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ENVIRONMENTAL BIOTECHNOLOGY

# Degradation of phenanthrene by *Novosphingobium* sp. HS2a improved plant growth in PAHs-contaminated environments

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**Abstract** At the same time that the European Union (EU) policy recommend to direct efforts towards reductions of heavy metals, polycyclic aromatic hydrocarbons (PAHs) and mining residues, there is the need to increase the cultivable areas within Europe to cope with the increasing demands for food and energy crops. Bioremediation is a good technique for the restoration of contaminated soils; however, it has not been used extensively because of the variability of the outcome. This variability is frequently due to a bad establishment of foreign degrading populations in soil. We have demonstrated that *Novosphingobium* sp. HS2aR (i) is able to compete with other root colonizers and with indigenous bacteria, (ii) is able to establish in high numbers in the contaminated environments and (iii) is able to remove more than 90 % of the extractable phenanthrene in artificially contaminated soils. Furthermore, we have demonstrated that the capacity to remove phenanthrene is linked to the ability to promote plant growth in contaminated environments. The fact that the presence of *Novosphingobium* sp. HS2aR improves the growth of plants in contaminated soil suggests that it may be a useful strain for utilization in amelioration of soil quality while improving the growth of economically important energy crops, thus adding value to the bioremediation strategy.

**Keywords** Bioremediation · *Novosphingobium* · Polycyclic aromatic hydrocarbons · Plant growth promoting bacteria

Sara Rodriguez-Conde and Lázaro Molina equally contributed to the manuscript.

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## Introduction

One of the undesirable side effects of the industrial era is the accumulation of contaminants in the environment. Although there is no comprehensive information about the extension and location of contaminated soils in the EU, it is estimated that 16–35 % of the territory suffers from the effects of contamination. The report about "The State of Soil in Europe" (Jones et al. 2012) estimated that at least 250,000 sites need urgent decontamination, and the document SWD (2012) 101/final/2 suggested the re-utilization of urban and industrial areas after decontamination to reduce the use of new virgin soils. Nevertheless, due to the high cost of conventional remediation techniques, many of such areas are not included in remediation plans. Bioremediation, the utilization of living organisms to clean up contaminated areas, is a promising technology that can be used in the rehabilitation of these areas because it is a relatively cheap technique (Escalante-Espinosa et al. 2005; Pandey et al. 2009). However, this technique has not been widely used, and it has been shown that reduced persistence of inoculated contaminant-degrading bacteria in soils is one of the main reasons for unsuccessful bioremediation (Bouchez et al. 2000; Pandey et al. 2009).

At the same time that the EU policy is recomending that efforts should be made to reduce heavy metals, polycyclic aromatic hydrocarbons (PAHs) and mining residues in the environment, there is also the need to increase the cultivable areas within Europe to cope with the increasing demands for

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food and energy crops. Polluted soils are considered useless for agriculture, not only due to the presence of contaminants that can alter the food properties but also because plants frequently cannot thrive or grow very poorly on them (Sverdrup et al. 2003; Reynoso-Cuevas et al. 2008; Murherjee and Bordoloi 2011). The utilization of contaminated soils for the production of energy crops could be a good solution to avoid the problems associated with adsorption of contaminants in food crops, although how to achieve high vegetal productivity in these contaminated environments remains a problem. Decreasing the contaminant concentration in soil at the same time as seedling growth is taking place is a good solution for improving plant growth; utilization of bacterial strains that are able to do so will be a great advantage in bioremediation strategies.

In this study, we have identified *Novosphingobium* sp. HS2aR, a rifampicin-resistant strain originated from *Novosphingobium* sp. HS2a (which was isolated from soil as a good phenanthrene and naphthalene degrader), as an excellent strain to eliminate phenanthrene in soils and to contribute to plant growth. This strain is able to persist in contaminated soils, one of the best properties for a strain to be used in bioremediation. Although members of the *Sphingomonas* group have been extensively described as excellent degraders of polychlorobiphenyls (PCBs), PAHs, herbicides and other contaminants (Shi et al. 2001; Böltner et al. 2008; Verma et al. 2014; Mulla et al. 2016), their biotechnological properties related with rhizospheric environments have not been studied in detail before.

Our results demonstrate that this strain contains the perfect combination of characteristics to use in the restoration of contaminated soils, thus promoting plant growth in contaminated environments. We have also demonstrated that the ability to promote plant growth is due to the capacity of this strain to degrade phenanthrene in soils. These results show that *Novosphingobium* sp. HS2a is an excellent strain to use in the restoration of polluted sites at the same time that the land is being used for the growth of economically attractive crops.

## Material and methods

### Bacterial strain

*Novosphingobium* sp. HS2a was isolated from soil samples taken near an industrial area in Huelva through enrichment using phenanthrene as the sole carbon source. Strain identification was done on the basis of the production of a yellow pigment on plates (typical of the *Sphingomonas* group) and 16S rRNA sequencing. Additional multilocus sequence analysis of *gyrB*, *trpF*, *edd* and *recA* genes confirmed that this isolate is a member of the *Sphingomonas* group, clearly different from other well described members of the group.

To be able to differentiate this strain from rhizospheric indigenous populations, one spontaneous mutant resistant to rifampicin was selected by growing the strain in increasing concentrations of rifampicin. The final strain was able to grow with 10 μg/ml of rifampicin and was named *Novosphingobium* sp. HS2aR. This strain has been deposited at the CECT and was accessioned CECT 9150.

### Phenanthrene degradation in laboratory media and the effects of root exudates in degradation

The bacteria were grown overnight in M9 minimal medium plus glucose as the sole carbon source and rifampicin. Flasks containing 25 ml of M9 minimal medium (Abril et al. 1989) and 2.5 mg phenanthrene (dissolved in 500 μl of hexane) were prepared the day before the inoculation and were left open for 1 h to allow hexane evaporation. Because phenanthrene solubility in water is 1.6 mg/l at 25 °C, after hexane evaporation, most of the phenanthrene was located on the surface of the liquid medium as a crystalline powder. Flasks were inoculated to reach an initial $OD_{660\ nm}$ of 0.05. Flasks were cultured at 30 °C on an orbital shaker (200 strokes/min). Samples were taken at different times (0, 11, 24, 35, 48, 72, 144 and 240 h) to measure culture turbidity and to quantify the phenanthrene and 1-hydroxy-2-naphthoic acid.

To study the effect of root exudates on phenanthrene mineralization, 40 surface-sterilized clover seeds were grown in a glass jar with 50 ml of glass beads and 25 ml of water with Fe-EDTA for 15 days. Plants were grown in a plant chamber at 24 °C with a light cycle of 14 h and 18 °C during the 10 h of darkness. Afterwards, the liquid was collected and passed through a 0.22-μm filter. Flasks with 20 ml of clover exudates plus/minus phenanthrene (2 mg) were used to monitor the growth of the strain and for phenanthrene quantification.

For phenanthrene and 1-hydroxy-2-naphthoic acid quantification, 500 μl of media was taken (avoiding phenanthrene crystals to measure only soluble phenanthrene) and this volume was mixed with 500 μl of methanol and analysed with high-performance liquid chromatography (HPLC) as previously described (Pinyakong et al. 2000).

To study the effect of roots and root exudates on gnotobiotic experiments, we used a similar experimental set up as above, but we inoculated the jars with an overnight culture to reach a final $OD_{660\ nm} \approx 0.005$. After 15 days, the plants were extracted and the bacteria in the exudates were counted by the drop-plating method. For rhizospheric bacterial counts, plants were taken from each jar, rinsed twice in 2 ml of sterile water to eliminate non-attached cells and vortexed for 30–40 s with sterile glass beads before serial dilutions were plated. Results show the mean value of three independent experiments. One volume of methanol was added to the jars to dissolve residual phenanthrene that was measured by HPLC. Residual phenanthrene was expressed as

the percentage of phenanthrene in each experiment compared with the corresponding control. Experiments were performed three times with two independent microcosms each time.

### Construction of a mutant in the phenanthrene dioxygenase of *Novosphingobium* sp. HS2aR mutant

The phenanthrene dioxygenase gene was identified by Southern blot using probes based on different phenanthrene dioxygenase sequences from GenBank. Primers bphA1-*Kpn*I (3′-GGGGTACCGCTGTTCGTAAACTGGCAT-5′) and bphA1-*Bam*HI (3′-CGGGATCCGAAGAATGCCGACA CCGAAAC-5′) were used to amplify a 586 bp DNA fragment and primers bphA2-*Bam*HI (3′-CGGGATCCGATC GGCGCGAGCCCCAGCT-5′) and bph2-*Sal*I (3′-GCGTCG ACAACGAGGCCAGACACAGCAAC-5′) to amplify a second DNA fragment (640 bp). These two fragments were cut with the appropriate restriction enzymes and cloned into pBML previously cut with *Kpn*I and *Sal*I. The resulting plasmid was cut with *Bam*HI and the Ωkm excised with *Bam*HI from pHP45 (Prentki and Krisch 1984) was cloned in between the two PCR fragments. Two hundred microgrammes of the final plasmid was introduced by electroporation into *Novosphingobium* sp. HS2aR to allow double recombination. Positive clones were identified by their the kanamycin resistance and checked by PCR; correct integration of the construction was examined by Southern blot (not shown).

### ß-Galactosidase assays

A DNA fragment of 504 bp, including the intergenic region between *phnA1f* and the putative protein or f114, was amplified with primers designed on the sequence of plasmid pNL1 from *Novosphingobium aromaticivorans* F199 using genomic DNA from strain *Novosphingobium* sp. HS2a. Oligonucleotides incorporated restriction sites to facilitate cloning in the appropriate direction (an *Eco*RI site in the primer designed to meet the 5′ end and a *Pst*I site in the primer designed to meet the 3′ end). Upon amplification, DNA was digested with *Eco*RI and *Pst*I and ligated into the low copy number pMP220 vector (Spaink et al. 1987), previously cut with the same enzymes to produce plasmid pPHS2 ($phnA1_{HS2a}$ promoter). The plasmid was sequenced to verify the promoter sequence. pPHS2 was electroporated into *Novosphingobium* sp. HS2aR. Transformants were selected in LB plates plus tetracycline (10 mg/l); individual colonies were grown in liquid media, and the plasmid was extracted and digested with *Eco*RI and *Pst*I for verification. Selected strains were grown overnight on M9 minimal media plus glucose and then diluted to an $OD_{660\ nm}$ of 0.1/ml in fresh M9 minimal media plus glucose. Protocatechuate, *o*-phthalate and salicylate were added at a final concentration of 5 mM; phenanthrene was used at 1 mg/10 ml, while naphthalene and 1-hydroxy-2-naphthoic acid were added as crystals. All of them were added immediately after inoculation, and ß-galactosidase assays (Miller 1972) were done 5 and 24 h later. Assays were run in duplicate and were repeated for at least three independent experimental rounds.

To test root exudates, cultures were grown overnight as before and the following day diluted to an OD of 0.1/ml in M9 plus glucose. Two hundred microliters were added to a 96-well plate, and 5, 10 and 25 μl of exudates (obtained as above) were added to the corresponding wells. Plates were done in duplicate, and in one of them, naphthalene (crystal power) was added into an empty well; naphthalene was used as inducer instead of phenanthrene to facilitate the assay because it is a highly volatile compound. After 16 h of incubation at 30 °C with constant agitation, ß-galactosidase assays were performed following the protocol developed by Marqués et al. (unpublished). Briefly, 15 μl of cells was mixed with 15 μl of mixed alkyltrimethylammonium bromide (MATAB) and kept on ice for 20 min. Then, 100 μl of buffer Z and 20 μl of orthonitrophenyl-galactopyranoside (ONPG) (as in Miller 1972) were added, and the plate was incubated at 30 °C until a yellow colour was developed. Reaction was stopped with 150 μl of calcium carbonate (0.5 M pH 7). Plates were read in a DTX800 Multimode Detector (Beckman Coulter, Brea, CA, USA) at 620 (original plate), 540 and 450 nm.

### Competitive root colonization

Ten surface-sterilized clover seeds were placed in sterile glass tubes containing glass beads. Cells grown on M9 minimal media plus 10 mM glucose overnight were diluted to an OD at 660 nm of 0.005 (approximately $10^6$ colony forming units (CFUs)/ml) in 2.5 mM Fe-EDTA solution, and 2 ml of this solution was added to each tube. In control tubes, 2 ml of the Fe-EDTA solution was added. Seeds were added to the corresponding tubes, and samples were taken immediately after inoculation and 4, 7 and 14 days later. Seeds germinated after 2–3 days. Samples were analysed for number of cells in supernatant and attached to the roots. For rhizospheric bacterial counts, plants were taken from each tube, rinsed twice in 2 ml of sterile water to eliminate non-attached cells and vortexed for 30–40 s with sterile glass beads before serial dilutions were plated. Results show the mean value of three independent experiments.

To study the colonization in competition with indigenous bacteria, the assays were performed as described above but seeds were not previously sterilized. The number of *Novosphingobium* sp. HS2aR CFUs inoculated was calculated to equal the number of bacteria on seeds. Samples were analysed for number of total CFUs by growing them on LB and LB plus rifampicin (10 μg/ml) plates. Results are the mean value of three independent experiments.

### Phenanthrene bioremediation in soil

Commercial organic solid vegetable support (COMPO*; Munster, Germany) 40 % (v) was mixed with 60 % (v) of washed sand. One gramme of phenanthrene dissolved in 3 ml of acetone was added to 200 g of soil and was thoroughly mixed and kept in a hood for at least 1 h to evaporate traces of acetone. Uncontaminated soil (1.8 kg) was later mixed with 200 g of contaminated soil. Soil (~100 g) was added to each pot. Thirty clover seeds were planted in each pot and grown in a plant chamber at 24 °C with a light cycle of 14 h and a dark cycle of 10 h at 18 °C. Pots with only contaminated soil and with contaminated soil plus clover were kept as controls. Overnight cultures grown on LB media plus rifampicin were diluted until $OD_{660\ nm}$ 0.01/ml in tap water; 20 ml of mixture was added to each microcosm. Because the LB media added to the tap water was always <100 μl, we considered that its effect in plant growth or microbial stimulation was negligible. Four control pots of contaminated and non-contaminated soil were used for measuring phenanthrene concentration. Four independent experiments with two replicas were set up. Samples were taken at day 0, 17 and 30 days. For phenanthrene extraction, 1 g of soil was dried at room temperature overnight, and 2 ml of hexane/acetone (2 ml of 2:1) was added to the soil, vortexed and sonicated for 10 min in a glass tube. After centrifugation (1000 rpm for 10 min at 4 °C), the supernatant was transferred to a clean tube and the extraction was repeated with the soil. Samples were evaporated to dryness under $N_2$ streams, and the extract was re-suspended in 1 ml of methanol/water (1:1) prior to HPLC analysis.

To analyse the survival of the strains in the microcosms, 1 g of soil was incubated overnight at 30 °C with agitation (200 strokes per minute) with 10 ml of M9 minimal media without carbon source. The following day, serial dilutions were plated on M9 minimal media plus rifampicin, cycloheximide and naphthalene as carbon source. Plates were incubated for 3 days at 30 °C and then the number of $Rif^R$-$Naph^+$ CFUs was counted.

### Changes in bacterial diversity in microcosms experiments

One gramme of soil from the different microcosms was used to isolate DNA with the FastDNA® Kit for soil (MP Biomedicals LLC, Solon, OH, USA). Hypervariable regions V3-V5 of the 16S bacterial genes were amplified and after labelling the fragments of each sample with a molecular identifier (MID), the samples were cleaned to eliminate primer-dimers. DNA was quantified using the Quant-iT™ PicoGreen (Invitrogen, Carlsbad, CA, USA). Equimolar quantities of the amplicons from the different samples were cleaned again to eliminate primer-dimers and used in Roche 454 pyrosequencing platform (LifeSequencing, Paterna, Valencia, Spain).

Sequences were first depleted of MID sequences, primers and short sequences (<300 bp length), and finally, chimera were eliminated using UCHIME program. Taxonomic assignation of each filtrated sequence was done with local alignments (BLASTN) using 16S ribosomic RNA database obtained from 16S RNA database of the NCBI that had been curated to minimize alignment errors and to reduce false positives. Rarefaction curves reached a plateau suggesting that the number of sequences is enough to detect all the variability present in the samples.

### GenBank accession numbers

GenBank accession number for *Novosphingobium* sp. HS2a 16S DNA is KF544932.1. Accession numbers for the *phnA1* dioxygenase fragment and promoter are KX272769 and KX272770. Accession numbers for *gyrB*, *edd*, *trpF* and *recA* sequences are KX761987, KX761986, KX761989 and KX761988 respectively. The pyrosequencing data were submitted to the SRA database under accession number SRP082638.

## Results

### Phenanthrene degradation

*Novosphingobium* sp. HS2a was isolated from soil near an industrial complex in Huelva (Spain). This strain was able to grow on minimal medium M9 with phenanthrene or naphthalene as the only carbon source (not shown). *Novosphingobium* sp. HS2aR was able to degrade 25 mg of phenanthrene in 72 h. Interestingly, phenanthrene dissolved in the media increased during the first 35 h (up to 43.56 ± 29.52 mg/l), while the concentration of soluble phenanthrene in control flasks without bacteria remained constant (1.78 ± 0.5 mg/l), suggesting the bacteria caused a solubilization effect. The intermediate degradation compound, 1-hydroxy-2-naphthoic acid, was detected after 11 h, but it was fully transformed after 144 h (Supplementary Fig. S1).

To test if phenanthrene degradation was inhibited by the presence of other carbon sources, the cultures were grown in the presence of glucose or clover root exudates. When the strain was grown with glucose as carbon source (5 mM), the cultures grew faster than with only phenanthrene and the presence of phenanthrene did not have any detrimental effect on growth. Because culture growth was faster, phenanthrene disappearance from culture medium was also faster than in cultures without glucose (no phenanthrene was detectable at 24 h). Clover root exudates by themselves did not support the growth of the strain; however, when phenanthrene was added to the exudates, *Novosphingobium* sp. HS2aR was able to grow at similar rates as those in M9 minimal medium plus

phenanthrene and the contaminant was degraded similarly (not shown).

In the latter experiment, exudates were filtrated through a 22-μm filter, so we could not exclude the possibility of high molecular weight compounds that could influence phenanthrene degradation being eliminated, and therefore, we decided to explore the phenanthrene degradation in gnotobiotic systems. Fifteen days after the addition of seed and bacteria to the system, almost 75 % of the initial phenanthrene concentration disappeared from the system (Fig. 1a). It should be noted that in the control experiment with plants but without bacteria, 40 % of the phenanthrene was eliminated probably because adsorption to the root (the plants were removed prior phenanthrene extraction). The number of colony forming units (CFUs) remained constant in the exudate, and its numbers in the root surface were approximately $10^6$ CFUs after 15 days (Fig. 1b).

All these results indicated that in *Novosphingobium* sp. HS2aR, the phenanthrene degradation pathway was active in the presence of different carbon sources and root exudates.

## Identification of the *phn* genes in *Novosphingobium* sp. HS2aR

After amplification and labelling of dioxygenase genes fragments from different *Sphingomonas* strains (Romine et al. 1999; Demanèche et al. 2004), we used these probes to hybridize with genomic DNA of *Novosphingobium* sp. HS2a. The only probe that gave positive signals in the Southern blot was that from *bphA1f* of *Sphingomonas aromaticivorans* F199. We amplified and sequenced a 567 bp fragment of the corresponding gene from our strain; sequence was 99 % identical at the nucleotide level to that of *bphA1f* gene from *S. aromaticivorans* F199 (Romine et al. 1999) and *phnA1f Sphingomonas* sp. strain LH128 (Schuler et al. 2009). To demonstrate that this dioxygenase was involved in phenanthrene degradation, we constructed a knocked-out mutant. This mutant strain was unable to grow in M9 minimal when phenanthrene was used as the sole carbon source (not shown). As this gene in *Novosphingobium* sp. HS2a was involved in phenanthrene degradation, we named it *phnA1f*.

## *phnA1f* gene expression in the rhizosphere

It has been reported that expression of contaminant-degrading genes can be induced or repressed in the presence of root exudates (Donnelly et al. 1994; Gilbert and Crowley 1997; Yi and Crowley 2007; Rentz et al. 2004). Although we observed phenanthrene degradation in the rhizosphere in gnotobiotic systems and in the presence of root exudates, we decided to directly measure gene expression by cloning the *phnA1f* gene promoter into pMP220 that carries a promoterless *lacZ* gene as reporter. The basal activity of the promoter (cultures grown on M9 minimal medium plus glucose) was 149.84 ± 14.89 MU (Miller units), while the promoterless plasmid showed a β-galactosidase activity of 47.40 ± 7.57 MU. We did not observe increased expression from the promoter in the presence of phenanthrene (135.40 ± 15.69) or naphthalene (88.99 ± 14.12) meaning that this gene was not induced by these contaminants, neither was this promoter induced by some of the reported metabolic intermediates, 1-hydroxy-2-naphthoic acid, protocatechuate, *o*-phthalate or salicylate.

To determine the effect of different roots exudates on *phnA1f* expression, we measured β-galactosidase activity in the presence and absence of naphthalene when root exudates were added to the culture media. We did not observe any increase or decrease in β-galactosidase activity when exudates from clover (*Trifolium repens*), mint (*Mentha spicata*), winter ray-grass (*Lolium perenne*) or grass (30 % ray-grass, 30 % *Festuca arundinacea* Tomahawk and 40 % *F. arundinacea* Rendition) were used.

These results indicate that different root exudates were not inhibiting *phnA1f* expression and, therefore, that this strain was a good candidate for rhizoremediation experiments.

## *Novosphingobium* sp. HS2aR is highly competitive in rhizosphere colonization

Soil is a highly complex environment where competition among different microorganisms can limit the survival of foreign strains. Therefore, we studied the ability of *Novosphingobium* sp. HS2aR to compete with other bacterial strains. First, we set up a simple experiments in which we co-inoculated our isolate with *Pseudomonas putida* KT2440 (described as a good root colonizer; Molina et al. 2000) into a test tube with 10 surface sterilized clover seeds. After 14 days, the proportions of bacteria inoculated were similar to the proportion at the beginning of the experiments, indicating that *Novosphingobium* sp. HS2aR was able to co-exist with *P. putida* KT2440. To test the ability of our isolate to thrive in the presence of indigenous bacteria, we inoculated the seeds that were not sterilized with this strain. At the beginning of the experiment, the number of CFUs in control tubes (only seeds) was about 20–40 CFUs/ml, and after 10 days, this number increased to $10^6$–$10^7$ CFUs/ml; no colonies were detected on LB plus rifampicin at any time. Tubes inoculated with 2–50 CFUs/ml of the rifampicin-resistant strains contained $10^6$–$10^7$ rifampicin-resistant CFUs/ml 10 days after inoculation suggesting that *Novosphingobium* sp. HS2aR was able to successfully colonize the rhizosphere in the presence of indigenous bacteria.

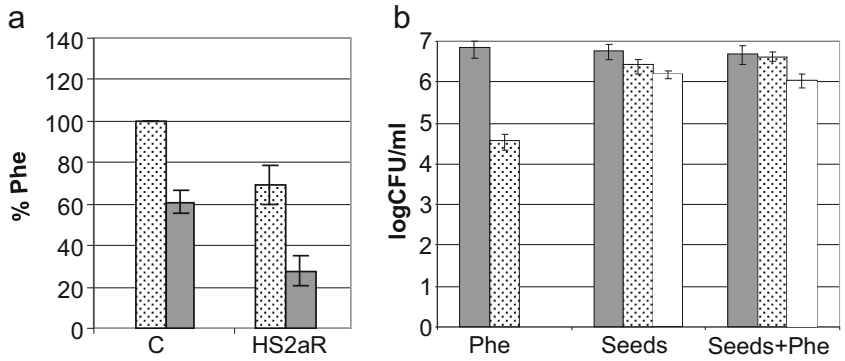


**Fig. 1** Phenanthrene disappearance and number of CFUs in gnotobiotic systems. **a** Percentage of phenanthrene (*Phe*) in the media after 15 days. *Dotted bars* without clover, *grey bars* with clover, *C* non-inoculated control. **b** Number of CFUs in the gnotobiotic system. *Grey bars* CFUs at the beginning of the experiment, *dotted bars* CFUs in the supernatant after 15 days, *white bars* rhizospheric CFUs after 15 days. Data represent the mean of three independent experiments

## Phenanthrene disappearance in soil microcosms

To assess the performance of the strain in soil, we set up microcosms artificially contaminated with phenanthrene. Our first observation was that phenanthrene exerted a clear detrimental effect on the development of clover that was already evident 15 days after planting (Fig. 2a, left panel). Our second observation indicated an important role for natural attenuation and/or complexation of phenanthrene with soil particles to render non-extractable complexes; 17 days after contamination, 40 % of the extractable phenanthrene was not detected in both vegetated and non-vegetated microcosms and extractable phenanthrene decreased with time especially in non-vegetated microcosms (Fig. 3). Inoculation with *Novosphingobium* sp. HS2aR accelerated phenanthrene degradation, and almost 93–97 % of the extractable phenanthrene was eliminated after 30 days (Fig. 3). The final outcome of the bioremediation was not significantly different between vegetated and non-vegetated microcosms.

Interestingly, the detrimental effect of phenanthrene on plant growth was relieved in microcosms inoculated with *Novosphingobium* sp. strain HS2aR (Fig. 2b).

To demonstrate that clover growth improvement in contaminated environments was due to phenanthrene degradation by *Novosphingobium* sp. HS2aR, the *phnA1f* knocked-out mutant was tested in bioremediation experiments; in this case, the detrimental effect to phenanthrene on plant growth was still evident (not shown) and the amount of detectable phenanthrene in the soil was similar to that of the control experiments without inoculation (Fig. 4).

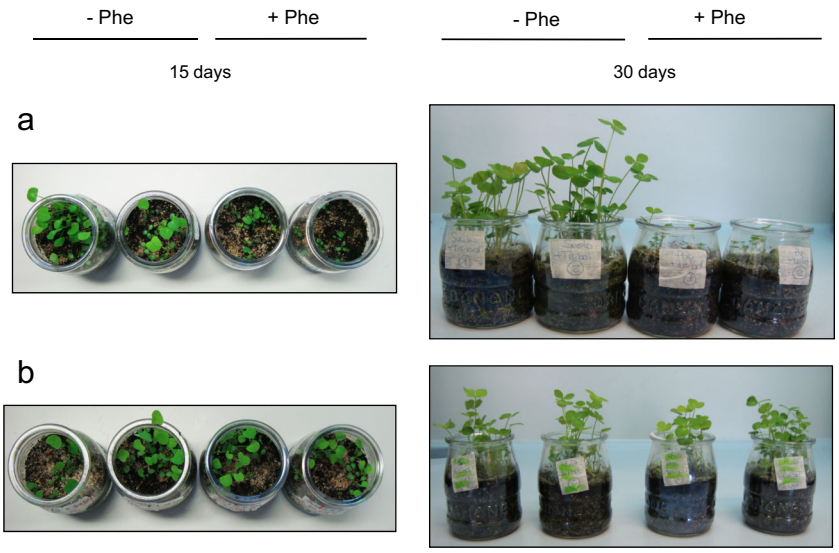


**Fig. 2** Growth of clover in microcosms with and without phenanthrene (*Phe*) after 15 days (*left-hand panels*) and 30 days (*right-hand panels*). **a** Control without addition of foreign bacteria. **b** Microcosms inoculated with *Novosphingobium* sp. strain HS2aR. Photographs of one representative experiment (two replicas) are shown

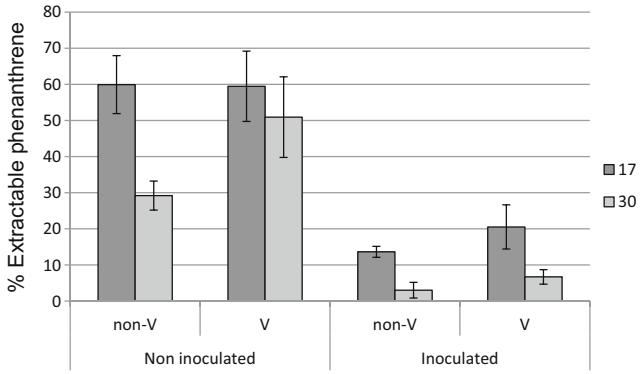


**Fig. 3** Elimination of phenanthrene in microcosms. Percentage of extractable phenanthrene present in microcosms 17 days (*dark grey*) and 30 days (*grey*) after inoculation. Percentages refer to the amount of phenanthrene extracted at day 0. *Non-V* non-vegetated microcosms, *V* vegetated microcosms. Percentages are based on the results of four independent experiments with two replicas each. Differences between treatments with and without *Novosphingobium* sp. HS2a (at both times) are statistically significant (Student's *t* test, $P < 0.05$), while differences between treatments with and without clover are not

### Persistence of *Novosphingobium* sp. HS2aR in soil microcosms

The presence of *Novosphingobium* sp. HS2aR in soil microcosms was assessed first by counting the number of naphthalene degrading bacteria resistant to rifampicin. Strains were recovered from vegetated and non-vegetated soils, with higher numbers in inoculated microcosms than in the non-inoculated ones (Fig. 5a). It should be noted that the presence of phenanthrene in soil stimulated the naphthalene degrading populations in both vegetated and non-vegetated soils as well as in non-inoculated and inoculated microcosms.

To further study the evolution of soil bacterial populations after bioaugmentation with *Novosphingobium* sp. HS2aR, soil DNA was extracted and 16S ribosomal DNA was amplified and sequenced. In uncontaminated soils, we did not observe significant alterations in the number of *Novosphingobium* sequences after the introduction of *Novosphingobium* sp. HS2aR. Microcosms with phenanthrene contained higher numbers of *Novosphingobium* than microcosms without the contaminant, and these numbers were especially high when microcosms were inoculated with *Novosphingobium* sp. HS2aR (Fig. 5b). The most abundant *Novosphingobium* sequences in microcosms with phenanthrene are related with *Novosphingobium resinovorum*, a strain closely related with *Novosphingobium* sp. HS2a (Supplementary Fig. S2). While in the non-inoculated microcosms, sequences related with *N. resinovorum* represents 3.3 % of the total sequences in microcosms inoculated with *Novosphingobium* sp. HS2a their proportion increased up to 21 % (Table 1); in microcosms with clover, the increase was from 0.18 % in non-inoculated microcosms to 11.5 % when they were inoculated. These results suggested that the presence of phenanthrene increased the persistence of our introduced bacteria in soil.

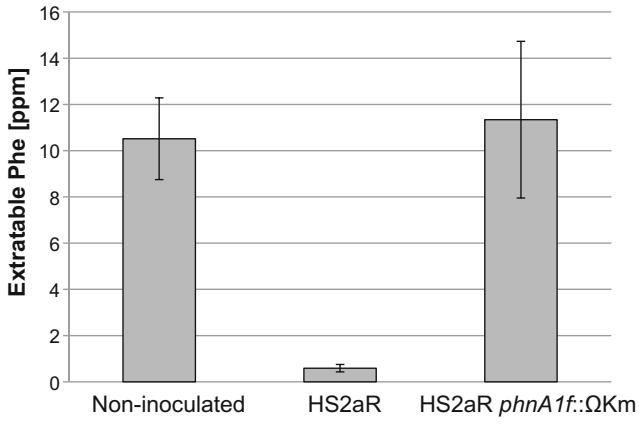


**Fig. 4** Amount of extractable phenanthrene (Phe) after inoculation with *Novosphingobium* sp. HS2aR and its derivative mutant in *phnA1f* 30 days after inoculation. Data are based in three independent experiments with two replicas each

## Discussion

Although bioremediation is a cost-effective solution for soil restoration, not all bacterial strains behave similarly in their establishment in the environment, their capacity of competition with other strains and their ability to degrade the contaminants after re-introduction. We have demonstrated that *Novosphingobium* sp. HS2aR fulfils all the requirements for being a good soil bioremediator: (i) it is able to compete with other root colonizers and with indigenous bacteria, (ii) it is able to establish in high numbers in the contaminated environments and iii) it is able to remove more than 90 % of the phenanthrene in artificially contaminated soils. Furthermore, the capacity to remove phenanthrene is linked to the ability to promote plant growth in contaminated environments. This characteristic makes the utilization of bacteria in contaminated soil even more economically interesting because these soils can be used for the production of energy crops at the same time that biodegradation is taking place.

One interesting observation is the capacity of the strain to solubilize phenanthrene. This effect was measured in the quantification of soluble phenanthrene in liquid media, but it is also observable in gnotobiotic systems; phenanthrene crystals are visually observed in control experiments without bacteria throughout the time of the experiment while they are not observable at 15 days when *Novosphingobium* sp. HS2aR was inoculated. PAHs have a low solubility in aqueous media and tend to bind to organic and inorganic soil fractions

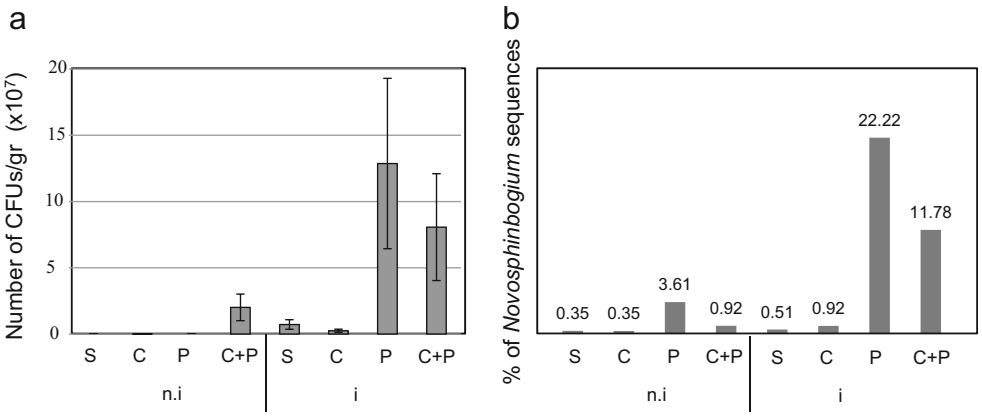


**Fig. 5** Persistence of strains in microcosms. **a** Number of $Naph^{+}Rif^{R}$ CFUs in soils. **b** Percentage of *Novosphingobium* sequences in soil. *S* soil, *C* soil with clover, *P* soil with phenanthrene, *C + P* soil with phenanthrene and clover, *n.i* non-inoculated soils, *i* inoculated soils

(Cavalca et al. 2008), factors that reduced their bioavailability and therefore their biodegradation (Kuiper et al. 2004). The capability to solubilize phenanthrene is another property that supports the good performance of this strain in the bioremediation of soils.

Although we observed that the presence of clover positively influenced the bioremediation outcome in gnotobiotic experiments (Fig. 1), in vegetated microcosms, we did not observe improved degradation when compared with non-vegetated microcosms. This result does not necessarily mean that the presence of plants had no effect on phenanthrene degradation. Contaminant-degrading bacteria thriving near the roots of plants can alleviate the toxicity of contaminants, promoting plant growth (Kirk et al. 2005; Graj et al. 2013) and that is what we observed in microcosms; *Novosphingobium* sp. HS2aR totally relieved the toxic effect of phenanthrene on the plant. Root exudates only diffuse a few millimetres from the root (Leigh et al. 2002) and although the beneficial effect on plant growth may be attributed to the elimination of the contaminant near the surface of the root during the first stage of plant growth, the higher decrease of phenanthrene near the root cannot be detected in bulk soil (influenced and non-influenced by plant) measurements.

*Sphingomonas* are commonly isolated from contaminated soils (Leys et al. 2004), and we have seen an increase in the number of *Novosphingobium* sequences in contaminated microcosms, even without inoculation (Fig. 5). As expected, this increase is higher in inoculated soils than in non-inoculated soils. This data suggests that the presence of this carbon

**Table 1** Percentage of *Novosphingobium* species identified in microcosms by 16S RNA gene sequences

| Strains | Non-inoculated | | | | Inoculated | | | |
|---|---|---|---|---|---|---|---|---|
| | Soil | Clover | Phe | Phe + clover | Soil | Clover | Phe | Phe + clover |
| *Novosphingobium hassiacum* | 0.15 | 0.06 | 0.11 | 0.27 | 0.09 | 0.18 | 0.17 | 0.13 |
| *Novosphingobium lentum* | 0.09 | 0.08 | 0.02 | 0.02 | 0 | 0.22 | 0.10 | 0.05 |
| *Novosphingobium nitrogenifigens* | 0.09 | 0.06 | 0 | 0.07 | 0.03 | 0 | 0.04 | 0 |
| *Novosphingobium subterraneum* | 0.01 | 0.02 | 0 | 0.09 | 0.03 | 0.04 | 0.04 | 0 |
| *Novosphingobium resinovorum* | 0.01 | 0.04 | 3.31 | 0.18 | 0.33 | 0.43 | 21.68 | 11.51 |
| *Novosphingobium tardaugens* | 0 | 0.07 | 0.02 | 0.05 | 0 | 0.02 | 0.02 | 0 |
| *Novosphingobium stygium* | 0 | 0.01 | 0 | 0.13 | 0 | 0.02 | 0 | 0 |
| *Novosphingobium pentaromativorans* | 0 | 0 | 0.11 | 0.09 | 0 | 0.02 | 0.06 | 0.02 |
| *Novosphingobium indicum* | 0 | 0 | 0.03 | 0 | 0 | 0 | 0.04 | 0 |
| *Novosphingobium panipatense* | 0 | 0 | 0.02 | 0.02 | 0.02 | 0 | 0.08 | 0.07 |

*Phe* phenanthrene

source acts as a major driving force for the proliferation of phenanthrene-degrading bacteria, thus contributing to the success of bioremediation strategies.

In conclusion, our results show that *Novosphingobium* sp. HS2aR is an excellent strain for utilization in the restoration of contaminated soils at the same time as the soil is used for growing plants. This is a very attractive option because it will allow the utilization of contaminated and non-productive areas in non-food crops suitable for the production of biofuels and other value-added goods, an activity with a high economic profit.

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**Compliance with ethical standards**

**Conflict of interests** The authors declare that they have no conflict of interests.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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