHORUS-DEGRADING BACTERIA FROM AGRICULTURAL MEDITERRANEAN
SOILS
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1071)
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AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     Direct Submission
JOURNAL   Submitted (18-FEB-2010) Dpt. Biologia Cellulare e dello Sviluppo,University of
Palermo, Viale delle Scienze Edif 16, Palermo, PA
90128, ITALY
FEATURES             Location/Qualifiers
     source          1..1071
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                     /mol_type="genomic DNA"
                     /strain="RBD4"
                     /db_xref="taxon:1950"
                     /note="PCR_primers=fwd_name: fD1"
 BASE COUNT      246 a    268 c    352 g    205 t
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        1 tgccgaacgg gtgagtaaca cgtggggaat ctgcccttca ctctgggaca agccctggaa
       61 acggggtcta ataccggata acactgcgga tcgcatggtc tgcggttgaa agctccggcg
      121 gtgaaggatg agcccgcggc ctatcagctt gttggtgggg tgatggccta ccaaggcgac
      181 gacgggtagc cggcctgaga gggcgaccgg ccacactggg actgagacac ggcccagact
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      301 cgcgtgaggg atgacggcct tcgggttgta aacctctttc agcagggaag aagcgaaagt
      361 gacggtacct gcagaagaag cgccggctaa ctacgtgcca gcagccgcgg taatacgtag
      421 ggcgcaagcg ttgtccggaa ttattgggcg taaagagctc gtaggcggct tgtcgcgtcg
      481 gatgtgaaag cccggggctt aaccccgggt ctgcattcga tacgggcagg ctagagtgtg
      541 gtaggggaga tcggaattcc tggtgtagcg gtgaaatgcg cagatatcag gaggaacacc
      601 ggtggcgaag gcggatctct gggccattac tgacgctgag gagcgaaagc gtggggagcg
      661 aacaggatta gataccctgg tagtccaccc ataaacgtt gggaactagg tgttggcgac
      721 attccacgtc gtcggtgccg cagctaacgt atttagttcc ccgcctgggg agtacggccg
      781 caaggctaaa actcaaaggg attgacgggg gcccgcacaa gcagcgagca tgtggcttaa
      841 ttcgacgcaa cgcgaagaac cttaccaagg cttgacttat acctgaaagc atcgagatgg
      901 tgctcccccc ttgtggtcag tatacagctg gtgccatggc tgttcatcag cttcatgtcg
      961 tgagatgttg ggtcaacgtc ccgcaacgaa ccgccacact tgtttcttgt tgccagcagc
     1021 cttcggggta atggggacct ccacagaaaa ctgcccggtt caatctcgaa g
LOCUS      GU902299          620 bp    DNA     linear   BCT 14-APR-2010
DEFINITION  Pseudomonas putida strain M2P3 16S ribosomal RNA gene, partial sequence.
ACCESSION GU902299
VERSION
KEYWORDS    .
SOURCE      Pseudomonas putida
ORGANISM  Pseudomonas putida
Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Pseudomonadaceae; Pseudomonas.
REFERENCE   1  (bases 1 to 620)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     ISOLATION OF ORGANOPHOSPHORUS-DEGRADING BACTERIA FROM AGRICULTURAL MEDITERRANEAN
SOILS
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 620)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     Direct Submission
JOURNAL   Submitted (18-FEB-2010) Dpt. Biologia Cellulare e dello Sviluppo,University of
Palermo, Viale delle Scienze Edif 16, Palermo, PA
90128, ITALY
FEATURES             Location/Qualifiers
     source          1..620
                     /organism="Pseudomonas putida"
                     /mol_type="genomic DNA"
                     /strain="M2P3"
                     /db_xref="taxon:303"
                     /note="PCR_primers=fwd_name: fD1"
BASE COUNT      160 a    136 c    197 g    127 t
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       61 tcagcggcgg acgggtgagt aatgcctagg aatctgcctg gtagtggggg acaacgtttc
      121 gaaaggaacg ctaataccgc atacgtccta cgggagaaag caggggacct tcgggccttg
      181 cgctatcaga tgagcctagg tcggattagc tagttggtgg ggtaatggct caccaaggcg
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      301 ctcctacggg aggcagcagt ggggaatatt ggacaatggg cgaaagcctg atccagccat
      361 gccgcgtgtg tgaagaaggt cttcggattg taaagcactt taagttggga ggaagggcat
      421 taacctaata cgttagtgtt ttgacgttac cgacagaata agcaccggct aactctgtgc
      481 cagcagccgc ggtaatacag agggtgcaag cgtttatcgg aattactggg cctaaagcgc
      541 gcgtaggtgg tttgttaagt tggatgcgaa agccccgggc tcaacctggg aactgaatca
      601 aaaactggca cctagagaac

LOCUS       GU902300        1078 bp    DNA     linear   BCT 14-APR-2010
DEFINITION  Azospirillum brasilense strain RCD2 16S ribosomal RNA gene, partial sequence.
ACCESSION GU902300
VERSION
KEYWORDS    .
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SOURCE      Azospirillum brasilense
ORGANISM  Azospirillum brasilense
Bacteria; Proteobacteria; Alphaproteobacteria;
Rhodospirillales; Rhodospirillaceae; Azospirillum.
REFERENCE   1  (bases 1 to 1078)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E.,Alonzo,G. and Quatrini,P.
TITLE     ISOLATION OF ORGANOPHOSPHORUS-DEGRADING BACTERIA FROM  AGRICULTURAL MEDITERRANEAN
SOILS
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1078)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     Direct Submission
JOURNAL   Submitted (18-FEB-2010) Dpt. Biologia Cellulare e dello Sviluppo,University of
Palermo, Viale delle Scienze Edif 16, Palermo, PA
90128, ITALY
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
                     /strain="RCD2"
                     /db_xref="taxon:192"
                     /note="PCR_primers=fwd_name: fD1"
 BASE COUNT      253 a    260 c    357 g    208 t
 ORIGIN
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       61 aacacgtggg aacctgcctt atggttcggg ataacgtctg gaaacggacg ctaacaccgg
      121 atgtgccctt cgggggaaag tttacgccat gagaggggcc cgcgtccgat taggtagttg
      181 gtggggtaat ggcccaccaa gccgacgatc ggtagctggt ctgagaggat gatcagccac
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      421 agcagccgcg gtaatacgaa gggggcgagc gttgttcgga attactgggc gtaaagggcg
      481 cgtaggcggc ccgatcagtc agatgtgaaa gccccgggct caacctggga actgcatttg
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      721 tgaatgctag acgctggggt gcatgcactt cggtgtcgcc gctaacgcat taagcattcc
      781 gcctggggag tacggccgca aggttaaaac tcaaaggaat tgacgggggc ccgcacaagc
      841 ggtggagcat gtggtttaat tcgaagcaac gcgcagaacc ttaccaaccc ttgacatgtc
      901 cattgccggt ccgagagatt ggaccttcag ttcggctgga tggaacacag gtgctgcatg
      961 gctgtcgtca gctcgtgtcg tgagatgttg ggttaagtcc cgcacgagcg caacccctac
     1021 cgccagttgc atcattcagt tggcacttct ggtgaaactg ccggtgacag ccggagga

LOCUS       GU902301         1102 bp    DNA     linear   BCT 14-APR-2010
DEFINITION  Rhizobium sp. strain RCD1 16S ribosomal RNA gene, partial sequence.
ACCESSION GU902301
VERSION
KEYWORDS    .
SOURCE      Rhizobium sp.
ORGANISM  Rhizobium sp.
Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
Rhizobiaceae; Rhizobium/Agrobacterium group; Rhizobium.
REFERENCE   1  (bases 1 to 1102)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     ISOLATION OF ORGANOPHOSPHORUS-DEGRADING BACTERIA FROM AGRICULTURAL MEDITERRANEAN
SOILS
JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1102)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     Direct Submission
JOURNAL   Submitted (18-FEB-2010) Dpt. Biologia Cellulare e dello Sviluppo,University of
Palermo, Viale delle Scienze Edif 16, Palermo, PA
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AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     ISOLATION OF ORGANOPHOSPHORUS-DEGRADING BACTERIA FROM AGRICULTURAL MEDITERRANEAN
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REFERENCE   2  (bases 1 to 1130)
AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     Direct Submission
JOURNAL   Submitted (18-FEB-2010) Dpt. Biologia Cellulare e dello Sviluppo,University of
Palermo, Viale delle Scienze Edif 16, Palermo, PA
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AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     ISOLATION OF ORGANOPHOSPHORUS-DEGRADING BACTERIA FROM AGRICULTURAL MEDITERRANEAN
SOILS
JOURNAL   Unpublished
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AUTHORS   Fodale,R., De Pasquale,C., Lo Piccolo,L., Palazzolo,E., Alonzo,G.and Quatrini,P.
TITLE     Direct Submission
JOURNAL   Submitted (18-FEB-2010) Dpt. Biologia Cellulare e dello Sviluppo,University of
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90128, ITALY
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Appendice B

L18 HS-SPME AND GC-MS AS VALID TOOLS TO ASSESS VOLATILE ORGANIC COMPOUNDS FROM SOIL NATURAL ORGANIC MATTER

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Introduction

Humic acids (HAs) which are involved in almost all physical, chemical, and biological processes occurring in soil system[1,2] represent the most abundant fraction of soil organic matter. In particular, HAs are well known to be very active in interacting to various extents and modalities with a variety of organic and inorganic chemical contaminants[3]. Knowledge of the composition, structure, and functionalities of HAs is therefore, essential for the understanding of their chemical behavior and reactivity in environmental matrices.

Chemical analyses on HAs are traditionally done by chromatographic and spectroscopic techniques. Recent advances in the chemistry of humic substances, revealed that a detailed characterization can be better achieved when a separation of the different components is obtained[4]. Here we suggest head space solid-phase micro-extraction (HS-SPME) coupled with gas-chromatography/mass spectrometry (GC-MS) as a new analytical tool for the characterization of the volatile components released from humic substances at 80 °C.

Materials and Methods

S o i l

Two surface horizons were sampled from the ancient caldera of Vico (nearby Rome) and Monte Faito (Naples) in Italy. Detailed characterization of the two soils are reported elsewhere[5].

HA Extraction

Humic acids (HAs) were extracted by using common procedures[6]. Namely, 100 g of each soil were suspended in 500 ml of 1M NaOH and 0.1M $Na_4P_2O_7$ and centrifuged at 7,000 rpm for 20 min. The supernatant was then treated with 37% HCl until pH 1 was reached to precipitate HAs. Humic acids were purified by a series of dissolutions in 1M NaOH followed by flocculations in 6M HCl. Each HA was then shaken twice in a 0.25M HCl/HF solution for 24 h, dialized agaist distilled water till Cl-free and freeze-dried.

CPMAS ^{13}C-NMR Spectroscopy

CPMAS ^{13}C-NMR experiments were performed on a Bruker Avance 400 spectrometer operating at 100.6 MHz on carbon-13 and equipped with a 4 mm standard bore solid state probe. The rotor spin rate was set at 13,000 Hz. Samples were packed in 4 mm Zirconia rotors with Kel-F caps. A contact time of 1 ms, a recycle delay of 2 s, an acquisition time of 35 ms and a RAMP sequence to account for inhomogeneities of the Hartmann-Hahn condition at high rotor spin rates were used. Spectra acquisition was done with Topspin 2.0, whereas data elaboration was done with Mestre-C 4.9.9.9 by using a line broadening (LB) of 50 Hz and an automatic baseline correction with a 3rd order polynomial and Bernstein algorithm. Semi-quantitative results were obtained by integrating the spectral regions in the intervals 184–159 ppm (COOH), 159–110 ppm (aromatic C), 110–88 ppm (anomeric C), 88–62 ppm (C–O), 62–48 ppm (C–N) and 48–0 ppm (alkyl C). All the areas were normalized to the total spectral areas and content percent was obtained.

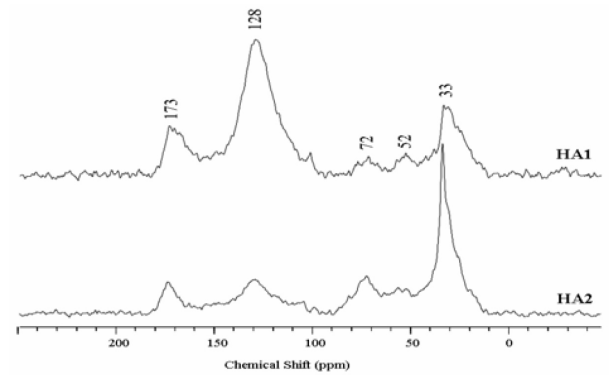

Fig. 1. **CPMAS ^{13}C-NMR spectra of the two humic acids used in the present study**

H S - S P M E E x t r a c t i o n

A polydimethylsiloxane fiber (PDMS 100 µm, Supelco, Bellefonte, PA – USA) was used to sample the head space in equilibrium with 20 mg of the two HAs placed in 15 ml glass vials. These were sealed with a poly(tetrafluoroethylene) silicon septum-lined cap (Supelco, Bellefonte, PA, USA) and heaten 30 min at temperatures of 80 °C. The sampling time for was 5 min maintaining the 80 °C temperature.

G C - M S A n a l y s i s

The gas-chromatographic analyses were run on a Hewlett-Packard 5890 GC system interfaced with a HP 5973 quadrupole mass spectrometer. A HP5–MS column was used (5% diphenyl – 95% dimethylpolysiloxane 30 m × 0.2 mm, 0.25 µm film, J&W Scientific, Folsom CA, USA). The fibre was manually inserted in a GC inlet port equipped with a specific glass liner for SPME injection (0.75 mm i.d.). Analyses were carried out in splitless mode with helium as carrier gas at 1 ml min^{-1}. Chromatographic conditions were: injector temperature 250 °C, oven temperature program: 1 min at 60 °C followed by a linear temperature increase of 5 °C min^{-1} up to 280 °C held for 15 min. The MS full-scan conditions were: source temperature 230 °C, interface temperature 280 °C. The electron impact ionization spectra were obtained at 70 eV, recording mass spectra from m/z 40 to 550.

Results and Discussion

The HS-SPME extraction method allowed the identification of 31 chemical components in each HA. Analyses at different temperatures have been done (data not repoted).

The optimal temperature conditions for the qualitative discrimination of HAs 80 °C giving a relative standard deviation [RSD, %] of ≤10 % for each identified component. Moreover, the amount of volatile components sampled in the head space was significantly representative of the whole HA systems as assessed by analyses with icreasing HA material. Table I reports the class of substances identified over the two solid state humic acids. Eight classes of organic compounds were discriminated. Among those, the largest contribution was from aromatic materials. In fact, HA1 revealed 66.77 % of aromatic moieties in the volatile mixture, whereas HA2 was 81.55 % (Table I). The amount of volatile aromatic molecules in HA1, smaller than in HA2, appears at variance with the content of aromatic systems obtained from CPMAS ^{13}C-NMR spectroscopy (Fig. 1, Table II). The aromatic C content by NMR (159–110 ppm) was the largest in HA1, thereby leading to the hypothesis that such material should produce a larger amount of volatile aromatic components than HA2. The contradictory HS-SPME GC-MS and CPMAS ^{13}C NMR results could be explained by considering the different natures of the soils from which the HAs were extracted. In fact, HA1 was extracted from a buried soil whereas HA2 was obtained from a surface horizon of a different volcanic soil. Organic material in buried conditions undergoes to anaerobic and abiotic degradation. Conversely, aerobic and biotic transformations occur in organic materials placed on surface soil horizons. The abiotic degradation in anaerobic conditions may produce highly condensed aromatic systems. Due to the larger molecular size of the condensed aromatic substances in HA1, these are less available for head space sampling than the corresponding aromatic systems formed in aerobic and biotic transformations. Except for the aromatic moieties which were in disagreement when comparing the gas-chromatographic data with the NMR results, the remaining components be have similarly. In fact, Tables I and II report the same trends for all the relative amounts revealed by the two analytical techniques used in the present study.

Table I
Composition of the volatile components released by the two HAs at 80 °C. The compounds were grouped according to their functional groups. The reported correspondent percent amounts [%] of identified compounds are the medium value of three different chromatographic analyses

	Content [%]	
Compounds	HA1	HA2
Acids	18.64	7.30
Aldehydes	3.56	1.19
Ketones	1.36	0.00
Alcohols	0.53	0.40
Amines	0.57	0.40
Olephins	1.21	1.11
Aromatics	66.77	81.55
Alkanes	7.19	8.27

Conclusions

In the present study we used a new analytical tool for the characterization of the volatile components in humic substances. Results showed that the amount of volatile aromatic moieties was depending on the nature and genesis of the soils from which the HAs were extracted. Moreover, a number of fatty acids, as well as alkane-systems were also found in the head space in equilibrium with the solid humic substances. The relevant novelty was that we expected fatty acids and hydrophobic alkanes to be confined mainly inside the hydrophobic and chemically protected core of the supramolecular humic acids. For this reason the volatilization to the head space was thought to be difficult. Conversely, our findings appeared to confirm CPMAS ^{13}C NMR results from Conte and Berns (2008)[7] which described the conformation of humic substances as made mainly by a hydrophobic aromatic core surrounded by long carbon chains. The HS-SPME-GC-MS technique appears to be a very promising tool in obtaining relevant information on the lighter fraction of organic components weakly bound to humic materials.

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Table II
Content [%] by CPMAS ^{13}C NMR of the different groups in HAs

Soil humic acid	COOH	Aromatic C	Anomeric C	C–O	C–N	Alkyl C
	184–159 ppm	159–110 ppm	110–88 ppm	88–62 ppm	62–48 ppm	48–0 ppm
HA1	12	55	5	4	4	21
HA2	9	21	3	14	8	45

ISOLATION OF ORGANOPHOSPHORUS-DEGRADING BACTERIA FROM AGRICULTURAL MEDITERRANEAN SOILS

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ABSTRACT

Pesticides are biologically active compounds designed to interfere with metabolic processes and environmental safety. The biodiversity of organophosphorus degrader bacteria in Mediterranean agricultural soils was evaluated. 47 strains were isolated from Sicilian soils under different management systems. The isolates were obtained from enrichment cultures containing parathion as an exclusive carbon source. They were grouped into 20 Operational Taxonomic Units (OTUs) on the base of the ribosomal Internal Transcribed Spacer (ITS) polymorphism. A positive and significative correlation was found between bacterial biodiversity and such soil chemico-physical characteristics (soil clay content, cationic exchange capacity and carbon percentage). The strains, identified by partial sequencing of 16S rRNA gene, were tested in order to evaluate their pesticide degrading ability in Mineral Salt Medium (MSM) and in three different standard soils distinguished by their different chemico-physical characteristics. Solid Phase Micro-Extraction (SPME) and solid liquid extraction methods coupled with gas-chromatograph and mass-spectrometer (GC-MS) instruments were used. Strains affiliated to *Sinorhizobium*, *Pseudoxanthomonas*, *Streptomyces iakyrus*, *Microbacterium takaoensis* and *Isoptericola dokdonensis* have never mentioned as organophosphorus degraders.

KEYWORDS: biodegradation, organophosphorus pesticides, solid phase micro-extraction

INTRODUCTION

Organophosphorus pesticides (OPs) have a notable importance in agriculture but are also considerable as a threat for human health and environment. They are potent acetylcholinesterase (AChE) inhibitors [1] and the specific relationship between OPs and AChE is commonly used as biomarker of OPs contamination [2]. Organophosphorus pesticides act by contact, ingestion and inhalation. The OPs alter the equilibrium of natural ecosystems causing air, water and soil pollution [3]. Several aspects are involved in the fate and behaviour of OPs in the environment: effluent irrigation [4], photodecomposition mechanisms [5], volatilization [6] and, finally, biodegradation [3, 7]. Due to the role of bacteria in the xenobiotic compounds degradation processes, a key significant knowledge could be reached by studying their fate and behaviour in pure and mixed strain cultures [8]. The first micro-organism that could degrade organophosphorus compounds was isolated in 1973 and identified as *Flavobacterium* sp., since then several bacterial species both Gram positive and Gram negative, have been isolated which can degrade a wide range of organophosphorus compounds [9].

The most significant step in detoxification of OPs is hydrolysis of P-O-alkyl and P-O-aryl bonds generally catalized by an organophosphate hydrolase or phosphotriesterase. Oxidation, alkylation and dealkylation reactions are also involved in OPs degradation processes [3].

Taking into account the risks caused by the use of pesticides, biodegradation is an efficient way to preserve human and environment safety. The aim of this study was to evaluate the biodiversity of naturally occurring OPs degrading bacteria in Mediterranean soils. Enrichment cultures, containing parathion as the sole carbon source, were set up to isolate OP degraders from agricultural soils cultivated under different management systems. Parathion degradation ability of selected isolates was evaluated in liquid cultures and standard soil microcosms.

MATERIALS AND METHODS

Soil sampling and analysis

The bacteria were isolated from cultivated soils of west Sicily under conventional and organic management systems. Soil samples were characterized after air-dried and sieved (< 2 mm) procedures. Their physico-chemical properties were determined. The pH was measured in distilled water by using a soil water solution (1:2.5 *w/v*) and a glass membrane electrode (Mettler-Toledo). Soil electron conductivity (EC) was measured in distilled water using a soil/water

ratio of 1:5 (w/v). Cation exchange capacity (CEC) was determined using barium chloride and triethanolamine solution at pH 8.2 as the extracting solution. The soil organic C % of each sample was determined by the Walkley–Black dichromate oxidation method [10].

Bacterial isolation

For bacterial isolation soil samples were directly used within 24 h from collection without any treatment, in order to preserve its biological properties. Each soil sample (2 g) was added to 20 ml of a Mineral Salt Medium (MSM) [11] pH 7, containing 400 µl of a filter (0.2 µm) sterilized standard solution of parathion in hexane/ethyl acetate (1:1) (20 µg ml^{-1} final concentration). The enrichment cultures were incubated at 30 °C on a rotary shaker at 200 r.p.m. for 4 days. Two ml aliquots of each culture were re-inoculated in fresh MSM containing double concentration of parathion and incubated in the same conditions. Aliquots of these enrichment cultures were serially diluted and plated onto MSM agar containing parathion as carbon source (40 µg ml^{-1}) and therefore incubated at 30 °C for 4 days. Colonies were streaked to purity on the same medium and characterized phenotypically for colony color and morphology and Gram staining. Phenotypically different isolates were maintained under glycerol 20% at -80°C.

Amplification of the 16S-23S ITS and 16S rRNA gene

Ribosomal Intergenic Spacer Analysis (RISA) was performed in order to analyze the diversity of our bacterial isolates. The 16S-23S ribosomal spacers (Internal Transcribed Spacer, ITS) were amplified by colony-PCR (Polymerase Chain Reaction) using ITSF/ITSReub primer set [12] in a 20 µl volume reaction. The PCR mixture contained 1 µl of a single colony lysate as template, 0.3 µM of each primer, 0.2 mM of deoxynucleoside triphosphate (dNTPs), 1X reaction buffer, 1.5 mM $MgCl_2$ and 0.05 u/µl of HotStarTaq® DNA Polymerase (QIAGEN). The PCR was carried out under the following cycling profile: 95 °C for 20 min; 30 cycles of 1 min at 95 °C, 1 min at 55 °C, 1.5 min at 72 °C; 72 °C for 10 min. PCR products were visualized on ethidium bromide-stained 1.5 % agarose gel in order to identify different ITS profile. Isolates with identical ITS profiles were grouped into the same Operational Taxonomic Units (OTUs). One or more representative isolate(s) for each OTU was randomly chosen for partial sequencing of 16S rRNA [13] and phyologenetic identification. The 16S rRNA was amplified using the fD1/rD1 primer set [14] with the same protocol as described above. The PCR amplification products were purified by using the QIAquick Gel Extraction Kit (QIAGEN) and commercially sequenced. Unambiguous sequences were used in BLASTn search [15]. The sequences produced in this work were submitted to GenBank/EMPL/DDBJ database under the accession numbers GU902282 to GU902303.

Statistical analyses

Principal component analysis (PCA) was carried out considering the principal components with an eigen value >1. The method of Pearson parametric correlation was used and 6 variables were considered: number of isolated bacteria, particle size, carbon percentage, pH, Electron Conductivity (EC) and Cationic Exchange Capacity (CEC) of soils. The amounts of parathion recovered from each microcosm were expressed in terms of % residues. The statistical differences in the amount of parathion recovered in different experimental microcosms were also analyzed by t tests. All statistical analyses were done using Statistical 6.0 for Windows (StatSoft Inc., Tulsa, OK, USA).

Degradation of parathion in liquid medium

The parathion degradation ability of nine different isolates was tested in liquid MSM. Each isolate was inoculated in Luria-Bertani broth (Difco) and incubated in the dark for 24 h at 30 °C on a rotary shaker at 150 r.p.m. Cells were harvested by centrifugation at 4000 r.p.m. for 20 min, washed twice with MSM and suspended to an OD 590 nm in MSM. 20 ml of MSM were contaminated with a filter (0.2 µm) sterilized standard solution of parathion in hexane/ethyl acetate (1:1), to achieve a final concentration of 15 µg ml^{-1}. After 24h of shaking and solvent evaporation under a gentle nitrogen stream 0.5 ml of inoculum were added in triplicate. Abiotic and non contaminated controls were also set up. The cultures were incubated in the dark, for 72 h, at 30 °C on a rotary shaker at 150 r.p.m.

Degradation of parathion in standard soils

The degradation ability of 2 selected strains was also tested on soil microcosms containing three standard soils namely 2.1, 6S and 3A sandy, sandy-clay and loam respectively [16]. The standard soils were purchased from Landwirtschaftiche Untersuchungs- und Forschhungsanstalt (LUFA) Speyer (Germany). Each standard autoclaved soil (2 g aliquots) was contaminated with a filter sterilized standard solution of parathion in hexane/ethyl acetate (1:1), to achieve a final concentration of 100 µg ml^{-1}. After 24h of shaking and solvent evaporation under a gentle nitrogen stream, the microcosms were inoculated with 0.5 ml of a bacterial suspension prepared as described above and then incubated on a rotary shaker in the dark, for 72 h, at 30°C. Abiotic and non contaminated controls were set up.

Analytical procedure

The degradation ability was studied by evaluating the parathion residues after incubation. It was expressed as % in respect to abiotic and non contaminated controls. The parathion residues in the liquid medium were extracted, purified and concentrated simultaneously by using a Solid Phase Micro-Extraction (SPME) method. The analytical procedure previously developed from De Pasquale et al. [16] was fully investigated in liquid cultures in order to evaluate the possible use of SPME. Three fibres PDMS (100 mm polydimethylsiloxane), PA (85 mm polyacrylate) and CW-DVB (65 mm carbowax-divinylbenzene) all equipped with holders for manual injection (Supelco UK) were tested. Prior to use, the PDMS fibres were conditioned at 250 °C for 30 min, the PA fibres at 300 °C for 2 h, and the CW-

DVB fibres at 220 °C for 30 min, each in a GC injector port by using a proper liner Ø 0.75 mm. Partitioning coefficient (*Kd*) for parathion were examined by determining Kd_{SPME} in the liquid medium.

The analytical procedure was evaluated at pH 5 with 10 % NaCl (*w/v*). The analysis were carried out in triplicate in non inoculated systems. Kd_{SPME} values were calculated by considering the nanograms of analyte absorbed or adsorbed on to the SPME fiber and the initial pesticide content in the system.

The pH of each liquid sample was adjusted by using NaOH or HCl solution (0.1, 0.5, 1 M). The fiber was directly immersed in liquid medium (20 ml) at room temperature (≅ 20 °C) for 15 min under magnetic stirring.

A different extraction method was used in order to evaluate the parathion residues in soil microcosms. 3 ml of deionised water were added to each sample. The sample was mixed by using ultra-turrax (IKA T18 Basic). 2g of Na_2SO_4 and 3g of $NaHCO_3$ were added and pesticide residue was extracted with 30 ml of ethyl acetate after 1h on a rotary shaker. The supernatant was filtered by using hydrophobic PTFE membrane filters 0.45 µm (ALBET) and a Millipore filtration glass unit. The extract was evaporated to low volume by using a rotary evaporator at 40 °C (HEIDOLPH LABOROTA 4000 EFFICIENT, WB ECO). Finally it was diluted by using ethyl acetate to a final volume of 5 ml.

The analyte residue was evaluated by using a gas-chromatograph and mass-spectrometer (GC-MS) system (Hewlett-Packard 5890 and 5973 MS). The column used was a HP5–MS (5% diphenyl- 95% dimetil polisiloxano 30 m x 0.2 mm, 0.25 µm film, J&W Scientific, Folsom CA, USA). The oven temperature program was: 80 °C for 1 min, with an increase of 5 °C min^{-1} to 220 °C for 5 min. Helium flow was 1 ml min^{-1}; injection mode was split 1/10 in the case of solvent injections and splitless in the SPME method. The injection port temperature was 280°C; injection volume for ETAC extract was 1 µl and two different liners Ø (0.75 and 2 mm) were used for SPME and solvent injections respectively.

MS full-scan method was used and the electron impact ionization spectra were obtained at 70 eV, recording mass spectra from m/z 42 to 550 UMA. The parathion mass spectra was compared with the data bank NIST 05 and the identification was confirmed by using a standard product (all 99% purity – Fluka, Sigma-Aldrich Chemie GmbH, Switzerland).

The degradation extents were calculated as the ratios of substrate degraded in test flasks to substrate recovered in abiotic controls.

RESULTS AND DISCUSSION

Eight cultivated soils under different management systems and characterized by their physico-chemical parameter (Table 1) were chosen in west Sicily as bacterial source. The mediated soil samples were used in enrichment cultures with parathion as the sole carbon source. This pesticide was chosen because of its high toxicity and persistence in the environment.

After plating on parathion-containing MSM agar medium, 47 phenotypically different colonies able to grow on parathion as unique carbon source were isolated.

The 47 new isolates, separated into Gram-positives and Gram-negatives, were grouped by analyzing the 16S-23S ribosomal internal transcribed spacer (ITS) into 20 different Operational Taxonomic Units (OTUs from A to T) (Table 2). Each OTU comprised of 1 to 9 isolates (Table 2) and each soil comprised of 2 to 8 different OTUs (Table 1). The highest number of OTUs (from 5 to 8) was found in soils APCH, RIBCON, RIBBIO and RIBFAN, all from Citrus groves (Tab. 1), the lowest bacterial diversity was detected in VCH and POV soils with 2 and 3 OTUs from a vineyard and a greenhouse, respectively (Table 1).

TABLE 1 – Physico-chemical characteristics of soils utilized in order to isolate strains grouped in Operational Taxonomic Unit (A-T);

Soil code	VCH	APCH	POV	KAD	RIBCON	RIBBIO	RIBFAN	RIBINO
Geographical origin	Partinico	Misilmeri	Partinico	Misilmeri	Ribera	Ribera	Ribera	Ribera
Culture	Vineyard	Citrus grove	Greenhouse Tomato	Persimmon orchard	Citrus grove	Citrus grove	Citrus grove	Citrus grove
Agricultural treatment	conventional	conventional	conventional	conventional	organic	organic	conventional	conventional
Clay %	6.3	33.3	9.7	37.5	40.8	40.2	43.3	38.9
Loam %	3.7	23.3	3.5	24.9	24.8	27.2	23.7	27.3
Sand %	90.0	43.4	86.8	37.6	34.4	32.6	33.0	33.7
Soil Type	Sand	clay loam	loamy sand	clay loam	clay	clay	clay	clay loam
Org C %	0.54	1.58	0.71	1.66	2.60	2.82	3.33	2.48
pH (H_2O)	7.2	8	6.4	8.1	7.8	7.8	8	7.9
EC (1:5) dS/m	0.2	0.4	0.3	0.5	0.3	0.4	0.4	0.4
CEC (cmol⁺/kg)	13.1	27.5	13.7	30.6	27.4	32.6	33.8	32.4
Isolated bacteria	2	6	4	4	8	9	9	5
OTU	C-K	B-D-F-N-P-Q	F-K-T	A-B-J-M	C-D-F-G-Q-R	D-I-J-M-O	B-D-E-G-H-L-O-S	B-C-D-K

TABLE 2 - Phylogenetic identification of the representative isolates from each Operational Taxonomic Unit (A-T);

OTU	Number of isolates in the OTU	Representative isolate (s)	16S rRNA gene aligned nucleotides	Most closely related sequence, accession number (% of identity)	Soil code
A	1	M1P2	732	*Sinorhizobium medicae* EU445262 (97%)	KAD
B	4	M1D3	955	*Arthrobacter* sp. EF612307 (99%)	KAD
C	3	RCP1	1109	*Rhizobium* sp. EU529842 (98%)	RIBCON
D	9	RFP2	1094	*Xanthomonas translucens* AY572961 (97%)	RIBFAN
		ROP3	1088	*Stenotrophomonas* sp. AB461831 (98%)	RIBINO
E	1	RFD1	1056	*Isoptericola dokdonensis* DQ387860 (99%)	RIBFAN
F	4	P2P1	1146	*Pseudoxanthomonas* sp.EU025131 (98%)	POV
G	2	RCP3	1027	*Stenotrophomonas maltophilia* AJ131117 (99%)	RIBCON
H	1	RFD4	1017	*Paracoccus* sp.AM403616 (99%)	RIBFAN
I	1	RBP2	1015	*Rhizobiales* EF219048 (98%)	RIBBIO
J	2	M1P1	1046	*Streptomyces iakyrus* AB184877 (99%)	KAD
K	5	P1P4	988	*Microbacterium takaoensis* AB201047 (98%)	VCH
L	1	RFP4	1083	*Agrobacterium* sp.GU085230 (98%)	RIBFAN
M	2	RBD1	995	*Sinorhizobium meliloti* AY196963 (100%)	RIBBIO
		M1D4	1026	*Sinorhizobium* sp.EU399910 (99%)	KAD
N	1	M2D3	1143	*Sinorhizobium* sp.DQ196475 (98%)	APCH
O	3	RBD4	1033	*Streptomyces peucetius* AB249907 (96%)	RIBBIO
P	1	M2P3	613	*Pseudomonas putida* AY395005 (98%)	APCH
Q	2	RCD2	1073	*Azospirillum brasilense* EF634031 (99%)	RIBCON
R	1	RCD1	1087	*Rhizobium* sp.EF437254 (97%)	RIBCON
S	2	RFP1	1119	*Agrobacterium tumefaciens* EU697966(97%)	RIBFAN
T	1	P2P3	1151	*Roseomonas fauriae* AY150046 (97%)	POV

A number of factors such as plant type, soil, agricultural practice, soil microbial community, roots constituents and chemical compounds applied to the soil and plants, influence the microbial activity and bacterial biodiversity of soils [17, 18]. The PCA reduces the number of total variables to only few retaining the major part of the information. The amount of variables was reduced to only two (PC1 and PC2) which retained 80.4% of the total variance (Fig. 1). Soils with the largest amount of clay, % C and biodiversity retained positive score on PC1. Sandy soils were placed in the directly opposite position with respect to the biodiversity loading (Fig. 1). Moreover by the increase of pH and EC soils were separated to each other on PC2. Soil clay content, C % and CEC, with values of 0.77, 0.89, 0.7 respectively, were significantly and positively correlated with bacterial biodiversity (Fig. 1).

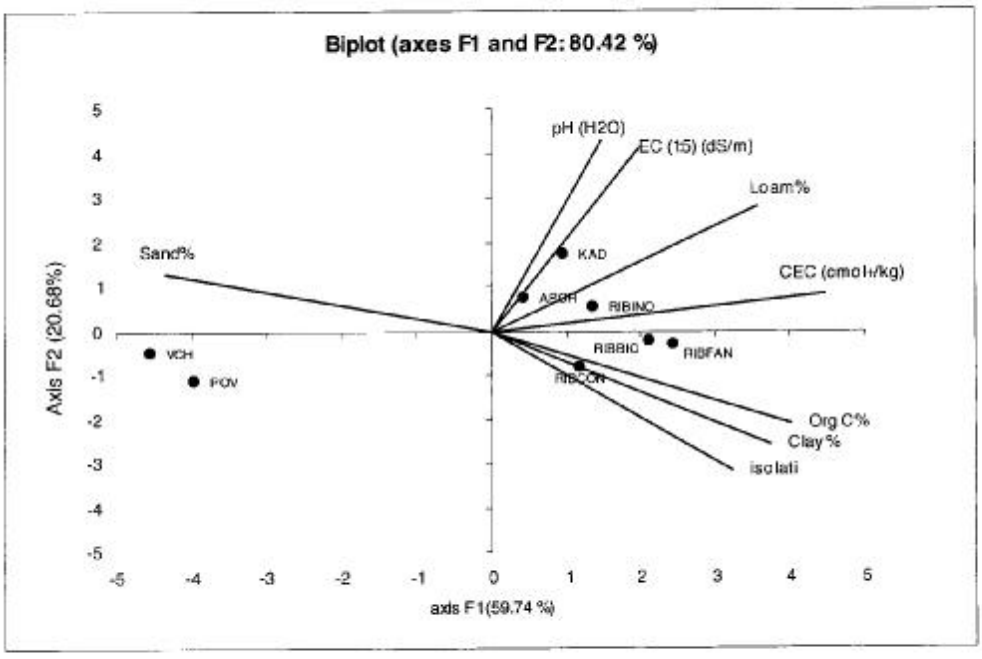

FIGURE 1 - Principal component analysis considering soil physico-chemical characteristics and bacterial biodiversity;

The 16S rRNA gene of one or more representatives for each OTU was sequenced. BLASTn search results are shown in Table 2. Although BLAST analysis was performed with 16S rDNA partial sequences, the closest species are retained as correct and used to identify the isolated strains.

The isolates were affiliated to 13 different genera (Table 2) that comprise both well known OPs degraders and taxa that were never cited as degraders. The number of Gram negative isolates is much greater than that of Gram positives; interestingly 8 OTUs out of the 20 are affiliated to the Rhizobiaceae family and are present in almost all the analysed soils (Table 1).

Among the isolated bacteria, members of the genus *Agrobacterium* and other rhizobiaceae are known as degraders of glyphosate, diazinon, coumaphos and parathion [3, 19, 20]. We also isolated four *Sinorhizobium* strains, genus known for degrading phosphonate compounds [21].

Other isolates belong to the Gram negative genera *Stenotrophomonas*, *Pseudomonas* [22, 23], *Xanthomonas*, *Roseomonas* (syn. *Azospirillum brasilense*) [24] and *Paracoccus* [25] that comprise members that are already known as OPs degraders. For some of these genera very few isolates were obtained in these last years and little information is available. For example an unique *Stenotrophomonas* isolate (YC-1) was recently reported as degrader of chlorpyrifos, parathion, methyl parathion and fenitrothion [26]. Among the Gram negative bacteria a strain of *Pseudoxanthomonas* phylogenetically related to *Xanthomonas* was also isolated. This genus is unknown as OPs degrader.

Isolates belonging to rhizobiaceae and those related to *Azospirillium brasilense* in particular, appear to be the most interesting strains and could be very useful in bioremediation practices of contaminated soils considering their symbiotic/associative lifestyle and their nitrogen fixation ability. *Azospirillum*-related bacteria are defined as PGPR (Plant Growth Promoting Rhizobacteria) as, besides performing nitrogen fixation, they can promote plant growth and protect plants from pathogens [27].

Among the Gram positive bacteria, members of the genus *Arthrobacter* degrade a wide range of organophosphorus compounds i.e. glyphosate [28], monocrotophos [29], chlorpyrifos, diazinon, EPN, fenitrothion, isofenphos, parathion and ethoprophos [30, 31]. Moreover some strains of *Arthrobacter* can degrade p-nitrophenol, which is the product of hydrolysis of parathion [3, 32]. Although *Arthrobacter* is a soil ubiquitous genus we isolated only one strain of *Arthrobacter* in this work. Members of this genus are generally adapted to resource-limited conditions and do not fluctuate in response to the addition of a carbon source. For this behaviour, they are considered K-strategists in comparison with r-strategists such as *Pseudomonas* and other Gram negative growing rapidly in the presence of a readily available carbon. Enrichment cultures may have favoured r-Strategists rather than K-strategists thus reducing the chance to isolate *Arthobacter* strains [33].

The species *Streptomyces iakyrus*, *Microbacterium takaoensis* and *Isoptericola dokdonensis*, to which some of our isolates belong, are not mentioned as organophosphorus degraders. Other members among *Streptomycetes* i.e. strain StC is able to degrade glyphosate via C-P bond cleavage [34] and a strain identified as *Microbacterium esteraromaticum* is capable to hydrolyse fenamiphos [35].

Although all the 47 isolates are able to grow on parathion as the sole carbon source, some strains were chosen to demonstrate their parathion degradation ability both in liquid medium and in soil microcosms.

Therefore to evaluate the degradation ability in the liquid medium, the partitioning coefficients (Kd_{SPME}) for parathion were examined by determining the fiber/liquid distribution coefficient. Data supplied by the manufacturer indicate that each SPME fiber consists of different amount of polymeric phase. The (*Kd*) of each fiber PDMS, PA and CW-DVB versus the parathion concentration in the solution was 808, 388.7, 404, respectively. On the above base it has been considered the RSD % (n=6) and the PDMS fiber was chosen for the extraction method in liquid medium.

The degradation ability in liquid medium was tested on four newly isolated putative OP degraders (namely: strains M1P1, P1P4, RFD1) and on isolates affiliated to genera or species already known as organophosphorus degraders (namely: strains M2D3, RCP3, P2P3, RFP1, RBP2, RCD2). The activity of the isolates in degrading parathion are shown in Figure 2. The amounts of parathion remaining in the cultures of strains P2P3, M2D3, RFP1, RBP2, RCD2 after 72 h incubation were lower than 30%. Strains P2P3, M2D3 metabolized more parathion with degrading nearly all the parathion (≅ 90-95 %) within 72h. Consequently, strain P2P3 which exhibited the highest parathion-degrading activity, was selected for further studies under the more realistic conditions of soil microcosms. Furthermore it was selected because of its interesting ability as above described. Strain RFD1 was also chosen for soil microcosms analysis to test the degradation ability of a strain as new OPs degrader. The results confirm the biodegradation ability of both strains although with different efficiency in each soil. In these conditions strain RFD1 is more efficient than strain P2P3 in all kind of soils thus upsetting the previous data obtained on mineral medium (Fig. 2). The biodegradation of organic chemicals in soil is a process resulting from the interaction between the soil microbial community and the chemico-physical environment [36]. Comparing the different soil matrices of the three standard soils the percentage of pesticide residue was considerably lower in the soils 2.1 and 6S in respect to soil 3A for both strains. Parathion residue was below 10%, between 10% and 20% and around 40% in the soil 2.1, 6S and 3A respectively (Fig. 3). In particular biodegradation was more efficient in the sandy soil 2.1 confirming that the soil texture is one main parameter affecting the biodegradation process.

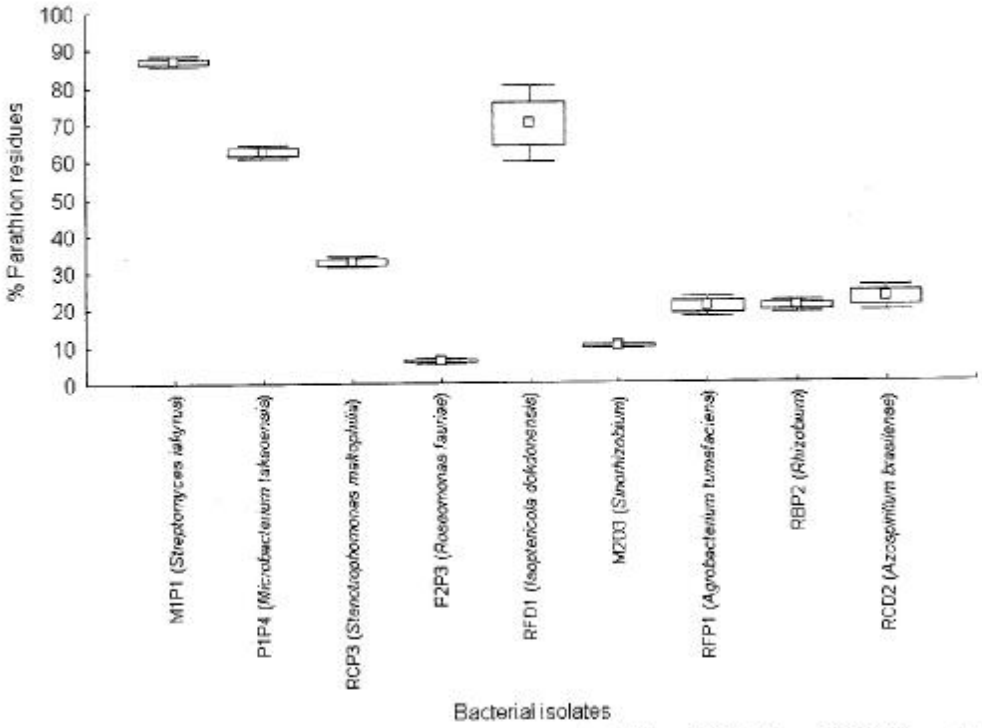

FIGURE 2 - Organophosphorus degradation ability of the selected bacterial strains in Mineral Salt Medium (MSM). The small square represents the average; the big square represents the average ± the standard error ; the error bar represents the average ± the standard deviation;

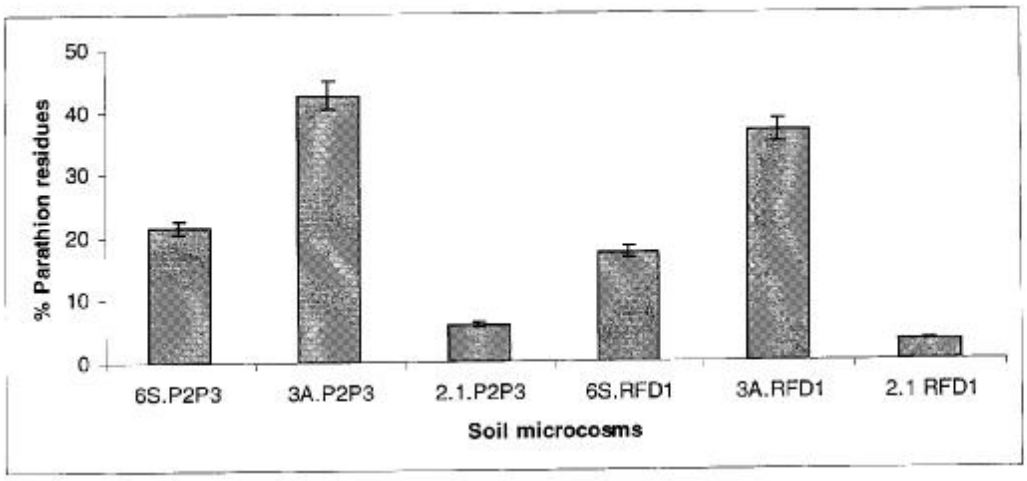

FIGURE 3 - Organophosphorus degradation ability of 2 selected bacteria (*Roseomonas fauriae* strain P2P3; *Isoptericola dokdonensis* strain RFD1) in three different soils (namely: 6S, 3A, 2.1) sandy-clay, loam and sandy respectively;

CONCLUSIONS

Mediterranean soils cultivated with different crops and under different agronomic practices host a large number of phenotipically different strains capable of growing on parathion as sole carbon source. The higher level of biodiversity appears to be related to soil physico-chemical characteristics such as soil clay and total organic carbon. Among the cultural systems analyzed citrus groves host the highest bacteria diversity, both under organic and conventional management. This could be considered as a result of the indigenous history of citrus tree and agriculture practice management in Sicily island.

The nitrogen fixation ability of some isolated strains is a remarkable point. The collection of the isolated strains is mainly composed by Gram negative among which a predominant number is represented by *Sinorhizobium* strains. Among the Gram negative 2 strains of *Roseomonas* were isolated and one of these was found to be good OPs degrader. Gram positive isolates, unknown as OPs degraders, were also obtained.

The study underlines a strong effect of the texture in the decontamination capability phenomena. The percentage of sand, infact, affects the biodegradation ability.

The isolated bacteria could be utilized in the bioremediation of contaminated sites; noteworthy are *Sinorhizobium* and *Roseomonas*, their employ could be useful and interesting in the organic farming practices of Mediterranean area by using their decontamination ability and beneficial effects on plants.

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Involvement of an Alkane Hydroxylase System of *Gordonia* sp. Strain SoCg in Degradation of Solid *n*-Alkanes▽

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Enzymes involved in oxidation of long-chain *n*-alkanes are still not well known, especially those in Gram-positive bacteria. This work describes the alkane degradation system of the *n*-alkane degrader actinobacterium *Gordonia* sp. strain SoCg, which is able to grow on *n*-alkanes from dodecane (C_{12}) to hexatriacontane (C_{36}) as the sole C source. SoCg harbors in its chromosome a single *alk* locus carrying six open reading frames (ORFs), which shows 78 to 79% identity with the alkane hydroxylase (AH)-encoding systems of other alkane-degrading actinobacteria. Quantitative reverse transcription-PCR showed that the genes encoding AlkB (alkane 1-monooxygenase), RubA3 (rubredoxin), RubA4 (rubredoxin), and RubB (rubredoxin reductase) were induced by both *n*-hexadecane and *n*-triacontane, which were chosen as representative long-chain liquid and solid *n*-alkane molecules, respectively. Biotransformation of *n*-hexadecane into the corresponding 1-hexadecanol was detected by solid-phase microextraction coupled with gas chromatography-mass spectrometry (SPME/GC-MS) analysis. The *Gordonia* SoCg *alkB* was heterologously expressed in *Escherichia coli* BL21 and in *Streptomyces coelicolor* M145, and both hosts acquired the ability to transform *n*-hexadecane into 1-hexadecanol, but the corresponding long-chain alcohol was never detected on *n*-triacontane. However, the recombinant *S. coelicolor* M145-AH, expressing the *Gordonia alkB* gene, was able to grow on *n*-triacontane as the sole C source. A SoCg *alkB* disruption mutant that is completely unable to grow on *n*-triacontane was obtained, demonstrating the role of an AlkB-type AH system in degradation of solid *n*-alkanes.

Fn2

Alkanes are saturated, linear hydrocarbons whose chain length can vary from 1 (in methane) to more than 50 carbon atoms. Alkanes constitute about 20 to 50% of crude oil, depending on the source of the oil, but living organisms, such as bacteria, plants, and some animals, also produce them as pheromones (4). As a result, alkanes are widespread in nature, and many microorganisms have evolved enzymes to use them as a carbon source. Alkanes, however, are chemically inert and must be activated before they can be metabolized. Under aerobic conditions, activation is usually achieved by oxidation of one of the terminal methyl groups to generate the corresponding primary alcohol by alkane hydroxylases (AHs) (18, 26). Although many microorganisms are capable of degrading aliphatic hydrocarbons and they are readily isolated from contaminated and noncontaminated sites, relatively little is known about the molecular characteristics of their alkane-degradative systems. Indeed, until recently, the alkane-degradative genes of only a small number of Gram-negative bacteria, namely, *Pseudomonas*, *Acinetobacter*, and *Alkanivorax*, had been described in detail. Among these, the *alk* system found in *Pseudomonas putida* GPo1, which degrades C_5 to C_{12} *n*-alkanes, remains the most extensively characterized alkane hydroxylase system (27). The initial terminal oxidation of the alkane substrate to a 1-alkanol is catalyzed by a three-component alkane hydroxylase complex consisting of a particulate nonheme integral membrane alkane monooxygenase (AlkB) and two soluble proteins, rubredoxin (AlkG) and rubredoxin reductase (AlkT). The *Pseudomonas putida alk* genes are located in two different loci (*alkBFGHJKL* and *alkST*) on the OCT plasmid, separated by 10 kb of DNA (27). Five chromosomal genes (*alkM*, *rubA*, *rubB*, *alkR*, and *xcpR*) in at least three different loci are required for degradation of C_{12} to C_{18} alkanes in *Acinetobacter* sp. strain ADP1 (17). Similar to the case for *P. putida* GPo1, the initial terminal alkane oxidation is also catalyzed by a three-component alkane hydroxylase system, which comprises an alkane monooxygenase (AlkM), rubredoxin (RubA), and rubredoxin reductase (RubB). *Acinetobacter* sp. strain M-1 was shown to possess two alkane monooxygenase genes (*alkMa* and *alkMb*) as well as single copies of *rubA* and *rubB*, located in three different loci. AlkMa is involved in degradation of long-chain *n*-alkanes up to C_{16} and AlkMb in degradation of very-long-chain *n*-alkanes up to C_{30} (23). More recently, a flavin-binding monooxygenase, AlmA, was found involved in oxidation of very long-chain *n*-alkanes up to C_{32} in *Acinetobacter* sp. strain DSM17874 (25).

Much less is known about the alkane-degradative systems of Gram-positive bacteria. Homologs of *alkB* were amplified from *Rhodococcus erythropolis* NRRL B-16531, *Amycolatopsis rugosa* NRRL B-2295, and *Mycobacterium tuberculosis* H37Rv (22) and from *Nocardiodes* sp. strain CF8 (7). The *M. tuberculosis alkB* homologs could be functionally expressed in an *alkB* knockout derivative of *Pseudomonas fluorescens* CHA0 and in *P. putida* GPo1 and were shown to oxidize alkanes ranging from C_{10} to C_{16} (21). Four alkane monooxygenase homologs (two as part of alkane gene clusters and two occurring as separate genes) were identified in two closely related *Rhodo-*

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coccus strains and analyzed by functional heterologous expression in *Escherichia coli* and *Pseudomonas* spp. (31). Moreover, genes encoding an AH system (alkane 1-monooxygenase, rubredoxins, and rubredoxin reductase) from *Gordonia* sp. strain TF6 were cloned, sequenced, and expressed in *E. coli*, where they were found to be the minimum components required to confer alkane hydroxylase activity in this strain on *n*-alkanes up to C_{13} (6). Alkanes longer than C_{16} support growth of many microorganisms, but the identity of enzymes involved in their oxidation is known for only a restricted number of isolates (27, 30), among which is only one Gram-positive strain, belonging to the genus *Geobacillus* (LadA) (5). *Rhodococcus* and other closely related G+C-rich, mycolic acid-containing actinomycetes, such as *Corynebacterium*, *Gordonia*, and *Nocardia*, are increasingly recognized as ideal candidates for the biodegradation of hydrocarbons because of their ability to degrade a wide range of organic compounds, hydrophobic cell surfaces, production of biosurfactants, and robustness and ubiquity in the environment (12).

Recently, a Gram-positive bacterium identified as *Gordonia* sp. strain SoCg, which is able to grow on and to degrade long and solid *n*-alkanes up to hexatriacontane, was isolated (16). In this work the alkane degradation system of *Gordonia* SoCg was investigated by cloning and sequencing the *alk* locus; gene expression was analyzed in relation to the time course of *n*-alkane consumption, and the metabolic intermediate of *n*-hexadecane hydroxylation was identified. Functional expression in heterologous hosts unable to use *n*-alkanes and the *Gordonia alkB* disruption mutant confirmed the role of an actinobacterial di-iron nonheme integral membrane alkane monooxygenase in degradation of *n*-alkanes longer than C_{16}.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and general methods. *Gordonia* sp. strain SoCg was isolated from a hydrocarbon-contaminated Mediterranean shoreline (16). It grows on the mineral medium Bushnell-Haas (BH) medium (Difco) with a wide range of *n*-alkanes, from C_{12} up to C_{36}, as the sole carbon source but does not grow on short-chain *n*-alkanes. *Gordonia* was routinely grown on JM medium (14) or on liquid mineral BH medium supplemented with 10 mM *n*-alkanes directly supplied in the liquid medium (*n*-hexadecane, C_{16}) or supplied as finely ground powder (*n*-triacontane, C_{30}). In biodegradation kinetic experiments, *n*-alkanes were added to BH medium as an *n*-hexane solution, once it was established that *n*-hexane is not toxic to or utilized by the strain. In solid cultures on BH agar, *n*-hexadecane was supplemented as vapor as described elsewhere (16).

The bacterial strains, commercial cloning vectors, and plasmids constructed in this study are described in Table 1. *E. coli* was routinely grown in Luria medium (19) and *S. coelicolor* in JM medium (14). T1

Plasmid and chromosomal DNA purification, enzymatic digests, ligations, and bacterial transformations were performed using standard molecular techniques (9, 19) or according to the manufacturer's instructions. All primers used for PCR amplification were synthesized by Invitrogen and are listed in Table 2. The 16S rRNA gene was amplified using primers rD1 and fD1 (29) in a 20-μl reaction mixture containing 1 μl of chromosomal DNA, 0.2 μM each primer, 0.2 mM deoxynucleoside triphosphates (dNTPs), and 1.5 U of recombinant *Taq* DNA polymerase (Invitrogen, Life Technologies). PCR was carried out in a Biometra thermocycler using the following program: 94°C for 5 min; 30 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C; and a final extension at 72°C for 7 min. Where not otherwise specified, the PCR was carried out under the same conditions but with an annealing temperature of 62°C. T2/AQ:A AQ: B

For pulsed-field gel electrophoresis (PFGE) analysis, undigested DNA plugs of *Gordonia* SoCg were prepared as described by Kieser et al. (9). PFGE was performed with 0.5× Tris-borate-EDTA (TBE) as the running buffer at 14°C by using a CHEF DRII PFGE system (Bio-Rad) at 160 V, 400 mA, n s20 s^{-1}, and e/w 20 s, for 18 h (2). For hybridization experiments, undigested PFGE-separated DNA was transferred to Hybond N nylon membranes (Amersham International plc, Buckinghamshire, United Kingdom), according to the protocol for large DNA fragment transfer (2). AQ: C

All Southern hybridizations were carried out using digoxigenin (DIG)-dUTP-labeled probes (Fig. 1) obtained by PCR and labeled using the digoxigenin system by Boehringer Manheim Biochemicals (Indianapolis, IN). F1

Growth curves on *n*-alkanes. Gram-positive strains were grown in 30 ml JM medium (14) in 250-ml buffled flasks for 48 h at 30°C. Cells were washed three times with BH medium and suspended in the same medium to give an optical density at 600 nm (OD_{600}) of 1.0. Afterwards, 1 ml (about 1 mg [dry weight]) of bacterial suspension was inoculated in 1-liter buffled flasks containing 120 ml BH supplemented with 10 mM *n*-alkane. The flasks were incubated at 30°C on a rotary shaker (200 rpm), and at each time point 1-ml aliquots were sampled and centrifuged at 4,000 × *g* and the pellet dried at 65°C to a constant weight. AQ: D

Time course of *n*-alkane consumption. To determine *Gordonia* SoCg *n*-alkane utilization, 300 μl of washed cells suspended in BH medium to an OD_{600} of 1.0 as described above was inoculated into 100-ml glass tubes containing 10 ml BH medium supplemented with 10 mM *n*-hexadecane or *n*-triacontane in an *n*-hexane solution. A total of 48 tubes were incubated at 30°C under shaking conditions; 12 tubes for each *n*-alkane were inoculated with *Gordonia* SoCg, and 12 were left uninoculated to be used as abiotic controls. Residual long-chain *n*-alkanes were *n*-hexane extracted as described elsewhere (16) from the whole tube content after 22, 46, 62, and 96 h of incubation, in triplicate, and analyzed by the gas chromatography-mass spectrometry (GC-MS) analytical technique with a Hewlett-Packard 5890 GC system interfaced with an HP 5973 quadrupole mass spectrometer detector. As the stationary phase, an HP5-MS capillary column (5% diphenyl–95% dimethylpolysiloxane; 30 m by 0.2 mm; 0.25-μm film thickness [J&W Scientific]) was used. The GC oven temperature program was as follows: 40°C for 5.00 min, increase of 10°C min^{-1} to 280°C, and holding for 20 min. Helium was used as the carrier gas with a constant flow rate of 1 ml min^{-1}. Electron impact ionization spectra were obtained at 70 eV, with recording of mass spectra from 42 to 550 amu, which allows 3.5 scans s^{-1}. The time course of consumption was expressed as residual *n*-alkane with respect to abiotic controls. AQ: E AQ: F

Analysis of the metabolic intermediates from the *n*-alkane oxidation pathways. The metabolic intermediates resulting from incubation of SoCg, M145-AH, and BL21-AH (expressing the Gordonia *alkB* gene) on C_{16} and C_{30} were analyzed by solid-phase microextraction (SPME) coupled with GC-MS. *Gordonia* SoCg, *Gordonia* SoCg Ω*alkB*, *S. coelicolor* M145-AH, and *S. coelicolor* M145 carrying the empty pIJ8600 were grown in JM medium, washed, and resuspended in BH medium to a final OD_{600} of 1.0 as described above. *E. coli* BL21-AH and *E. coli* BL21 carrying the empty pRSET-B were grown overnight in LB medium, washed three times with phosphate buffer (pH 7.2), and suspended in the same volume. One milliliter of cells was inoculated in 100-ml glass tubes with 10 mM each *n*-alkane in the presence of inducers (isopropyl-β-D-thiogalactopyranoside [IPTG] in *E. coli* tubes according to the instructions for the Ni-nitrilotriacetic acid [NTA] purification system [Invitrogen] and thiostrepton [10 ng ml^{-1}] in *Streptomyces* tubes) and incubated at 37°C for 6 h with shaking. Abiotic controls were incubated under the same conditions and analyzed in parallel.

The entire suspensions were analyzed by immersing the SPME fiber, which was coated with 85-μm polyacrylate (PA) and equipped with a holder for manual injection. The time needed to reach equilibrium between the amount of analyte adsorbed by the polymeric film and the initial concentration of the analyte in the sample matrix during the SPME sampling is dependent on the properties of both the analyte and the matrix (4) and in our study was 20 min at 45°C. Prior to use, the fiber was conditioned at 300°C for 2 h in the GC injector port. An HP-5MS 5% phenyl methyl siloxane capillary column was used to perform the gas chromatographic separations. The initial oven temperature was 80°C with a constant helium flow, corresponding to the nominal head pressure of 9.37 lb/in^2. The temperature increase was 5°C min^{-1} to 280°C, and then the temperature was held for 20 min. The ionization spectra were obtained as described above. Analytical identification and quantifications were carried out using standard-grade compounds purchased from Sigma-Aldrich and the commercial NIST 2005 mass spectrum library search database.

Cloning and sequence analysis of *Gordonia* SoCg alkane hydroxylase genes. The probe alkCg23 was obtained by PCR from palkCg23 using the pair of primers AH+for and AH+rev (16) and was used in a Southern analysis to identify suitable restriction fragments in BamHI/BglII-digested SoCg chromosomal DNA (Fig. 1). Fragments in the range of 8 to 10 kb were cut out from a preparative agarose gel, purified, and ligated into BamHI-digested and dephosphorylated pUC18. The ligation mixture was used to transform *E. coli* DH10B (Invitrogen) by electroporation. *E. coli* transformants were selected on Luria agar supplemented with IPTG, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and ampicillin (19). The transformants were screened by colony

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
Gordonia sp.		
SoCg	Long-chain *n*-alkane degrader, *alkB*$^+$	16
SoCg Ω*alkB*	SoCg disruption mutant, *alkB*$^-$ Apra^r	This study
Escherichia coli		
DH10B	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK* λ$^-$ *rpsL nupG*	Invitrogen
ET12567	F$^-$ *dam-13*::Tn*9 dcm-6 hsdM hsdR zjj-202*::Tn*10 recF143 galK2 galT22 ara14 lacY1 xyl-5 leuB6* Cml^r Tet^r Kan^r	9
BL21(DE3)/pLysS	F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *dcm gal* λ(DE3) pLysS Cam^r	Invitrogen
BL21-AH	*E. coli* BL21 containing the recombinant expression vector pRalkB; *alkB* Amp^r	This study
Streptomyces coelicolor		
M145	Wild type; SCP1$^-$ SCP2$^-$	9
M145-AH	*S. coelicolor* M145 containing the recombinant integrative pIalkB; *alkB* Thio^r Apra^r	This study
Cloning and expression vectors		
pUC18	*E. coli* cloning vector, Amp^r	Invitrogen
pGEM-T Easy vector	*E. coli* cloning vector, Amp^r	Promega
TOPO-TA	*E. coli* cloning vector, Amp^r	Invitrogen
pRSET-B	*E. coli* expression vector, Amp^r	Invitrogen
pIJ8600	Streptomycete expression vector, Apra^r, promoter induction by thiostrepton	9
pIJ773	Streptomycete cloning vector, Apra^r, used to extract apramycin resistance cassette *aac*(3)IV with its *oriT*	9
Plasmids containing DNA from *Gordonia* SoCg		
palkCg23	pGEM-T Easy vector derivative containing a 570-bp *alkB* fragment (GenBank accession no. EF437969)	16
palk68	pUC18 derivative containing an 8-kb fragment of *Gordonia* SoCg including the *alk* cluster	This study
pGalkB1	pGEM-T Easy vector derivative containing *alkB* (1.2 kb), amplified by PCR with primers alkNIFor and alkBHIRev	This study
pRalkB	pRSET-B derivative containing *alkB* (1.2 kb)	This study
pIalkB	pIJ8600 derivative containing *alkB* (1.2 kb)	This study
palkapra	palk68 derivative containing the apramycin resistance cassette cloned into a unique AleI restriction site of *alkB*	This study

TABLE 2. Primers used in this study

Primer	Sequence[a]	Reference or source
AHqRTFor	5′-GGACCGATGCTGGTCTATGT-3′	This study
AHqRTRev	5′-CAGATAACAGGCCATGACGA-3′	This study
rubA3qRTFor	5′-CTACCGTGTCCGGTCTGTG-3′	This study
rubA3qRTRev	5′-CCAGTCGTCGGGAATGTC-3′	This study
rubA4qRTFor	5′-CTGCGAGGTCTGCGGATT-3′	This study
rubA4qRTRev	5′-GGCCACCTCGACCATCTC-3′	This study
rubBqRTFor	5′-GGGTGTTGATCCAGTTCAGG-3′	This study
rubBqRTRev	5′-TATCTGGCACATCACCAACG-3′	This study
alkUqRTFor	5′-GCGTTCACCGAGTACTTCAC-3′	This study
alkUqRTRev	5′-ATCGACAACCACGTCGACTC-3′	This study
alkNIFor	5′-AACATATGCTCGTGAGAGGAGCGTGC-3′	This study
alkBHIRev	5′-AAGGATCCCCGGACAACGGTAGGCGC-3′	This study
CF	5′-ATGTTYATHGCNATGGAYCCNC-′	11
CR	5′-NARNCKRTTNCCCATRCANCKRTG-′	11
apra750FR	5′-ATTCCGGGGATCCGTCGACC-′	This study
apra750RV	5′-TGTAGGCTGGAGCTGCTTC-′	This study
ladAFR	5′-GGCGTSTACGMCRWCTACGGYRGG-′	This study
ladARV	5′-GAYCTACCAGGYCGGGTCGTCG-′	This study
alkCG341FR	5′-CCGAGGACCCGGCGAGCTC-′	This study
alkCG341RV	5′-CTCCGGGGTGCACCGCTC-′	This study

[a] Underlining indicates ●●●●.

hybridization with the same probe. The recombinant plasmid designated palk68 was isolated from an *alk*$^+$ clone using the Miniprep column kit (Qiagen) and analyzed by restriction fragment length polymorphism (RFLP) to estimate the insert size. A 4,472-bp region of the insert was commercially sequenced on both strands by primer walking. Nucleotide and deduced amino acid sequences were compared with EMBL/SwissProt/GenBank databases using BLASTN and BLASTX at NCBI, and open reading frames (ORFs) were identified using Chromas Pro 2.33.

A LadA-related monooxygenase gene in the SoCg genome was searched for by PCR using a degenerate primer pair (ladAFR and ladARV [Table 2]) designed from the consensus sequences of the *ladA* genes from *Geobacillus thermodenitrificans* NG80-2 (NCBI accession no. YP_001127577.1) (5), *Mycobacterium smegmatis* MC2 155 (NCBI accession no. YP_886406.1), *Mycobacterium avium* 104 (NCBI accession no. 883212.1), and *Nocardia farcinica* IFM 10152 (NCBI accession no. YP_117411.1). A touch-down PCR protocol was carried out with the following program: an initial denaturation step at 94°C for 2 min; 16 cycles of 45 s at 94°C, 1 min at annealing temperatures decreasing from 72 to 58°C (with a 2°C decremental step from cycle 2 to 8), and 1 min at 72°C; 26 cycles of 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C; and a final 7-min extension at 72°C.

Genes encoding cytochrome P450 alkane hydroxylases were searched for using primers CF and CR (Table 2) designed from the conserved domains in the N-terminal and C-terminal regions of CYP153A family enzymes.

Cloning of *alkB* and construction of expression plasmids. The 1.2-bp *alkB* gene was amplified from *Gordonia* SoCg genomic DNA using primers alkNIfor and alkBHIrev (Table 2) (containing an NdeI restriction site immediately up-

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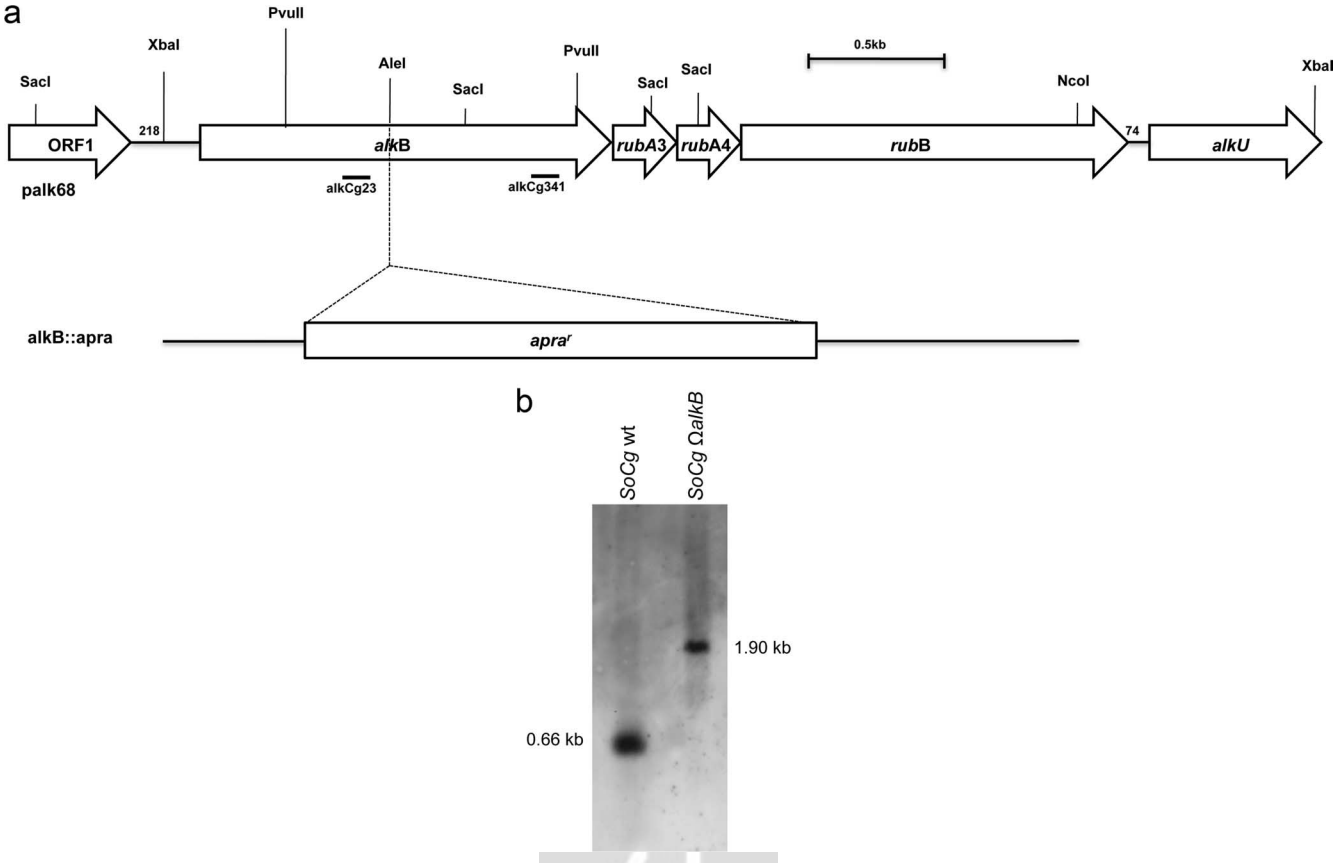

FIG. 1. Schematic representation of the 4,472-bp region of *Gordonia* sp. strain SoCg carried by palk68. (a) Genetic organization of the gene cluster and restriction map. The orientations of identified genes are indicated by arrows. Probes alkCg23 and alkCg341 were used in Southern hybridization experiments for *alkB* localization and disruption mutant analysis, respectively. The 3.0-kb *alkB*::*apra* fragment was used to disrupt the *alkB* gene in *Gordonia* SoCg by double-crossover homologous recombination. The apramycin resistance cassette (1,342 bp) was inserted into the unique AleI site of the *alkB* gene sequence. (b) Southern analysis using DIG-labeled alkCg341 as a probe of the PvuII-digested genomic DNA extracted from the disruption mutant *Gordonia* SoCg Ω*alkB*, and wild-type (wt) *Gordonia* SoCg.

stream of the putative ATG start codon of *alkB* and a BamHI restriction site downstream of the putative TGA stop codon, respectively) using a touch-down PCR protocol as described previously. The amplicon was purified using NucleoSpin extract II (Macherey-Nagel GmbH & Co. KG) and ligated with pGEM-T-Easy vector (Promega). The resulting plasmid, pGalkB1 (Table 1), was checked by sequencing and the *alkB* insert was ligated as a PstI-NcoI fragment into the pRSET-B expression vector (Invitrogen) in frame with the T7 RNA polymerase promoter. The ligation mixture was used to transform *E. coli* BL21(DE3)/pLysS (Invitrogen), and the recombinant clones were selected on LB supplemented with ampicillin (200 mg ml^{-1}). Clone *E. coli* BL21-AH containing the recombinant plasmid pRalkB (Table 1) was selected and used for *alkB* expression analysis.

To clone *alkB* in *S. coelicolor* M145, the entire gene from pGalkB1 was cloned as an NdeI-BamHI fragment into pIJ8600 (Table 1) in frame with the *tipA* promoter. The ligation mixture was used to transform *E. coli* DH10B; the derived pIalkB (Table 1) was isolated and then transformed into *E. coli* ET12567 by electroporation. From the recombinant clone of *E. coli* ET12567, pIalkB was transferred to *S. coelicolor* M145 by conjugation (9). The resulting exconjugants were selected on apramycin (50 mg ml^{-1}) and thiostrepton (200 mg ml^{-1}), and the correct integration was verified by Southern hybridization. BamHI-digested genomic DNA was probed using the 1.2-kb *alkB* fragment and PCR amplified from pGalkBI with primers alkNIfor and alkBHIrev as previously described, and the 750-bp apramycin resistance cassette fragment was PCR amplified from pIJ773 using primers apra750FR and apra750RV.

Heterologous expression analysis. Crude extracts of *E. coli* BL21-AH and *E. coli* BL21 transformed with the empty vector pRSET-B were collected after incubation in LB medium supplemented with ampicillin to an OD_{600} of 0.6 in the presence or absence of IPTG as an inducer, according to the Ni-NTA purification system instructions (Invitrogen). About 0.5 μg of each soluble and insoluble protein fraction from each extract was loaded for SDS-PAGE and run in a Mini-Protean Tetra cell (Bio-Rad) at 20 mA and 150 V for 50 min using the SeeBluePlus2 prestained standard (Invitrogen) as a molecular size marker. After electrophoresis, proteins were electrotransferred from the gel to the Hybond-C Extra membrane (Amersham) using a Hoefer mini-VE semidry blotting apparatus (Amersham Pharmacia Biotech) at 150 V and 20 mA for 1 h. Immunostaining was carried out using alkaline phosphatase-conjugated anti-His tag monoclonal antibodies (Invitrogen) followed by detection with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Invitrogen).

To evaluate *alkB* gene expression in *S. coelicolor* M145-AH, total RNA was extracted and used in a reverse transcription-PCR (RT-PCR) assay as described below.

RNA isolation, RT-PCR analysis, and absolute quantitative RT-PCR. For RNA isolation, SoCg was grown for 22 h in BH medium supplemented with 10 mM hexadecane, triacontane, and fructose as described above. M145-AH was grown in JM medium supplemented with thiostrepton as an inducer. After incubation for 22 h at 30°C with shaking, cells were suspended in P buffer (9) and lysed using lysozyme (6 mg ml^{-1}). Total RNA was extracted using the RNeasy midikit (Qiagen). DNase I (Roche) treatment was performed at 37°C for 1 h, and after ethanol precipitation and a washing step with 70% ethanol, the air-dried RNA pellet was resuspended in 50 μl of sterile distilled water.

RT-PCR was performed by using the Superscript one-step RT-PCR kit (Invitrogen) with about 0.1 μg of total RNA as a template, the primer pair AHqRT-for and AHqRTRev designed from the internal region of *alkB* (Table 1), and the conditions indicated by the supplier, routinely using 35 PCR cycles. For each reaction, a negative control with *Taq* polymerase and without reverse transcriptase was included in order to exclude DNA contamination.

The expression of the *Gordonia* SoCg *alk* cluster genes was quantitatively analyzed by quantitative reverse transcription-PCR using the Applied Biosystems 7300 real-time PCR system (Applied Biosystems). A high-capacity cDNA archive kit (Applied Biosystems) was used, according to the manufacturer's instructions, to reverse transcribe 5 μg of total DNA-free RNA. Then, 3 μl of the cDNA was mixed with 10 μl of SYBR green PCR master mix (Applied Biosystems) and 5 pmol of each primer in a final volume of 20 μl. In addition to AHqRTfor and AHqRTRev, the primer pairs rubA3qRTFor/rubA3qRTRev, rubA4qRTFor/rubA4qRTRev, rubBqRTFor/rubBqRTRev, and alkUqRTFor/alkUqRTRev were specifically designed from the genes *rubA3*, *rubA4*, *rubB*, and *alkU*, respectively. PCR was performed, in triplicate for each gene, under the following conditions: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 62°C. Eventually, a dissociation reaction was performed under the following conditions: a 1-min step with a temperature gradient increase of 1°C per step from 55 to 99°C. A negative control (distilled water) was included in all real-time PCR assays. Standards for the *alk* genes were constructed from purified palk68 that was quantified using a Qbit fluorometer (Invitrogen) and diluted in a 10-fold series to create a six-point standard curve (0.5×10^2 to 0.5×10^6 molecules) that was run in duplicate with each set of samples. The number of copies per microliter was calculated as follows: molecular mass of palk68 (standard template) = 11,433 bp × 660 Da = 7.54×10^6 g mol^{-1}; 1 molecule or 1 copy of fragment = $7.54 \times 10^6/6.02 \times 10^{23} = 1.25 \times 10^{-17}$ g; therefore, 10 ng of template contains $10 \times 10^{-9}/1.25 \times 10^{-23}$ copies = 8×10^{14} molecules.

Construction of *Gordonia* SoCg *alkB* disruption mutant. In order to obtain *Gordonia* SoCg electrocompetent cells 2 mg (wet weight) of cells was inoculated and left for 3 days in 25 ml YEME (9) with 2 g $liter^{-1}$ of glycine at 30°C. The rich biomass was collected at the bottom of the 50-ml tube and pretreated for 15 min in an ultrasonic batch. The pellet obtained after centrifugation at 5,500 × *g* for 30 min was immediately incubated with 10 g $liter^{-1}$ chilled glycerol on ice for 1 h. The cells were then washed three times with 10 g $liter^{-1}$ chilled glycerol and finally resuspended in 3 ml of 10 g $liter^{-1}$ glycerol, aliquoted into 200-μl samples, and stored at −80°C.

The apramycin resistance cassette, including its own promoter and *oriT*, was extracted from plasmid pIJ773 by digestion with EcoRI and HindIII and cloned into pUC18. The correct recombinant plasmid was checked by sequencing, and the cassette was excised using EcoRI and filled in, using the Klenow fragment enzyme (Roche), to obtain blunt ends. The apramycin resistance cassette was inserted into the unique AleI site within the *alkB* gene of palk68. The resulting plasmid (palkapra [Table 1]) was XbaI-NcoI digested to obtain an *alkB*::apra linear fragment (Fig. 1), which was introduced by electroporation into *Gordonia* SoCg electrocompetent cells. Apramycin-resistant transformants were selected on apramycin, and gene disruption by double-crossover homologous recombination was confirmed by Southern analysis using the DIG-labeled alkCg341 as a probe.

Nucleotide sequence accession numbers. The *Gordonia* SoCg 16S rRNA gene sequence and the 4,472-bp palk68 insert have been submitted to GenBank under accession no. AY496285.2 and HQ026811, respectively.

RESULTS

Identification and properties of strain SoCg. The *n*-alkane degrader *Gordonia* sp. strain SoCg was isolated from a hydrocarbon-contaminated Mediterranean shoreline. This strain is able to grow on *n*-alkanes of different lengths, from dodecane (C_{12}) to hexatriacontane (C_{36}), as the sole C source (16); it is unable to grow on *n*-octane or shorter *n*-alkanes, but it is not inhibited by short-chain *n*-alkanes, e.g., *n*-hexane. The analysis of the complete 16S rRNA gene sequence of SoCg showed the highest similarity (98% identity, 1,442/1,470 nucleotides) to the DNA sequence of *Gordonia amicalis* strain T3 (GenBank accession no. EU427321.1), which is a *tert*-amyl methyl ether degrader recently isolated from a hydrocarbon-contaminated soil (15). The presence of an *alkB* homolog gene in the SoCg genome has been previously demonstrated by PCR using degenerate primers (16). Pulsed-field gel electrophoresis (PFGE) of undigested DNA extracted from strain SoCg revealed the presence of a large cryptic plasmid; the PFGE-separated DNA was probed with DIG-labeled probe alkCg23 (Fig. 1) in a Southern hybridization, and the *alkB* gene was localized on the chromosome of *Gordonia* SoCg (data not shown). The same probe hybridized to only one band in the genomic DNA digested with various restriction enzymes (data not shown). Southern hybridization analysis confirmed that strain SoCg harbors in its chromosome a single copy of the *alkB* gene, as previously suggested by sequencing of cloned PCR fragments (16). As SoCg degrades a large range of long-chain *n*-alkanes, we also tried to find in its genome other, *alkB*-unrelated putative genes involved in long-chain alkane degradation, using PCR. The primer pair designed from the consensus sequence of *ladA* from *Geobacillus thermodenitrificans* NG80-2 and other Gram-positive strains gave two different amplification products whose sequences were unrelated to known alkane hydroxylase genes. A second pair of primers, CF and CR (11), used to amplify the conserved region of the p450-CYP153 family genes gave no amplification product.

SoCg grows on long-chain *n*-alkanes and rapidly degrades them. Growth of *Gordonia* sp. strain SoCg in mineral broth supplemented with *n*-hexadecane or *n*-triacontane as the sole carbon source was followed for 96 h. An increasing biomass accumulation was observed on both *n*-alkanes up to 46 h, followed by a growth decline (Fig. 2a and b). Comparison of the two curves reveals that *n*-hexadecane supports higher biomass accumulation (3.4 mg ml^{-1}) than *n*-triacontane (2 mg ml^{-1}), as measured after 46 h of growth. GC-MS analysis of residual *n*-alkanes showed that substrate consumption is followed by biomass increase. Both *n*-alkanes, in fact, have almost completely disappeared within 62 h (with 11.1% residual *n*-hexadecane and 1.9% *n*-triacontane), with a rapid decline in the first 22 h (Fig. 2c). Consumption of *n*-triacontane is more rapid than that of *n*-hexadecane, in contrast with lower biomass accumulation on the longest alkane. It appears that the efficiency of the AH system is reduced with increasing chain length, as previously reported for other strains (5, 20, 21, 24, 28). F2

SoCg encodes an AlkB-type alkane hydroxylase system. In order to isolate the alkane hydroxylase gene and its flanking region, a BamHI-BglII fragment was cloned into pUC18, giving the plasmid palk68 (Table 1). An internal fragment of palk68 was completely sequenced, and a 4,472-bp nucleotide sequence that exhibited overall identities of 79% with the AH-encoding system of *Gordonia* sp. strain TF6 (6) and 78% with those of *Rhodococcus* sp. strains Q15 and NRRL B-16531 (31) was obtained. Sequence analysis revealed six consecutive open reading frames (ORFs) (Table 3) which were designated as encoding Orf1 (a conserved hypothetical protein), AlkB (alkane 1-monooxygenase), RubA3 (rubredoxin), RubA4 (rubredoxin), RubB (rubredoxin reductase), and AlkU (putative TetR-like regulator) according to the sequence homology with other known genes (6, 31). T3

Expression of SoCg *alk* genes is induced by long-chain *n*-alkanes. The expression of *alk* genes in the presence of long-chain and solid *n*-alkanes was analyzed by quantitative reverse transcription-PCR. Total RNA was extracted from *Gordonia* SoCg cultures after 22 h of growth at 30°C in mineral broth supplemented with *n*-hexadecane or *n*-triacontane as the sole carbon source. Total mRNA from fructose-grown cells was extracted and analyzed as a control. cDNA molecules were reverse transcribed from the total DNA-free RNA and used as

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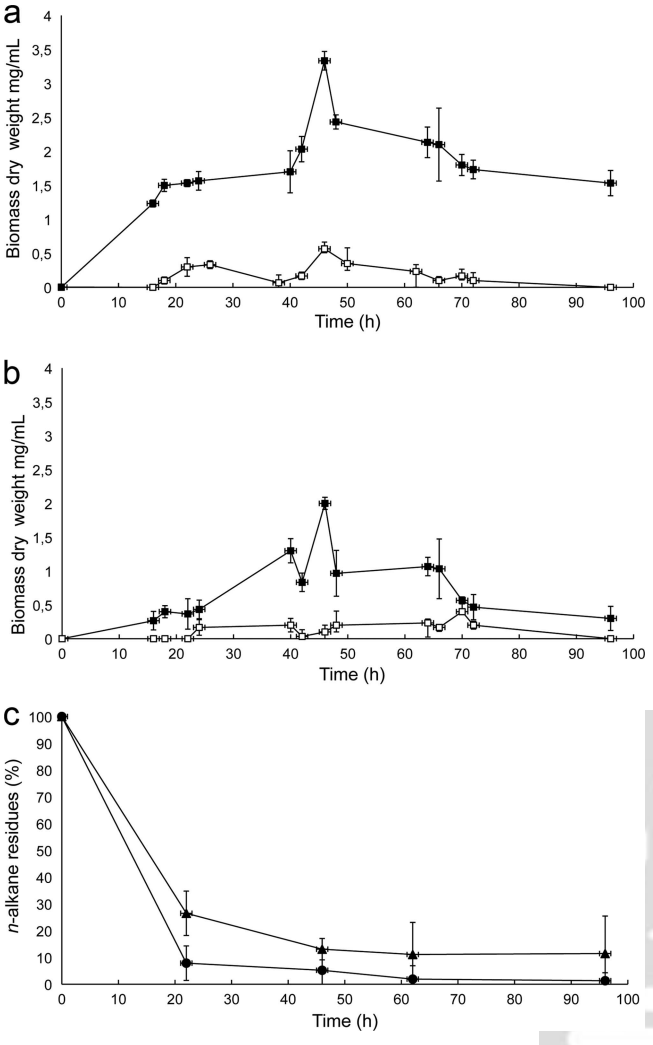

FIG. 2. Growth on long-chain *n*-alkanes and degradation kinetics of *Gordonia* sp. strain SoCg. (a and b) Time courses of growth of wild-type *Gordonia* sp. strain SoCg (■) and *Gordonia* sp. strain SoCg Ω*alkB* (□) on mineral BH medium supplemented with 10 mM *n*-hexadecane (a) and *n*-triacontane (b) as the sole carbon source; growth was measured as an increase in dry biomass in cultures over time. (c) Time courses of consumption of hexadecane (▲) and *n*-triacontane (●, determined by GC-MS and expressed as residual *n*-alkane with respect to abiotic controls. Standard errors were calculated from three independent determinations.

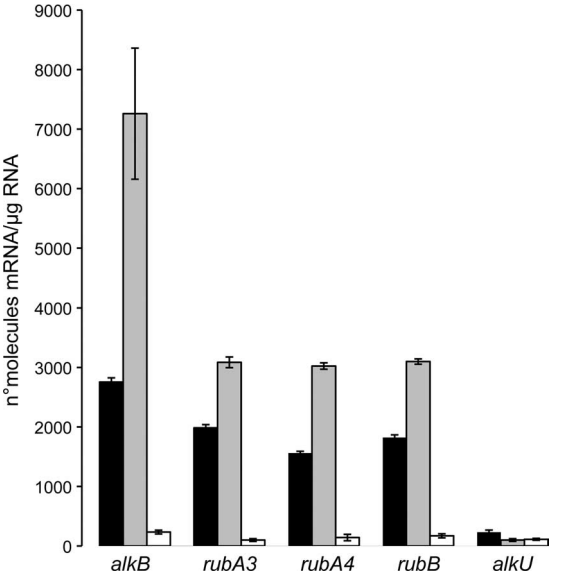

FIG. 3. Absolute real-time RT-PCR analysis of *Gordonia* SoCg *alk* genes. mRNA levels after 22 h of incubation in the presence of *n*-hexadecane (black bars), *n*-triacontane (gray bars), or fructose (white bars) are expressed as number of molecules μg^{-1} total RNA. Standard errors were calculated from three independent determinations of mRNA abundance in each sample.

templates to quantify *alkB*, *rubA3*, *rubA4*, *rubB*, and *alkU* transcripts. The amounts of *alkB*, *rubA3*, *rubA4*, and *rubB* transcripts were larger on *n*-alkanes than on fructose (Fig. 3) and larger on *n*-triacontane than on *n*-hexadecane. The results show that both long-chain *n*-alkanes induce the expression of all the *alk* genes except *alkU*. *alkU* has been found downstream of the *alkB-rubA-rubB* cluster in Gram-positive *n*-alkane degraders (7, 21, 31) and also in the genome of *Nocardia farcinica* (8); it possesses helix-turn-helix DNA-binding motifs and shows deduced amino acid similarity to putative regulatory proteins of the TetR family; however, its expression is not influenced by *n*-alkanes, and further investigations are needed to assess its involvement in *n*-alkane degradation.

The SoCg AH system is functional on liquid long-chain *n*-alkanes. Metabolic intermediates were extracted by SPME from SoCg cells incubated with *n*-alkanes and identified by GC-MS. After 6 h of incubation in the presence of *n*-hexadecane as the sole C source, 1-hexadecanol was identified by comparing its Kovats index and the electron impact mass spectra with those obtained by the injection of the authentic standards (Fig. 4a). When SoCg was grown on *n*-triacontane, in contrast, we were unable to determine the corresponding primary long-chain alcohol. A longer incubation time or other extraction methods (i.e., hexane extraction) did not lead to triacontanol detection (data not shown). Almost all alkane hydroxylase activities described so far have been analyzed in-

TABLE 3. Genes identified and sequence similarities in the *Gordonia* sp. strain SoCg *alk* locus

Gene	Product length (amino acids)	Best BLASTP alignment (accession no.)	Overlap (% identity)
orf1	124	Conserved hypothetical protein, *Rhodococcus equi* ATCC 33707 (06829834.1)	48/124 (38)
alkB	411	Alkane 1-monooxygenase, *Gordonia* sp. strain TF6 (BAD67020.1)	287/323 (88)
rubA3	55	Rubredoxin 3, *Gordonia* sp. strain TF6 (BAD67021.1)	45/54 (83)
rubA4	61	Rubredoxin 4, *Gordonia* sp. strain TF6 (BAD67022.1)	34/59 (57)
rubB	400	Rubredoxin reductase, *Gordonia* sp. strain TF6 (BAD67023.1)	249/349 (71)
alkU	160	Putative transcriptional regulator, TetR family, *Mycobacterium abscessus* (YP 001704325.1)	87/168 (51)

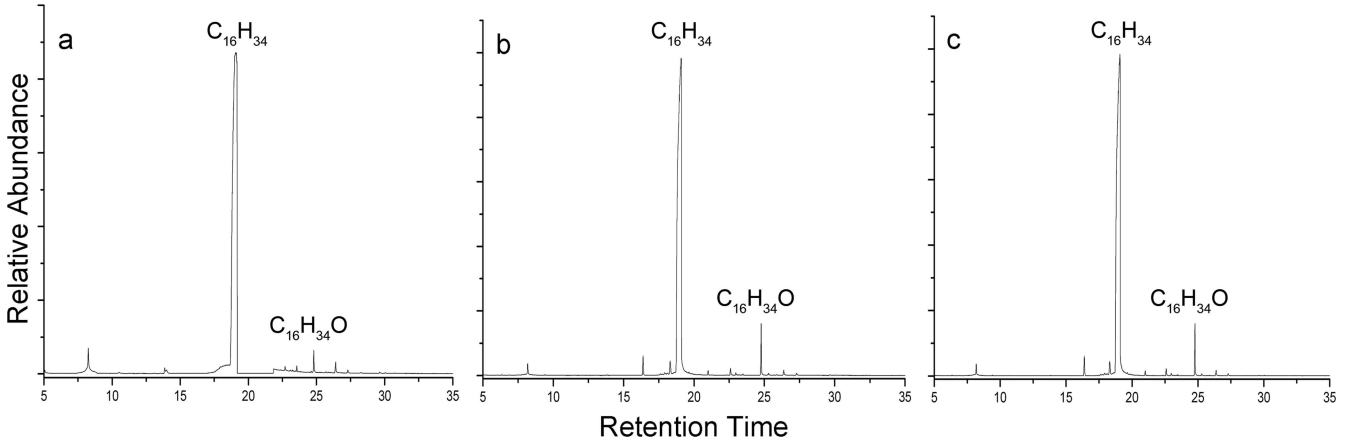

FIG. 4. Hydroxylation of *n*-hexadecane by *Gordonia* SoCg (a), *E. coli* AH (b), and *S. coelicolor* M145 AH (c). GC chromatographs show conversion of hexadecane to 1-hexadecanol.

AQ: G directly by mineralization of ^{14}C labeled *n*-alkanes (31) and growth assays (21, 25). Only a few authors have reported the detection of metabolic intermediates of long-chain *n*-alkanes metabolism in a naturally occurring *n*-alkane degrader (32) or in a heterologous host (6) or the activity of the purified protein (5). Here, biotransformation activity on hexadecane to the corresponding alcohol by a strain of *Gordonia* is reported. These results clearly indicate that *n*-hexadecane is metabolized via the terminal oxidation pathway, like in other *n*-alkane degrading bacteria (6). As no long-chain alcohol could be detected on triacontane, it can be hypothesized that it is immediately used in the following reactions (10) or that it is undetectable because of its insolubility (23).

The *Gordonia* SoCg *alkB* is heterologously expressed in *E. coli* and *S. coelicolor*. The unique *alkB* gene was heterologously expressed in *E. coli* BL21 using the expression vector pRSET-B. The expression of His-tagged AlkB in the resulting recombinant strain *E. coli* BL21-AH (Table 1) was confirmed by Western blotting using alkaline phosphatase-conjugated anti-His tag monoclonal antibodies. The protein was detected mainly in the insoluble fraction of a crude extract of *E. coli* BL21-AH after 4 h of induction with IPTG (data not shown). SPME/GC-MS analysis of bacterial cultures that were IPTG induced for 4 h revealed that *E. coli* BL21-AH was able to transform *n*-hexadecane into 1-hexadecanol (Fig. 4b). No hexadecanol or other products were detected using *E. coli* carrying pRSET-B. Although *alkB*-related alkane hydroxylase activity is known to be rubredoxin and NAD(P)H dependent (26), hexadecane hydroxylation was obtained in *E. coli* expressing only *alkB.* Fujii and colleagues defined *alkB*, *rubA3*, *rubA4*, and *rubB* as the minimum component genes of the alkane hydroxylase systems (6). In fact, those authors obtained biotransformation of *n*-octane to 1-octanol in *E. coli* TOP10 carrying plasmid pAL526, which contained the *Gordonia* TF6 *alk* cluster composed of the four genes. However, the relative AlkB activity was not completely eliminated in the absence of the other alkane hydroxylase system components. Similarly, two *E. coli* recombinants which expressed the *Rhodococcus opacus* B-4 *alkB1* and *alkB2* genes were able to convert *n*-alkanes (C_5 to C_{16}) to their corresponding alcohols in anhydrous organic solvents (20).

When *n*-triacontane was used as a substrate for *E. coli* BL21-AH, the corresponding primary long-chain alcohol could not be revealed using SPME/GC-MS analysis. As *E. coli* may not be the appropriate host, *S. coelicolor* M145 was used to express the *Gordonia alkB* gene. This strain does not grow on *n*-alkanes, but *n*-hexadecane-degrading *Streptomyces* species have recently been isolated (1). Using the integrative plasmid pIJ8600, the recombinant strain *S. coelicolor* M145-AH was obtained, in which the expression of *alkB* confers the ability to grow on *n*-hexadecane (data not shown). SPME/GC-MS analysis showed the presence of 1-hexadecanol in *S. coelicolor* M145-AH cultures on *n*-hexadecane (Fig. 4c). This is the first study to achieve biotransformation of *n*-hexadecane to 1-hexadecanol using *S. coelicolor* expressing an alkane hydroxylase gene. Wild-type *S. coelicolor* M145 and *S. coelicolor* carrying pIJ8600 were unable to biotransform *n*-hexadecane (data not shown). However, using *S. coelicolor* M145-AH, the corresponding primary long-chain alcohol *n*-triacontanol could not be detected.

***S. coelicolor* M145-AH expressing SoCg *alkB* grows on *n*-triacontane.** In order to analyze the activity of *Gordonia* AlkB on solid *n*-alkanes, a growth assay was set using *S. coelicolor* M145-AH in the presence of *n*-triacontane. *E. coli* was not used for growth assays because it lacks the metabolic pathway for alcohol metabolization. The growth curve of M145-AH on triacontane (Fig. 5) shows that *Gordonia alkB* confers the F5 ability to grow on solid *n*-alkanes and also that this strain possesses in its own genome the genes involved in utilization of fatty alcohols. *S. coelicolor* M145 was thus revealed to be a good system to express the alkane hydroxylase genes from long-chain alkane degraders.

The SoCg *alkB* disruption mutant is unable to grow on *n*-triacontane. To investigate the relevance of AlkB for degradation of long-chain *n*-alkanes by *Gordonia* SoCg, *alkB* was inactivated by introducing an apramycin resistance cassette in a unique AleI restriction site that is present at position 576 of its nucleotide sequence (Fig. 1a). The recombinant strains were selected on apramycin solid medium, and site-specific apramycin cassette insertion was first analyzed by PCR amplification of internal fragments of the resistance cassette with primers apra750FR and apra750FR (Table 2) and of the re-

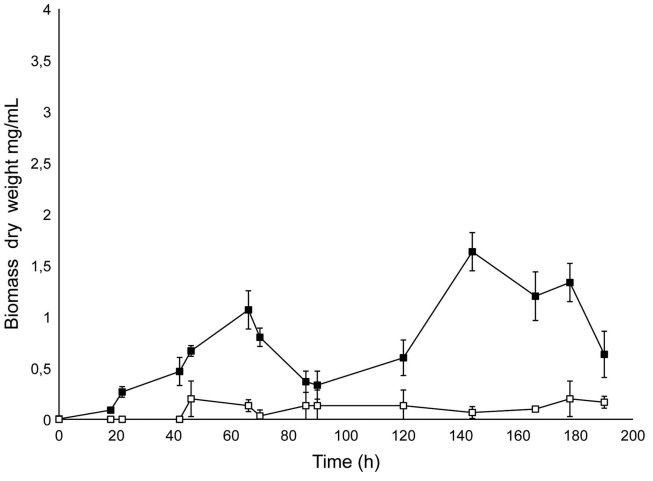

FIG. 5. Growth curves of the recombinant strain *Streptomyces coelicolor* M145-AH (■) and wild-type *S. coelicolor* M145 (□) in BH mineral medium supplemented with 10 mM *n*-triacontane as the sole C source. The solid *n*-alkane was added as finely ground powder, and growth was measured as increase in dry biomass in cultures over time. Standard errors were calculated from three independent determinations.

gion between the 5′ end of *alkB* and the 5′ end of *rubA3* (data not shown). Finally, the PvuII-digested genomic DNAs of four positive clones were analyzed in a Southern hybridization experiment using the DIG-labeled alkCg341 probe (Fig. 1b). One strain showing the correct insertion of the cassette was named *Gordonia* SoCg Ω*alkB* and used for further experiments. Strain SoCg Ω*alkB* exhibited poor growth on *n*-hexadecane (7-fold lower than that of the wild-type strain) and, interestingly, no growth on triacontane, making it evident that *alkB* disruption had a negative effect on solid *n*-alkanes metabolic pathway. When the disruption mutant was incubated in the presence of 1-triacontanol as the sole C source it was able to grow even better than the wild type on triacontane (data not shown). On the other hand, *Gordonia* SoCg Ω*alkB* was still able to transform *n*-hexadecane into the corresponding alcohol (data not shown), suggesting that oxidation of *n*-hexadecane in the absence of *alkB* must be carried out by an unknown oxidation system that in any case does not allow efficient growth of the strain.

DISCUSSION

Many bacteria capable of degrading long-chain alkanes have been isolated, and the enzyme systems that oxidize long-chain *n*-alkanes up to C_{16} have been characterized (see references 18, 26, and 30 for reviews). Although long-chain alkanes are more persistent in the environment than shorter-chain alkanes, genes involved in degradation of *n*-alkanes longer than C_{16} had not been reported prior to the work of Throne-Holst et al. (25) and Feng et al. (5). A flavin-binding monooxygenase involved in oxidation of very-long-chain *n*-alkanes up to C_{32} has been characterized in *Acinetobacter* sp. strain DSM17874 (25), and LadA from *Geobacillus thermodenitrificans* NG80-2 is the first long-chain *n*-alkane monooxygenase functional on alkanes in the range from C_{15} to C_{36} to be cloned and structurally characterized from a Gram-positive strain (5). Both enzymes show little or no homology with the widespread and well-characterized AlkB-type alkane hydroxylases usually reported as being functional on long-chain *n*-alkanes up to C_{16} in Gram-positive and Gram-negative strains (21, 30, 31).

Here the unique functional AlkB-type alkane hydroxylase system that allows growth on long-chain liquid and solid *n*-alkanes in the Gram-positive *Gordonia* strain SoCg is described. To date the only long-chain alkane hydroxylase system of this genus that has been characterized is that of *Gordonia* TF6, which was found to be functional on *n*-alkanes from C_5 to C_{13} (6).

The ability of *Gordonia* SoCg AlkB to biotransform *n*-hexadecane into the corresponding primary alcohol was assessed by SPME/GC-MS analysis in SoCg and in two heterologous hosts expressing the SoCg *alkB* gene. *S. coelicolor* M145 was successfully used as a heterologous host for an alkane hydroxylase gene. Although the *n*-triacontane biotransformation product, triacontanol, could not be detected in any of the heterologous systems, the role of SoCg AlkB in triacontane metabolization was demonstrated by growth assays. *S. coelicolor* M145-AH expressing the *Gordonia alkB* gene acquired the ability to grow on triacontane, while the disruption mutant SoCg Ω*alk*B lost this ability. Moreover, the SoCg alkane hydroxylase-encoding genes are induced by both liquid and solid *n*-alkanes, which is in accordance with the ability of this strain to grow on and rapidly metabolize *n*-alkanes up to C_{36} (16).

Taken together, these results suggest that the identified alkane oxidation system plays a central role in the degradation of long-chain and solid *n*-alkanes by *Gordonia* SoCg. Moreover, at least one other, less efficient enzyme that is responsible for oxidation of *n*-hexadecane exists. This second AH system seems to be unrelated to other known alkane hydroxylase systems characterized so far. AQ: H

Many bacterial strains contain multiple, and quite divergent, integral membrane AlkBs (31) that have different substrate ranges (23, 24) or are activated during different growth phases (13). The strategy of *Gordonia* SoCg seems to be based on a single *alkB* gene, which is induced by a wide range of long and solid *n*-alkanes throughout the time course of growth (L. Lo Piccolo, unpublished results), encoding an enzyme with highest activity on hexadecane and reduced activity on triacontane. Growth of SoCg on triacontane would be poorer that that on hexadecane for this reason and also because a second, unknown system, that is functional on C_{16} but not on C_{30}, would contribute to overcome the limiting step of *n*-alkane degradation on C_{16}. The alkane hydroxylase, in fact, catalyzes the initial attack and determines the size range of *n*-alkanes to be degraded; its specific activity is generally reduced with increasing chain length (5, 20, 21, 24, 28).

The relationship between the AlkB protein structure and its function has been investigated; it has been proposed that AlkB is made of six transmembrane helices that are assembled in a hexagonal structure forming a deep hydrophobic pocket where four conserved histidine residues that chelate the iron atoms necessary for its activity are located on the cytoplasm surface (28). The alkane molecule should slide into the pocket until the terminal methyl group is correctly

positioned relative to the His residues. Amino acids with bulky side chains protruding into the pocket would limit the size of the *n*-alkane to be hydroxylated, while less bulky side chain amino acids allow longer alkanes to deeper enter into the hydrophobic pocket (28). *Pseudomonas putida* GPo1 and *Alkanivorax borkumensis* AP1 AlkB mutant derivatives oxidize alkanes longer than C_{12} when tryptophan is replaced by serine, cysteine, or other small amino acids at position 55 or 58 of the two proteins (28). Amino acid sequence alignment of AlkB proteins showed a valine residue in the corresponding amino acid position of *Gordonia* SoCg AlkB, confirming the possibility of accepting long-chain alkanes in the active site, although other residues/mechanisms could be involved in *n*-alkane recognition.

Bacteria appear to degrade chemicals only when they are dissolved in water, and dissolution of solid substrates is generally considered a prerequisite for their biodegradation (33). Long-chain and solid *n*-alkanes are insoluble in water and, although we know how *n*-alkanes are oxidized, we still poorly know how they are recognized and how they enter the cells, especially when they are in the solid state. Two mechanisms for accessing medium and long-chain liquid alkanes have been recognized in bacteria: (i) biosurfactant-mediated accession by cell contact with emulsified hydrocarbons and (ii) interfacial accession by direct contact of the cell surface with the hydrocarbon (3). *Gordonia* belongs to the *Corynebacterium*/*Mycobacterium*/*Nocardia* (CMN) complex, which is characterized by mycolic acid-containing cell walls that confer hydrophobicity to these bacteria and allow cell adherence to the *n*-alkanes by direct contact of cells with hydrocarbons, generally with no or low biosurfactant production. Our observations confirm that the strategy of SoCg for accessing liquid hydrocarbons is by direct contact and that this strategy is also used for solid alkanes. In fact, a massive adhesion of SoCg cells to triacontane (supplemented as finely ground powder) was observed, while the culture liquid phase was almost clear for a long period of growth. Direct contact with the solid substrate might favor growth of *Gordonia*, as it can have direct access to the substrate without its previous solubilization in the aqueous environment.

The recent first report of expression of *Rhodococcus alkB* genes in anhydrous organic solvents corroborates these observations (20) and suggests new biotechnological applications in water-free environments.

The alkane hydroxylase from *Gordonia* SoCg is active on a wide range of long-chain liquid and solid *n*-alkanes and is able to use other electron transfer systems in the absence of its two specific components, rubredoxin and rubredoxin reductase. *Gordonia* sp. strain SoCg is the first actinobacterial strain that is able to grow on solid *n*-alkanes to be characterized.

ACKNOWLEDGMENTS

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