# A STUDY OF THE BIOTECHNOLOGICAL APPLICATIONS OF *NOVOSPHINGOBIUM PUTEOLANUM* PP1Y.

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*A zia Nanna*

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## RIASSUNTO

I composti aromatici sono i contaminanti ambientali più diffusi. Questo, probabilmente, spiega perché ceppi batterici capaci di degradare gli idrocarburi sono così largamente diffusi nell'ambiente.
Tra questi, i cosiddetti "batteri idrocarbonoclastici obbligati" (OHCB), come *Alkanivorax*, *Marinobacter* e *Oleispira* sono efficienti degradatori del petrolio, utilizzandone prevalentemente, o esclusivamente, la frazione satura. I ceppi più efficienti nella degradazione dei composti aromatici appartengono invece all'ordine degli Sfingomonadali, Pseudomonadali, e Micobatteri.
Recentemente, nel laboratorio in cui è stato sviluppato questo progetto di tesi, un nuovo ceppo batterico, *Novosphingobium puteolanum* PP1Y, è stato isolato dalle acque superficiali di una piccola baia all'interno del porto di Pozzuoli, in una zona caratterizzata dal transito di imbarcazioni da diporto e da un notevole inquinamento da idrocarburi delle acque superficiali.
Le analisi effettuate sul batterio isolato hanno permesso di identificarlo come appartenente all'ordine degli Sfingomonadali. Questo ceppo, non solo, si è mostrato in grado di utilizzare un numero sorprendentemente ampio di idrocarburi aromatici mono- e policiclici (quali ad esempio pirene, naftalene, fenantrene) quale unica fonte di carbonio e di energia, ma ha manifestato anche un efficace adattamento alla crescita su miscele complesse di molecole aromatiche disciolte in fasi non polari (come il gasolio e la benzina), apparentemente mediato dalla produzione di un biofilm che studi preliminari hanno mostrato essere funzionale alle sue capacità degradative.
Tali caratteristiche hanno stimolato un'attività di ricerca volta allo studio di possibili applicazioni biotecnologiche del ceppo batterico, sia per le sue particolari capacità degradative che per la produzione di un particolare tipo di biofilm, apparentemente funzionale alle strategie di degradazione del batterio.
È pertanto ragionevole ipotizzare un utilizzo del microorganismo per la *bioremediation* di siti contaminati, in modo particolare quando i contaminanti ambientali siano di natura aromatica.
A tal proposito occorre ricordare che il biofilm è un sistema biologico, in cui i batteri sono strutturati e organizzati in una comunità funzionale, che può essere formata da una singola specie o da diverse specie biologiche. Tali comunità sessili sono ricoperte da una matrice extracellulare, composta prevalentemente da omo- ed eteropolisaccaridi, prodotti dai batteri stessi, ma che può presentare anche altri elementi quali piccoli peptidi, sostanze nutritive, e DNA. Il ruolo fisiologico di questa barriera tra l'ambiente e il microorganismo, nei sistemi nei quali è stato studiato, è sostanzialmente riconducibile ad un ruolo primario nell'attuazione dei meccanismi di infezione dell'ospite, nel prevenire l'eccessiva disidratazione, nel favorire l'adesione a specifiche superfici oppure substrati o semplicemente come sostanze di riserva. Ovviamente questo ruolo fisiologico non può essere quello svolto in microorganismi come *Novosphingobium puteolanum*. PP1Y, nel quale il ruolo del biofilm deve essere investigato e chiarito *ex-novo*.
Gli scopi del lavoro di ricerca descritto nella presente tesi di dottorato sono stati: (**1**) definire le caratteristiche microbiologiche del ceppo *Novosphingobium puteolanum* PP1Y e verificare l'applicabilità del suo uso per processi di *bioremediation* su microcosmi inquinati naturali e artificiali, (**2**) analizzare il

dimorfismo planctonico/sessile del ceppo PP1Y focalizzando l'attenzione sull'identificazione dei determinanti molecolari extracellulari alla base del processo di formazione del biofilm, (**3**) completare il sequenziamento e l'annotazione del genoma del ceppo PP1Y, al fine di dotarsi di uno strumento di indagine diverso da quello dell'isolamento e dell'identificazione delle putative molecole necessarie alla strutturazione del biofilm, e per studiare più nel dettaglio i complessi pattern metabolici che sono alla base delle sue capacità degradative.

**(1) Definizione delle caratteristiche microbiologiche del ceppo *Novosphingobium puteolanum* PP1Y; verifica dell'applicabilità del suo uso per processi di *bioremediation* su microcosmi inquinati naturali e artificiali:**

Nell'ambito di questo obiettivo la nostra attenzione è stata focalizzata sull'approfondimento delle caratteristiche microbiologiche e delle potenzialità degradative del ceppo PP1Y.

A tal proposito è stata analizzata la capacità di *N. puteolanum* PP1Y di degradare miscele di idrocarburi aromatici, utilizzandoli come unica fonte di carbonio ed energia. Il microorganismo ha mostrato la capacità di degradare miscele complesse di idrocarburi aromatici (quali gasolio o benzina), disciolti in fase oleosa o forniti direttamente nel mezzo di coltura. Il ceppo PP1Y è stato inoltre in grado di operare indistintamente sia in terreni liquidi sia in modelli di suolo artificialmente contaminati. Infatti, la contemporanea analisi dei diversi campioni al microscopio ottico, ha permesso di osservare che il batterio è capace di creare uno specifico microambiente che riveste le gocce d'olio, i composti puri o le particelle di terreno, permettendo al ceppo PP1Y di concentrare il substrato di crescita, impedendone la libera dispersione nell'ambiente, e di metabolizzarlo più rapidamente, se confrontato con altri ceppi adesso impiegati nei processi di *bioremediation*.

L'utilizzo del ceppo PP1Y in interventi di biorisanamento *in situ* è particolarmente indicato poiché la capacità mostrata dal microorganismo di formare biofilm su superfici di varia natura potrebbe favorire l'adesione del ceppo ai substrati da trattare (per esempio terreno o sabbia), riducendo la dispersione e il dilavamento delle cellule da parte di agenti meteorici e limitando la necessità di ripetere frequentemente gli inoculi, con un notevole risparmio economico. Inoltre, utilizzando la sua capacità di formare emulsioni stabili, si riduce la necessità di aggiungere detergenti e surfattanti. Incapsulando piccole gocce di olio ed estraendone gli idrocarburi aromatici, il ceppo PP1Y può limitare la dispersione nell'ambiente, cosa che invece non si verifica alla presenza di altri ceppi che degradano efficientemente gli idrocarburi saturi e aromatici.

Sulla base dei risultati sperimentali ottenuti durante i primi due anni di attività in terreno liquido e su microcosmi inquinati artificialmente, il lavoro di ricerca si è incentrato, durante il terzo anno, sulla valutazione delle potenzialità degradative del ceppo PP1Y in ambienti inquinati non artificiali. Sulla base di ciò sono stati scelti appositi siti contaminati per saggiare, non solo le capacità metaboliche del biosistema in un ambiente naturale, ma anche e soprattutto la sua capacità di competere/cooperare con organismi autoctoni già adattati a quella particolare nicchia ecologica.

Gli esperimenti condotti su questa tipologia di microcosmo hanno evidenziato la capacità mostrata dal ceppo PP1Y di degradare efficientemente un inquinante

modello quale il fenantrene e di competere positivamente con i ceppi autoctoni del terreno scelto.

**(2) Il dimorfismo planctonico/sessile del ceppo PP1Y:**
*Novosphingobium puteolanum* PP1Y mostra un complesso dimorfismo planctonico/sessile e può formare in soluzione flocculi amorfi o un biofilm strutturato che gli consente di aderire a superfici idrofobiche o a interfacce acqua/gasolio. Dati preliminari suggeriscono che il biofilm del ceppo possa essere modulato nella sua organizzazione e composizione in funzione del substrato disponibile e delle condizioni di crescita. Questa caratteristica lascia intravedere la possibilità di utilizzare il biofilm stesso per applicazioni industriali.
Allo scopo di individuare quali tra i componenti del gasolio possano essere i "segnali" chimici che consentono a *N. puteolanum* PP1Y di optare per la strutturazione in flocculi amorfi o in un biofilm organizzato, sono state approntate diverse crescite in mezzo minimo contenente uno o più idrocarburi normalmente presenti nel gasolio al fine di poter valutare la crescita dell'organismo nelle diverse condizioni utilizzate. È stato osservato che è possibile riprodurre un fenotipo gasolio-simile utilizzando tetradecano contenente il 5% in peso di una miscela di idrocarburi aromatici che include un idrocarburo monociclico (*p*-xilene, 0,8%), un idrocarburo biciclico (naftalene, 1,92%), un idrocarburo triciclico (fenantrene, 1,28%) ed un idrocarburo tetraciclico (pirene, 1%). E' stato inoltre dimostrato che alcuni composti aromatici, quali il pirene, il propilbenzene, il tetraidronaftalene, l'isopropilbenzene, il butilbenzene, il dibenzotiofene, il carbazolo (dibenzopirrolo) ed il dibenzofurano, non sono in grado di indurre la formazione di flocculi, come accade con la maggior parte degli altri idrocarburi aromatici, bensì di pellicole traslucide. Queste, osservate al microscopio, appaiono costituite da una prevalenza di materiale extracellulare e da un esiguo numero di cellule, occasionalmente riunite in corte catenelle.
Per valutare nel dettaglio le proprietà chimico-fisiche del materiale extracellulare prodotto dal ceppo PP1Y e stabilire gli effetti di tali molecole sui differenti substrati di crescita, sono stati effettuati innanzitutto test di solubilizzazione e di evaporazione su diversi composti aromatici disciolti nei vari mezzi di coltura del batterio. In tutti i casi analizzati, il materiale extracellulare prodotto dal microorganismo ha mostrato un notevole effetto ritardante sui normali processi di solubilizzazione in fase acquosa e di evaporazione dei differenti substrati. Ciò è risultato molto interessante se confrontato con differenti dati di letteratura. Diversi studi, condotti su altri tipi di biosurfattanti di origine batterica, come ad esempio l'alasano prodotto da *Acinetobacter radioresistens* KA53, hanno mostrato, infatti, comportamenti discordanti rispetto a quelli evidenziati per il ceppo PP1Y. Nello specifico, i biosurfattanti di sintesi chimica o di origine batterica hanno la proprietà di rendere facilmente disponibili i substrati aromatici per i microorganismi che li producono. Il loro ruolo è di abbassare la tensione superficiale in un liquido polare, principalmente l'acqua, e di migliorare quindi la solubilità in fase acquosa di composti prevalentemente apolari, in modo da permettere al batterio, che vive in un ambiente acquoso, di poterli metabolizzare più facilmente.
Il biofilm prodotto da PP1Y ha invece mostrato la capacità opposta di fungere da filtro biologico per tali molecole, intrappolandole e concentrandole, in modo da impedirne la naturale dispersione nell'ambiente esterno e garantire una migliore biodisponibilità per il microorganismo stesso. È apparso pertanto evidente che la

formazione del biofilm sia strettamente funzionale alla capacità del batterio di "isolare" e utilizzare la fonte di carbonio ed energia disponibile, e tale caratteristica è quindi da tenere nella massima considerazione nella progettazione di processi di *bioremediation*.
Nell'ambito di questo obiettivo la nostra attenzione è stata inoltre focalizzata sull'identificazione e sull'analisi di molecole extracellulari, che potrebbero essere alla base del processo di formazione dei flocculi e del biofilm, prodotte dal batterio in diverse condizioni di crescita.
L'analisi chimica delle fasi acquosa e oleosa, isolate meccanicamente da colture contenenti come unica fonte di carbonio ed energia differenti substrati aromatici forniti singolarmente o in miscele, ha permesso di evidenziare la presenza di elementi extracellulari sia di natura carboidratica sia di natura proteica.
Per quanto riguarda la componente carboidratica, è stato utilizzato il protocollo di estrazione dei metil-glicosil-acetilati, ed analizzando i campioni così trattati mediante GC-MS, in collaborazione con il gruppo del prof. A. Molinaro (Dipartimento di Chimica Organica e Biochimica, Università degli studi di Napoli Federico II), è stato possibile risalire alla composizione qualitativa delle fasi acquose ottenute nelle diverse condizioni di crescita. Tali analisi hanno mostrato la presenza di zuccheri a cinque (non ancora definiti) e a sei atomi di carbonio (mannosio, galattosio e glucosio), nonché di ammino-zuccheri (quali l'N-acetilglucosammina e l'N-acetilmuramico) e acidi grassi monoinsaturi (prevalentemente C-18:1, ma anche C12:1, C14:1 e C16:1). Tali molecole sono evidenziabili non solo in colture contenenti idrocarburi aromatici, ma anche in terreni di coltura minimi contenenti substrati di crescita come l'acido glutammico. Occorre sottolineare che in tutti i casi analizzati i sopranatanti ottenuti mediante l'allontanamento delle cellule del batterio dal terreno di coltura sono in grado di stabilizzare la formazione di gocce di olio di paraffina prodotte dall'agitazione vigorosa del terreno, di impedirne la coalescenza e di garantire un'emulsione duratura.
In contemporanea, sono state effettuate anche analisi per SDS-PAGE di aliquote dei terreni di coltura ottenuti durante la crescita di *N. puteolanum* PP1Y negli esperimenti sopra elencati. Ciò ha evidenziato la presenza di un profilo proteico molto diverso rispetto a quello relativo all'estratto cellulare del batterio. Mediante l'utilizzo della spettrometria di massa, in collaborazione con la prof.ssa L. Birolo (Dipartimento di Chimica Organica e Biochimica, Università degli Studi di Napoli Federico II), è stato possibile procedere all'identificazione delle proteine extracellulari prodotte *N. puteolanum* PP1Y. Si tratta per la maggior parte di proteine tipiche delle membrane esterna ed interna di microorganismi batterici, come ad esempio le proteine *Flagellin-like* o il complesso OmpA/MotB.
Confrontando i risultati ottenuti con differenti dati di letteratura, è stata avanzata l'ipotesi secondo la quale la contemporanea presenza da un lato, di proteine esclusive della membrana plasmatica, e dall'altro di composti quali sfingolipidi e peptidoglicano, evidenziati dalle analisi chimiche condotte in collaborazione con il gruppo del Prof. Molinaro, possa essere riconducibile ad un meccanismo di secrezione vescicolare da parte del microorganismo.
Recenti studi hanno infatti dimostrato che differenti classi di batteri sono in grado di secernere delle cosiddette "*outer membrane vesicles"* (OMVs), contenenti differenti tipi di macromolecole e che assolvono diversi ruoli biologici. Queste OMVs sono utilizzate dai microorganismi, per secernere molecole insolubili in

aggiunta o in complesso con materiale solubile, oppure particolari enzimi, destinati a target specifici, in modo da proteggerli dall'ambiente esterno ed averli pronti per l'utilizzo in concentrazioni appropriate.
In conclusione, il biofilm prodotto dal ceppo PP1Y è fondamentalmente diverso dai "canonici" biofilm batterici in cui la matrice extracellulare è di natura prevalentemente carboidratica. Esso potrebbe essere invece il risultato di un complesso fenomeno di vescicolazione che comporta la formazione di una rete tridimensionale funzionale al particolare tipo di interfaccia acqua/gasolio al quale il batterio si è adattato nell'ambito della sua nicchia ecologica.

**(3) Analisi del genoma del ceppo PP1Y:**
Nell'ambito del presente obiettivo, è stato completato il sequenziamento e l'annotazione dell'intero genoma di *N. puteolanum* PP1Y, in collaborazione con i gruppi del Prof. F. Salvatore del CEINGE S.r.l. e del Prof. G. Paolella del Dipartimento di Biochimica e Biologia Molecolare, Università degli Studi di Napoli Federico II.
Successivamente, in collaborazione con il Dr. Eugenio Notomista presso il Dipartimento di Biologia Strutturale e Funzionale, è stata portata avanti un'analisi bioinformatica del genoma sequenziato e annotato di *N. puteolanum* PP1Y volta da un lato, ad identificare i pattern metabolici responsabili delle capacità degradative del microorganismo, e dall'altro, allo studio dei possibili meccanismi biosintetici alla base della strutturazione del biofilm prodotto.
I risultati ad oggi disponibili, ricordando che il corredo genico del ceppo PP1Y è organizzato in un cromosoma di circa 4.2 Mbp, affiancato da 3 mega plasmidi rispettivamente di 1.2, 0.2 e 0.05 Mbp, indicano la presenza di circa 160 *orf* putative, distribuite tra il cromosoma e il mega plasmide maggiore, codificanti per altrettanti sistemi multicomponente impegnati nelle vie di degradazione dei vari substrati aromatici. Tra questi possiamo sicuramente annotare delle diossigenasi ossidrilanti (40), decarbossilasi (2) e xylene monoossigenasi (2), a costituire il cosiddetto "*upper pathway*" responsabile dell'attivazione dell'anello aromatico, nonché diossigenasi *ring cleavage* (10) e 3,4 diidrossidenzoato 4,5 diossigenasi (4) che favoriscono il primo step del "*lower pathway*" nella via di degradazione dei composti aromatici.
L'analisi del genoma del ceppo PP1Y ha in definitiva evidenziato un numero sorprendentemente elevato di potenziali sequenze codificanti per enzimi dell'*upper* e del *lower pathway*. Questo ampio e versatile macchinario biochimico fornisce una possibile spiegazione per l'ampia gamma di composti aromatici che possono essere utilizzati da tale microorganismo quale unica fonte di carbonio e di energia.
E' interessante sottolineare inoltre la frequente presenza, tra le principali classi di ossigenasi del ceppo PP1Y, di enzimi strettamente correlati per i quali analisi di *homology modeling* indicano differenze nei siti attivi. Ciò suggerisce una possibile strategia generale, responsabile delle peculiarità metaboliche mostrate dal microorganismo, che si basa contemporaneamente sull'acquisizione di nuovi geni –probabilmente mediante trasferimento genico orizzontale- e sulla diversificazione dei geni acquisiti mediante eventi di duplicazione/divergenza.

## SUMMARY

Environmental pollution caused by the release of a wide range of xenobiotic compounds has assumed serious proportions. Bioremediation techniques, utilizing microorganisms to reduce the concentration and toxicity of various chemical pollutants such as petroleum hydrocarbons, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, industrial solvents and pesticides, are the most promising strategies for the restoration of polluted environments.
Several bacterial strains with promising degradative abilities have already been isolated and characterized. However, the full exploitation of the potential of bioremediation strategies requires not only the isolation of a large number of strains with wide degradative abilities but also an accurate characterization of these strains both at the microbiological and biochemical/genetic level. This knowledge is necessary to perform a rational planning of bioremediation interventions.
The present work describes isolation of new strains able to degrade aromatic hydrocarbons directly from polluted environments by means of appropriate selection procedures.
Novosphingobium sp. PP1Y, isolated from a surface seawater sample collected from a closed bay in the harbor of Pozzuoli (Naples, Italy), uses fuels as its sole carbon and energy source. Like some other Sphingomonads, this strain can grow as either planktonic free cells or sessile-aggregated flocks. In addition, this strain was found to grow as biofilm on several types of solid and liquid hydrophobic surfaces including polystyrene, polypropylene and diesel oil. Strain PP1Y is not able to grow on pure alkanes or alkane mixtures but is able to grow on a surprisingly wide range of aromatic compounds including mono, bi, tri and tetracyclic aromatic hydrocarbons and heterocyclic compounds.
During growth on diesel oil, the organic layer is emulsified resulting in the formation of small biofilm-coated drops, whereas during growth on aromatic hydrocarbons dissolved in paraffin the oil layer is emulsified but the drops are coated only if the mixtures contain selected aromatic compounds, like pyrene, propylbenzene, tetrahydronaphthalene and heterocyclic compounds.
These peculiar characteristics suggest strain PP1Y has adapted to efficiently grow at the water/fuel interface using the aromatic fraction of fuels as the sole carbon and energy source.
The whole PP1Y genome sequence could provide clues about the metabolism of this species and about the possibility of manipulating it for bioremediation purposes.
The PP1Y genome is comprised of a single 3.9 Mbp circular chromosome and of 3 plasmids, one megaplasmid (Mpl; 1.16 Mbp), one large plasmid (Lpl; 0.19 Mbp), and one small plasmid (Spl; 0.05 Mbp). Notably, this is the first time that a megaplasmid of such size has been identified in a sphingomonad. Our results will help to clarify the molecular basis of the unusual features of this strain and to engineer strains with enhanced cultural and bioremediation abilities.

# I
# INTRODUCTION

## 1.1. Antropic pollution and bioremediation.

Aromatic compounds are among the most widespread and hazardous pollutants in the environment. Several polycyclic aromatic hydrocarbons (PAH), like naphthalene and phenanthrene, are very toxic to aquatic organisms [**1**, **2**], whereas benzene, chrysene, benzo[*a*]anthracene, benzo[*a*]pyrene and aromatic amines are carcinogenic and represent a direct risk for human health [**3**, **4**]. Coal, petroleum and their derivatives are the main sources of aromatic compounds released into the environment. Depending on the source, fuel contains 20–40% of aromatic hydrocarbons [**5-7**]. Gasoline contains up to 25% and 10% of mono- and polycyclic aromatic hydrocarbons, respectively [**8**], whereas diesel oil contains up to 20% PAHs and only 5–6% alkylbenzenes [**9**]. The aromatic fraction of these fuels is extremely complex and is often characterized by the presence of (poly)alkyl-benzenes, PAHs, (poly)alkyl-PAHs, naphthenes (i.e. polycyclic compounds with fused aromatic and saturated rings) and heterocyclic compounds containing nitrogen, sulphur or oxygen.

Along with the rapid development of modern biotechnologies, bioremediation, which involves the use of microorganisms to reduce the concentration and toxicity of various chemical pollutants, has become nowadays one of the most promising approaches for environmental restoration [**10**, **11**]. Bioremediation procedures, compared to traditional methods of chemical and physical treatment of polluted sites, offer a low-impact, cost-effective, and safe tool for environmental clean-up [**12**].

There are more than 100 known microbial species capable of degrading hydrocarbons [**13**]. The most effective degraders of aromatic compounds belong to Sphingomonads ($Gram^-$), Pseudomonads ($Gram^-$), Mycobacteria ($Gram^+$) [**14-17**] and to the genus *Cycloclasticus* [**18-20**].

Sphingomonads are $\alpha$–proteobacteria whose outer membrane contain glycosphingolipids, instead of the more common lipopolysaccharides [**21**]. Recently, these microorganisms have been the focus of several studies because of their ability to degrade a wide array of xenobiotics, including mono- and polycyclic aromatic compounds, chlorinated compounds, herbicides and pesticides [**14**, **17**]. Notable examples include *Novosphingobium aromaticivorans*, which can use alkylbenzenes as the sole carbon and energy sources [**13**], N*ovosphingobium pentaromativorans* US6-1T*,* which degrades PAHs containing three to five aromatic rings [**22**], *Sphingomonas paucimobilis* EPA505, able to mineralize several polycyclic compounds [**23**], *S. paucimobilis* 20006FA which can use phenanthrene as the sole carbon and energy source [**24**], and *Sphingomonas wittichii* RW1T, endowed with the ability to grow using dibenzofuran and dibenzo-*p*-dioxin as the sole carbon and energy source and to co-metabolize mono- and dichloro-derivatives of these toxic aromatic compounds [**25**].

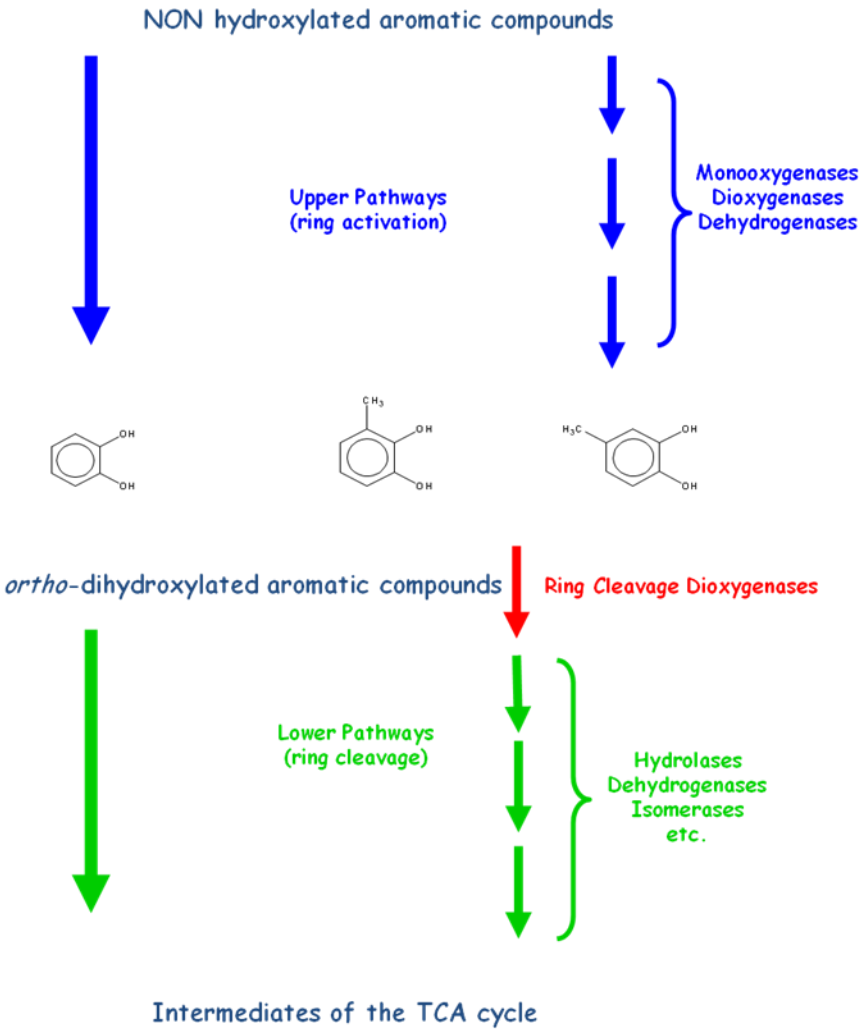
These bacteria show a remarkable metabolic versatility due to the wide spectrum of substrates recognized by their catabolic enzymes. In fact, this low substrate specificity allows the microorganisms to degrade structurally related molecules through the same catabolic pathways.

Although these organisms possess a remarkable variety of enzymes for the initial attack of substrates, the catabolic pathways usually converge and substrates are transformed into a limited number of key intermediates, which are then metabolized

by a single central pathway (**Figure 1-1**). This results in simpler control circuit and a lower energetic "cost" in terms of metabolic processes [**4**].
The catabolic pathways that allow bacteria to degrade aromatic compounds can generally be divided into two parts, indicated as *upper* and *lower pathways* [**26**] (**Figure 1-1**). The reactions of the upper pathways involve the activation of the aromatic ring by addition of two hydroxyl groups on two adjacent carbon atoms [**26**, **27**]. This addition can be performed simultaneously, if catalyzed for example by dioxygenases as the complex of the toluene dioxygenase from *Pseudomonas putida* F1 [**28**], or in two successive steps when catalyzed by monooxygenases such as toluene *o*-xylene-monooxygenase (ToMO) and phenol hydroxylase (PH) from *Pseudomonas sp.* OX1 [**29**, **30**], or toluene-2-monooxygenase (T2MO) from *Pseudomonas cepacia* G4 [**31**]. The products of the *upper pathways* are aromatic compounds such as protocatechuate or variously substituted catechols (**Figure 1-1**).
In the lower pathway, dihydroxylated aromatic molecules are converted into intermediates of the tricarboxylic acid cycle (TCA) that are readily metabolized by any type of organism. The first enzymes of this pathway are the catechol dioxygenases (CDO) responsible for the ring-fission reaction which can be either intradiolic or extradiolic depending on the metabolic pathway involved.
The genes coding for the enzymes of the upper and lower pathway are usually clustered in one, two or more independent operons whose expression is controlled by several regulatory proteins whose function is triggered by the presence of aromatic compounds [**26**].
Bacteria rapidly adapt to changing environments and to the available energy sources. This adaptation is often achieved through the transfer of genetic material from one microorganism to another. In fact, it is well known that the transfer of genetic material plays a crucial role in the evolution of bacterial degradation pathways [**32**, **33**]. As the genes coding for enzymes of many catabolic pathways are located on plasmids [**34**], plasmid transfer by conjugation greatly contributes to the spread of catabolic genes between bacteria belonging to different species. A substantial contribution to the spread of such metabolic properties is also due to mobile elements such as transposable elements [**35**]. The recombination events contribute to the evolution of different combinations of the catabolic operons, which provide the microorganism with novel metabolic properties, suitable for the degradation of new molecules. As a matter of fact the combination of different upper and lower operons increases not only the number of degradation pathways, but also the spectrum of substrates that can be used for growth [**36**], thus contributing to the evolution of new catabolic pathways [**32**, **37**]. It is worth noting that the capability shown by bacteria to rapidly adapt to use new chemical compounds as growth substrates is a topic of growing interest not only for bioremediation purposes but also for the development of biosynthesis of industrial interest [**38**].

## 1.2. Microbial biofilm.

A biofilm is an aggregate of microbial cells attached to a surface and embedded in a self-produced extracellular polymeric matrix whose structure is mainly composed by exopolysaccharides (EPS). Although the EPS component varies in its physical and chemical properties, it is primarily composed of polysaccharides which can be neutral or polyanionic, as in Gram- bacteria. The functional characteristics of the matrix are




**Picture 1-1:** The *catabolic pathways* responsible for the degradation of aromatic compounds in bacteria.

mainly related to the composition and structure of the polysaccharides which defin the shape of the primary biofilm.
The EPS, whose amount varies depending on the microorganism and increases with the increasing age of the biofilm, are also responsible for the resistance of the biofilm to antibiotics. EPS act in fact as a physical barrier to the diffusion of antibiotics into the matrix and therefore hamper the permeation of these molecules through the bacterial cell walls [**39**]. The bacteria within a biofilm show a higher resistance not only to antibacterial chemicals and to natural antibiotics, but also to bacteriophages and to the phagocytic amoebae.
Other than EPS, the matrix of the biofilm is composed also by proteins and by extracellular DNA, as reported in **Table 1.1**.

| **Table 1.1** | |
|---|---|
| **Composition of the biofilm matrix** | |
| **Components** | **%** |
| Water | > 97% |
| Bacterial Cells | 2-5%<br>(more species) |
| Polysaccharides | 1-2%<br>(neutral o polyanionic) |
| Proteins<br>(produced by cell lyses) | < 1-2%<br>(including enzymes) |
| DNA and RNA | < 1-2%<br>(produced by cell lyses) |
| Ions | Bound or free |

Some bacteria, including *Pseudomonas aeruginosa*, produce a considerable amount of extracellular DNA (eDNA) through a mechanism that is not related to cell lyses. In this case, the DNA appears to be carried outside the membrane through exocytosis. In *P*. *aeruginosa*, the eDNA has a functional role in the biofilm formation. Addition of DNase I to the culture medium, for example, strongly inhibits biofilm formation; in addition, DNase I can dissolve mature biofilm [**40**].
The matrix of the biofilm is a dynamic environment where nutrients accumulate and can be stored and where microbial cells preserve the appropriate homeostasis. The matrix is highly heterogeneous and different microenvironments co-exist within it.
The nature of the matrix is not only related, as already mentioned, to the structure of the EPS, but depends also on various extrinsic factors, such as the chemical and physical properties of the environment surrounding the biofilm. As a consequence, the structure of the biofilm matrix may change depending on the physiological state of the cells, the availability of nutrients, and the environment in which it is formed [**41**].
All this means that biofilm are not simply structures adhering to a surface, but they are biological systems characterized by a high degree of organization and where microorganisms are structured and coordinated in functional communities able, if necessary, to cooperate in reproductive and infectious processes [**42**].

Biofilm are ubiquitous; analysis of bacterial populations present in a wide variety of natural systems has shown that most of these microorganisms do not grow as planktonic cells but structured as a biofilm. In natural environments, planktonic cells are uncommon with the exception of ecosystems characterized by a reduced amount of nutrients, such as oceans [**43**].
Biofilm may harbor multiple microbial species (**Figure 1-2)** and/or multiple cell types; it has been in fact proposed that within biofilm individual cells of a single species can undertake different developmental pathways, thus resulting in a functional and morphological heterogeneous population.
In recent years the development of biofilm has also been studied by a genetic point of view, and several genes involved in this process of differentiation have been identified and characterized. The formation of the biofilm requires coordination, interaction and communication between different microbial species [**42**]. This complex sequence of events shows how bacterial cells, capable of independent life, are able to organize themselves into complex communities.
The formation of a biofilm is a dynamic and complex process which is divided into several phases:

I. Adhesion to a surface;
II. Formation of microcolonies;
III. Biofilm maturation.

The adhesion to the substrate and the formation of microcolonies are events influenced by environmental signals such as osmolarity, pH, metals availability, oxygen, temperature and nutrient availability [**42**], whereas the maturation of the biofilm, is mainly regulated by a phenomenon generally referred to as *quorum-sensing*: this latter is a communication system preserved across several bacterial species capable of responding to an increased density of population by strongly modifying gene expression [**43**].

***I. Surface adhesion****.* The first step in the biofilm formation involves the adhesion of bacterial cells to a surface or to a solid-liquid interface. This process depends on the direct contact of bacteria with the surface followed by the formation of specific cell-surface interactions that can overcome the common repulsive forces generated between the surfaces [**42**]. Although the initial contact of a bacterial cell with the surface is not genetically controlled, there is at least evidence in the recent literature showing the existence of mechanisms regulating the formation of stable interactions between cells and the surface. This observation derives from the study of the CPX system in *E. coli,* able to "sense" the interaction with a surface and to respond by regulating gene expression and promoting stable cell-surface interactions [**44**, **45**]**.** These studies also showed that the cell-surface interactions are strongly influenced by environmental conditions such as osmolarity.

***II. Microcolonies formation****.* Microcolonies are bacterial communities with a thickness of about three to five cells that develop following the interaction of some cells with the surface **[44**]. The stable interaction with the surface is not sufficient to become microcolonies, and more complex and stable cell-cell interactions are required [**46**]. Several bacteria show a more complex behavior in which a specific type of cell motion on the surface is responsible for the formation of microcolonies [**47**]. The clonal development is mainly controlled by nutrients; however, specific


**Figure 1-2:** Development of a multispecies biofilm formed by *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Flavobacterium* spp. Emission of fluorescence obtained by staining with acridine orange.

signal transduction pathways need to be activated to produce surface structures responsible for motility, such as pili, and for the production of the matrix of exopolysaccharides, the predominant structure that mediates stable cell-cell interactions [**48-51**].

***III. Biofilm maturation.*** After the initial stage of adhesion, microcolonies develop into a mature biofilm, and this step is associated with the production of EPS (**Figure 1-3**).
The mature biofilm can be made of a single layer of cells, or can be composed of a complex structure of cells characterized by the presence of water channels which allow the diffusion of nutrients throughout the sessile community (**Figure 1-4**) [**42**]. As underlined before, the structure of a mature biofilm, both mono-and multi-species, changes not only according to the different bacterial species that compose it, but also as a function of different parameters that characterize the environment [**52**].

The "catabolite repression", which represents a response of the cells to the high availability of nutrients, is a control system for the formation of biofilm in both $Gram^+$ and $Gram^-$ bacteria. Planktonic cells expose to the environment a greater surface area than sessile cells in the biofilm, thus in an environment rich in nutrients, aggregation does not represent a real advantage for growth [**52**]. The depth of the mature biofilm seems to be set up to control the acquisition of nutrients. The catabolic repression indicates that there is no advantage in developing a biofilm and induces the cells to remain in a planktonic form in the presence of high concentrations of nutrients.
The knowledge and the understanding of the biological dynamics of a biofilm formation have increased with the development of new techniques. Much of the early work was based on the use of scanning electron microscopy (**Figure 1-5**). In addition, the use of electron microscopy coupled with ruthenium-mediated staining of polysaccharides allowed to shed light on the nature of extracellular fibers in biofilm and their association with the cells. However, the preparation of samples for electronic microscopy may lead to artifacts; this drawback can be overcome with the use of “ESEM” (Environmental Scanning Electron Microscopy), an experimental approach that allows the observation of wet samples under pressure values ranging from 1 to 20 Torr and in conditions of controlled humidity and temperature [**53**].
The development of confocal laser scanning microscopy (CLSM) in the 80's has provided the ability to examine the biofilm *in situ*, although with a lower resolution compared to other techniques, without the limitations shown by the scanning electron microscope. The decrease in resolution is counterweighted by the possibility of examining an intact and unaltered matrix of the biofilm. The use of both CLSM and epifluorescence microscopy requires that microorganisms in the biofilm are fluorescent. The traditional staining procedures with fluorochromes are coupled today with modern techniques that involve the use of recombinant bacterial strains expressing the well-known green fluorescent protein (GFP) from *Aequorea victoria* [**54**].

## 1.3. Bioremediation and biofilm.

As previously mentioned, environmental contamination by heavy metals and/or organic substances of different nature is one of the major concerns for pollution. The type of pollution depends both on the nature of the industrial activity which causes the contamination

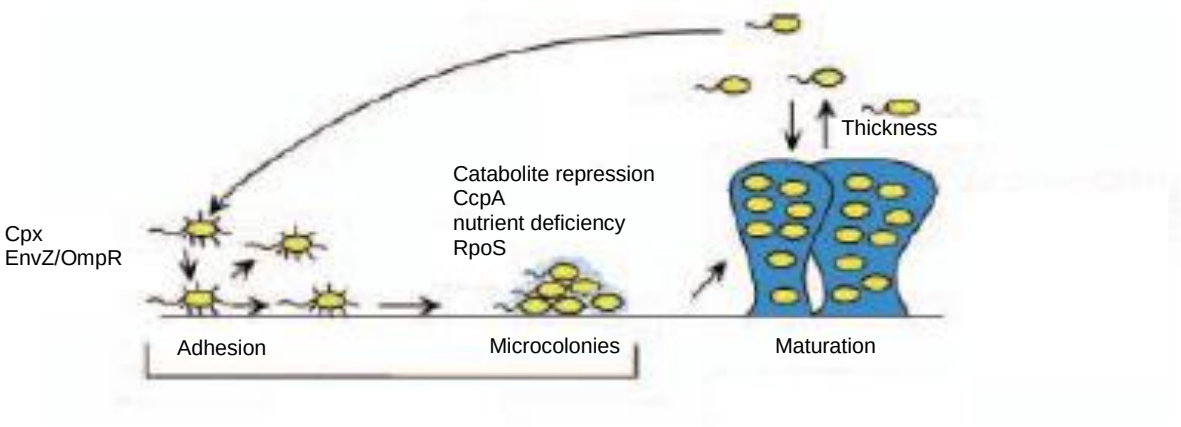



**Figure 1-3:** Schematic representation of the cycle of biofilm formation.

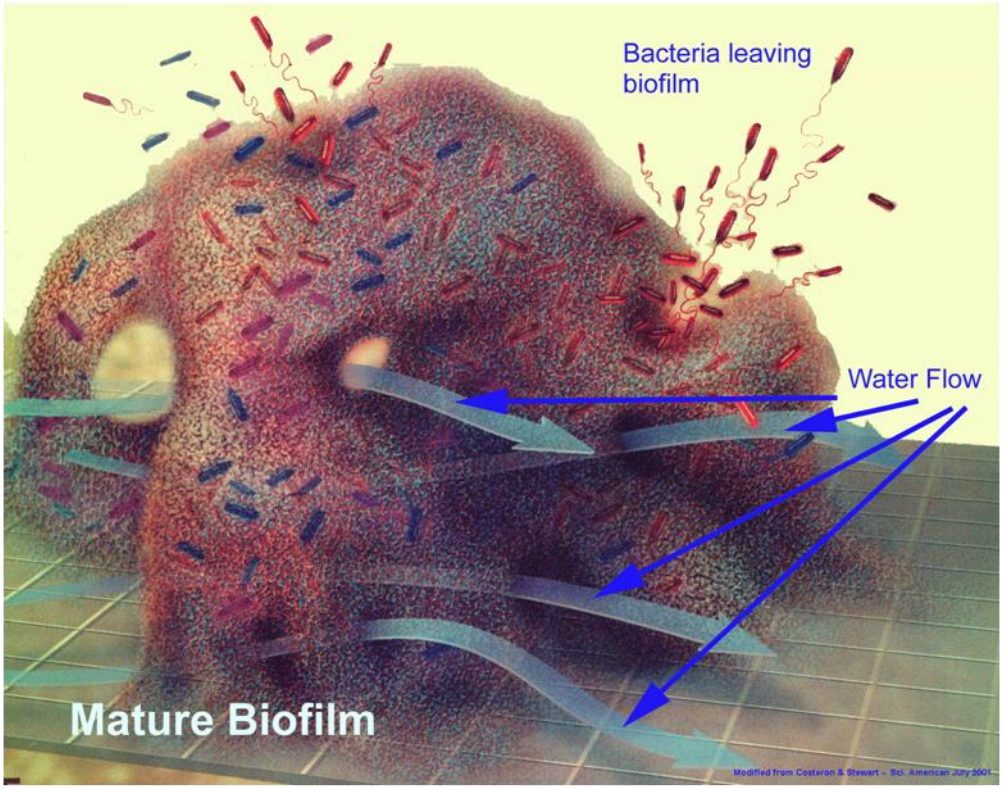



**Figure 1-4:** Mature biofilm. Water channels and the cellular release are shown .

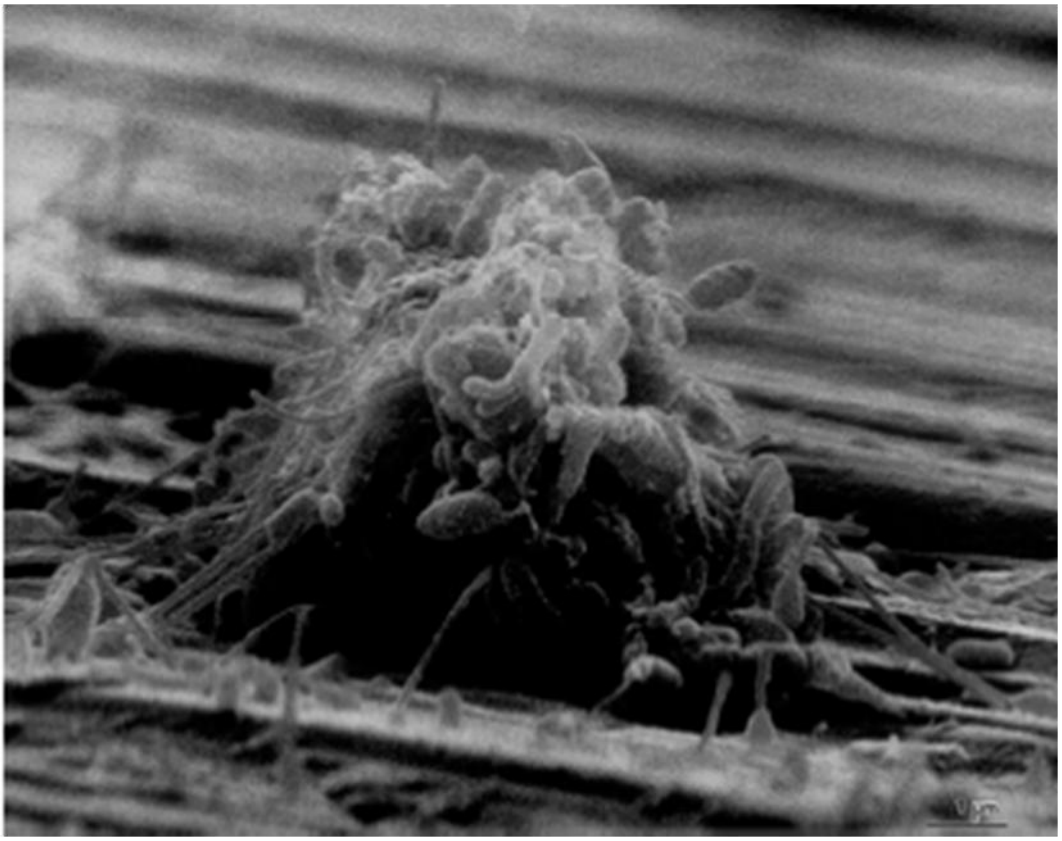


**Figure 1-5:** Scanning electron micrograph of a biofilm developed in an industrial water system.

(petrochemical, chemical, steel, mining and quarrying), and on the disposal of waste material from the production processes. Therefore, in most of the polluted sites, combined contaminations due to heavy metal, semimetal and organic substances of natural or xenobiotic origin are commonly found [**12**].

Today, the recovery of polluted sites, regardless of the type of contamination, is performed almost exclusively by using chemical and physical procedures. However, in recent years much attention has been given to the possibility of using the metabolic activity of certain microorganisms (bacteria, fungi and microalgae) for the recovery of contaminated sites through the degradation and/or the immobilization of organic pollutants, or through a change in the oxidation state of both heavy metals and semimetals [**12**]. Chemical and physical approaches for the restoration of polluted sites, other than being challenging from an economic point of view, are difficult to perform on a large scale, and are often responsible for additional environmental problems [**55-58**].

As a consequence, the development of biological systems for the remediation of polluted environments is an appealing alternative, presenting itself as a minimally invasive strategy for the environment, suitable for the treatment of large areas and competitive, if compared to chemical and physical procedures [**10**, **58-60**].

So far, microorganisms suitable for bioremediation purposes have been characterized in their planktonic form; however, as noted above, in most natural environments microorganisms are structured as communities embedded in biofilm [**39**, **61**, **62**]. Therefore it seems inappropriate to analyze only planktonic cultures to gain useful information for the design and implementation of bioremediation procedures of polluted sites [**63**].

Biofilm, as already mentioned, may consist of a single microbial species or, as it happens far more frequently, of different microorganisms such as bacteria, fungi, algae and protozoa [**64**]. The biofilm matrix harbors microbial communities which, once embedded in this protective framework, become resistant to conventional biocides, to antibiotic treatment, and to natural stress factors like the presence of heavy metals and contaminants [**65**, **66**].

The specific mechanisms responsible for the tolerance of the biofilm to all the compounds described above has not yet been completely understood; nonetheless, the matrix-mediated removal of the toxic compounds and the involvement, in the biofilm microbial community, of specialized cells called "persistent" might be responsible for the tolerance of biofilm to high concentrations of toxic agents [**67**].

Furthermore, the formation of biofilm is an interesting system for the degradation of organic contaminants, since the diffusion processes that take place within the biofilm generate redox gradients that can promote the degradation through a combination of aerobic and anaerobic processes. Moreover, the heterogeneous micro-environments of the biofilm matrix allow the persistence of slow-growing degrading strains whose survival as planktonic cells would be otherwise greatly hindered [**64**].

Almost all of the studies aimed at understanding the molecular determinants responsible for the formation and maturation of a biofilm have been conducted on single microbial strains of clinical interest, although in the environment biofilm consist of microorganisms of different species of eukaryotes and prokaryotes.

Cooperation between species within a biofilm is a key element to understand the physiological characteristics and the degradative abilities of the microorganisms involved; it has been observed, for example that mixed biofilm (*Penicillium/Bacillus*) lead to an increased degradation rate of polyethylene in the environment [**68**].

The interaction among microorganisms plays a major role when considering environments simultaneously polluted with heavy metals and organic pollutants. Heavy metals might strongly influence the degradative ability of a specific microbial group by altering both the physiology and the ecology [**69**]. This aspects must be taken into account when organizing the bioremediation of sites polluted with heavy metals such as arsenic, barium, cadmium, chromium, lead, mercury, nickel and zinc and organic substances such as petroleum, chlorinated solvents, pesticides and herbicides [**70**].

In conclusion it is straightforward to understand that biofilm, for their ability to adhere to substrates, for the resistance they confer to microbial communities towards heavy metals and organic pollutants, for the high microbial density that facilitates the mineralization of organic matter and for the ability to immobilize organic and inorganic matter by biosorption (removal of pollutants through the specific interaction between pollutants and microbes), bioaccumulation (increase of the concentration of the contaminant in the cell) and biomineralization (formation of insoluble precipitates deriving from the interaction between pollutants and microbial metabolic products), are good candidates for the bioremediation of soil organic compounds, heavy metals and semimetals [**64**, **71**, **72**].

The set-up of an effective bioremediation process is influenced by several factors that must be thoroughly analyzed in order to optimize the methodology and to keep a constant monitoring of the process itself.

In this context the main factors that need to be considered are: the characteristics of the soil, the indigenous microbial flora and the nature of the contaminant (physical and chemical properties, bioavailability, potential degradation products, etc...).

Bioremediation experiments performed in *microcosms* are a fundamental step to understand the main aspects influencing the biodegradation of a specific pollutant and allow reproducing on a laboratory scale, environmental conditions as close as possible to what happens in the field. This kind of experiments are performed in vessels or flasks in which aliquots of contaminated soil, withdrawn from polluted sites that need to be restored, are treated in different conditions in which several variables (such as the presence/absence of essential nutrients, selected microflora, specific biosurfactants, etc.) are individually modified and analyzed. Soil aliquots are then monitored over time and analyzed to evaluate the modification of different parameters such as the concentration of the contaminant, the number of microorganisms, the pH and the moisture level of the treated soil.

Several soil characteristics, such as organic matter content, clay and other elements, in fact, strongly affect the adsorption of contaminants in the solid phase. The organic matter, whose content greatly varies in different soil types, influences the adsorption of organic compounds in the soil. As an example, carbonaceous materials such as carbon black, with its strong adsorption capacity, is considered a "super-sorbent" [**73**], and is therefore a major contributor to the adsorption / distribution of some hydrophobic organic compounds such as PAHs [**74**], and as a result of their persistence in the soil.

Although in recent years much attention has been paid to the accumulation of toxic substances in the soil, the fate of these compounds and the details of the role of microbial activity in their degradation are still poorly understood [**75**].

Based on what described so far it is clear that a superficial approach to bioremediation is often the cause of bioremediation rising costs. An intervention of bioremediation, therefore, cannot be planned by considering only the presence of a

specific contaminant and its potential degradability, but must follow a complex and gradual process that, in addition to what indicated by the specific laws, provides:

- Physical-chemical characterization of the contaminated matrix;
- Characterization of the native flora;
- Trials and testing *in vitro* (microcosms);
- Trials and testing *in situ* (pilot plant).

A balanced distribution of resources between testing and application allows optimizing the effectiveness and the cost/benefit ratio of any remediation intervention.

## 1.4. *Novosphingobium puteolanum* PP1Y.

Recently, in the laboratory in which this project has been developed, a novel strain has been isolated and partially characterized, named *Novosphingobium puteolanum* PP1Y [**76**].

The microorganism was isolated from surface waters of a small dock bay in the harbor of Pozzuoli which is used for the storage of small boats and is characterized by a severe pollution of the water by aromatic hydrocarbons.

The microbiological analysis of the bacterium indicated that the microorganism belongs to the order of Sphingomonadales. The microorganism, however, shows different characteristics if compared to other members of the order, as it appears not only to be able to grow using, as the sole source of carbon and energy, a wide range of mono-and polycyclic aromatic substrates such as pyrene, naphthalene and phenanthrene, but also to have evolved an effective adaptation to the growth on complex mixtures of aromatic molecules dissolved in non-polar phases (such as diesel and gasoline). More specifically, with the exception of benzene and 1,2,3-trimethylbenzene, all common alkylbenzenes present in petroleum products can be used as the sole carbon and energy sources; among them, toluene, ethylbenzene, ethyltoluene and xylene, 1,2,4- and 1,3,5-trimethylbenzene. The PAHs that can be used as the sole source of carbon and energy by strain PP1Y include biphenyl, naphthalene, 1- and 2-methylnaphthalene, 1,2- 1,3- and 2,6-dimethylnaphthalene, phenanthrene, anthracene, pyrene, chrysene, benzo [*a*] anthracene, fluoranthene, acenaphthene, fluorene, tetralin (tetrahydronapthalene), dibenzofuran [**76**].

It is therefore reasonable to hypothesize a possible use of this microorganism for the bioremediation of contaminated sites, especially in the presence of complex mixtures of aromatic compounds.

*Novosphingobium puteolanum* PP1Y also showed the ability to produce a biofilm that preliminary data have suggested to be functional to its degradative capabilities and to be modulated in its organization and composition by the substrate and the growth conditions used. This observation has suggested the possibility of using the biofilm matrix of strain PP1Y for industrial applications [**76**].

In conclusion, all the microbiological characteristics of strain PP1Y make this microorganism suitable for its use in "*in situ"* bioremediation interventions.

## 1.5. Aim of the project.

In this work we have explored specific aspects of the biotechnological potential of *Novosphingobium puteolanum* PP1Y, focusing our attention on the use of this microorganism **i)** for the bioremediation of oil-polluted soils and **ii)** for the isolation of new catalytic activities intended for industrial applications.

*Novosphingobium puteolanum* PP1Y, isolated from seawater samples of the harbor of Pozzuoli (Naples, Italy), is able to grow on a wide range of aromatic compounds,

including mono-, bi-, tri-, and tetracyclic aromatic hydrocarbons and heterocyclic compounds. These peculiar features suggest that PP1Y has adapted to grow efficiently at the water/oil interface using the aromatic fraction of the fuel oils as a source of carbon and energy. Like some other Sphingomonads, this strain can grow as either planktonic free cells or sessile-aggregated flocks. In addition, this strain was found to grow as biofilm on several types of solid and liquid hydrophobic surfaces including polystyrene, polypropylene and diesel oil.

Specific objectives of this project have been:

**A)** To complete the microbiological characterization of *Novosphingobium puteolanum* PP1Y and to investigate, in model microcosms, several aspects of its use for bioremediation interventions. In this context the preliminary annotation of the whole genome of strain PP1Y has also been carried out, in collaboration with Dr. E. Notomista at the Department of Structural and Functional Biology (University Federico II of Naples, Italy), Prof. G. Paolella and Prof. F. Salvatore (CEINGE- Advanced Biotechnology, Naples, Italy and Department of Biochemistry and Medical Biotechnologies, University of Naples Federico II, Naples, Italy). The identification of the different catabolic pathways involved in the degradation of pollutants in strain PP1Y has provided significant clues about the molecular basis of the unusual features of this strain, and will help the future design of strains with enhanced cultural and bioremediation abilities.

**B)** To characterize the chemical composition and the morphology of the biofilm produced by *N. puteolanum* PP1Y, and to investigate the influence that such matrix could have in bioremediation procedures and its possible exploitation as a new biomaterial.

# II
# MATERIALS AND METHODS

**2.1. Culture Media.** LB broth was prepared according to Sambrook et al. [**77**]. Sea-Salts medium contained the indicated amount (0.5 – 5%, w/v) of sea salts (Sigma–Aldrich) and 1 g/L $NH_4Cl$. M9 medium comprised 7 g/L $Na_2HPO_4$, 3 g/L $KH_2PO_4$, 1 g/L $NH_4Cl$ and 0.5 g/L NaCl and was adjusted to pH 6.9. After autoclaving, 5 mL of a trace element solution was added to each liter of cooled M9. The trace element solution contained: 30.1 g/L $MgSO_4$, 27.3 g/L $FeSO_4$ x $7H_2O$, 5.4 g/L MgO, 1.0 g/L $CaCO_3$, 0.72 g/L $ZnSO_4 \times 7\ H_2O$, 0.56 g/L $MnSO_4 \times H_2O$, 0.125 g/L $CuSO_4 \times 5\ H_2O$, 0.14 g/L $CoSO_4 \times 7\ H_2O$, 0.03 g/L $H_3BO_3$, 0.004 g/L $NiCl_2 \times 6\ H_2O$, 0.006 g/L $Na_2MoO_4 \times 2\ H_2O$ and 25.6 mL/L HCl (36%). Potassium phosphate minimal medium (PPMM) contained 20 mM potassium phosphate at pH 6.9, 1 g/L $NH_4Cl$ and the indicated amount of NaCl (0–5%, w/v). The trace element solution was added as described for M9 medium.

**2.2. PAH-Agar Plates.**
Twenty mL of liquid M9 (or PPMM)/agar (1.5%) were added to a Petri dish and allowed to solidify. Next, 20 mg of phenanthrene or 7 mg of pyrene were dissolved in 0.6 mL of methanol and added to 5 mL of liquid M9 (or PPMM)/agar (1%). The resulting milky solution was spread on the agar layer and allowed to solidify. Control plates were prepared omitting the addition of PHAs to methanol. Anthracene-containing plates were prepared by spraying a saturated solution of anthracene in diethyl ether on a 25-mL PPMM-agar (1.5%) layer.

**2.3. Optimal Salt Concentration, pH and Temperature for Growth of Strain PP1Y.**
To determine the optimal salt concentration for growth, 10 µL aliquots of a culture grown in LB (about 1 O. D. at 600 nm) were inoculated in 10 mL of PPMM containing 5 g/L tryptone, 2.5 g/L yeast extract and NaCl from 0% to 5% (w/v) or in 10 mL of Sea-Salts medium (0.5–5%, w/v) containing 5 g/L tryptone, 2.5 g/L yeast extract and incubated at 30°C. The optimal pH was determined using PPMM containing 5 g/L tryptone, 2.5 g/L yeast extract and 10 mM NaCl. The pH of the solutions was adjusted at the desired value (5.5–8.5). The optimum temperature was determined in LB medium incubating the cultures at different temperatures. Growth was monitored by measuring the optical density at 600 nm.

**2.4. Growth on Fuels.**
Growth of strain PP1Y on diesel oil, gasoline and paraffin-dissolved gasoline was performed using PPMM containing 10–250 mM NaCl or Sea-Salts medium (0.5–2%). Growth of strain US6-1T on diesel oil and gasoline was performed using PPMM containing 200–400 mM NaCl or Sea-Salts medium (3%). Cultures were incubated at 30°C with orbital shaking (220 rpm). Bacterial growth was followed by measuring total proteins, carbohydrates and DNA of the cultures as described below.

**2.5. Growth on Single Hydrocarbons.**

Utilization of individual aromatic compounds as growth substrates was determined by supplementing 10 mL of PPMM containing 10 mM NaCl with 8 mg of an aromatic compound in liquid or crystalline form (depending on the hydrocarbon) or with 8 mg of the pure aromatic compound dissolved in 400 µL of either a n-dodecane (C12), n-tetradecane (C14), low viscosity paraffin (LVP) and high viscosity paraffin (HVP) mixture or silicon oils (polydimethylsiloxane or polymethylphenylsiloxane). For each aromatic substrate, several cultures were incubated in 50 mL polypropylene tubes at 30°C under orbital shaking (220 rpm). At different time points, cultures were freeze-dried, washed twice with 5 mL of hexane and twice with 5 mL of water-saturated diethyl ether/methanol/water = 8:3:1 (v/v/v) to remove oils, lipids and salts [**78**]. The washed material was dried under nitrogen and dissolved in 5 mL of 20 mM $H_2SO_4$ containing 10% ethanol. Protein content was assayed with the Bradford reagent (Sigma), using bovine serum albumin as a standard. Total carbohydrate content was assayed by the phenol/$H_2SO_4$ method [**79**] using glucose as a standard. DNA content was assayed with ethidium bromide using plasmid pGEM-3Z (Promega) as a standard. Samples were diluted in 0.1 M Tris acetate, pH 8.0, containing 10 mM EDTA and mixed with an ethidium bromide solution (Sigma) in a ratio 10,000:1 (v/v) according to the manufacturer's instructions. Fluorescence of the samples was measured using a Chemidoc XRS densitometer (Bio-Rad).

To determine the biochemical composition of the mucilaginous sheets produced by cultures containing LVP-dissolved propylbenzene, mucilaginous material was allowed to stratify at the surface of a 10 mL culture, and the liquid phase below the mucilage was removed. Mucilaginous material was washed twice with 10 mL of fresh PPMM, freeze-dried and treated as described above.

### 2.6. Phase Contrast Microscopy.

An Olympus BX-51 phase-contrast microscope equipped with a DP-70 camera was used to obtain images of flocks, oil drops and mucilaginous sheets. Samples were neither fixed nor stained unless otherwise noted.

### 2.7. Removal of Oil-Dissolved Aromatic Hydrocarbons by Strain PP1Y.

Typically, 15 mL of PPMM containing 10 or 100 mM NaCl were inoculated with 15 or 150 µL aliquots of cells grown in LB to an O.D. at 600 nm ~1. Cultures were supplemented with substrate, which was either 0.10 mL diesel oil in 1.4 mL of C14, 0.10 mL of gasoline in 1.4 mL of C14, 1.5 mL of paraffin (C12, C14, LVP or HVP) containing 0.1% (w/v) of a single PAH, or 1.5 mL of paraffin containing a mixture of 2–4 aromatic hydrocarbons at the indicated concentrations. These mixtures were incubated at 30°C under orbital shaking (220 rpm). Control samples were prepared and incubated as described above in the absence of bacteria. At increasing times, 200 µL aliquots of the PPMM/oil drop emulsion and 500 µL of the water phase were collected and stored at −20°C. For control samples in which no emulsion was present, 60 µL aliquots of oil phase and 600 µL aliquots of water phase were collected and stored. Before analysis, emulsion containing samples were thawed and centrifuged at 14,000×g for 10 min at 4°C. This procedure separates the mixture into four phases: a cell pellet, an aqueous phase, a disc of gelatinous heterogeneous material and an oil phase. It should be noted that by omitting

the freeze/thawing cycle, longer centrifugation times were needed to obtain complete stratification of the samples. The aromatic fraction of the oil phases (derived either from bacterial cultures and control samples) was analyzed by normal-phase HPLC on a nitrile-bonded phase (Waters Spheris-orb 5 µm CN-NP, 4.6×250mm) to obtain the so-called group or class separation of aromatic compounds [**21**, **80**, **81**]. Typically 4 µL of sample and 4 µL of a 0.1% solution of nitrobenzene (internal standard) in C12 (v/v) were mixed with 240 µL of hexane containing 6% isopropyl alcohol and loaded through a 200 µL loop. The HPLC system was equipped with a Waters 1,525 binary pump coupled to a Waters 2,996 photodiode array detector.

Analyses were carried out at a flow rate of 0.5 mL/min by using a two-solvent system comprised of 0.1% isopropyl alcohol in hexane (solvent A) and 20% isopropyl alcohol in hexane (solvent B). PAHs were separated using a 10 min isocratic step with 100%of solvent A, followed by a linear 0% to 90% solvent B gradient in 15 min and an isocratic 90% solvent B step. A spectrum of the eluate was measured between 210 and 500 nm. The relative amount of the monocyclic aromatic fraction of diesel oil and gasoline was measured by integrating chromatograms recorded at 262 nm, because at this wavelength, the molar extinction coefficients of the main monocyclic aromatic compounds are very similar (**Table 2.1**). For the same reason, the amount of the bicyclic and the tricyclic aromatic fractions were measured at 275 and 257 nm, respectively (**Table 2.1**). Pyrene was quantified at 334 nm. The aromatic fraction of the water phase was extracted twice with a half volume of C12 and analyzed as described above. To measure evaporation from the oil phase, cultures and control samples were prepared as described above, supplemented with 0.10 mL of gasoline in 1.4 mL of C14 and incubated 90 h at 30°C with orbital shaking. Samples were uncapped and incubated under static conditions at 25°C.

Air was gently pumped at the top of the tubes to allow for rapid gas exchange in the headspace. At increasing time points, samples were collected and analyzed as described above.

## 2.8. Removal of Aromatic Hydrocarbons from artificially polluted soils:

**2.8.1.** ***Growing conditions:*** 3 µL of frozen cells were inoculated into 10 mL of LB medium and the culture was incubated at 30°C for 15 h. The cells (3-4 O.D. /mL at 600 nm) were diluted in 20 mL of LB medium to a final concentration of 0.1 $O.D._{600}$/mL and incubated at 30°C for 5-7 h until obtaining an optical density of 0,8 to 1.6 $O.D._{600}$/mL (exponential phase). Cell cultures were harvested by centrifugation at 2,000xg for 15 min at 4°C and washed twice in PPMM medium.

After that, cells were suspended in deionized water to a final concentration of 4.6 $O.D._{600}$/mL and kept on ice until use.

**2.8.2.** ***Preparation of microcosms:*** Universal soil (composted mixed) Agri-Flor for ornamental plants and garden (producer by Gerovital Service Ltd.) was used for these experiments; characteristics of this soil are shown in **Table 2.2**.

| Table 2.1 | | | | | | |
|---|---|---|---|---|---|---|
| | | Hydrocarbon[a] | Solubility in water (mg/l) | Log $K_{ow}$ | $\varepsilon_{mM}$[b] | Growth[c] |
| | | *diesel-oil* | | | | F,C,T |
| | | *gasoline* | | | | F,T |
| (alkyl)benzenes | 1-R | *benzene* | 1760 | 2.13 | | - |
| | | *toluene* | 515 | 2.69 | 0.21±0.01 | F,c |
| | | *o-xylene* | 178 | 2.8-3.1 | 0.26±0.01 | F,c |
| | | *m-xylene* | 161 | 3.2 | 0.24±0.01 | F,c |
| | | *p-xylene* | 162 | 3.15 | 0.32±0.01 | F,c |
| | | *ethylbenzene* | 152 | 3.15 | 0.18±0.01 | f,c,s |
| | | *2-ethyltoluene* | 93 | 3.53 | 0.26±0.02 | F,c |
| | | *3-ethyltoluene* | 94 | 3.88 | 0.27±0.01 | f,c,s |
| | | *4-ethyltoluene* | 95 | 3.90 | 0.37±0.02 | F,c,s |
| | | *propylbenzene* | 60 | 3.6 | 0.21±0.01 | S |
| | | *isopropylbenzene* | 61 | 3.66 | 0.16±0.01 | S (*slow*) |
| | | *1,2,3-trimethylbenzene* | 75 | 3.66 | 0.21±0.01 | - |
| | | *1,2,4-trimethylbenzene* | 58 | 3.65 | 0.37±0.02 | F,c |
| | | *1,3,5-trimethylbenzene* | 48 | 3.42 | 0.22±0.02 | F,c |
| | | *butylbenzene* | 50 | 4.4 | 0.20±0.01 | c/S (*slow*) |
| naphthenes | 1-R | *Indan*[d] | 109 | 3.33 | 0.92±0.03 | f |
| | | *tetrahydronaphthalene* | 47 | 3.5-3.8 | 0.43±0.02 | S |
| | 2-R | acenaphthene | 4 | 3.92 | 4.5±0.2 | S |
| | | fluorene | 2 | 4.18 | 12.6±0.7 | f,c (*slow*) |
| polycyclic aromatic | 2-R | naphthalene | 32 | 3.36 | 6.0±0.3 | F,c |
| | | *1-methylnaphthalene* | 28 | 3.87 | 6.1±0.3 | F,c |
| | | 2-methylnaphthalene | 25 | 3.86 | 5.8±0.2 | F,c |
| | | *1,2-dimethylnaphthalene* | 15 | 4.31 | 4.8±0.2 | F,c |
| | | *1,3-dimethylnaphthalene* | 8 | 4.6 | 5.4±0.2 | F,c |
| | | *1,7-dimethylnaphthalene* | | 4.44 | 5.0±0.3 | f,c,T |
| | | 2,3-dimethylnaphthalene | 3 | 4.6 | 5.2±0.2 | F,c |
| | | 2,6-dimethylnaphthalene | 2 | 4.31 | 5.3±0.2 | F,c |
| | | 2,7-dimethylnaphthalene | | | 5.3±0.2 | F,c |
| | | biphenyl | 7.5 | 4.09 | 4.0±0.3 | F,c |
| | 3-R | phenanthrene | 1.3 | 4.57 | 15.8±0.6 | F,c,T |
| | | anthracene[d] | 0.07 | 4.5 | 16.7±0.8 | c,s (*slow*) |
| | 4-R | fluoranthene | 0.26 | 5.22 | | S |
| | | pyrene | 0.13 | 4.88 | | S |
| | | benzo[a]anthracene[d] | 0.014 | 5.91 | | c,s (*slow*) |
| | | chrysene[d] | 0.002 | 5.86 | | c,s (*slow*) |
| hetero cyclic | | dibenzofuran | 10 | 4.17 | | S |
| | | carbazol | 1.8 | 3.72 | | S |
| | | dibenzothiophene | 1.47 | 4.38 | | S |

[a] Aromatic hydrocarbons that are liquid at room temperature are reported in italics. The number of aromatic rings – from one to four – is indicated with the abbreviations 1-R/4-R, respectively.
[b] Extinction coefficients (mM) were measured in hexane containing 0.1% isopropyl alcohol at 262 nm, 275 nm and 257 nm for 1-R, 2-R and 3-R hydrocarbons, respectively.
[c] F = amorphous flocks and/or free cells (≥ 0.5 $O.D._{600nm}$); f = amorphous flocks and/or free cells (<0.5 $O.D._{600nm}$); C = emulsion containing coated oil drops (high cell density); c = emulsion containing coated oil drops (low cell density); T = biofilm on the polypropylene tube; S = formation of mucilage containing sheets visible by the naked eye; s = formation of sheets; - = no growth.
[d] Indan was used at 1% (w/v), whereas, due to their low solubility in paraffins (<0.5%), anthracene, benz[a]anthracene and chrysene were used as saturated solutions in $C_{14}$.

| Table 2.2 | |
|---|---|
| **Wet** | **<50%** |
| **pH (unit)** | **6-7,5** |
| **Organic Carbon (C)** | **>25%** |
| **Humic and fulvic acids** | **>7,5%** |
| **Organic nitrogen (N) (% S.S.)** | **1%** |
| **Carbon / nitrogen ratio (C/N)** | **25** |
| **Copper (Cu) (mg/Kg S.S)** | **<150** |
| **Zinc (Zn) (mg/Kg S.S)** | **<500** |
| **Salt content (dS/m)** | **4 dS/m** |

Experiments were also conducted on urban soil samples taken from an area characterized by intense city traffic and by heavy metals and PAHs pollution (**Table 2.3**). Microcosms were prepared with 10-20 g of soil in 50 mL polypropylene tube fitted with stopper.
To mimic the PAH contamination, the soils were artificially contaminated with phenanthrene (4 mg/g soil), used as a model of PAH compound, and with an artificial mixture of phenanthrene, 2,6 dimethylnaphthalene and pyrene (respectively at 4, 2 and 2 mg/g soil). In addition, were added to some microcosms increasing concentrations of heavy metals $NiCl_2$, $CuCl_2$, $ZnCl_2$, $PbCl_2$ (Sigma–Aldrich) at 0.5 – 20 mg/g soil.
The compounds were added to the soil dissolved in LVP or acetone. The soils were mixed manually with a spatula for 2 min before the addition of cells.
For each microcosm, were added 3 mL of cells suspended in deionized water (4.6 O.D./mL) prepared as described in the previous paragraph.

| Table 2.3 | | | | | |
|---|---|---|---|---|---|
| | **Cu µg/g** | **Ni µg/g** | **Cr µg/g** | **Pb µg/g** | **Cd µg/g** |
| **Via Acton** | 112.23 ±3.43 | 3.67 ±0.07 | 5.51 ±0.18 | 228.07 ±10.69 | 0.12 ±0.007 |
| **Legal limits** | 120 | n.a | n.a | 100 | n.a |

For each sample set was a microcosm of control to which were added 3 mL of no cells deionized water. The samples were incubated at 25°C.

**2.8.3.** ***Removal of aromatic hydrocarbons from soil by strain PP1Y:*** The evaluation of the degradation of phenanthrene by HPLC was performed on the

microcosms with added phenanthrene dissolved in LVP or acetone as described previously.

The extraction of various pollutants was conducted on 0.5 g of soil using 2.4 mL of hexane. To improve the efficiency of extraction, the samples were subjected to sonication for 5 min in ice and centrifuged at 10,000xg for 10 min, 14 μL of the organic supernatant were added to 481 μL of hexane and 5 μL of nitrobenzene (0.1% in C12) as internal standard. The analysis by HPLC was carried out as described previously.

**2.9. Heavy metals resistance.**

The growth in presence of heavy metals were performed using minimal medium containing 20 mM Mops salt pH 6.9, NaCl 100 mM, 1 g/L $NH_4Cl$ and glutamic acid 1.0% as sole carbon and energy source. The trace element solution was added as described in paragraph 2.1. Heavy metals tested were $NiCl_2$, $CuCl_2$, $ZnCl_2$, $PbCl_2$, $CoCl_2$ and $CdCl_2$ (Sigma–Aldrich).

**2.10. Analysis of the Extracellular Products:**

Strain PP1Y was grown in LB or PPMM containing 4 g/L of glutamic acid and 100 mM NaCl or PPMM containing 4% (w/w) of diesel oil. Cultures at the beginning of the stationary phase (about 5 O.D. at 600 nm) were centrifuged at 5,000×g for 30 min at 4°C to separate cells and supernatants.

**2.10.1. Proteins analysis:** Proteins contained in the supernatants were precipitated by trichloroacetic acid [**82**] and analyzed by SDS-PAGE. Gel electrophoresis under denaturing condition was performed as described by Laemmli. The resolving gel was prepared at 18% acrylamide and the stacking gel was prepared at 6% acrylamide [**83**].

**2.10.1.1. Mass spectrometric analysis:** Identification of proteins contained in the supernatants was carried out on trypsin digested samples either by digestion in solution or in situ after separation by polyacrilamide gel electrophoresis. Proteins were detected on the gel using a colloidal Coomassie kit (Invitrogen Life Technologies). Excised bands were destained, reduced with 10 mM dithiothreitol (DTT), carbamidomethylated with 55 mM iodoacetamide in 0.1 M $NH_4HCO_3$ buffer, pH 7.5 and subjected to tryptic in-gel digestion for 16 h at 37°C, by adding 100 ng of trypsin. Reactions were quenched by lowering the pH to about 1 with formic acid, and the resulting peptide mixtures were concentrated and purified using a reverse phase Zip Tip pipette tips (Millipore). The peptides were eluted with 20 μL of a solution comprising 50% acetonitrile and 0.1% formic acid in deionized water. Peptide mixtures were analyzed either by capillary liquid chromatography with tandem mass spectrometry detection (LC-MSMS).

The peptide mixtures were analyzed using a CHIP MS 6,520 QTOF equipped with a capillary 1,200 HPLC system and a chip cube (Agilent Technologies, Palo Alto, Ca). After loading, the peptide mixture (8 μl in 0.1% formic acid) was first concentrated and washed at 4 μL/min in a 40 nL enrichment column (Agilent Technologies chip), with 0.1% formic acid in 2% acetonitrile as the solvent.

The sample was then fractionated on a C18 reverse-phase capillary column (75 μm x 43 mm in the Agilent Technologies chip) at flow rate of 400 nL/min with a linear gradient of eluent B (0.1% formic acid in 95% acetonitrile) in A (0.1% formic acid in 2% acetonitrile) from 7% to 60% over 50 min. Peptide analysis was performed using data-dependent acquisition of one MS scan (mass range from 300 to 2000 m/z) followed by MS/MS scans of the three most abundant ions in each MS scan.

Raw data from nanoLC-MSMS analyses were employed to query, using MASCOT software (Matrix Science, Boston, USA) and by comparing the experimental data to the PP1Y complete genome database [**84**] and this thesis, chapter 3.2).

**2.10.2. Carbohydrate analysis:** Total carbohydrate content was assayed by the phenol/$H_2SO_4$ method [**79**].

**2.10.2.1. Acetylated methyl glycosides:** With the analysis of acetylated methyl glycosides it is possible to detect different types of monosaccharides, that is, deoxyhexoses, hexoses, uronic acids, aminosugars, 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo), neuraminic acid; however, sugars, as fructose and 2-deoxy ribose, are completely destroyed [**85**].

Each class of monosaccharides (hexoses, deoxyhexoses, etc.) is eluted in a particular range of the chromatogram, and displays the same electronic impact (e.i.) fragmentation pattern (fragmentation of different types of monosaccharides. For a given mass spectrum, the peaks most relevant for the recognition of the type of residue are those at high mass values. It must be noted that the molecular ion is never observed because it is unstable and prone to fragmentation. The most diagnostic ion usually observed, even if with low intensity, is the oxonium ion descending from the molecular ion after loss of the anomeric carbon substituent, together with the secondary fragments arising from the loss of acetic acid (60 u), acetic anhydride (120 u), or ketene (42 u).

Method provides dry the sample (0.5–1.0 mg of crude product or 0.2 mg of purified sample) over a drying agent, under continuous vacuum, for a couple of hours (see Notes and tips). Add 1 mL of methanolic hydrochloric acid and close the tube tightly. Incubate the sample at 80°C overnight (see Notes and tips).

Cool the sample and add n-hexane until the two layers are almost equal. Collect the hexane phase (top) separately and repeat the extraction twice, combining all top layers. Dry the methanolic phase in a stream of air, eventually warming it gently (40°C).

Acetylate the dried methanolic extract with pyridine (200 μL) and $Ac_2O$ (100 μL), 80°C, 30 min. Dry the products in a stream of air. Last traces of pyridine can be removed by adding few drops of toluene and evaporation. Dissolve the product in acetone (100 μL) and analyze (1–3 μL) by GCMS with the temperature program 150°C 3 min, 3°C/min up to 280°C, 280°C 10 min.

*Notes and tips:*

a) *Due to the kind of chemical cleavage, each monosaccharide will give rise to different O-methyl glycosides (a- and b-, furanose and pyranose ring) and more than one peak will be observed in the chromatogram.*

*b) Overnight incubation is necessary for samples of unknown composition. If occurrence of 2-amino-2-deoxy-sugars can be ruled out, methanolysis can proceed for 2 h.*

**2.10.3. Emulsification procedures:** For the emulsification assay, supernatants were centrifuged at 18,000×g for 30 min at 4°C and used without further purification. Typically, 1.5 mL of the supernatants and 0.5 mL of LVP were mixed vigorously for 2 min in glass tubes (5×100 mm). The emulsions were allowed to stand for 14 days at room temperature.

**2.11. Genome Analysis.**

We performed high-throughput *de novo* sequencing and assembly of the whole *Novosphingobium* genome using the Roche 454 genome sequencer 20 (GS20) system [**86**]. The genomic DNA was randomly sheared into small fragments, purified, and assessed for quality. The library obtained was hybridized on the surfaces of microscopic beads, clonally PCR amplified within the droplets of a water-in-oil emulsion (emPCR), and bidirectional sequenced using a pyrosequencing protocol. The resulting sequencing reads were analyzed and processed into contigs generating a consensus sequence. PCR amplifications were performed to fill gaps generated by contig assembly. Four sequencing runs were performed, which yielded more than 120 Mb, being equivalent to 1,224,396 sequencing reads. Because the PP1Y genome is about 5.3 Mb, we obtained a coverage of 22.6x. The average read length was 101.8 bp, and the average GC content was 61.7%. The vast majority (98.8%) of the sequencing reads were used for the assembly, which resulted in 286 contigs, 108 of which were larger than 500 bp. The largest contig was 368,742 bp. We used two PCR strategies to complete the assembly. The results were assembled with the GS20 data so that the entire genome sequence was obtained.

Subsequently, a single GS-FLX-titanium run was done, and the resulting longer reads were assembled with the GS20 reads, which showed there was no misassembly. Thus, the final coverage was higher than 88x.

**2.12. Other Methods.**

Cell pigments were analyzed as described previously [**87**, **88**]. Analysis of fatty acids was carried out by the Identification Service, DSMZ, Braunschweig, Germany. SDS-PAGE was performed as described by Sambrook et al. [**77**]. The kinetics of phenanthrene solubilisation by supernatants of the cultures of strain PP1Y was followed for 4 h as described for alasan [**89**].

# III
# RESULTS AND DISCUSSION

**3.1. Characterization of *Novosphingobium puteolanum* PP1Y.** Strain *Novosphingobium puteolanum* PP1Y was isolated as recently described (**Figure 3-1**) [**76**]. The 16S rRNA analysis showed that strain PP1Y is closely related to *Novosphingobium pentaromativorans* US6-1$^T$ (two differences in a 1,481 bases long alignment), a strain isolated in Korea and able to degrade 3-5 rings PAH [**22**], *Sphingomonas* sp. SB5 (three differences) [**90**] and to *Novosphingobium* sp. Phe-8 (five different nucleotides). Therefore, on the basis of the 16S rRNA sequences, strain PP1Y could be considered a new strain of species *Novosphingobium pentaromativorans* US6-1$^T$.A thorough microbiological characterization of strain PP1Y performed during my thesis project and described in this chapter adds important details to the preliminary data described in the Introduction section and shows that strain PP1Y can be considered a new *Novosphingobium* sp.

### 3.1.1. Phenotypic characterization of strain PP1Y and growth conditions.

Cells of *Novosphingobium* sp. PP1Y, observed by phase contrast microscopy, appear as short rods. They can be motile (likely flagellate) or non-motile (**Figure 3-2 A**). *Novosphingobium* sp. PP1Y grows efficiently in LB medium and in saline media (PPMM, Sea-Salts or M9) containing casaminoacids, single aminoacids or glucose as carbon sources [**76**].

*Novosphingobium* sp. PP1Y is able to grow at NaCl concentrations ranging from 0% to 4% (w:v). Highest growth rates can be measured at NaCl concentrations between 0.5% and 1.5% (w:v). Growth is very slow in the presence of NaCl concentrations above 5%. Very similar results are obtained using either PPMM containing different NaCl concentrations or Sea-Salts at concentrations between 0.5% and 5% (w:v). These features suggest that strain PP1Y is able to grow either in seawater or in brackish water, like river estuaries and costal lagoons and that these environments could be the main ecological niche of strain PP1Y. It is interesting to note that the behavior of strain US6-1$^T$ is different. In fact, the highest growth rate for this strain is observed at 2.5%NaCl. Moreover, the strain is not able to grow at NaCl concentrations below 1% [**22**].

Strain PP1Y is able to grow in a wide range of temperatures (from about 18°C to 42°C) and of pH values (from 5 to about 7.5). Highest growth rate is observed at 32-34°C and pH 6.0-7.0 in rich media and at 29-30°C and pH 7.0 in minimal media (data not shown). As comparison, the optimal temperature and pH for strain US6-1$^T$ are 30°C and 6.5, respectively [**22**].

Like other Sphingomonads, strain PP1Y produces an a typical outer membrane containing glycosphingolipids (GLS) instead of lipopolysaccharides [**21**].

The analysis of the fatty acids composition (Materials and Methods section and **Table 3.1**) shows that the lipidic composition of PP1Y strain is more similar to that of *Novosphingobium subarcticum* JCM10398$^T$ [**91**] than to that of *Novosphingobium pentaromativorans* US6-1$^T$ [**22**]. Moreover, this analysis shows some distinctive features, such as the absence of fatty acid 17:1 and the presence of fatty acid 18:1 methylated at position 11.

Liquid cultures of strain PP1Y, both in rich and in minimal media with several growth substrates, are very viscous due to the production of an abundant extracellular

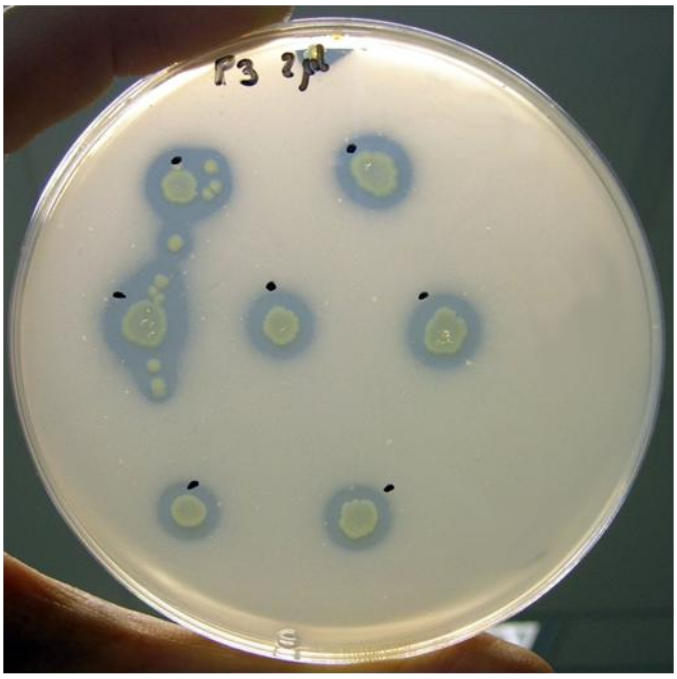



**Figure 3-1.** PP1Y colonies surrounded by a clear halo on M9G-Agar plate containing phenanthrene as the sole carbon source and incubated 7 days at 25°C.

material likely containing, among others, exopolysaccharides (EPS). In fact, it is known that several strains of sphingomonadales produce EPS containing repeated tetrasaccharidic units $(A\text{-}B\text{-}C\text{-}B)_n$ where A is usually rhamnose or mannose, B is glucose and C is glucuronic acid. Sugars at positions A and B usually bind "side-chains" constituted by other sugars or carboxylic acids like acetate and glycerate. Several of these polysaccharides are patented as jellifying agents or thickeners for food industry or scientific research. As an example, *Gelrite*, the gel used for the preparation of plates for thermophilic bacteria, is the commercial form of "gellan", the EPS produced by *Sphingomonas elodea* [**92**].

**Table 3.1**

| Fatty Acids | Strain | | |
|---|---|---|---|
| | **US6-1$^T$** | **JCM10398$^T$** | **PP1Y** |
| 12:0 2-OH | 0.8% | - | 0.06% |
| 13:0 2-OH | - | - | - |
| 14:0 | 0.6% | | 0.8% |
| 14:0 2-OH | 19,7% | 7.6% | 4.45% |
| 15:0 | - | - | - |
| 15:0 2-OH | 0.3% | - | - |
| 16:0 | 1.0% | 10.2% | 9.5% |
| 16:0 2-OH | 2.5% | 2.1% | 2.9% |
| 18:0 | - | - | 0.4% |
| 16:1 | 8.8% | 15.5% | 15.1% |
| 17:1 | 2.0% | 3.3% | |
| 18:1 | 64.0% | 61.1% | 65.0% |
| 18:1 11-$CH_3$ | - | - | 1.1% |
| Others | 0.3% | 0.2% | 0.7% |

### 3.1.2. The planktonic-sessile dimorphism of *Novosphingobium puteolanum* PP1Y and the analysis of the extracellular products.

Cells of *Novosphingobium* sp. PP1Ycan be motile (likely flagellate) or non-motile (**Figure 3-2 A**). Non-motile cells can form aggregates of different dimensions from 50-100 µm up to macroscopic "flocks" of 1-10 mm length. The number and dimension of flocks is influenced by several parameters including shaking, temperature and growth substrates. For example, formation of flocks is favored by slow shaking, or by high temperature (34°-38°C), or by the presence of glutamate and high phosphate concentration. Flocks, when dried and stained with methylene blue, show the presence of bunches of cells trapped inside a blue-stained matrix (**Figure 3-2 B**).

A similar behavior has already been described for some other *Sphingomonads* that show the so-called "planktonic/sessile dimorphism". These dimorphic strains can exist either in a sessile form made up of aggregated cells embedded in a polysaccharidic extracellular matrix, and a planktonic form of free cells. About 5% of the free cells are motile, possessing a single polar flagellum [**93**].

It is interesting to note that we did not observe any planktonic/sessile dimorphism in *N. pentaromativorans* US6-1$^T$ (DSM17173) grown in the presence of NaCl concentrations up to 4%, and using different carbon sources (tryptone, yeast extract, glutamate or propylbenzene) (data not shown).

Flocks formed by strain PP1Y showed some peculiar features.

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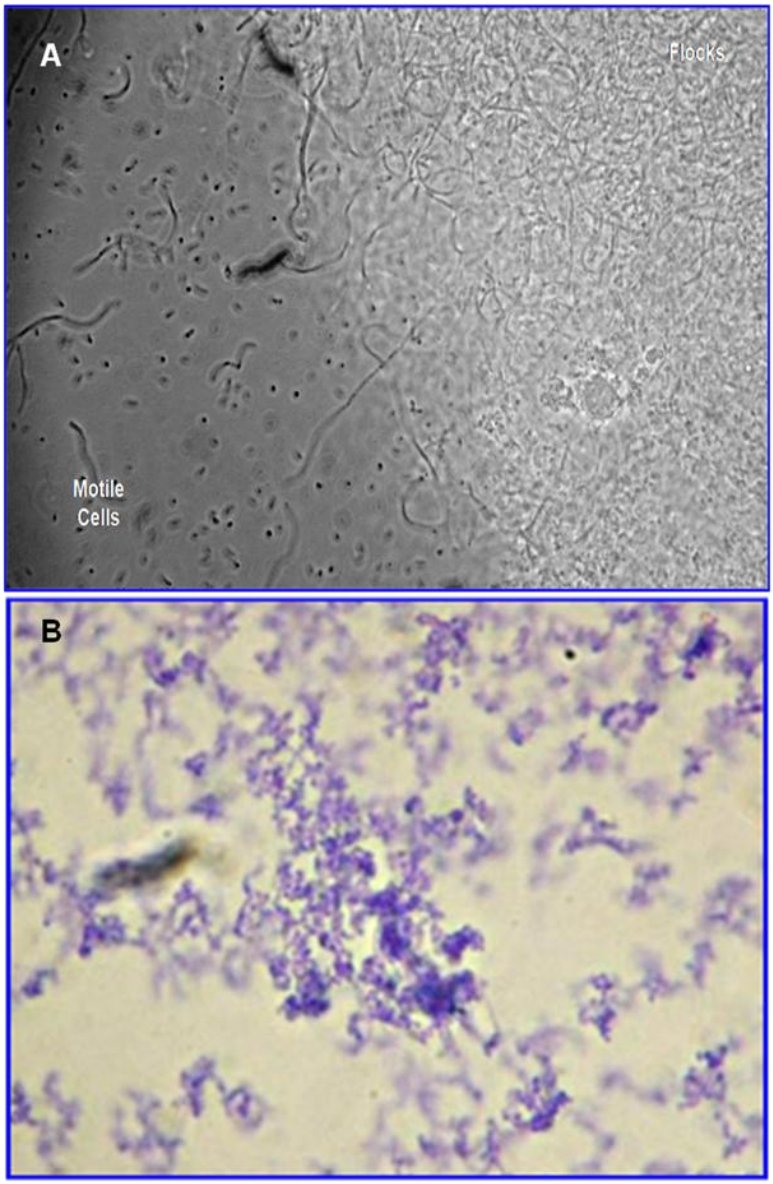


**Figure 3-2: A)** Phase contrast microscope picture showing the border of a flock (not stained). **B)** Phase contrast microscope picture showing a flock dried and stained with methylene blue.

They adsorbed and concentrated hydrophobic molecules like Red Sudan III and PAH (**Figure 3-3 A**) and adhered to hydrophobic surfaces as plastic polymers (for example polyethylene and polystyrene).
*Novosphingobium* sp. PP1Y forms on several types of hydrophobic surfaces what appears to be a more structured and complex biofilm. A yellow biofilm layer is formed for example in 24 h by *Novosphingobium* sp. PP1Y cultured in polystyrene Petri plates containing liquid rich medium. After about 48-72 h the plates contain a homogeneous gelatinous layer 3-4 mm thick. The biofilm, detached from the plates by vigorous shaking, forms gelatinous sheets (**Figure 3-3 B-C**).
*Novosphingobium* sp. PP1Y is able to use diesel oil and gasoline as the sole carbon and energy source (**Figure 3-4 A-B-C** and [**76**]). It is worth to note that formation of biofilm was observed also at the boundary between the aqueous phase and the oil phase when strain PP1Y was grown in the presence of either diesel oil or gasoline.
To shed light on the nature of the different types of sessile phenotypes expressed by strain PP1Y (i.e. flocks and structured biofilm) we analyzed the chemical composition (carbohydrates and proteins) of the soluble extracellular products of strain PP1Y produced in different growth conditions and which are presumably responsible of the viscosity of the exhausted growth medium (this chapter, paragraph 3.1.1).
At this stage we wanted to identify in the extracellular medium both protein and carbohydrate "building blocks" that might be first produced by the microbial strain in the extracellular medium and then organized in what we observe as a more complex and structured biofilm.
As the sessile phenotypes of PP1Y are evident when the microorganism is grown either in water/oil biphasic systems or in minimal media, we performed the experiments in two different growth conditions: (**i**) we used PPMM minimal medium containing 1% glutamic acid as carbon and energy source, or (**ii**) a biphasic system composed of PPMM minimal medium and a mixture of diesel oil, as carbon and energy source, dissolved in LVP paraffin.
The chemical analysis of the aqueous phases of these two systems and of the oil phase of the biphasic system was performed as described in the Materials and Methods section. Strain PP1Y was allowed to grow for 48 hours to maximize the amount of extracellular material produced (data not shown).

- **Carbohydrate analysis of the extracellular soluble products of strain PP1Y.**

For this analysis cells were harvested at 4°C and the supernatants were lyophilized and analyzed using the procedure of the methyl-glycol-acetylated (MGA) extraction (Material and Methods and [**85**]). The resulting samples were analyzed by GC-MS, in collaboration with Prof. A. Molinaro at the Department of Organic Chemistry and Biochemistry, University Federico II of Naples. The qualitative analysis highlighted the presence of pentose (still undefined) and hexose sugars (mannose, galactose, glucose), of amino-sugars (like N-acetylglucosamine and N-acetylmuramic acid) and of monounsaturated fatty acids (mainly C18:1, but also C12:1, C14:1 and C16:1) (**Figure 3-5 A-B**).
Moreover, these molecules were present in the aqueous phase, independently of growth conditions, and in the oil phase of the biphasic system. No significant differences were observed between the different samples (aqueous and oil phases).
Interestingly, these analyses showed that all culture exhausted media always contained a low total carbohydrate level and polysaccharides were never detected.

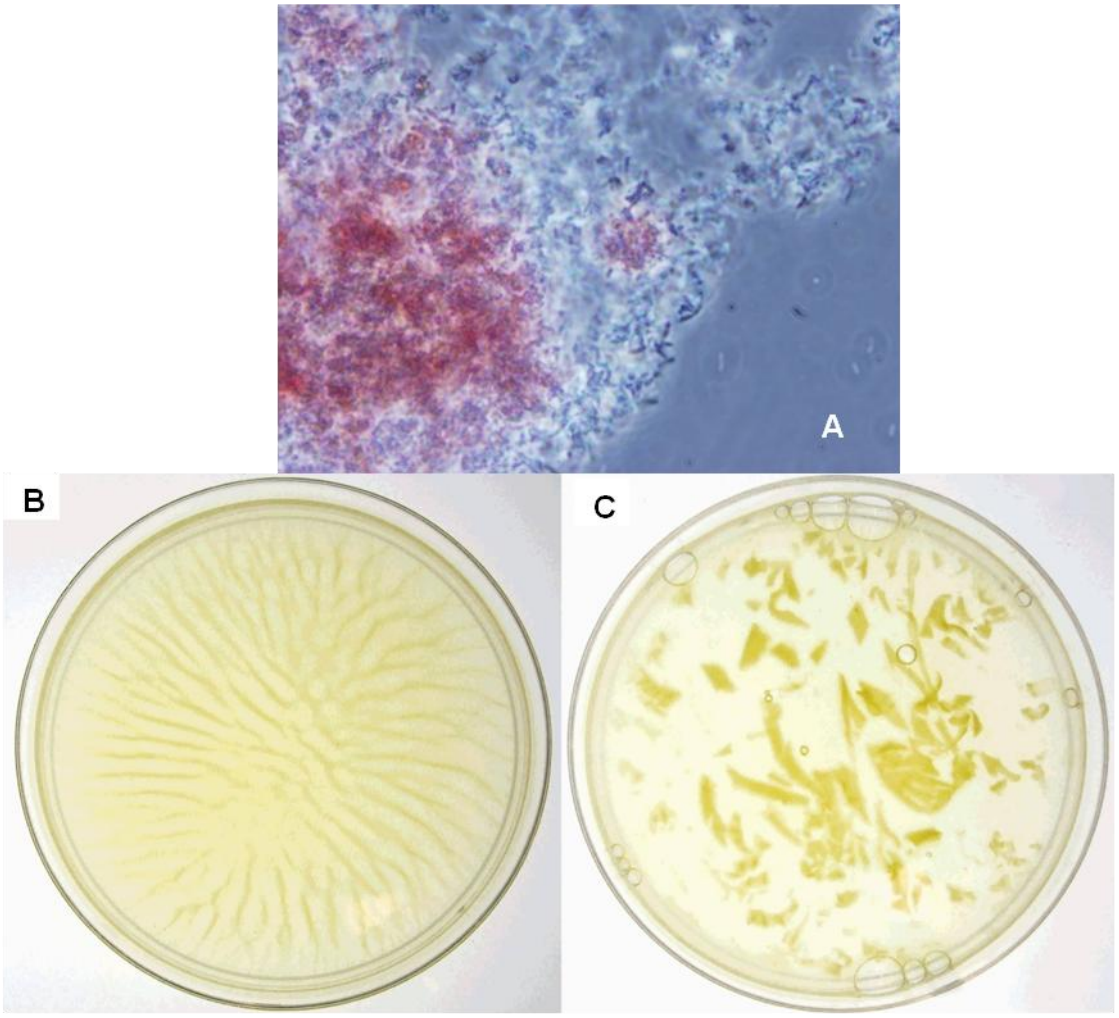


**Figure 3-3: A)** Phase contrast microscope picture showing the border of a flock grown in the presence of Sudan III. **B)** Polystyrene Petri plate containing 10 mL of LB medium in which *N. puteolanum* PP1Y formed in about 24 h a yellow biofilm layer. **C)** PP1Y biofilm detached from the plate by vigorous shaking.

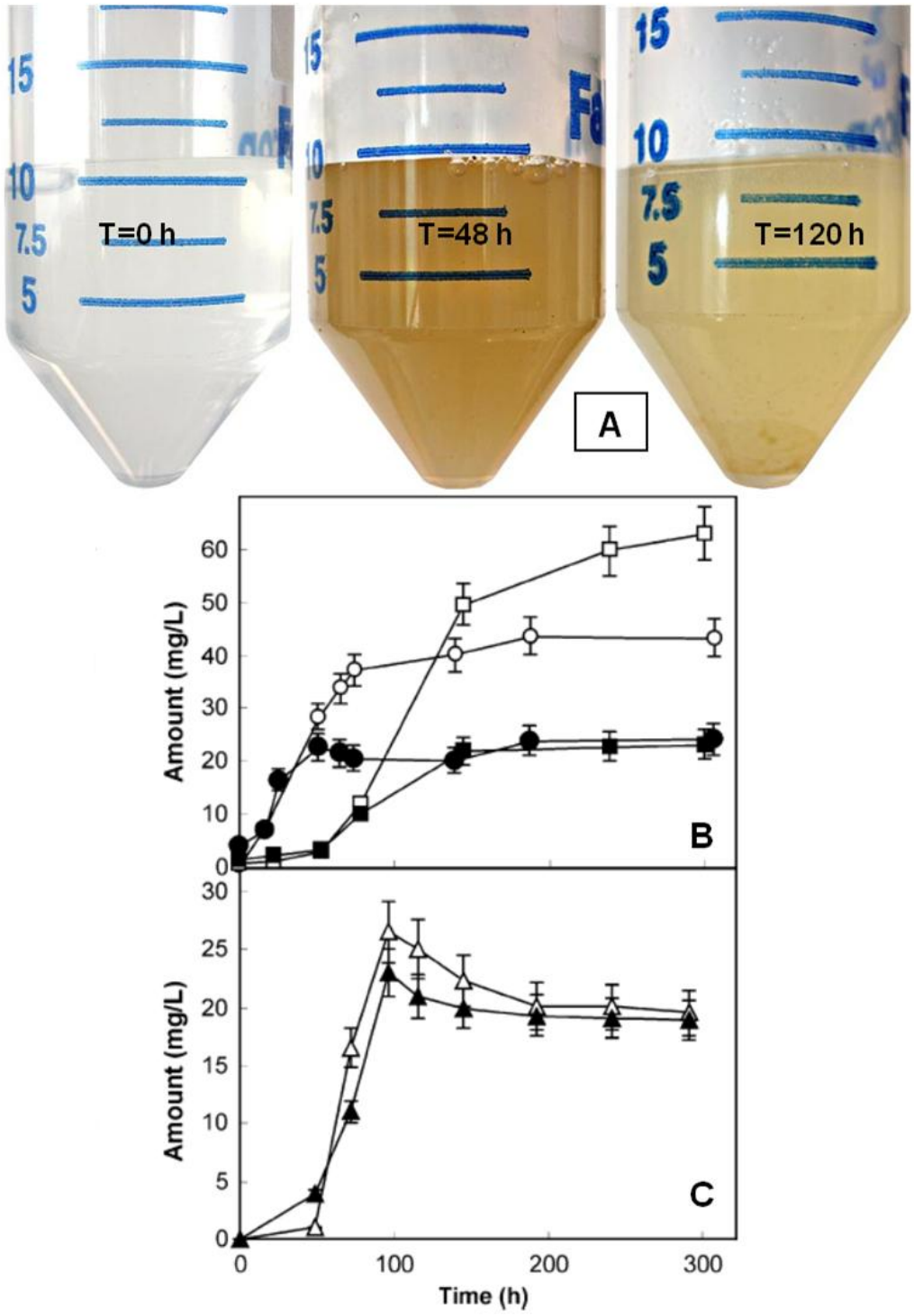


**Figure 3-4: A)** Growth of *Novosphingobium* sp. PP1Y on fuels as the sole carbon and energy source. **B)** Composition of cultures grown on diesel oil (empty circles, total proteins; filled circles, total carbohydrates) or on C14 containing 1% (w/v) *p*-xylene, 2% naphthalene, 1% phenanthrene and 1% pyrene (empty squares, total proteins; filled squares, total carbohydrates). In both cases cultures were grown at 0.04:1 oil/water ratio in PPMM containing 10 mM NaCl. **C)** Composition of a culture grown on gasoline (empty triangles, total proteins; filled triangles, total carbohydrates) at 0.0025:1 gasoline/water ratio in PPMM containing 100 mM NaCl.

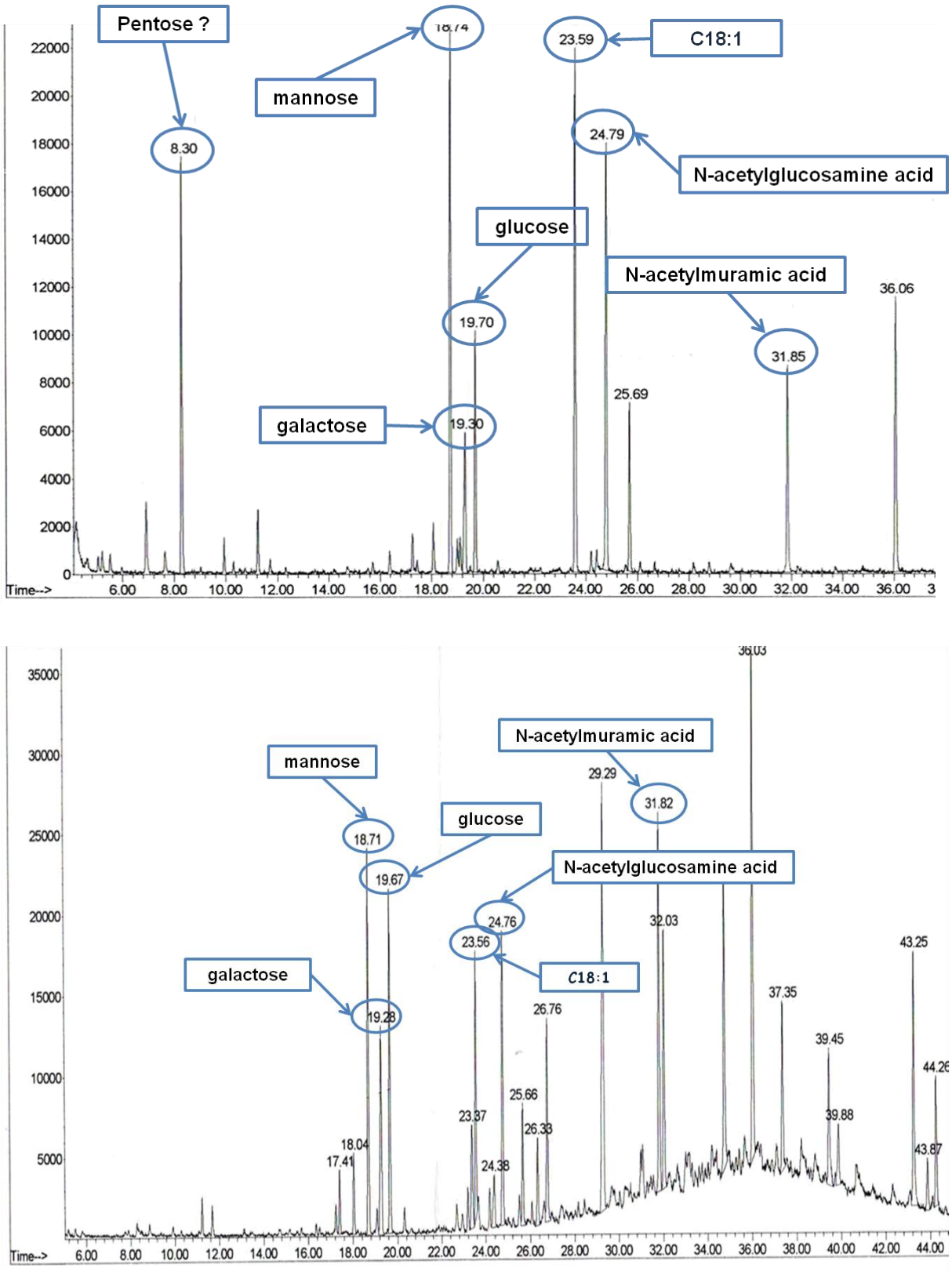


**Figure 3-5**: Chromatographic profile of a sample of exhausted growth medium of strain PP1Y treated with the extraction protocol of methyl-glycosyl-acetylated and analyzed by GC-MS: **A**) Glutamic acid supernatant; **B**) Diesel oil phase.

- **Protein analysis of the extracellular soluble products of strain PP1Y.**

Aliquots of the exhausted extracellular media of PP1Y, obtained as described above, were analyzed also by SDS-PAGE to detect the presence of extracellular proteins (**Figure 3-6 A-B**).
It should be noted that no data could be obtained from the electrophoresis analysis of oil phases due to the presence of organic molecules.
The protein profile shown in **Figure 3-6 A,** lane 3**,** is relative to the exhausted growth medium when PP1Y strain is grown in minimal medium containing 1% glutamic acid as sole carbon and energy source. When compared to a crude cell extract of strain PP1Y (**Figure 3-6 A**, **lane 2**), the protein profile of PP1Y medium shows some differences and the presence of additional bands, which are absent in the crude cell extract. The difference between the two protein profiles shown in **Figure 3-6A** exclude the hypothesis that the proteins present in the extracellular medium (**Lane 3**) might simply origin from PP1Y cells lyses.
The main protein bands visible after the SDS-PAGE of an aliquot of the exhausted growth medium of strain PP1Y (**Figure 3-6 B**, **lane 5**) were identified by Mass Spectrometry in collaboration with Prof. L. Birolo at the Department of Organic Chemistry and Biochemistry, University Federico II of Naples.The identity of the protein bands was assessed by a proteomic approach. Bands were excised from the polyacrylamide gel, digested with trypsin and analyzed by capillary LC-MSMS, as detailed in the Materials and Methods section. Proteins were then identified using the MASCOT software and by comparing the experimental data to the PP1Y complete genome database ([**82**] and this thesis, chapter 3.2). Results are shown in **Table 3.2**.
It is evident from the analysis of these data that most of the bands identified are proteins typical of the bacterial inner and outer membranes like for example TonB membrane receptors [**94**], flagellin-like proteins and the OmpA/MotB complex [**95**].
It is important to underline in this context that (**i**) unexpectedly, very few proteins typical of the intracellular *milieu* were identified in these samples, and (**ii**) insoluble cell debris arising from cell lyses had already been removed prior to analyse these samples, indicating that the extracellular proteins identified by Mass Spectrometry are soluble.

The carbohydrate and protein analysis performed on the extracellular products of PP1Y highlight from one side the presence of membrane sphingolipids, sugars typical of the peptidoglycan and proteins typical of the inner and outer membrane and from the other the unexpected absence of polysaccharides. Interestingly, these data are compatible with the presence, in the exhausted growth medium of strain PP1Y, of what has been recently described in literature as "*outer membrane vesicles"* (OMVs) [**96**].
Recent studies have been reported which analyze the biological role of these OMVs in different classes of bacteria [**97-100**]. These studies suggest that OMVs are used by microorganisms to secrete and, at the same time, to protect insoluble molecules or specific enzymes from environmental harsh conditions.
Moreover, the hypothesis has been advanced that these vesicles play a fundamental role in the survival of bacteria. Their production is in fact usually related to stress conditions, like the accumulation of nutrients in limited bioavailability, biofilm formation [**101**]**,** and infection of host organisms [**102**].

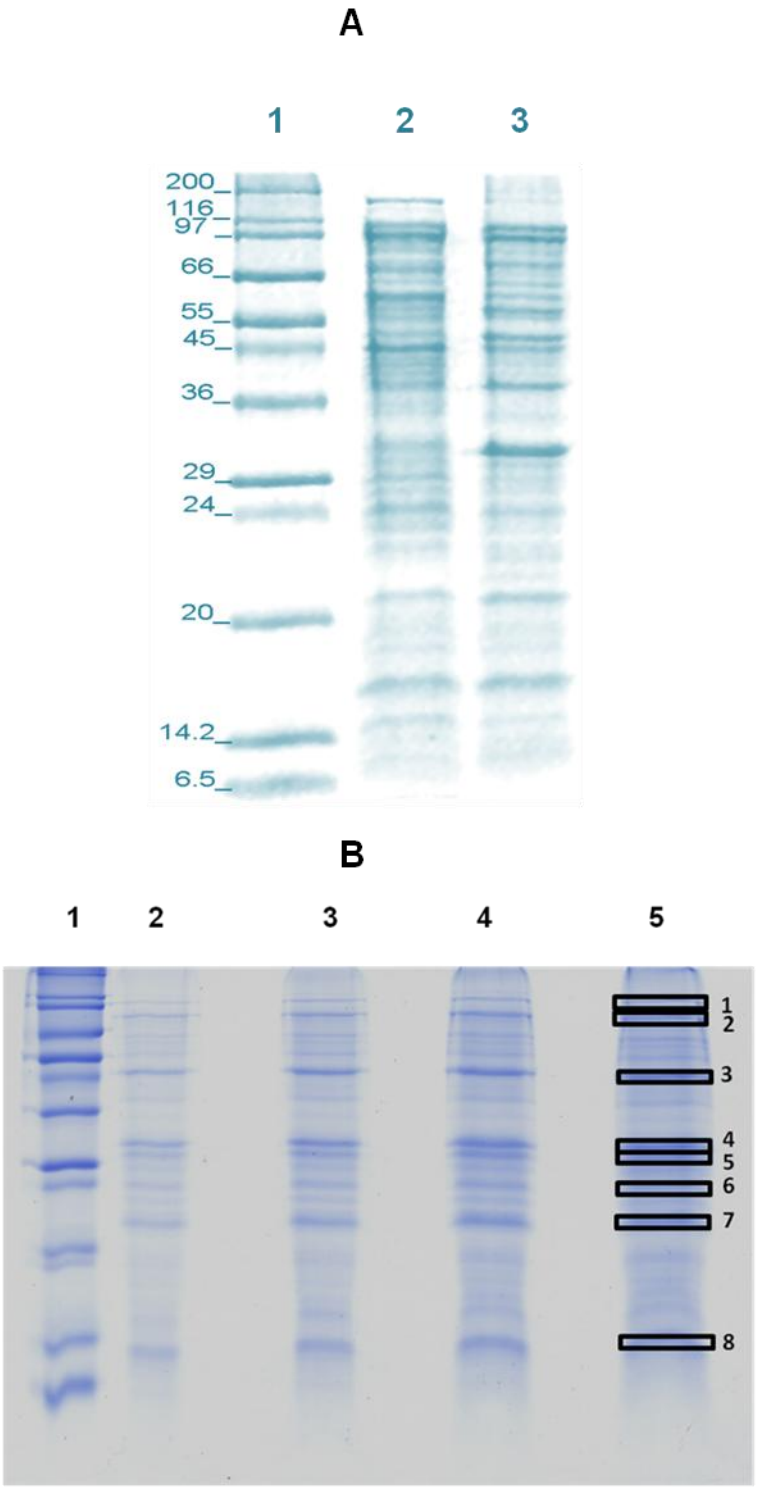


**Figure 3-6**: SDS-PAGE of aliquots of the exhausted growth medium (PPMM containing 1% glutamic acid) of strain PP1Y. **A) Lane 1**, molecular weight standard ranging from 200 kDa to 6.5 kDa; **Lane 2**, approximately 10 μg of PP1Y cell extract; **Lane 3**, 5 mL of cell-free, exhausted growth medium of strain PP1Y precipitated with 50% TCA. **B) Lane 1**,molecular weight standard ranging from 200 kDa to 6.5 kDa; **Lane 2**, **3**, **4** and **5**, increasing quantities (10, 20, 30 and 40 μL respectively) of exhausted growth medium. Highlighted in **Lane 5** are the protein bands excised and subjected to tryptic digestion and LC-MSMS analysis.

| Tabella 3.2 | | | | | |
|---|---|---|---|---|---|
| **Bands** | **Acc. Numb.** | **N° peptides** | **Score** | **Sequence coverage** | **BLAST Search** |
| **1** | A16329-696786660-2700_1 | 62 | 3146 | 80 | TonB-dependentreceptors |
| | A35552-696786660-2628_1 | 16 | 548 | 48 | Surfaceantigen (D15) |
| | B3779-1814974317-2373_1 | 8 | 126 | 12 | TonB-dependentreceptors |
| | A1220-696786660-825_1 | 4 | 120 | 5 | - |
| **2** | B3779-1814974317-2373_1 | 70 | 2960 | 90 | TonB-dependent receptors |
| | A3883-696786660-2172_1 | 44 | 1865 | 70 | TonB-dependent receptors |
| | A3861-696786660-2481_1 | 55 | 1815 | 80 | TonB-dependent receptors |
| | A16329-696786660-2700_1 | 18 | 385 | 30 | TonB-dependent receptors |
| | A34737-696786660-2445_1 | 9 | 184 | 15 | TonB-dependent receptors |
| | A34773-696786660-2274_1 | 6 | 104 | 10 | - |
| **3** | A9368-696786660-1107_1 | 73 | 2233 | 89 | - |
| | A15020-696786660-1359_1 | 32 | 848 | 50 | PpiC-type peptidyl-prolyl cis-transisomerase |
| | A18441-696786660-1191_1 | 17 | 535 | 48 | elongation factor Tu |
| | A8020-696786660-1380_1 | 10 | 223 | 30 | peptidase M28 |
| | A16329-696786660-2700_1 | 9 | 197 | 13 | TonB-dependent receptors |
| | B3779-1814974317-2373_1 | 7 | 179 | 14 | TonB-dependent receptors |
| | A2128-696786660-1563_1 | 10 | 176 | 19 | peptidase S1C, Do (trypsin-like) |
| | A26531-696786660-1362_1 | 5 | 145 | 11 | Pyrrolo-quinoline quinine |
| | C1710--54233742-1968_1 | 3 | 131 | 4 | - |
| | A30032-696786660-1389_1 | 7 | 122 | 13 | translocation protein TolB |
| **4** | A1220-696786660-825_1 | 65 | 3699 | 74 | flagellin-likeprotein |
| | A14257-696786660-828_1 | 37 | 1740 | 72 | flagellin-likeprotein |
| | A15877-696786660-864_1 | 16 | 334 | 47 | Predicted methyltransferase |
| | A34737-696786660-2445_1 | 4 | 130 | 5 | TonB-dependent receptor |
| | A3995-696786660-852_1 | 5 | 103 | 15 | 2-hydroxymuconic semialdehyde hydrolase |
| **5** | A1220-696786660-825_1 | 23 | 1042 | 60 | flagellin-likeprotein |
| | A15877-696786660-864_1 | 24 | 594 | 58 | Predicted methyltransferase |
| | A5388-696786660-753_1 | 41 | 444 | 14 | Putative outer membrane protein |
| | A9368-696786660-1107_1 | 19 | 401 | 38 | - |
| | C1915--54233742-804_1 | 31 | 329 | 10 | putative fimbrial assembly protein |
| **6** | A1220-696786660-825_1 | 17 | 530 | 64 | flagellin-like protein |
| | A28028-696786660-744_1 | 11 | 43 | 447 | - |
| | A6554-696786660-696_1 | 18 | 308 | 34 | - |
| | A22224-696786660-675_1 | 7 | 263 | 35 | OmpA/MotB |
| | A14257-696786660-828_1 | 11 | 219 | 28 | flagellin-like protein |
| | A20212-696786660-771_1 | 11 | 206 | 35 | MotA/TolQ/ExbBproton canne |
| | A35570-696786660-687_1 | 10 | 195 | 49 | outer membrane chaperoneSkp (OmpH) |
| **7** | A1220-696786660-825_1 | 25 | 890 | 68 | flagellin-likeprotein |
| | A6053-696786660-603_1 | 11 | 523 | 44 | - |
| | A11681-696786660-717_1 | 13 | 477 | 36 | protein of unknown function DUF1520 |
| | A14257-696786660-828_1 | 18 | 264 | 7 | flagellin-like protein |
| **8** | A35277-696786660-345_1 | 18 | 325 | 86 | - |
| | A1220-696786660-825_1 | 7 | 183 | 36 | flagellin-likeprotein |
| | A12255-696786660-390_1 | 7 | 163 | 28 | cytochrome c, class I |

### 3.1.3. The flocks produced by strain PP1Y.

Flocks used in the following analysis were produced during a growth of *N. puteolanum* PP1Y in PPMM minimal medium in the presence of 1% glutamic acid as the sole source of carbon and energy. The growth was carried out for 48 h to maximize flocks accumulation in the medium. Flocks were separated from the exhausted medium by centrifugation at 1,000xg at 4°C for 5 minutes, washed using 50 mM phosphate buffer at pH 7.0 and centrifuged under the same conditions to remove residual medium and free cells. Washing procedure was repeated to obtain a clean supernatant (about 3-4 washes).

The pellet was suspended in a solution containing 1% formaldehyde and incubated at 4°Cundercontinuous shaking for 60'. The solution was centrifuged and the pellet washed at least twice to remove excess formaldehyde. At the end of this procedure the pellet was suspended in a 0.2 N NaOH solution and incubated under continuous shaking at 4°C for 3 hours.

The procedure described allows fixing cells embedded in the flocks, thereby preventing lyses and release of cytoplasm material in the culture medium. Moreover, the procedure separates the extracellular matrix from the remaining cell paste [**103**].

The treated flocks were lyophilized, subjected to the MGA extraction protocol and analyzed by GC-MS.

The chromatographic profile obtained after GC-MS and shown in **Figure 3-7** highlights the presence of molecules that have not been identified during the analysis carried out on the soluble extracellular products and previously described. Among other differences, it is worth to note the presence of dideoxy hexoses, rhamnose, glucose, fatty acids (C16 and monounsaturated C18).

These results suggest that although there are many common elements between the extracellular soluble products and the flocks produced by PP1Y, these latter cannot be considered only as macroscopic aggregates of the OMVs produced by strain PP1Y since they show the presence of additional elements that might be important for the organization of a more structured biofilm.

### 3.1.4. Growth on diesel oil, gasoline and artificial diesel and biofilm formation.

*Novosphingobium* sp. PP1Y is able to use diesel oil and gasoline as the sole carbon and energy source (**Figure 3-4**). Diesel oil is well tolerated, at least up to 10:1 (diesel oil/water), whereas gasoline is more toxic. The highest concentration of gasoline allowing growth of the strain was found to be 0.02:1 (gasoline/water). This low value is likely due to the fact that gasoline is a light fraction of petroleum and contains low molecular weight, hydrosoluble hydrocarbons, like hexane and cyclohexane which damage cell membranes and proteins [**22**, **104**]. In fact, gasoline diluted 3–5-fold (v/v) in LVP, a mixture of medium and long chain alkanes that can reduce partitioning of small hydrocarbons to the water phase, is less toxic than pure gasoline and allows obtaining higher growth rates and cell densities. Mixtures of diesel oil and gasoline are also good growth substrates. For example, an 8:2 diesel oil/gasoline mixture allows growth rates higher than diesel oil alone (data not shown).

As it is well known that salts reduce the solubility of hydrocarbons in water and can exert a protective effect on cells [**22**], the ability of strain PP1Y to grow using gasoline as sole carbon and energy source was also studied as a function of the NaCl concentration.

To this purpose different conditions have been tested in which both gasoline:water ratios and NaCl concentrations have been varied in the growth medium.

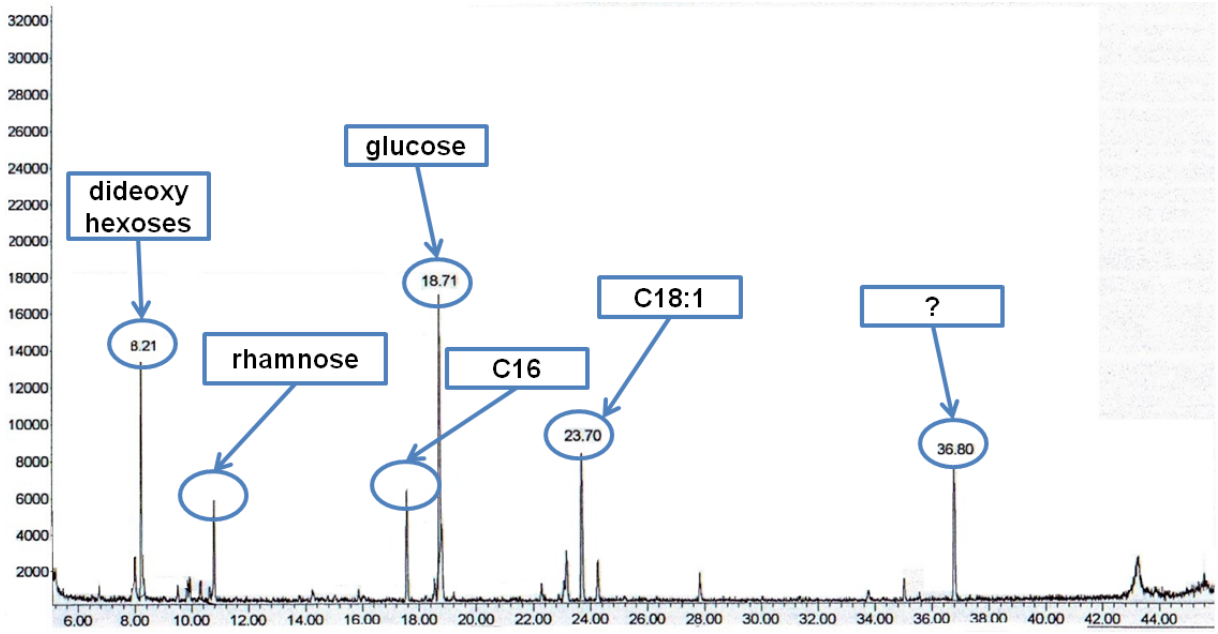


**Figure 3-7**: Chromatographic profile of a sample of flock of exhausted growth medium of strain PP1Y treated with the extraction protocol of methyl-glycosyl-acetylated and analyzed by GC-MS.

As evident from **Figure 3-8,** at a ratio 0.0025:1 gasoline/water, NaCl concentration has a little effect on the amount of total proteins and carbohydrates of the stationary cultures. On the contrary, at a ratio 0.005:1 gasoline/water, the amount of total proteins and carbohydrates at 100 and 250 mM NaCl is significantly higher than the amount obtained at 10 mM NaCl. This observation confirms that specific concentrations of NaCl in the growth medium can compensate the toxic effect of increased gasoline/water ratio.

Interestingly, we have observed that strain US6-1$^T$ is not able to use diesel oil or gasoline as the sole carbon and energy source, at least in the range 0.002:1–0.04:1 fuel/water (v/v).

During growth under orbital shaking in biphasic systems with either water/diesel oil or water/gasoline-paraffin, *Novosphingobium* sp. PP1Y emulsifies the oil phase.

The resulting oil drops have a diameter ≤1 mm. When shaking is stopped, drops stratify at the surface but do not join together to form homogeneous oil phase.

Analysis of the diesel oil drops by phase contrast microscopy reveals that each drop is coated by bacterial cells. The density of cells bound to the surface of the drops increases along with incubation time (**Figure 3-9 A–E**). Under UV light, they have a blue fluorescence typical of PAHs (**Figure 3-9 B–C**). This finding confirms that cell-coated drops contain diesel oil. In aged cultures, “super-aggregates” were detected. These aggregates are formed by two or more bacteria coated drops clearly glued to each other by translucent extracellular material (**Figure 3-9 F**). These findings indicate that *Novosphingobium* sp. PP1Y has developed a complex biofilm which is responsible of a “mechanical” emulsification strategy based on the physical entrapment of small quantities of oil phase. Finally, in static cultures containing a diesel oil phase stratified at the surface of the water phase, strain PP1Y forms a continuous biofilm layer entirely covering the interface (data not shown).

The composition of the aromatic fraction of cell-coated diesel oil and gasoline drops was analyzed by HPLC [**80**, **81**] as a function of time, as described in the Materials and Methods section. Aromatic compounds were identified on the basis of their elution times and of their UV spectra [**80**, **81**].

Incubation with strain PP1Y significantly changes the composition of the aromatic fractions of diesel oil and gasoline (**Figure 3-10** and **3-11 A-B**). The peaks of mono- and bicyclic aromatic compounds show the largest decrease, both in diesel oil and gasoline (**Figure3-10 A-B**). The reduction is more significant on the descending part of the peaks thus suggesting a preferential degradation of the less substituted aromatic rings [**80**, **81**] (**Figure 3-10**). On the other hand, in the regions corresponding to the tricyclic aromatic compounds and (alkyl)pyrenes only specific peaks show a significant reduction (**Figure 3-10**).

In order to measure evaporation rates, a culture containing gasoline was incubated under orbital shaking for 90 h to obtain a stable emulsion of the gasoline phase. The culture was then uncapped and exposed to air for 7 h under static conditions. A cell-free control sample containing gasoline and PPMM minimal medium was also set up in parallel and treated as described above.

**Figure 3-11 C** shows that the emulsion of cell-coated drops loses mono and bicyclic aromatic hydrocarbons much more slowly than gasoline in the abiotic control sample. The difference is particularly relevant in the case of the monocyclic aromatic fraction.

It is worth to underline that neither the concentration of NaCl (10 or 100 mM) nor a different initial density of the cells (0.001 or 0.01 $O.D._{600\ nm}$) influence the results described above.

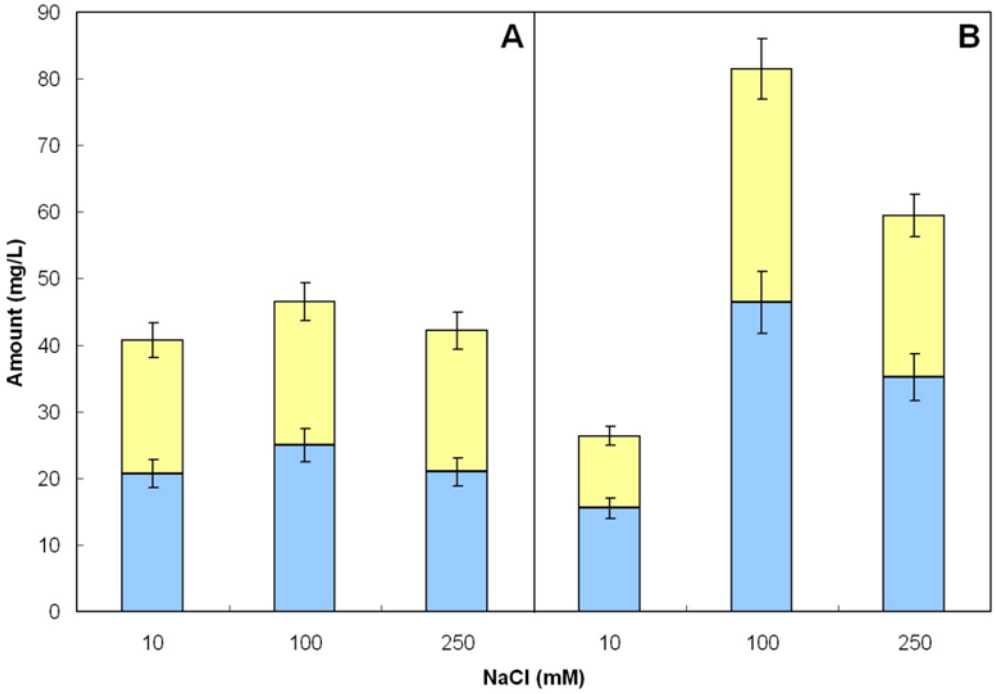


**Figure 3-8:** Growth on gasoline as the sole carbon and energy source at different NaCl concentrations. Cyan bars, total proteins; yellow bars, total carbohydrates. Proteins and carbohydrates were analyzed after 144 h of incubation when all the cultures were in the stationary phase. Cultures were grown at a ratio 0.0025:1 gasoline:water (**A**) or 0.0050:1 gasoline:water (**B**).

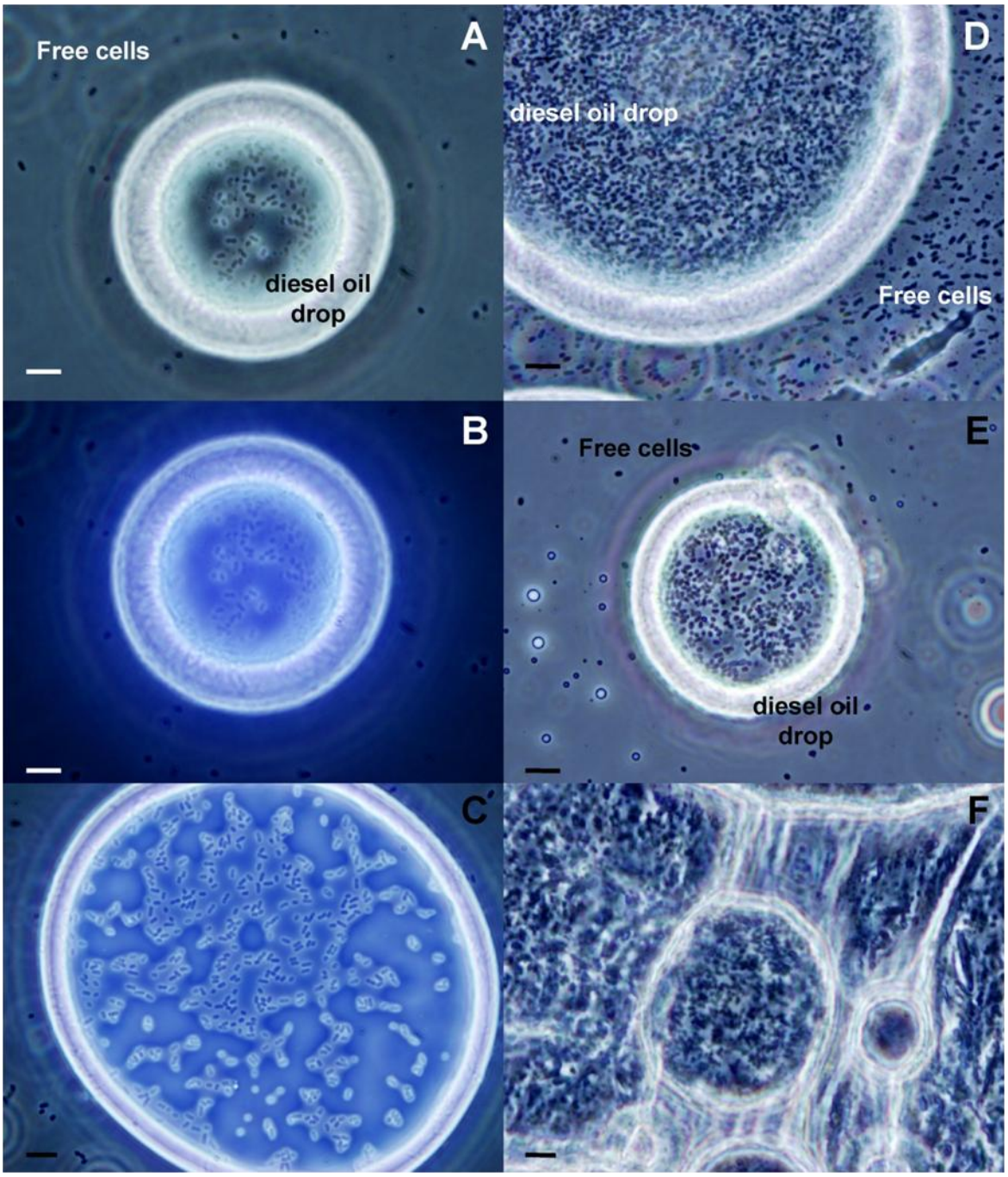


**Figure 3-9:** Phase contrast microscopy analysis of coated diesel oil drops. **A)** and **B)** Coated diesel oil drop after 2 days of incubation observed using visible light only or UV/visible light, respectively. **C)** Coated diesel oil drop after 2 days of incubation observed using UV/visible light. **D)** and **E)** Coated diesel oil drops after 10 days of incubation. **F)** Super-aggregates of coated drops. Bar = 10 µm.

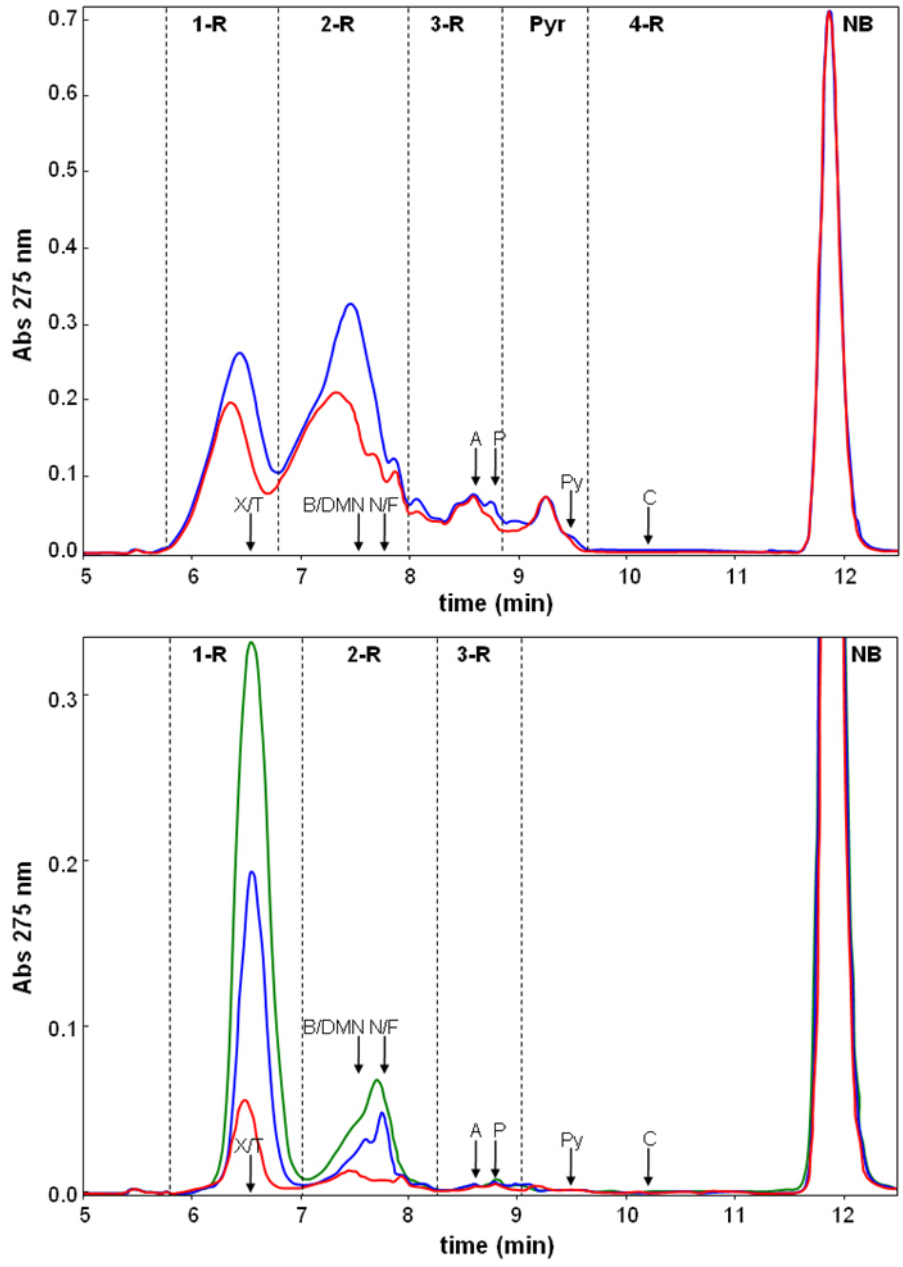


**Figure 3-10:** Normal-phase HPLC analysis of the aromatic fraction of cell-coated fuel drops. **A)** Analysis of the aromatic fraction of cell-coated diesel oil drops (red line) and an untreated diesel oil sample (blue line) incubated 125 h. **B)** Analysis of the aromatic fraction of cell-coated gasoline drops incubated 240 h (red line), of an untreated gasoline sample incubated 240 h (blue line) and of a not incubated, untreated gasoline sample (green line). Black dotted lines define the peaks of aromatic hydrocarbon groups. Abbreviations **1-R**/**4-R** indicate the number of aromatic rings – from one to four, respectively – **Pyr** indicates (alkyl)pyrenes and **NB**, nitrobenzene.

Aromatic hydrocarbon groups were identified on the basis of their elution times and UV spectra. Black arrows indicate elution times of pure standards (**X**, xylenes; **T**, tetrahydronaphthalene; **B**, biphenyl; **DMN**, dimethylnaphthalenes; **N**, naphthalene; **F**, fluorene; **A**, anthracene; **P**, phenanthrene; **Py**, pyrene; **C**, chrysene).

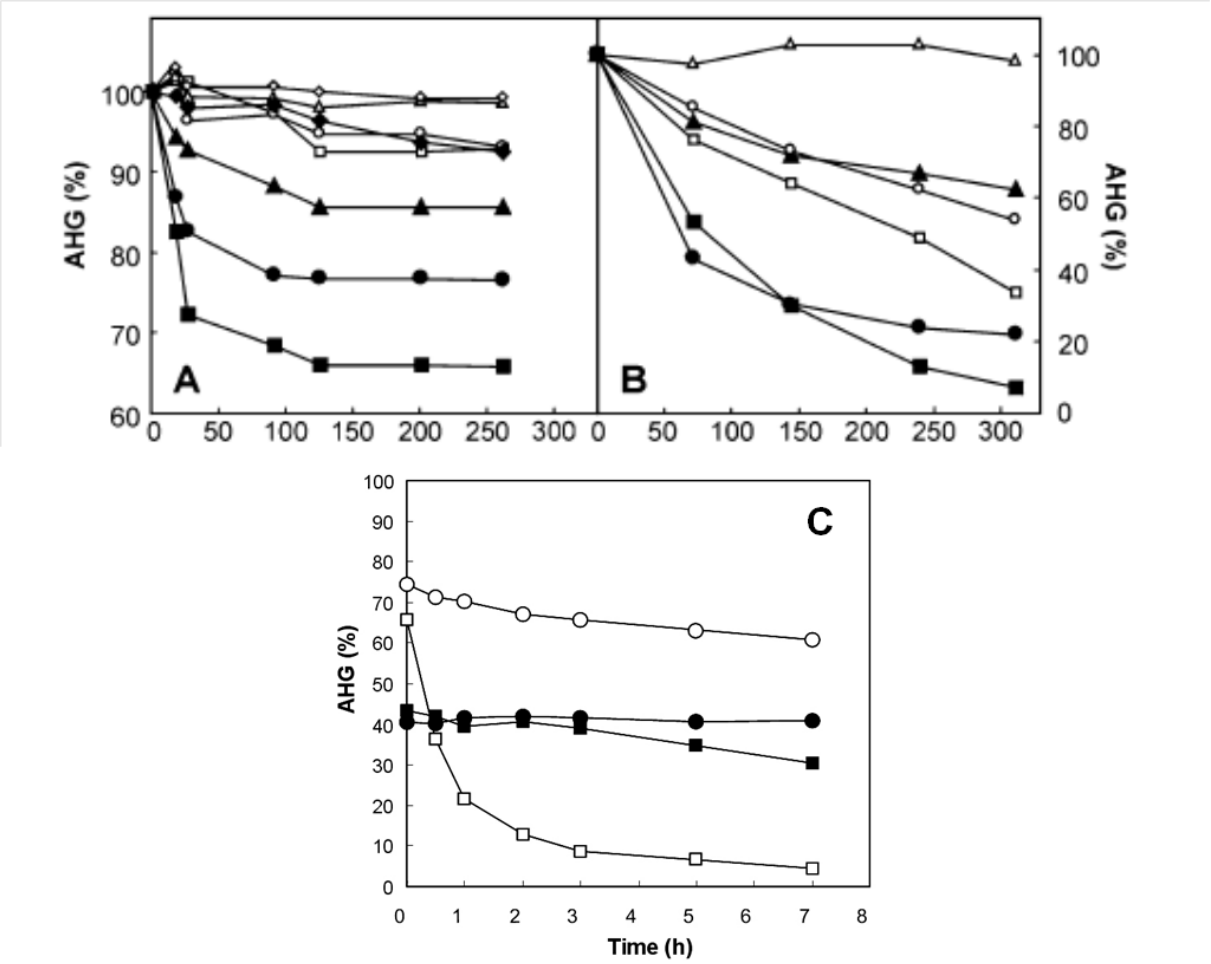


**Figure 3-11: A-B)** Removal of hydrocarbons containing one (filled squares), two (filled circles), or three aromatic rings (filled triangles) and of (alkyl)pyrenes (filled diamonds) from diesel oil and gasoline, respectively. AHG, aromatic hydrocarbon group. The cultures with an initial optical density of 0.01 $O.D._{600\ nm}$ were incubated in PPMM containing 100 mM NaCl. **C)** Evaporation of aromatic hydrocarbons from cell-coated gasoline drops and untreated gasoline. Filled and empty squares, relative concentration of hydrocarbons containing one aromatic ring in cell-coated gasoline drops and untreated gasoline, respectively; filled and empty circles, relative concentration of hydrocarbons containing two aromatic ring in cell-coated gasoline drops and untreated gasoline, respectively. AHG, aromatic hydrocarbon group. Relative error was always lower than 10%. Error bars were omitted for clarity.

These results suggest that strain PP1Y might have evolved mechanisms to reduce the loss of the most volatile aromatic hydrocarbons from the oil phases, which, in turn, are also the optimal growth substrates for this microbial strain.
Gasoline and diesel oil contain hundreds of aromatic and saturated hydrocarbons. On the other hand, our experiments aimed at identifying single molecules which can be used by the microorganism as the sole carbon and energy source have been carried out only on a limited number of compounds [**76**]. Moreover, the results collected so far indicate that the growth of strain PP1Y (morphology of the cells, resistance to salts/temperature/pH, etc.) depends on the concentration and type of its carbon and energy source (diesel oil or gasoline). We thus tried to understand if there were individual diesel oil/gasoline components which could be responsible for the different microorganism growth behavior we had observed.
When we tested single aromatic compounds as growth substrates, we always observed that they were able also to produce the emulsification of the oil phase. However, the analysis of the emulsion by phase contrast microscopy (data not shown) indicated that no aromatic compound, when used alone, was able to reproduce the features of the diesel oil emulsions, which contain oil drops homogeneously coated by biofilm and super-aggregates of coated drops.
Two different behaviors were instead observed. The majority of mono-, bi- and tricyclic aromatic hydrocarbons yielded emulsions containing large oil drops covered by few cells (data not shown) and no super-aggregates. Moreover, these incompletely coated paraffin drops, differently from coated diesel oil drops, showed coalescence when compressed between glass slides, thus suggesting that they were scarcely covered by extracellular material.
Nonetheless, it is worth to underline that some aromatic hydrocarbons like propylbenzene, isopropylbenzene, tetrahydronaphthalene, acenaphthene, pyrene, fluoranthene and heterocyclic aromatic compounds induce the production of a mucilaginous emulsion containing large coated oil drops (diameter 2 to 3 mm) and thin transparent layers of extracellular matrix (from less than 1 to 3 to 4 mm), from now on indicated as “sheets”. Analysis of the sheets by phase contrast microscopy shows that they are composed of a translucent material containing few isolated cells and some short chains of cells (**Figure 3-12**)**.** These findings suggest that these aromatic compounds may induce the production of the extracellular matrix.
Using *a trial and error* procedure, we were able to reproduce the features observed in diesel oil grown cultures only using as growth substrate a mixture of C14 (or LVP) dissolved aromatic hydrocarbons containing a single representative of each class of (poly)cyclic aromatic hydrocarbons (mono-, bi-, tri- and tetra-cyclic) contained in diesel oil. For example, an optimized mixture of C14 containing 1% *p*-xylene (an alkylbenzene), 2% naphthalene (a bicyclic aromatic hydrocarbon), 1% phenanthrene (tricyclic) and 1% pyrene (tetracyclic), in spite of its simple composition, induces a behavior very similar to that shown by PP1Y cultures grown in diesel oil.
We observed that this mixture, from now on indicated as “artificial diesel”, provides homogeneously coated oil drops very similar to diesel oil coated drops. In addition, the protein and carbohydrate content of cultures grown on diesel oil and on the “artificial diesel” are very similar (data not shown).
We also varied the composition of the “artificial diesel” and the resulting coated oil drops were analyzed to point out differences, if any, which could depend on the composition of the “artificial diesel”.

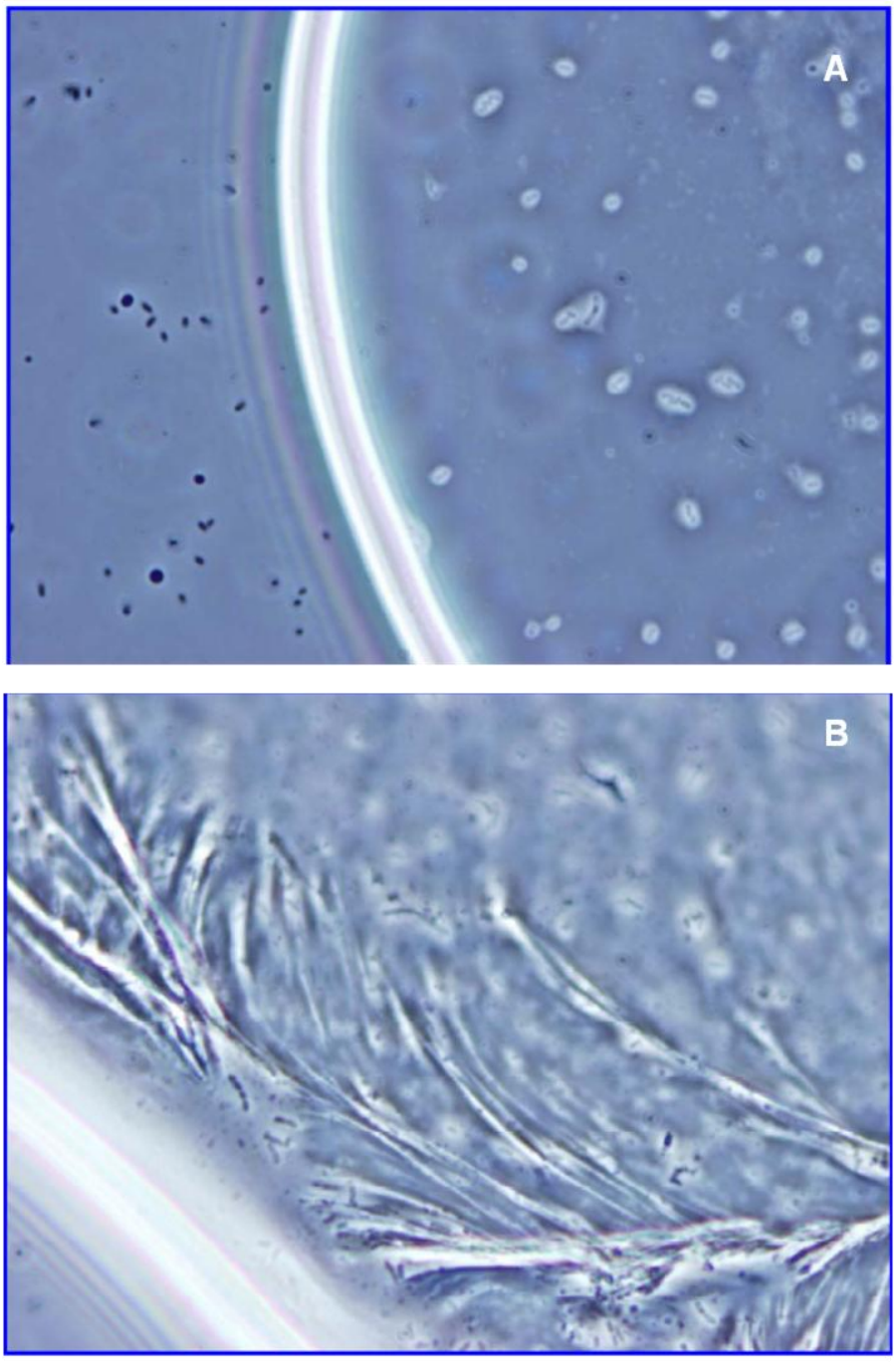


**Figure 3-12: Figure 3-12:** Phase contrast microscopy analysis of cultures grown using C14-dissolved *p*-xylene or pyrene. **A)** Coated *p*-xylene containing-C14 drop. **B)** Refractive cell-containing sheet formed by strain PP1Y during growth on pyrene containing-C14 drops.

We determined that mixtures devoid, or with lower concentrations of pyrene, yielded oil drops with an incomplete coating and fewer cells attached on the surface (**Figure 3-13 A-B**).
Interestingly, higher concentrations of pyrene (1.6%) yielded drops with an irregular coating containing translucent regions, presumably composed of extracellular organic material, where cells were absent (**Figure 3-13 D**).
Substituting naphthalene with 2,6-dimethylnaphthalene reduced the concentration of pyrene necessary to obtain the diesel like coating from 1% to 0.2–0.4% (**Figure 3-13 E-G**).Cultures grown on 2,6-dimethylnaphthalene and 2% pyrene showed instead only amorphous aggregates (**Figure 3-13 H**).
We observed also that pyrene can be substituted by other heterocyclic compounds. For example, a mixture containing 1% *p*-xylene, 2% naphthalene, 1% phenanthrene and 0.4% dibenzothiophene (this latter as a substitute of pyrene) in C14 yields homogeneously coated oil drops (**Figure 3-14 A-B**). As it happens for pyrene, higher concentrations of dibenzothiophene induce the formation of several super-aggregates of coated drops with abundant extracellular material between drops (**Figure 3-14 C-F**). On the contrary, using tetrahydronaphthalene instead of pyrene we observed a marked decrease of the extracellular matrix production.
In fact, a mixture containing 1% *p*-xylene, 2% naphthalene, 1% phenanthrene and 2% tetrahydronaphthalene is emulsified to a heterogeneous mixture of completely (**Figure 3-14 G**) and partially coated drops (**Figure 3-14 H**).
In conclusion, the experiments described above and the analysis of the different culture characteristics (i.e. phenotype) of the coated oil drops show that the colonization of the oil drop surface by *N. puteolanum* PP1Y depends on the specific composition of the aromatic fraction of the oil drops.
The results obtained by using “artificial diesel” allow to identify, among its components, some aromatic molecules that influence more than others the phenotype of the biofilm produced by strain PP1Y around the coated oil drops.

## 3.2. Genome analysis of *Novosphingobium puteolanum* PP1Y.

As previously described [**76**], strain PP1Y shows a very peculiar adaptation to grow on complex mixtures of mono and polycyclic hydrocarbons dissolved in oil phases.
This includes the ability to stabilize oil in water emulsions, to form a regular biofilm layer on the oil drops and to use several aromatic hydrocarbons as sources of carbon and energy even when present in complex mixtures.
Recently the genome of *Novosphingobium puteolanum* PP1Y has been completely sequenced [**84**]. The PP1Y genome is comprised of a single 3.9 Mbp circular chromosome and of 3 plasmids, one megaplasmid (Mpl; 1.16 Mbp), one large plasmid (Lpl; 0.19 Mbp), and one small plasmid (Spl; 0.05 Mbp).
In this chapter a preliminary genome analysis will be described. This work has been done in collaboration with Dr. E. Notomista at the Department of Structural and Functional Biology (University Federico II of Naples, Italy), Prof. G. Paolella and Prof. F. Salvatore (CEINGE- Advanced Biotechnology, Naples, Italy and Department of Biochemistry and Medical Biotechnologies, University of Naples Federico II, Naples, Italy). The analysis of the genome of the strain revealed several genomic features which shed light on most of the unusual phenotypic traits described in the previous chapter.

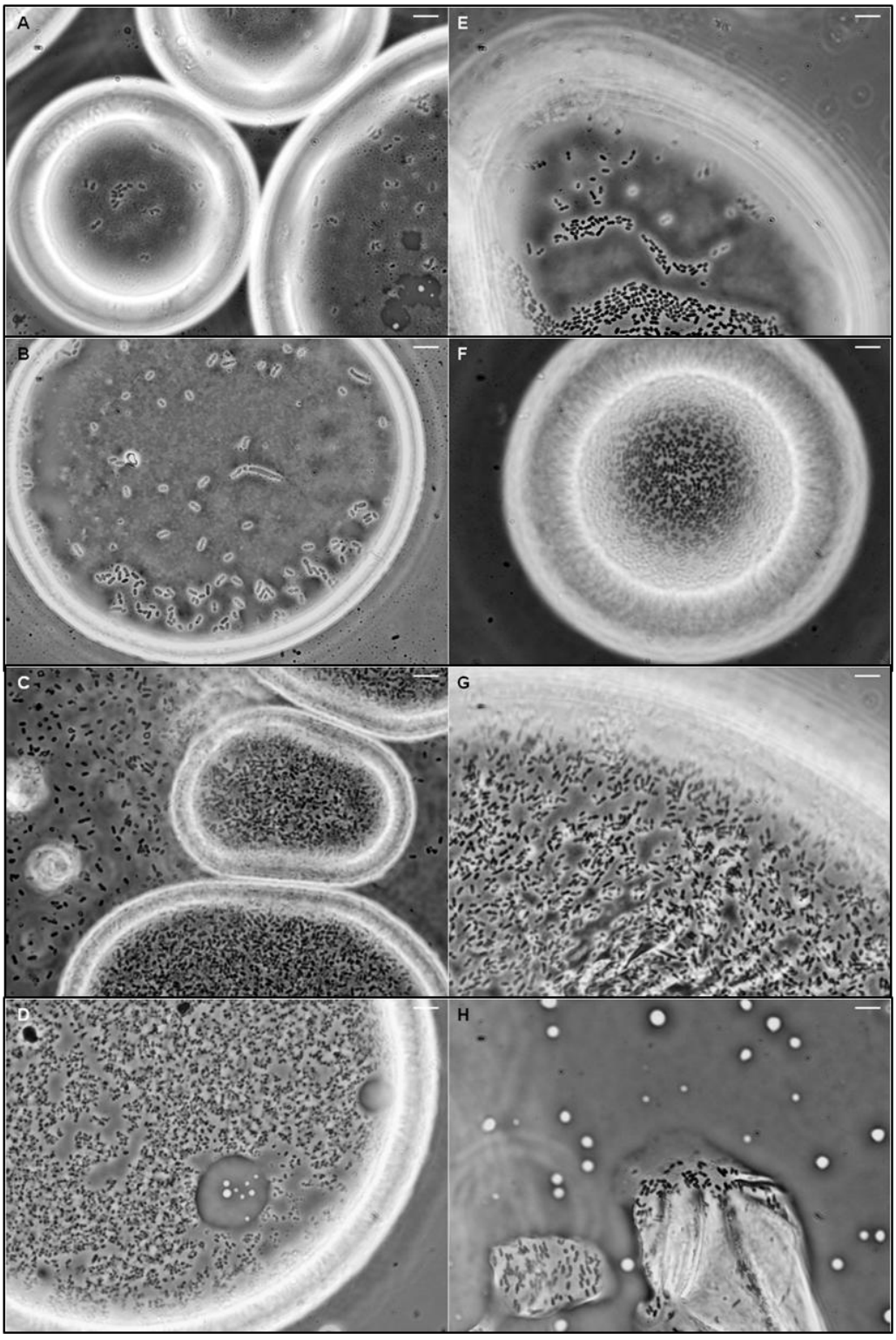


**Figure 3-13:** Phase contrast microscopy analysis of the emulsions produced by cultures grown using C14-dissolved aromatic hydrocarbon mixtures. **A-D)** Coated drops of C14 containing 1% *p*-xylene, 2% naphthalene, 1% phenanthrene and 0%, 0.4%, 1%, or 1.6% pyrene, respectively. **E-H)** Coated drops of C14 containing 1% *p*-xylene, 2% 2,6-DMN, 1% phenanthrene and 0%, 0.4%, 1%, or 2% pyrene, respectively. Bar = 10 µm.

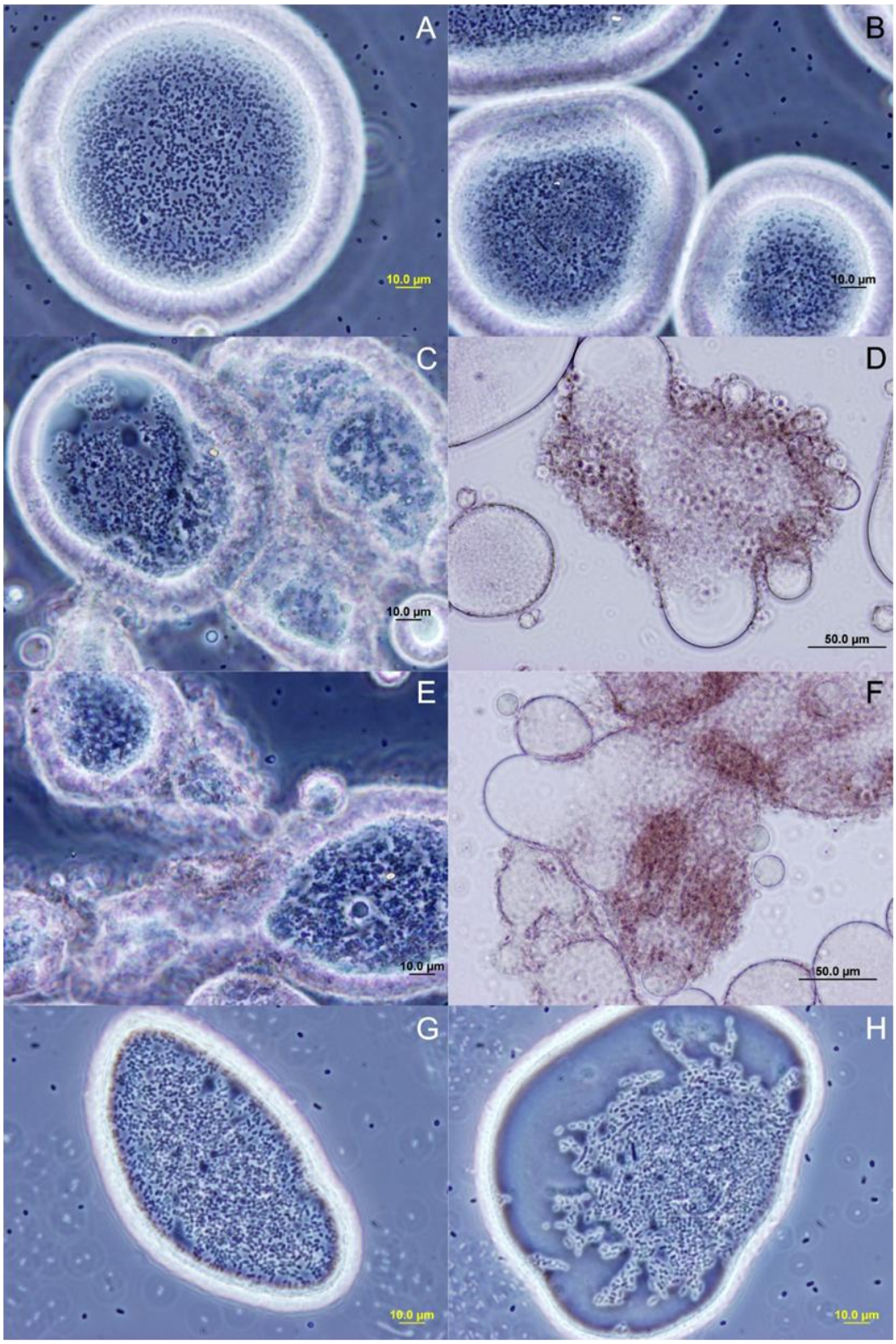


**Figure 3-14:** Phase contrast microscopy analysis of the emulsions produced by cultures grown using C14 containing 1% *p*-xylene, 2% naphthalene, 1% phenanthrene and 0.4% dibenzothiophene (**A**, **B**), 1.2% dibenzothiophene (**C**, **D**), 1.6% dibenzothiophene (**E**, **F**) or 2% tetrahydronaphthalene (**G**, **H**). The reddish color of biofilm in **D** and **F** is due to the accumulation of a red dye during growth on high concentrations of dibenzothiophene.

### 3.2.1. Genetic basis of aromatic compounds degradation in strain PP1Y.

Degradation of aromatic hydrocarbons requires the activation of the aromatic ring. This generally occurs by dihydroxylation reactions catalyzed by monooxygenases or dioxygenases/dehydrogenases which constitute the so-called *upper pathway* (**Figure 3-15**) [**105**]. Ring activation is followed by ring cleavage, a reaction catalyzed by specialized dioxygenases (intra and extradiol dioxygenases) which triggers the so-called *lower pathway* (**Figure 3-16**) [**106**]. In the case of methylated aromatic compounds the initial step can be a monooxygenation reaction of the methyl group followed by its oxidation to carboxylate. These reactions can be catalyzed by soluble dioxygenases or by membrane monooxygenases. The arylcarboxylate eventually undergoes ring dihydroxylation and cleavage. **Figure 3-15** shows the possible degradation *pathways* for toluene.

The analysis of the genome of the strain PP1Y revealed a surprisingly high number of potential sequences coding for both upper and lower pathway enzymes (**Figure 3-17**). Interestingly, no soluble multicomponent monooxygenase related to the well characterized methane monooxygenases (MMO) from *Methylococcus capsulatus* (Bath) [**107**] and toluene *o*-xylene monooxygenase (ToMO) from *Pseudomonas* sp. OX1 [**106**] were found. However, 38 potential gene sequences coding for 34 different aromatic hydroxylating multicomponent dioxygenases were identified. This number is considerably higher with respect to the number of potential dioxygenases coded by the genome of *N. aromaticivorans* F199 (about 20 dioxygenases), a closely related strain frequently used in bioremediation interventions (**Table 3.3**).

Moreover, in this preliminary analysis the following coding sequences were identified (**Table 3-3**):

- **<u>Two potential membrane monooxygenases</u>** (methyl hydroxylating enzymes). These two enzymes show 96% identity (at protein level) between each other thus suggesting a recent event of gene duplication. Interestingly the two monooxygenases from PP1Y show several mutations in the hypothetical substrate binding region thus suggesting that these enzymes could possess different substrate specificity.

**Table 3.3**

| **Proteins Complexes** | ***Novosphingobium sp.* PP1Y** | ***Novosphingobium aromaticivorans F199*** |
|---|---|---|
| ring hydroxylating dioxygenases | 34 | 20 |
| membrane-bound monooxygenases | 2 | 1 |
| Mo-depending MO | 5 | 3 |
| Flavine monooxygenases | >19 | ??? |
| 2-oxoglutarate-dependent oxygenases (taurine-dioxygenase like) | 1 | 0 |
| 2-oxoglutarate-dependent oxygenases | 2 | 1 |
| extradiol ring-cleavage dioxygenases | 7 | 4 |
| protocatechuate 4,5-dioxygenases (extradiol ring-cleavage) | 4 | 5 |

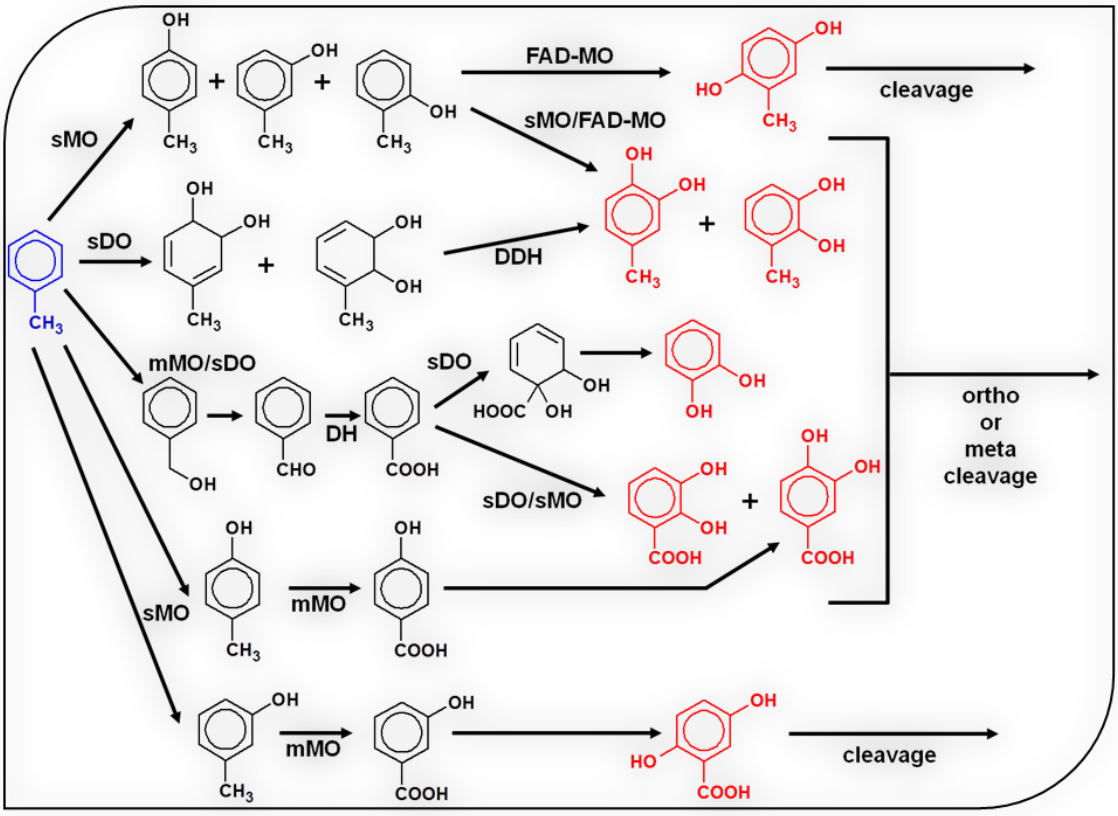


**Figure 3-15:** Metabolic pathways involved in the degradation of toluene (*upper pathway*).

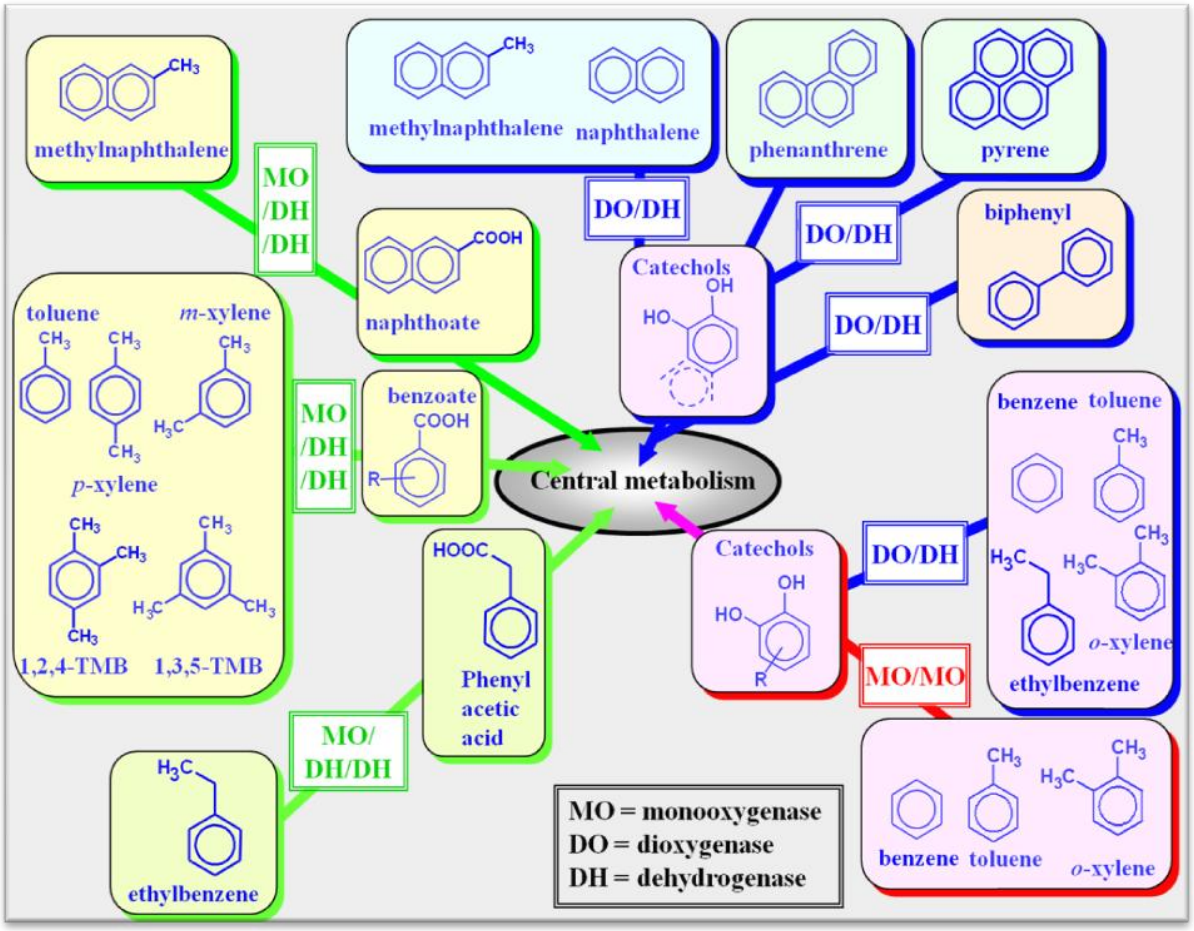


**Figure 3-16:** Metabolic pathways involved in the degradation pathway of aromatic compounds (*lower pathway*).

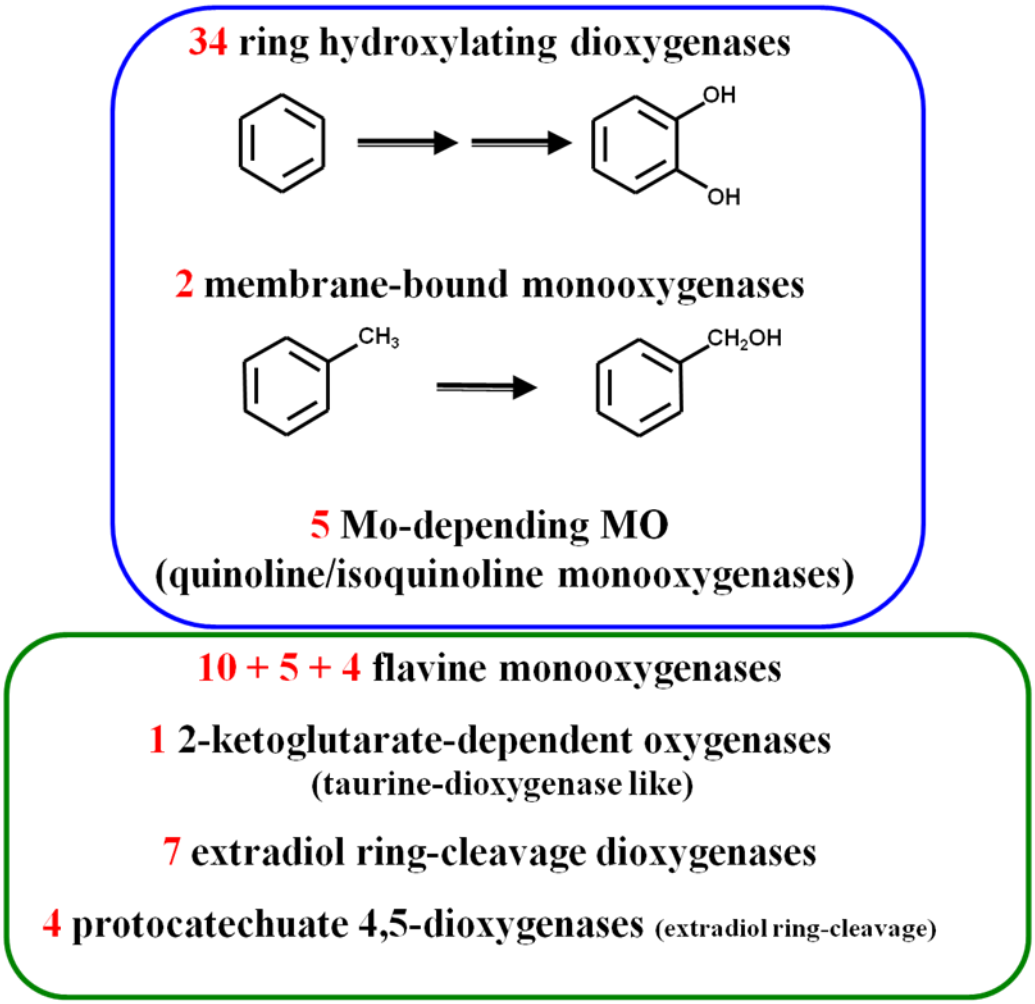


**Figure 3-17:** Potential coding sequences for both *upper* and *lower pathway* enzymes.

- **Seven homomultimeric extradiol ring cleavage dioxygenases and four potential coding sequences for eterodimeric extradiol ring cleavage dioxygenases (ERCD) related to 3,4-dihydroxybenzoate dioxygenases** (**Figure 3-18**)**.** It is worth to underline that the enzymes belonging to the ERCDs family usually cleave catechol rings characterized by the presence of substituents with carboxylate groups like dihydroxybenzoates and dihydroxyphenylacetates.
- **Three potential Baeyer Villiger monooxygenases** (BVMO, ketone monooxygenase)**.** These enzymes catalyze the monooxygenation of ketones to esters which can be successively hydrolyzed to a carboxylic acid and an alcohol thus performing a crucial role in the degradation of several xenobiotic compounds. **Figure 3-19** shows the relationships among the enzymes of strain PP1Y and some well characterized BVMOs. Unfortunately, neither the phylogenetic relationship nor the available BVMO structures allow to hypothesize the substrate specificity of the BVMO from strain PP1Y.
- **Five potential cytochrome P450 monooxygenases.** These enzymes are versatile monooxygenases which can be involved in growth substrate activation, detoxification of toxic compounds and production of secondary metabolites. The majority of the sphingomonads show from 1 to 5 coding sequences for cytochrome P450 monooxygenases with the exception of strains RW1 and F199 whose genomes contain 15 cytochrome P450-MO [**108**, **109**]. Cytochrome P450-MO can catalyze also the demethylation of aromatic metoxy or (di)methylamino groups. Interestingly, coding sequences AT30146 and the adjacent AT30157 code for a cytochrome P450-MO and an arylamine-N-acetyltransferase, respectively. Together these enzymes could catalyze the detoxification of the toxic and mutagenic (di)methyl-arylamines according to the scheme:

**(di)methyl-arylamine➔methyl-arylamine➔arylamine➔acetanilide (N-acetyl-arylamine).**

- **Five coding sequences for hypothetical Molybdopteryn-dependent oxygenases** (MoMO)**.** These latter are peculiar enzymes which use molybdenum containing cofactor as redox center. These enzymes can catalyze diverse and difficult reactions as the monooxygenation of nitrogen containing heterocyclic compounds like xantine, quinoline and isoquinoline, the oxidation of carbon monoxide to $CO_2$ but also the oxidation of several aldehydes (**Figure 3-20**). It is worth noting that the phylogenetic tree of MoMOs (not shown), like that of cytochrome P450-MOs, shows a heterogeneity among sphingomonads likely due to independent horizontal gene transfer events. Unfortunately, the phylogenetic tree and the available structures do not allow to hypothesize the substrate specificity of the PP1Y enzymes. Therefore we tested quinoline, isoquinoline and several methylquinoline as the sole carbon and energy source. Our data indicate that strain PP1Y is not able to use isoquinoline and 3-methylquinoline whereas it is able to grow, albeit slowly, using quinoline, 2-, 4-, 6-, 7- and 8-methylquinoline as the sole carbon and energy sources. Interestingly these quinolines induce a phenotype characterized by an excessive production of extracellular material similar to that induced by pyrene (paragraph 3.1.4) and the heterocyclic compounds dibenzofuran, dibenzothiophene and carbazole [**76**]. The range of methylquinolines which can be used as carbon source allow to hypothesize two distinct initial hydroxylation events as shown in **Figure 3-21**.

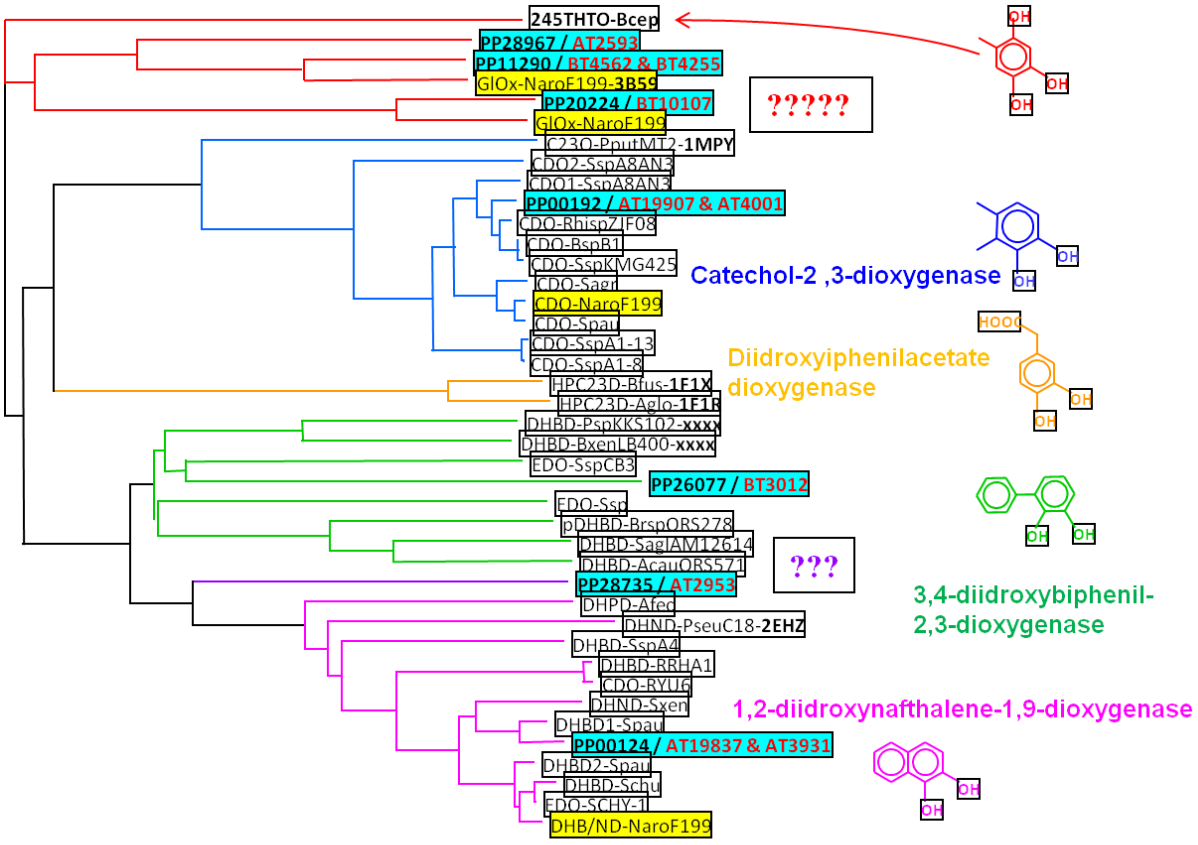


**Figure 3-18:** Seven potential CDSs for homomultimeric extradiol ring cleavage dioxygenases.

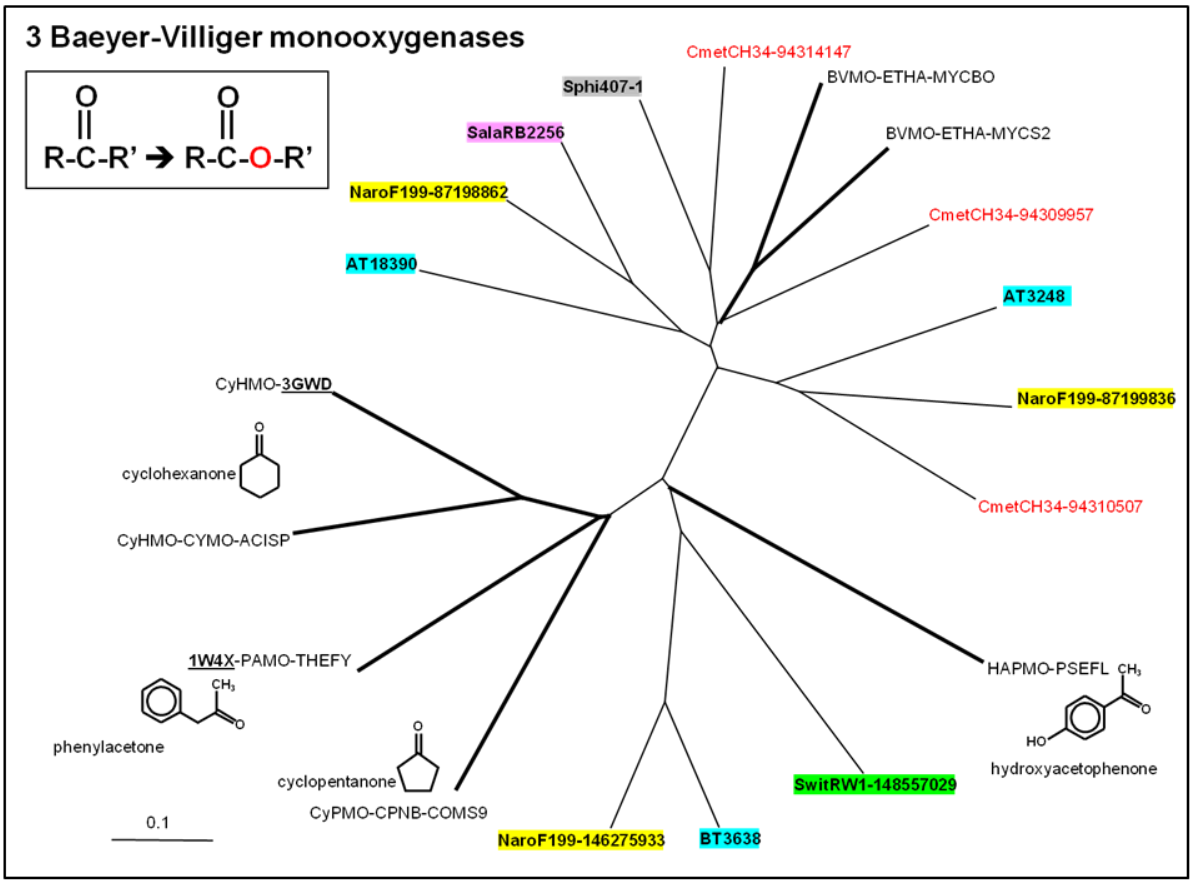


**Figure 3-19:** Three potential coding sequences for Baeyer Villiger monooxygenase (BVMO, ketone monooxygenase) were identified. These enzymes catalyze the monooxygenation of ketones to esters which can be successively hydrolyzed to a carboxylic acid and an alcohol thus performing a crucial role in the degradation of several xenobiotic compounds.

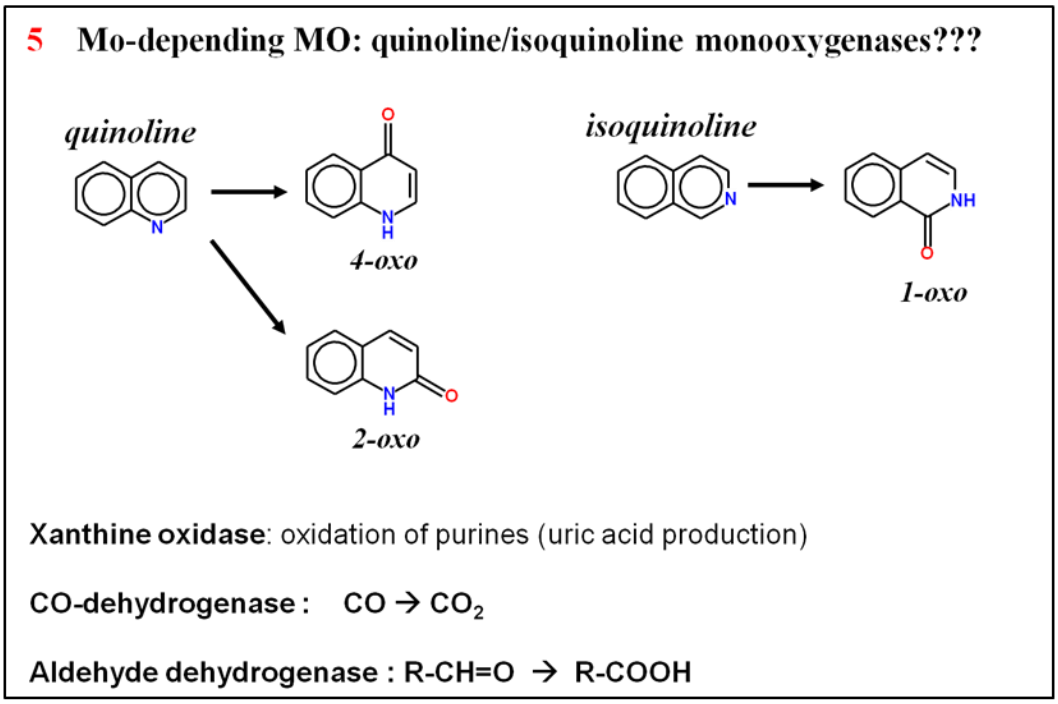


**Figure 3-20:** Molybdopteryn-dependent oxygenases (MoMO) are peculiar enzymes which use a molybdenum containing cofactor as redox center. These enzymes can catalyze diverse and difficult reactions as the monooxygenation of nitrogen containing eterocyclic compounds like xantine, quinoline and isoquinoline, the oxidation of carbon monoxide to $CO_2$ but also the oxidation of several aldehydes;

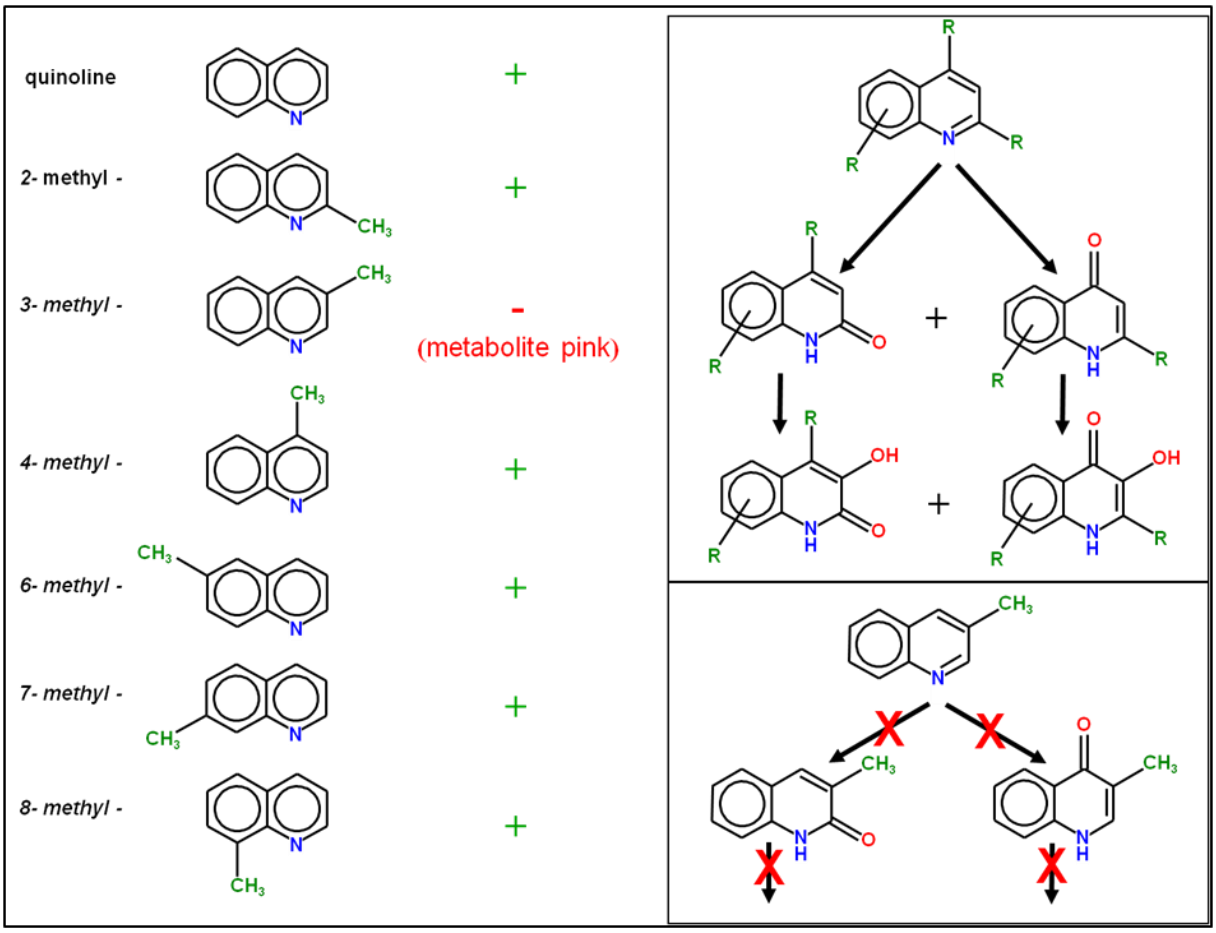


**Figure 3-21:** The range of methylquinolines which can be used as carbon source allow to hypothesize two distinct initial hydroxylation events.

It should be noted that both initial hydroxylation at positions 2 and 4 have been already described for quinoline degrading bacteria (**Figure 3-20**). This hypothetical mechanism would also explain the fact that 3-methylquinoline is not a growth substrate (**Figure 3-21**).

The surprisingly diversified and versatile biochemical machinery of strain PP1Y, revealed by this preliminary analysis, provides a possible explanation for the very large number of aromatic compounds that can be used by this strain as the sole source of carbon and energy.
The frequent presence, among the main classes of oxygenases of strain PP1Y, of strictly related enzymes for which homology modeling reveals differences in the active site pockets (Notomista E. et al, *manuscript in preparation*), suggests a general strategy responsible for the microorganism metabolic peculiarities based both on "gene collecting" – likely by horizontal transfer – and on the diversification of the acquired genes by duplication/divergence.

### 3.2.2. Genetic basis of strain PP1Y resistance to organic solvents.

The ability to grow in close contact with a diesel oil phase is one of the most intriguing PP1Y features described in chapter 3.1. Usually, resistance to organic solvents and/or aromatic compounds is mediated by two mechanisms which protect cell membranes and decrease the intracellular and intra-membrane concentrations of toxic solvents: (**i**) alteration of the cell surface and/or the membrane lipids which reduces the entrance of organic solvent and the perturbation of lipidic bilayer features like fluidity and semipermeability; (**ii**) the production of membrane efflux pumps which actively excrete the toxic molecules.
The production of extracellular polymers will be discussed in the next paragraph (3.2.3). As for the second mechanism the genome of strain PP1Y shows the presence of several coding sequences for the resistance-nodulation-cell division superfamily type (RND-type) Efflux Pumps. These complex membrane pumps are composed by three types of subunits: the inner membrane subunit, the outer membrane subunit and the so called membrane fusion component located into the periplasmic space (**Figure 3-22**). The inner membrane component mediates the translocation of toxic ions or neutral molecules through the inner membrane in a process dependent on the $H^+$ gradient, the membrane fusion and outer membrane components form a sort of tunnel which allows the excretion of toxic compounds outside the cell.
The genome of strain PP1Y contains 8 potential gene sequences coding for inner membrane components and even more sequences for outer and membrane fusion components thus suggesting possible formation of hybrid pumps.
Interestingly, RND-type efflux pumps are not only responsible for microbial tolerance to organic solvents, but also to heavy metals. Moreover, another metal detoxification system is represented by P-type ATPases, ATP dependent efflux pumps known to be involved in the excretion of metal cations [110]. The genome of strain PP1Y contains 8 gene sequences potentially coding for P-type ATPases, a number similar to that of *Cupriavidus metallidurans* CH34 (9 coding sequences), formerly known as *Ralstonia metallidurans* CH34 [111], a gram-negative, non-spore forming bacillus that flourishes in millimolar concentrations of toxic heavy metals [111]. In PP1Y, two hypothetical P-type ATPases belonging to a subfamily specific for copper and two belonging to a subfamily able to excrete several divalent toxic metals like cobalt,

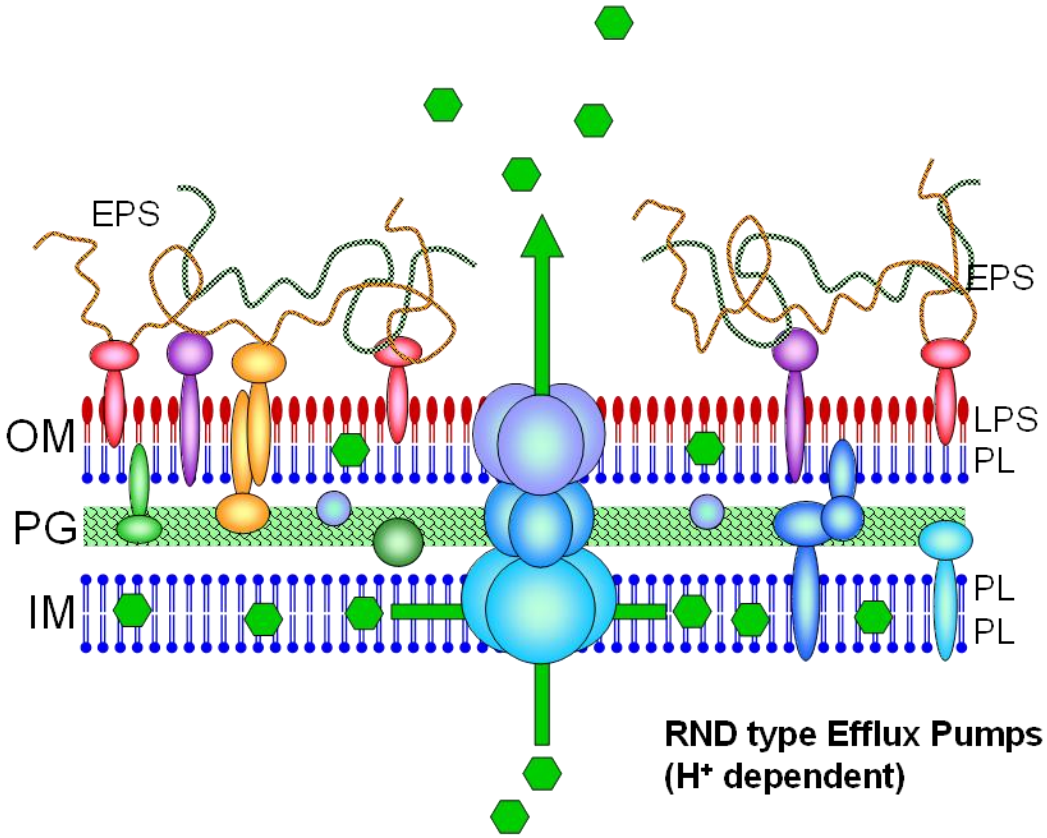


**Figure 3-22:** A schematic representation of a typical RND-type efflux pump. **OM**, outer membrane. **PG**, peptidoglycan. **IM**, inner membrane.

nickel, lead, cadmium, mercury, and zinc were identified. Three P-type ATPases belonging to uncharacterized subfamilies were also found. PP1Y genome shows the presence of an additional P-type ATPase closely related to a subfamilies of $H^+$ transporters found only in plants.
On the basis of these findings we tested the ability of strain PP1Y to grow in the presence of high concentration of metal cations.
For these experiments growth of strain PP1Y was performed in PPMM minimal medium containing 1% glutamic acid, in the presence of millimolar concentrations of selected heavy metals (Materials and Methods section).
The results presented in **Figure 3-23** show the ability of strain PP1Y to tolerate heavy metals concentrations comparable to those reported for *C. metallidurans* CH34 thus suggesting another technological use of this microorganism in environments characterized by heavy metals pollution.

### 3.2.3. Genetic basis of the production of extracellular carbohydrates in strain PP1Y.

As described in chapter 3.1, strain PP1Y shows a very complex “social” behavior and is able to form different types of multicellular amorphous aggregates and ordered biofilm. Almost all the cultures, both in rich and minimal media, contain variable amounts of amorphous flocks and often, when the test tube is made of hydrophobic polymers like polypropylene and polystyrene, biofilm on the tube. When growing on diesel oil the strain forms also biofilm at the surface of diesel oil: in static cultures it colonizes the entire interface, whereas, during orbital shaking it stabilizes the oil/water emulsion by coating oil drops with biofilm.
Several sphingomonads produce soluble acidic polysaccharides known as *sphingans* (examples are gellan, diutan and welan) used for a wide spectrum of industrial applications [**112**]. **Figure 3-24** shows the repeated tetrasaccharidic unit most commonly found in these polysaccharides and the structure of some popular sphingans which differ, essentially, in the nature of the ramifications and/or modifications of the common backbone. The genes necessary for the synthesis of gellan, diutan and sphingans in *Sphingomonas* strain S88 are known [**113**]: they form three very similar gene clusters in strains *Sphingomonas elodea*, *Sphingomonas sp*. ATCC 53159 and *Sphingomonas* ATCC 31554. No similar cluster is present in the genome of strain PP1Y. Even if several coding sequences for sphingan synthesis have distantly related homologs in PP1Y genome (with % identity lower than 30-40%) some essential genes are missing, like, for example, the transferase of glucuronic acid, the polymerase which assembles the tetrasaccharidic units and two of the four genes for the synthesis of dTDP-rhamnose precursor.
Therefore, it can be concluded that strain PP1Y likely does not produce a sphingan-like polysaccharide. This is in agreement with our data on the presence of extracellular carbohydrates, described in chapter 3.1 (paragraph 3.1.2 - 3.1.3).
We should underline, however, that several gene clusters potentially coding for the synthesis of extracellular polysaccharides are distributed among the three larger replicons of PP1Y (the main chromosome, the 1.16 Mbp megaplasmid and the 0.19 Mbp large plasmid). Several biofilm forming strains secrete cellulose as matrix component. The cellulose synthase from *Acetobacter xylinum* is a prototype among the bacterial cellulose synthases [**114**]. These two or three component membrane enzyme synthesizes and, at the same time, secretes and deposits cellulose fibrils outside the cell [**114**]. The 0.19 large plasmid of strain PP1Y and the genome of

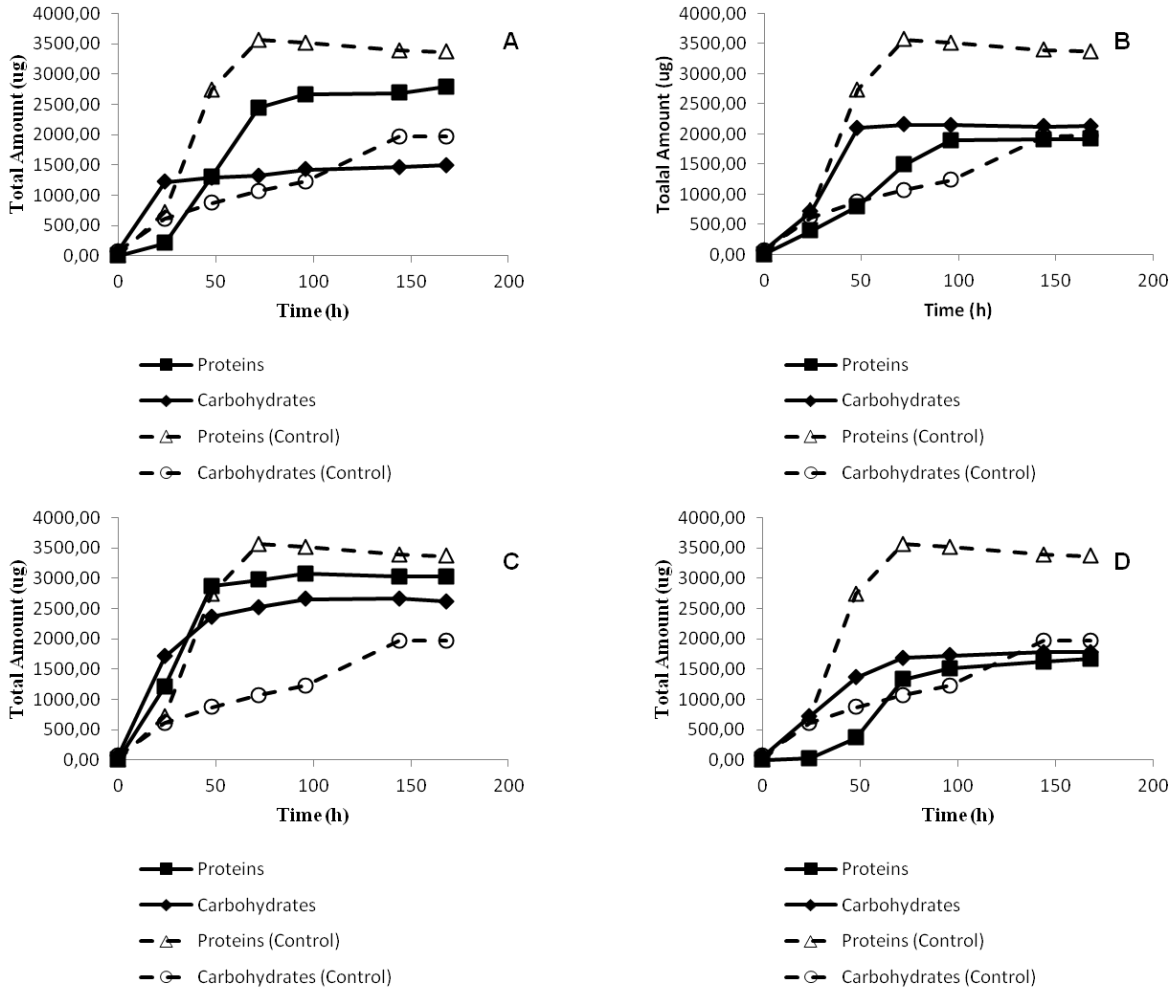


**Figure 3-23:** PP1Y growth curves in the presence of millimolar concentrations of heavy metals. **A)** 2.5 mM $NiCl_2$; **B)** 10 mM $PbCl_2$; **C)** 10 mM $CuCl_2$; **D)** 5 mM $ZnCl_2$;. The control growth presented in all graphs has been performed in 1% glutamic acid.

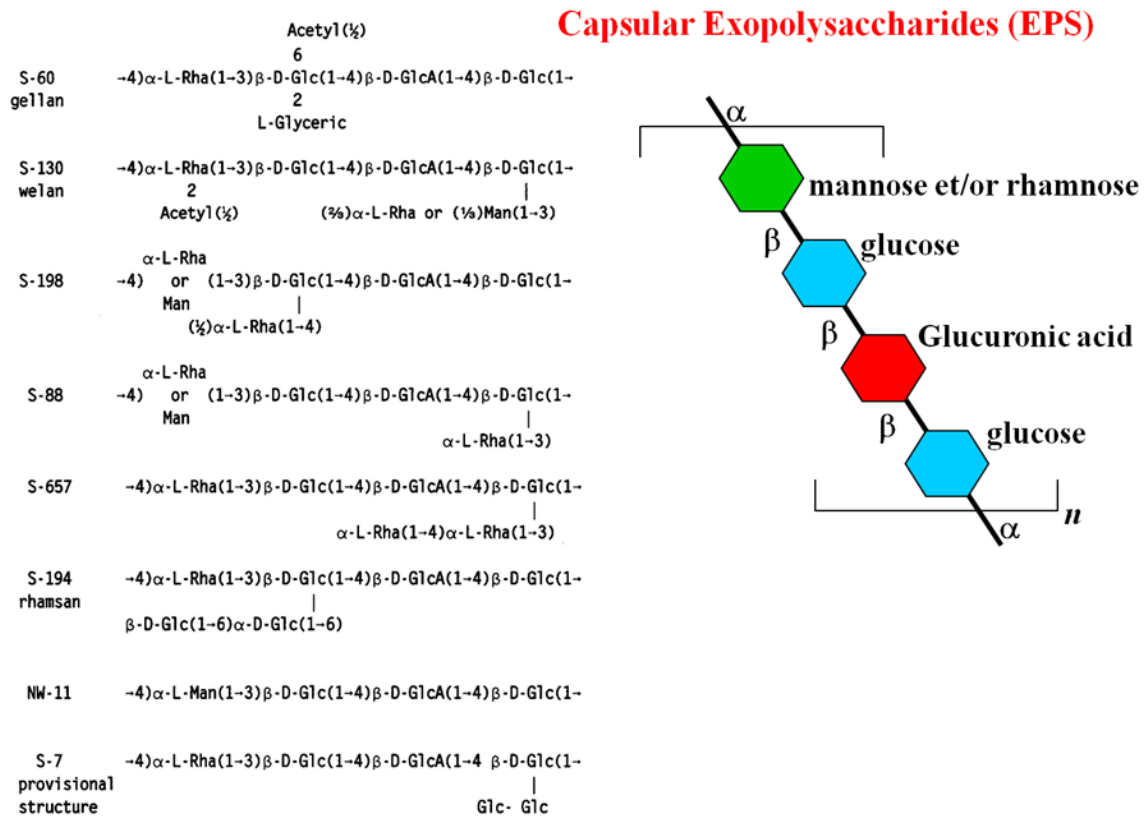


**Figure 3-24:** The structure of some popular sphingans which differ, essentially, in the nature of the ramifications and/or modifications of the common backbone.

strain *Sphingobium japonicum* UT26S share a gene cluster coding for a two subunit type cellulose synthase and a hypothetical cellulase thus suggesting a possible role both in biofilm synthesis and in its remodeling.

## 3.3. The biotechnological potential of *Novosphingobium puteolanum* PP1Y.

### 3.3.1. Biotechnological potential of strain PP1Y for the bioremediation of contaminated soils: a pilot experiment.

Sphingomonads have been studied for their ability to degrade several pollutants, in particular aromatic compounds, and for their planktonic/sessile dimorphism. Both these features are present in strain PP1Y as reported above.
In fact, we have shown that the strain is able to use a wide range of mono and polycyclic aromatic compounds as carbon and energy sources and is tolerant to the presence of high concentrations of heavy metals in the growth medium (chapter 3.2). Its degradative ability is comparable to or greater than the most versatile sphingomonads like *S. paucimobilis.* Moreover, it is worth to remind that strain PP1Y efficiently colonizes several types of hydrophobic surfaces including water/oil interfaces. In addition to these features, this microorganism has a surprisingly high tolerance to diesel oil, which allows its growth in biphasic diesel oil/water cultures containing more oil than water. To the best of our knowledge, this latter behavior has never been reported for a Sphingomonad. As an example, *Sphingomonas sp.* Ant 17, known to use fuels as the sole carbon and energy source, has been reported to grow in 1:1,000 (v/v) fuel/water biphasic systems [**115**].
To evaluate the potential use of strain PP1Y in the bioremediation of environments contaminated with PAH, pilot experiments of bioaugmentation were performed in (**i**) artificially contaminated soils (from now on indicated as artificial microcosms), and (**ii**) polluted soils from natural environments.

### 3.3.2. Removal of Aromatic Hydrocarbons from artificially polluted soils.

For this kind of experiments a commercially available gardening soil was used, as described in the Materials and Methods section. Artificial microcosms were prepared using 20 g of soil to which phenanthrene, used as a model pollutant, was added to a final concentration of 4 mg/g of soil. Phenanthrene was added by dissolving crystals in acetone or in LVP and strain PP1Y was used at a concentration of 0.7 $OD_{600}$/g dry soil; soil was incubated at 25°C. **Figure 3-25 A** shows that phenanthrene was degraded at ca. 50 hours and ca. 150 hours when dissolved in acetone and LVP, respectively.
Interestingly, even though strain PP1Y has been isolated from seawater and its ability to grow in the presence of NaCl suggests that the microorganism has adapted to a marine environment, the comparison of phenanthrene degradation rates in soil and liquid (**Figure 3-25 B**) indicates a higher degradation rate in soil. It can be hypothesized that cells in soil might have access to additional carbon sources other than phenanthrene, and/or that soil texture may function as a protective matrix [**116**, **117**].
The efficiency of the microorganism in removing other aromatic molecules from artificially polluted soils was also studied in the presence of artificial complex mixtures of pollutants.
To this purpose degradation experiments in artificially contaminated soils were performed using mixtures of aromatic compounds containing phenanthrene,

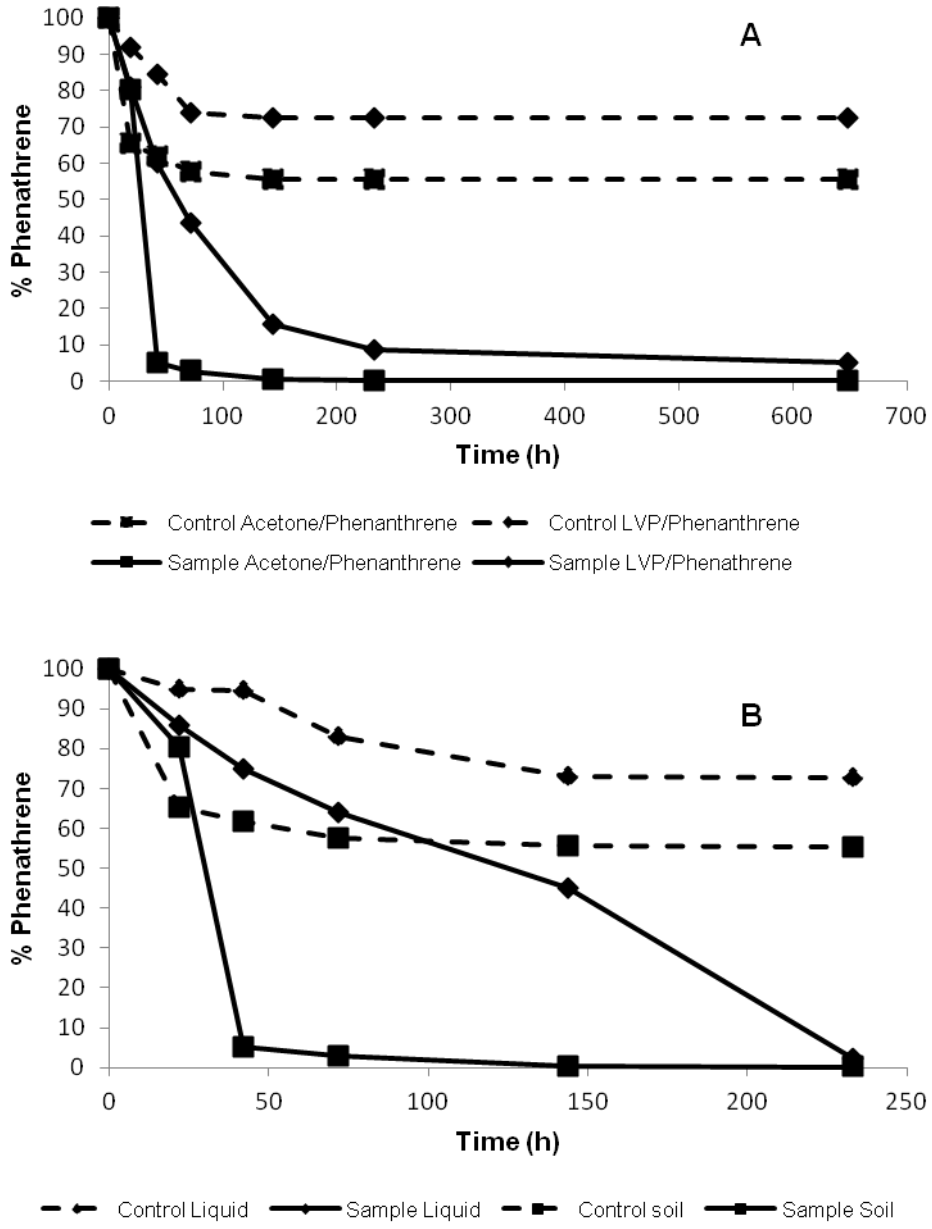


**Figure 3-25: A)** PP1Y-mediated degradation of phenanthrene in artificial microcosms (phenanthrene was added by dissolving crystals in acetone or in LVP at a concentration of 4 mg/g of soil). **B)** Comparison of degradation of phenanthrene in artificial microcosms and in liquid medium.

2,6 dimethylnaphthalene and pyrene, at concentrations of 4, 2 and 2 mg/g of soil, respectively. The compounds were added to microcosms, as described previously, dissolved in acetone and LVP.
In this case (**Figure 3.26**) strain PP1Y has been shown to degrade the components of this complex mixture at rates comparable to those observed for the individual compounds and in any case higher than those observed for the same compounds during growth in the liquid medium (data not shown). More specifically, strain PP1Y proved able to degrade about 50% of phenanthrene and 2,6 dimethylnaphthalene in about 40 hours (150 and 80 hours respectively when growing in liquid medium, data not shown).
In the case of pyrene, the microorganism was able to metabolize approximately 34% of the molecule in 300 hours, whereas less than 20% is degraded in the case of growth in liquid medium.
We tested also the ability of strain PP1Y to remove phenanthrene (final concentration of 4 mg/g of soil) from artificial microcosms in the presence of increasing concentrations of heavy metals (from 0 to 20 mg/g).
As shown in **Figure 3-27** in the case of artificially polluted soils prepared in the presence of phenanthrene and $NiCl_2$, in all cases tested PP1Y was able to metabolize phenanthrene present in the microcosm. It has to be underlined that the degradation potential of the microorganism is a function of the increasing concentration of metal. Increasing the amount of nickel in the soil on one hand increases the time needed from the strain to degrade the aromatic hydrocarbons present, but on the other seems to stimulate a more rapid adaptation of the microorganism itself to drastic environmental conditions. It can be observed, interestingly, that for low concentrations of nickel, in the range of 1-5 mg/g of soil, the bacterium shows in the first 24 hours an increased metabolic activity compared to the growth in a phenanthrene polluted sample without metal (**Figure 3-27)**.

### 3.3.3. Removal of Aromatic Hydrocarbons from natural polluted soils.

To verify that the results described in the preceding paragraphs can be reproduced in real environmental conditions, it was decided to operate targeted sampling in areas with a high rate of urban pollution. Degradation experiments carried out on artificial microcosms were repeated using naturally polluted soils. Soil samples were collected from flower beds on a public site in the city of Naples, in an area adjacent to the harbor of Naples characterized by heavy city traffic and by high concentrations of PAHs and heavy metals (for more details on soil characteristics, see Materials and Methods).
Samples were prepared following the procedure used for the artificially polluted garden soil. Phenanthrene was added to a final concentration of 4 mg/g of soil and dissolved in acetone.
**Figure 3-28**, shows that strain PP1Y is able to completely degrade phenanthrene in a time comparable that measured in the case of artificial soil (about 140 hours). The same experiment was repeated in a control sample in which the polluted soil was not subjected to previous sterilization, to maintain the endogenous flora, and was not inoculated with strain PP1Y. As shown in **Fig. 3-28** an almost complete disappearance of phenanthrene, not exclusively due to evaporation was observed in about 250 hours, suggesting the presence of endogenous phenanthrene-degrading microorganisms in the collected soil.

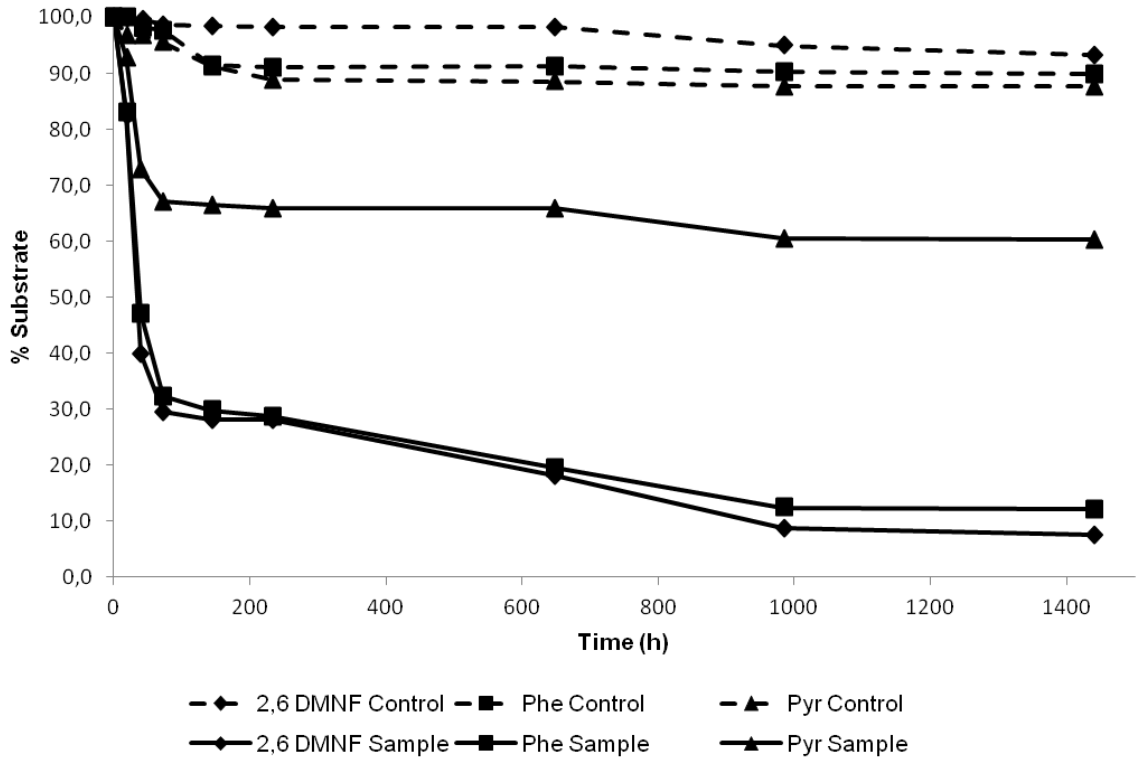


**Figure 3-26:** Degradation curves of an artificial mixture containing 2,6 dimethylnaphthalene, phenanthrene and pyrene (respectively 4, 2 and 2 mg/g of soil) in an artificially contaminated soil.

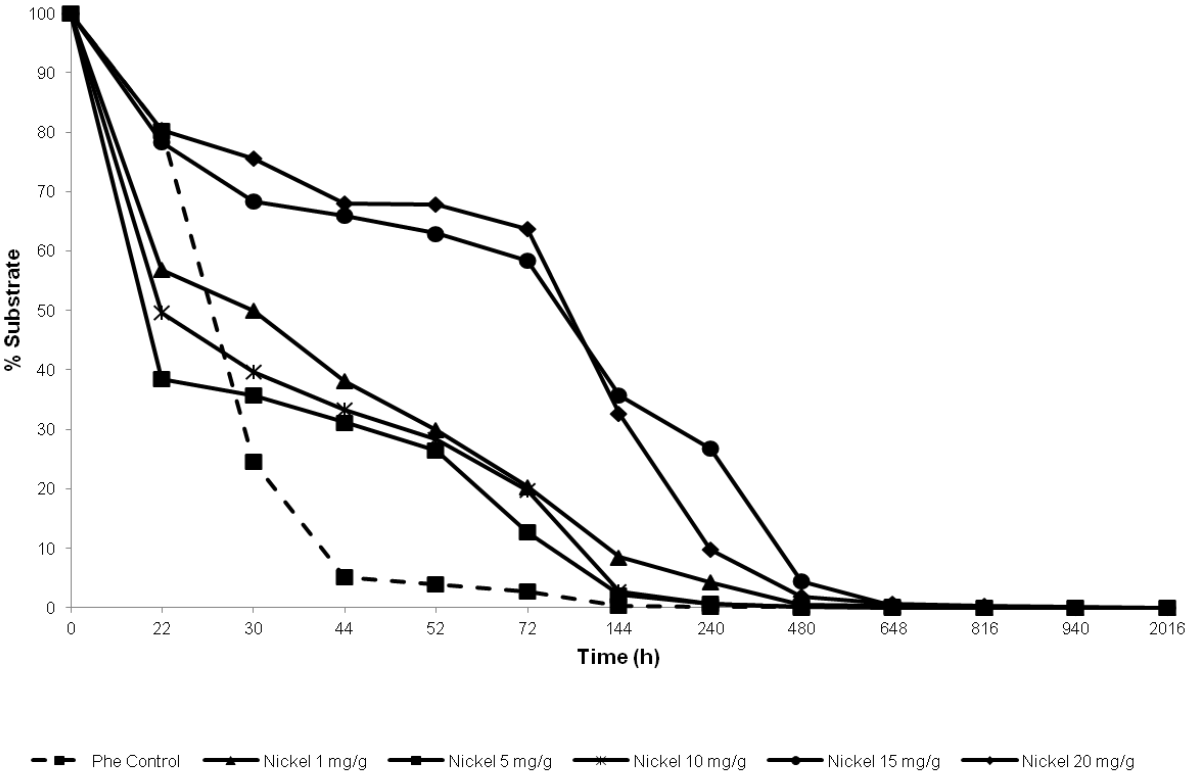


**Figure 3-27:** Degradation curve of phenanthrene in artificially contaminated soils and in the presence of different concentrations of nickel.

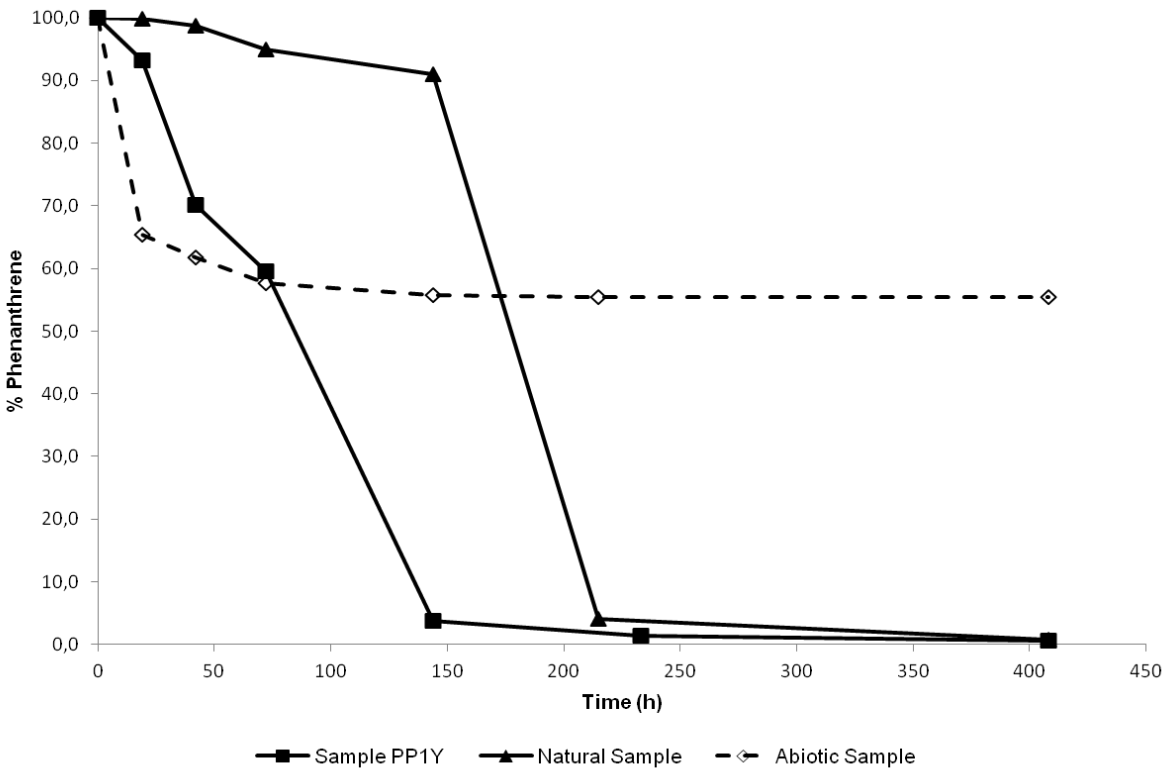


**Figure 3-28:** Degradation curve of phenanthrene in natural soil samples.

These results, although preliminary, clearly indicate the possibility of using *N. puteolanum* PP1Y to enrich the endogenous microflora of polluted soils to degrade exogenous pollutants in bioremediation protocols.

# IV
# CONCLUSIONS

Sphingomonads have been studied for their ability to degrade several pollutants, in particular aromatic compounds, and their planktonic/sessile dimorphism. Both of these features are very well developed in *N. puteolanum* PP1Y.

The strain is able to use a very wide range of mono and polycyclic aromatic compounds as carbon and energy sources. Its degradative ability is comparable to or greater than the most versatile sphingomonads like *S. paucimobilis* EPA 505, *N. aromaticivorans* F199 and *Sphingomonas* sp. Ant 17 [**115**]. Moreover, strain PP1Y shows a very high propensity to adopt the sessile phenotype and efficiently colonize several types of hydrophobic surfaces including water/oil interfaces. In addition to these features, strain PP1Y has a surprisingly high tolerance to diesel oil, which allows its growth in biphasic diesel oil/water cultures containing more oil than water [**115**]. To the best of our knowledge, such behavior has never been reported for a Sphingomonad.

It should be noted that the majority, if not all Sphingomonads, with the ability to degrade aromatic hydrocarbons have been isolated from soil or fresh water and seawater sediments [**13**, **22**, **118**]. By contrast, strain PP1Y has been isolated from the sea surface, and accordingly, it shows specific adaptations to grow at water/fuel interfaces including (**1**) a surprisingly high tolerance to diesel oil, (**2**) the ability to emulsify oil and to adhere to the oil surface and (**3**) the ability to use a very wide range of aromatic compound, -even when present in complex mixtures.

Other findings further support the hypothesis that water/fuel interface is a very well-suited environment for this strain: (**1**) oil colonization depends on the presence of specific aromatic compounds contained in petroleum-derived fuels, (**2**) the secreted emulsifier of strain PP1Y is not able to increase the water solubility of PAHs and (**3**) the biofilms formed on fuel drops reduce evaporation of mono- and bicyclic aromatic compounds and prevent the dispersion of good growth substrates.

The data reported in the chapter “Results and Discussion” allow us to propose an intriguing mechanism for the formation of the cell-coated oil drops. Extracellular biomolecules secreted by strain PP1Y bind and stabilize oil drops generated by shaking. Polysaccharides are not involved in this step of the colonization process as strain PP1Y does not secrete soluble polysaccharides in any condition tested. Cells then colonize the surface of the drops and start to produce an extracellular matrix that likely contains exopolysaccharides not soluble in water. The biofilm and the extracellular matrix increase the mechanical stability of the drops. Moreover, the surface colonization step and/or the secretion of the extracellular matrix are stimulated by the presence in the oil phase of selected aromatic growth substrates like propylbenzene, ethyltoluene, tetrahydronaphthalene, pyrene, fluoranthene and heterocyclic compounds. These compounds that are usually present in gasoline and diesel oil at very low concentrations might act as signal molecules stimulating cells to colonize a nutrient rich oil phase, whereas, at high concentrations, they could induce the formation of biofilms with an excess of extracellular matrix

Using a similar mechanism, strain PP1Y could colonize a static water/oil interface. In some ways, strain PP1Y has properties more similar to OHCBs (**o**bligate **h**ydro**c**arbonoclastic **b**acteria) rather than to other Sphingomonads. In fact, these marine bacteria use petroleum and fuels as carbon and energy sources and form

biofilms at water/oil interfaces [**119**, **120**]. With the exception of the genus Cycloclasticus, OHCBs preferably, if not exclusively, degrade saturated hydrocarbons. Thus, with regard to the total degradation of petroleum, OHCBs and strain PP1Y complement each other very well.
The peculiar features of strain PP1Y could allow it to grow in several fuel-polluted environments with low–medium salinity and at the same time make it a very promising tool for *in situ* bioremediations. In fact, the presence of several aromatic hydrocarbons in complex mixtures improves its growth and degradation capabilities rather than impairing them. The ability of strain PP1Y to spontaneously form biofilm on several surfaces could allow the adhesion of cells to soil, sand and mud particles, thus reducing its washing out by atmospheric agents. Moreover, its ability to form emulsions reduces the need to use detergents which are often required in bioremediation treatments and its capacity to encapsulate oil drops and to preferentially remove the aromatic fraction may avoid the dispersion of toxic aromatic hydrocarbons in the environment. It is worth to remind that the ability of this microorganism to grow in a wide range of temperature and salinity values allows for its use in a variety of environments, including costal lagoons and river estuaries. Finally, in collaboration with Dr. Eugenio Notomista at the Department of Structural and Functional Biology (Naples, University Federico II), Prof. F. Salvatore (CEINGE-Advanced Biotechnology S.c.ar.l., Naples, Italy) and Prof. G. Paolella (Department of Biochemistry and Medical Biotechnologies, University of Naples Federico II, Naples, Italy), we are currently completing the analysis of the recently completed genome of strain PP1Y [**84**]. Our results will help clarifying the molecular basis of the unusual features of this strain, described in this thesis, and will be an invaluable tool to engineer strains with enhanced cultural and bioremediation abilities.

# V
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## VI
## PUBLICATIONS


**1)** Izzo V., Notomista E., Scognamiglio R., **Troncone L.**, Donadio G., Di Donato A. *The Catalytic Potential of Recombinant Bacterial Multicomponent Monooxygenases ToMO and PH For the Synthesis of Antioxidant Tyrosol and Hydroxytyrosol in the Strain E.coli/JM109.* In Metabolism and molecular systems for the biotransformation of aromatic molecules (2010), 33-40. Open Archive of the University of Naples (*http://www.fedoa.unina.it/3695*).

**2)** Notomista E., Pennacchio F., Cafaro V., Izzo V., **Troncone L.**, Varcamonti M., Di Donato A. *Novosphingobium sp. PP1Y Has Adapted to Use the Aromatic Fraction of Fuels Oils as the Sole Carbon and Energy Source.* In Metabolism and molecular systems for the biotransformation of aromatic molecules (2010), 41-49. Open Archive of the University of Naples *(http://www.fedoa.unina.it/3695).*

**3)** Izzo V., Leo G., Scognamiglio R., **Troncone L.**, Birolo L., Di Donato A. *PHK from phenol hydroxylase of Pseudomonas sp. OX1. Insight into the role of an accessory protein in bacterial multicomponent monooxygenases.* Arch Biochem Biophys, (2011). **505**(1): p. 48-59.

**4)** Notomista E., Pennacchio F., Cafaro V., Smaldone G., Izzo V., **Troncone L.**, Varcamonti M., and Di Donato A. *The marine isolate Novosphingobium sp. PP1Y shows specific adaptation to use the aromatic fraction of fuels as the sole carbon and energy source. Microb Ecol, (2011).* ***61****(3): p. 582-94.*

**5)** Izzo V., Notomista E., Scognamiglio R., **Troncone L.**, Donadio G., Pezzella A., Di Donato A. *Tuning the specificity of the recombinant multicomponent toluene o-xylene monooxygenase from Pseudomonas sp. strain OX1 for the biosynthesis of tyrosol from 2-phenylethanol.* Appl Environ Microbiol, (2011). **77**(15): p. 5428-37.

## VII
## PRESENTATIONS IN SCIENTIFIC MEETINGS

**1)** Notomista E., Cafaro V., Izzo V., **Troncone L.**, Smaldone G., D'Urso N., Garzillo F., Varcamonti F., and Di Donato A. *Adaptation of Novosphingobium sp. PP1Y to grow on complex mixture of aromatic compounds dissolved in oil phases.* III International Conference on Environmental and Applied Microbiology (BioMicroWorld 2009). Lisbon, Portugal, 2-4 December 2009.

**2)** Cafaro V., Notomista E., Izzo V., Scognamiglio R., **Troncone L.** and Di Donato A. *Molecular dissection of aromatic hydrocarbons biodegradation.* The 4th Korea-Italy S&T Forum. Naples, Italy, 2-4 June, 2010.