

## Brief report

# Rhizoremediation of lindane by root-colonizing *Sphingomonas*

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### Summary

We used a two-step enrichment approach to isolate root-colonizing hexachlorocyclohexane (HCH)-degrading microorganisms. The first step consists of the use of classical liquid enrichment to isolate  $\gamma$ -HCH degraders. The  $\gamma$ -HCH-degrading microbes were attached in mass to corn seeds sown in soil with  $\gamma$ -HCH, and after plant development we rescued bacteria growing on root tips. Bacteria were then subjected to a second enrichment round in which growth on liquid medium with  $\gamma$ -HCH and inoculation of corn seeds were repeated. We then isolated bacteria on M9 minimal medium with  $\gamma$ -HCH from root tips. We were able to isolate four *Sphingomonas* strains, all of which degraded  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -HCH. Two of the strains were particularly good colonizers of corn roots, reaching high cell density in vegetated soil and partly removing  $\gamma$ -HCH. In contrast, these bacteria performed poorly in unplanted soils. This study supports the hypothesis that the removal of persistent toxic chemicals can be accelerated by combinations of plants and bacteria, a process generally known as rhizoremediation.

### Introduction

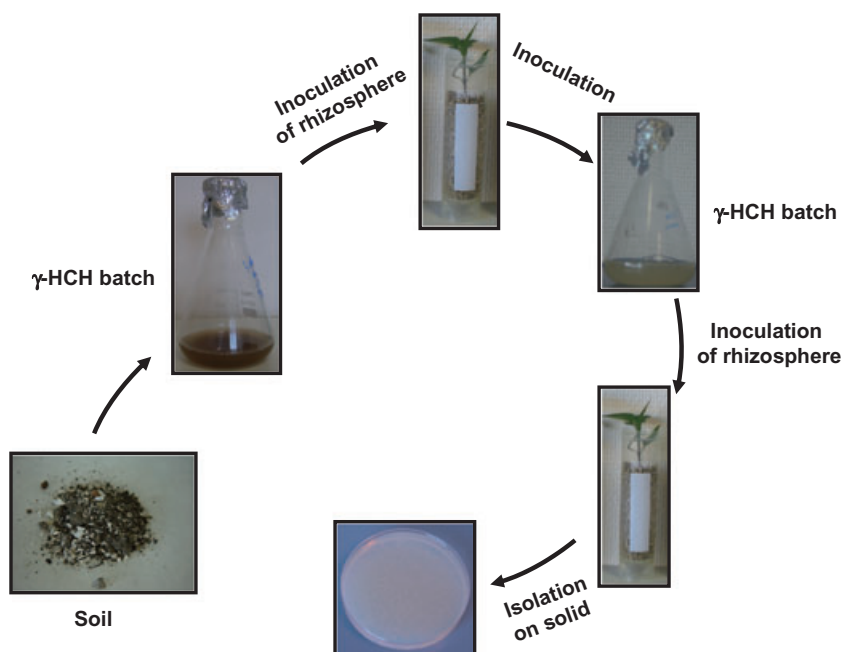
The organochlorine pesticide lindane [ $\gamma$ -hexachlorocyclohexane (HCH)] and its non-insecticidal isomers ( $\alpha$ -,  $\beta$ -,  $\delta$ -HCH) continue to pose serious environmental

and health concerns, particularly in sites where they have been produced and inappropriately stored or disposed of. Despite being banned commercially for decades (Simonich and Hites, 1995), HCH remains a persistent contaminant in many soils. The  $\gamma$ -isomer of HCH, known as lindane, has been widely used as a pesticide. This compound is highly recalcitrant, has dispersed throughout the biosphere and has bioaccumulated in many species. Contaminated environments frequently contain mixtures of several HCH isomers (mostly  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -HCH) which differ in their toxicity, water solubility (and thus mobility and bioavailability) and recalcitrance (Mohn *et al.*, 2006). The  $\gamma$ -isomer has insecticidal effects, whereas  $\beta$ -HCH is less harmful but extremely recalcitrant to aerobic degradation. Several groups have found that the  $\delta$ -isomer inhibits bacterial growth, thus possibly limiting bioremediation (Dogra *et al.*, 2004; Mohn *et al.*, 2006; our unpublished results).

Natural attenuation of HCH is attributed to microbial activity and as a result, bioremediation is considered a potential strategy for the long-term *in situ* attenuation of HCH contamination. Support for this strategy comes from studies suggesting that HCH degradation occurs in a variety of soil types, including aerobic and anaerobic ones (MacCrae *et al.*, 1969; Tu, 1976; Jagnow *et al.*, 1977; Doelman *et al.*, 1985; Bachmann *et al.*, 1988), and that the organisms responsible for HCH degradation are enriched with repeated applications of lindane (Wada *et al.*, 1989; Nagata *et al.*, 1999). However, direct application of *Sphingomonas* sp. UT26 and related strains into soil had very little effect, mainly because of poor survival of these microorganisms in soil and their high sensitivity to low water content (our unpublished results).

Rhizoremediation, the degradation of contaminants by microorganisms in the rhizosphere (the soil affected by plant roots), holds great potential for the remediation of contaminated soil (Kuiper *et al.*, 2004a,b) In the 'rhizosphere effect' plants provide nutrients in the form of root exudates, oxygen and favourable redox conditions to soil microorganisms, and this in turn results in increased bacterial diversity, population density and activity compared with bulk soil (Molina *et al.*, 2000; Vílchez *et al.*, 2000; Espinosa-Urgel and Ramos, 2001). Moreover, plants may be used as bio-injectors of pollutant-degrading

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**Fig. 1.** Two-step root colonization and recovery of lindane-degrading microorganisms. About 10 g of soil was suspended in 100 ml of M9 minimal medium with  $\gamma$ -HCH isomer. When  $\gamma$ -HCH disappeared 1 ml of suspension was mixed with corn seeds. After washing, the seeds were planted in 40 g of vermiculite with 10 mg of  $\gamma$ -HCH per gram of vermiculite. Bacteria that colonized 1 cm root tip were removed and grown on M9 minimal medium with  $\gamma$ -HCH. When growth was apparent we inoculated the corn seeds again, and after 10 days bacteria were recovered by spreading the suspension on M9 minimal medium plates with  $\gamma$ -HCH as the sole carbon source.

microorganisms into contaminated soil (Ramos-González *et al.*, 2005). Kuiper and colleagues (2004a) described an approach to select bacteria able to degrade a pollutant and colonize the rhizosphere of plants. This approach involves a two-step enrichment process in which bacteria are isolated in liquid culture from the roots of plants growing in contaminated sites, and these cultures are used to re-colonize plant roots so that biodegrading microbes can be isolated once again. Here we used this two-step process to enrich HCH-degrading bacteria able to colonize the roots of maize plants (Fig. 1). We established enrichments to isolate bacteria capable of degrading not only insecticidal  $\gamma$ -HCH, but also recalcitrant  $\beta$ -HCH and growth-inhibiting  $\delta$ -HCH. In particular, we attempted to enrich 'rhizodegraders', root-colonizing bacteria able to degrade HCH isomers. The soil samples came from Ansio (Vizcaya, Spain) and Chemnitz-Schweizerthal (Germany), and varied greatly in levels of HCH contamination and the presence of the various HCH isomers. At some points the level of HCH was  $> 1$  mg of HCH per gram of soil.

#### Two-step enrichment of HCH-degrading bacteria that colonize the rhizosphere ('rhizodegraders')

Neufeld and colleagues (2006) found that differences in soil microbial composition and diversity were associated with the presence of HCH. To find out whether the presence of pollutants favoured the proliferation of HCH-degrading microorganisms, we used a two-step enrichment approach adopted from Kuiper and col-

leagues (2004a) to select HCH degraders able to colonize the roots of maize plants. The first step was growth in batch using modified M9 mineral medium (Abril *et al.*, 1989; Duque *et al.*, 1993) and  $\gamma$ - or  $\beta$ - or  $\delta$ -HCH as the sole carbon source ( $10 \text{ mg ml}^{-1}$ ), and microorganisms were isolated from rhizosphere soil (soil surrounding the roots of plants) from HCH-contaminated sites in Germany and Spain, or bulk soil from the same locations (Table 1). Cultures were incubated at  $28^\circ\text{C}$  with shaking for 2 weeks and monitored for HCH degradation as described previously (Böltner *et al.*, 2005). We found HCH degradation in all cultures with  $\gamma$ -HCH, but no degradation in cultures with  $\beta$ - or  $\delta$ -HCH as the sole carbon source, suggesting that  $\delta$ - and  $\beta$ -HCH were not used as carbon or energy source when used in enrichment cultures.

After a first enrichment step in batch culture with  $\gamma$ -HCH-containing medium as described above, an aliquot of the culture was used to inoculate surface-sterilized maize seeds (Fig. 1). Corn seeds were surface-sterilized through rinsing with sterile deionized water and washed twice for 10 min with 70% (v/v) ethanol and once with 20% (v/v) bleach, followed by thorough rinsing with sterile deionized water. Surface-sterilized seeds were germinated on a Petri dish in the presence of penicillin G ( $500 \mu\text{g ml}^{-1}$ ) (Oehrle *et al.*, 2000) at  $30^\circ\text{C}$  for 2 days in the case of root colonization assays, or for 3 days if seedlings were to be transferred to a tube with soil or vermiculate. Around  $5 \mu\text{l}$  of bacterial culture containing about  $10^8$  colony-forming units (cfu)  $\text{ml}^{-1}$  was used to inoculate the seeds. After incubation for 1 h without shaking at  $30^\circ\text{C}$ , the seeds were washed and planted in

**Table 1.** Description of soil samples used to isolate HCH-degrading microorganisms: name and characterization of HCH-degrading *Sphingomonas* strains isolated in this study.

Soil sample source	Sample type	Isolate	HCH isomers degraded	Closest relative (16S rDNA)	Identities
Chemnitz-Schweizerthal 'Dump site', Germany	Bulk soil	DS-204B	$\alpha$ , $\beta$ , $\gamma$ , $\delta$	<i>Sphingomonas herbicidovorans</i> AB042233	1383/1386 (99%)
Chemnitz-Schweizerthal 'Old factory', Germany	Bulk soil	OF-178A	$\alpha$ , $\beta$ , $\gamma$ , $\delta$	<i>Sphingomonas</i> sp. DS3-1 AJ87127	1016/1022 (99%)
Chemnitz-Schweizerthal 'Old factory', Germany	Rhizosphere soil from grasses	GOF-203	$\alpha$ , $\beta$ , $\gamma$ , $\delta$	<i>Sphingomonas taejonensis</i> AF131297	1016/1022 (99%)
Bilbao, Ansio site, Spain	Rhizosphere soil from <i>Plantago lanceolata</i>	Ans-PL0	$\alpha$ , $\beta$ , $\gamma$ , $\delta$	<i>Sphingomonas</i> sp. D12 AB105809	519/530 (97%)
Bilbao, Ansio site, Spain	Bulk soil	Ans-PL2	$\alpha$ , $\beta$ , $\gamma$ , $\delta$	<i>Sphingomonas</i> sp. D12 AB105809	519/530 (97%)

The enrichment strategy, strain identification based on 16S rDNA sequencing and HCH isomers degraded by the isolates are described in the text.

pots containing vermiculite or used to determine the number of bacteria attached to the seed. Subsequently, the seedlings were collected and the root tips were removed. The attached rhizosphere bacteria were suspended in M9 buffer by vigorous shaking of the root tips with glass beads. Then the bacterial suspension was used to inoculate a new batch culture with  $\gamma$ -HCH. These bath cultures were used to inoculate corn seeds that were sown in vermiculite with HCH to favour enrichments in root-colonizing HCH degraders. After the second round, rhizosphere bacteria were spread on solid medium containing different HCH isomers. We found only small colonies (around 1 mm diameter) that appeared 1–2 weeks after inoculation. Putative HCH degraders were recognized as those surrounded by a clear zone in the HCH film.

The appearance of all colonies obtained with a given source of soil was identical, and we therefore kept a single isolate from each enrichment. Then we assayed  $\gamma$ -HCH degradation by gas chromatography-mass spectrometry (GC-MS) as described before (Böltner *et al.*, 2005). Each of the isolates (Table 1) was able to degrade the insecticide.

With this approach we isolated three *Sphingomonas* strains from the 'Old factory' soil sample from the Chemnitz-Schweizerthal site, and named the strains DS-204B, OF-178A and GOF-203. On the basis of 16S rDNA sequences, these strains belonged to the genus *Sphingomonas* and exhibited high identity with strains isolated previously (Table 1 and Fig. 2).

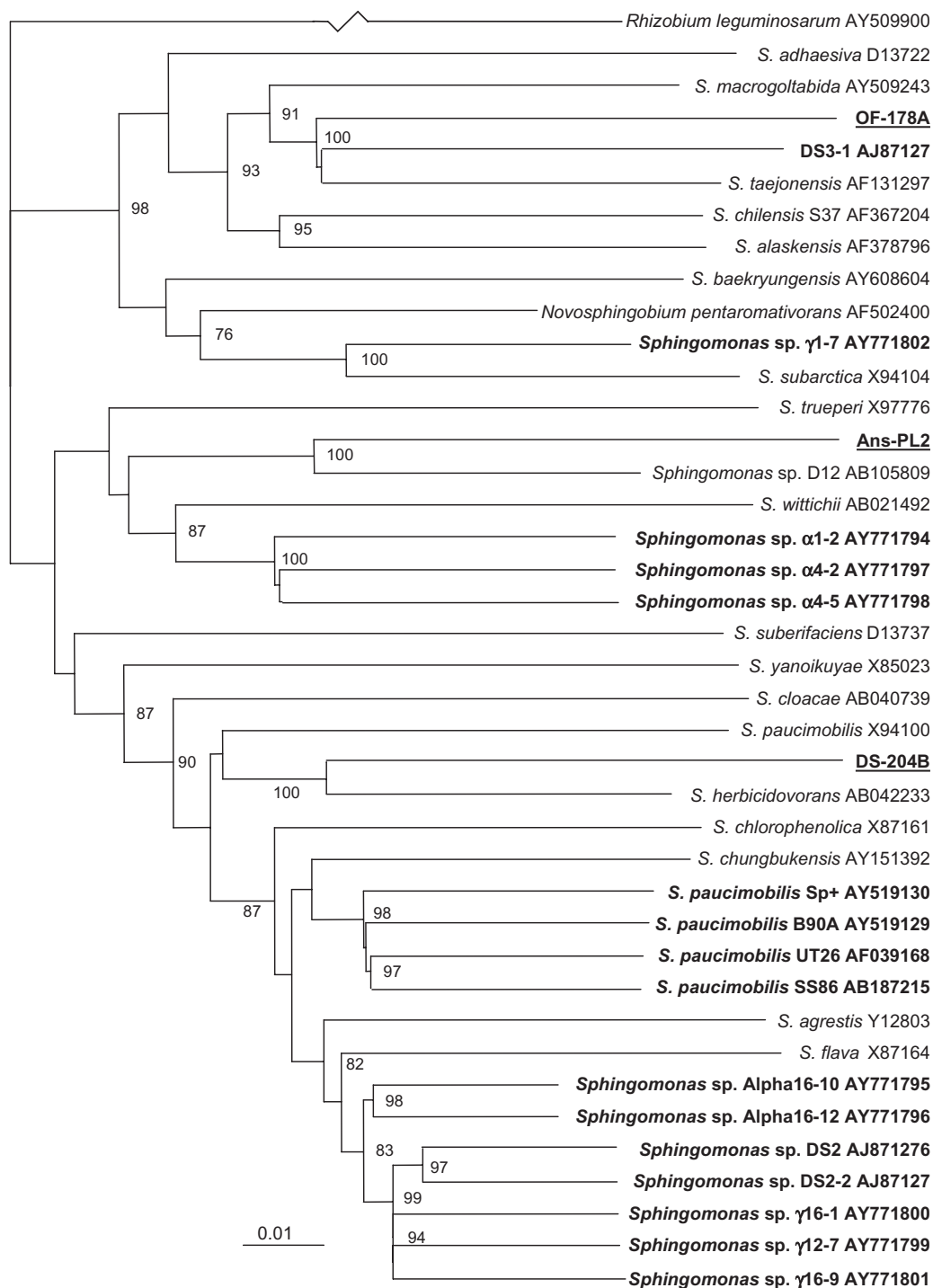
The same two-step enrichment with bulk soil and rhizosphere soil from *Plantago lanceolata* plants collected at the Ansio site in Bilbao (Spain) yielded two new isolates, called Ans-PL0 and Ans-PL2, which were identical based on analysis of their 16S rDNA sequences. These

members of the genus *Sphingomonas* were related to the HCH-degrading  $\alpha$ 1-2,  $\alpha$ 4-2 and  $\alpha$ 4-5 strains previously isolated by W.W. Mohn and V. de Lorenzo from the same location (pers. comm.). This suggested that the Ansio strain is well adapted to HCH degradation and root colonization.

The four newly isolated strains were subjected to degradation tests with different HCH isomers in minimal medium supplemented with low concentrations of amino acids. This source of carbon was chosen because these series of experiments were designed with the aim of testing rhizoremediation of lindane and because we previously showed that plant exudates are rich in amino acids and support bacterial growth (Vílchez *et al.*, 2000; Revelles *et al.*, 2004; Ramos-González *et al.*, 2005). We found that all four strains were capable of removing  $10 \mu\text{g ml}^{-1}$   $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -HCH within 96 h in 25 ml of batch culture assays carried out as described earlier by Böltner and colleagues (2005) (Table 1 and Fig. 3).  $\beta$ -HCH and  $\delta$ -HCH are apparently unable to support growth when used in enrichment cultures, although they can be removed by a number of strains isolated by enrichments with  $\gamma$ -HCH. This is particularly relevant as it offers the opportunity to remove chemicals that may not support microbial growth.

#### Presence of *lin* genes and IS6100 in HCH rhizodegraders

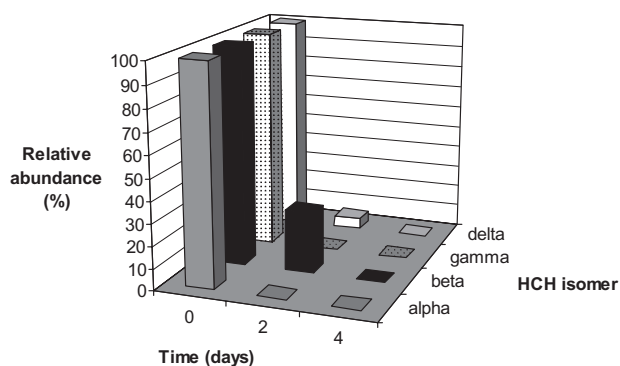
*Sphingomonas paucimobilis* UT26 and other HCH-degrading *Sphingomonas* strains from remote geographic locations use a well-characterized pathway encoded by the *lin* genes (Nagata *et al.*, 1999; Kumari *et al.*, 2002; Dogra *et al.*, 2004; Böltner *et al.*, 2005). Polymerase chain reaction with primers described previously (Böltner



**Fig. 2.** Phylogenetic tree of the *Sphingomonas* isolates identified in this study. The tree was constructed with the PILE UP program.

*et al.*, 2005) was used to generate amplicons for the structural genes *linA*, *linB*, *linC*, *linD*, *linE* and the transcriptional regulator *linR*, as well as the transposable element IS6100 supposedly involved in horizontal *lin* gene transfer (data not shown). We sequenced the *lin* genes of strains OF-178, DS204-B, GOF-203 and Ans-PL0, and found that

the sequences were 99–100% identical to the *lin* genes of UT26. This is consistent with the finding that *lin* genes spread via horizontal gene transfer among *Sphingomonas* strains (Dogra *et al.*, 2004; de Felipe *et al.*, 2005). The pathway for  $\gamma$ -HCH degradation in *S. paucimobilis* UT26 has been well characterized (Nagata *et al.*, 1999)



**Fig. 3.** Degradation of four HCH isomers by a culture of *Sphingomonas* GOF-203. Gas chromatography-mass spectrometry determination of lindane isomers was performed with an HP 6890 series gas chromatograph fitted with an HP 6890 mass selective detector, using a capillary column (HP-5MS, 30 m  $\times$  0.25 mm  $\times$  0.25  $\mu$ m). The injector and detector temperatures were 260°C and 280°C, respectively, and the carrier gas (helium) flow rate was 1.3 ml min<sup>-1</sup>. The temperature programme started at 50°C, then temperature was increased at a rate of 8°C min<sup>-1</sup> between 50°C and 190°C, followed by a final stage at 190°C for 2.5 min. Cultures of strain GOF-203 in modified M9 minimal medium (Abril *et al.*, 1989) were supplemented with 10  $\mu$ g ml<sup>-1</sup> of the indicated HCH isomer and 0.3% (w/v) casamino acids. At the indicated times the concentration of HCH isomers was determined as indicated above. HCH isomers used from the outside to the inside of the figure were  $\alpha$  (grey bars),  $\beta$  (black bars),  $\gamma$  (dotted bars) and  $\delta$  (open bars) respectively.

and we assume that the same pathway operates in the rhizodegrader *Sphingomonas* strains isolated in this study.

### Colonization rate of strains GOF-203 and Ans-PL0

To determine the colonization rate of two of the new isolates found in association with plant rhizospheres, we first determined the strains' ability to adhere to corn seeds. Assays were performed exactly as described by Espinosa-Urgel and colleagues (2000) with *Pseudomonas putida*. We found that the number of bacteria capable of adhering to corn seeds was in the range of 10<sup>4</sup>–10<sup>5</sup> cfu per seed, which is about one order of magnitude below that of *P. putida*, but at least one to two orders of magnitude higher than adherence of the UT26 strain that is a poor root colonizer. Ten pre-germinated corn seeds covered with each of the two *Sphingomonas* strains under study (GOF-203 and Ans-PL0) were transferred to Falcon tubes with sterile vermiculite to which 30 ml of plant nutritive solution was added. Plants were incubated at 24°C with light exposure for 12 h in a 24 h period. We then determined the number of cfu per gram of root. Colonization by both strains was uniform, with an average value of around  $5 \pm 1 \times 10^8$  cfu per gram of root. We also determined the cfu throughout the root system. We found that in the first centimetre of the root end tip, the number of cfu

was on the order of 10<sup>9</sup> per gram of root, whereas in the upper root segments the number of cfu ranged between  $8 \times 10^8$  and  $1.5 \times 10^8$  per gram of root. To a certain extent this indicates a differential root colonization rate, which may be attributable to differences in the availability of nutrients in different root segments (Loper and Henkels, 1997; Jaeger *et al.*, 1999; De Angelis *et al.*, 2005). This hypothesis is in agreement with findings in other microorganisms (Yang and Crowley, 2000; Marschner *et al.*, 2002; van Diepeningen *et al.*, 2005; Green *et al.*, 2006).

Similar assays to those reported above with vermiculite were performed in a loamy silt soil with the following characteristics (wt/wt): 38% sand, 43% silt and 19% clay; pH 7.9; organic matter content 2.1% and 8% CaCO<sub>3</sub> content. We found that GOF-203 and Ans-PL0 also colonize the root of corn plants, reaching almost 10<sup>8</sup> cfu per gram of root. Then we performed a series of assays in which we spiked the soil to about 0.5 mg of  $\gamma$ -HCH per gram of soil, a concentration often found in polluted soils, and incubated pre-germinated corn seeds covered with *Sphingomonas* GOF-203 and Ans-PL0. We monitored the number of cfu in bulk soil and associated to the plant roots, as well as lindane concentration in the soil. [Lindane concentration was determined in soil extracts obtained with a mixture of hexane/acetone (1 : 1) and subjected to microwave pulses (2 min at 600 W, plus 9 min at 1200 W) followed by GC-MS of the treated extract]. After 25 days of incubation with lindane, we found that in the root soil the number of *Sphingomonas* cells was around 10<sup>7</sup> cfu per gram of root. The concentration of lindane decreased by about 30% with respect to the initial concentration, whereas in the control inoculated with the strain in unplanted soil the number of cfu per gram of soil was below 10<sup>3</sup>, and the decrease in lindane concentration was less than 2%. In sterile planted soil, lindane decreased by less than 3%. In another control with uninoculated and unplanted soil, the decrease in lindane concentration after 25 days was less than 1%. These results indicated a positive effect of plants on survival of the *Sphingomonas* strains in soil, and an enhancement of their degradative properties.

Field studies will be needed to reveal the full potential of this approach, but it should be mentioned that phytoremediation and phytorhizoremediation have been shown to be efficient ways to remove nitro-organic compounds and polyaromatic hydrocarbons, and effective in immobilizing heavy metals in the environment (Kuiper *et al.*, 2001; 2004b; van Dillewijn *et al.*, 2007). We therefore propose that rhizoremediation holds great potential for the treatment of persistent and recalcitrant organic chemicals, although the efficiency of the process can be influenced by a number of factors that may require adjustment to achieve optimal results.



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## References

- Abril, M.A., Michán, C., Timmis, K.N., and Ramos, J.L. (1989) Regulator and enzyme specificities of the TOL plasmid-encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate of the pathway. *J Bacteriol* **171**: 6782–6790.
- Bachmann, A., Walet, P., Wijnen, P., de Bruin, W., Huntjens, J.L., Roelofsen, W., and Zehnder, A.J. (1988) Biodegradation of alpha- and beta-hexachlorocyclohexane in a soil slurry under different redox conditions. *Appl Environ Microbiol* **54**: 143–149.
- Böltner, D., Moreno-Morillas, S., and Ramos, J.L. (2005) 16S rDNA phylogeny and distribution of *lin* genes in novel hexachlorocyclohexane-degrading *Sphingomonas* strains. *Environ Microbiol* **7**: 1329–1339.
- De Angelis, K.M., Ji, P., Firestone, M.K., and Lindow, S.E. (2005) Two novel bacterial biosensors for detection of nitrate availability in the rhizosphere. *Appl Environ Microbiol* **71**: 8537–8547.
- De Felipe, K.S., Pampou, S., Javanovic, O.S., Pericone, C.D., Ye, S.F., Kalachikov, S., and Shuman, H.A. (2005) Evidence for acquisition of *Legionella* type IV secretion substrates via interdomain horizontal gene transfer. *J Bacteriol* **187**: 7716–7726.
- Doelman, P., Haanstra, L., Deruiter, E., and Slange, J. (1985) Rate of microbial-degradation of high-concentrations of alpha-hexachlorocyclohexane in soil under aerobic and anaerobic conditions. *Chemosphere* **14**: 565–570.
- Dogra, C., Raina, V., Pal, R., Suar, M., Lal, S., Gartemann, K.-H., et al. (2004) Organization of *lin* genes and IS6100 among different strains of hexachlorocyclohexane degrading *Sphingomonas paucimobilis*: evidence for horizontal gene transfer. *J Bacteriol* **186**: 2225–2235.
- Duque, E., Haidour, A., Godoy, F., and Ramos, J.L. (1993) Construction of a *Pseudomonas* hybrid strain that mineralizes 2,4,6-trinitrotoluene. *J Bacteriol* **175**: 2278–2283.
- Espinosa-Urgel, M., and Ramos, J.L. (2001) Expression of a *Pseudomonas putida* aminotransferase involved in lysine catabolism is induced in the rhizosphere. *Appl Environ Microbiol* **67**: 5219–5224.
- Espinosa-Urgel, M., Salido, A., and Ramos, J.L. (2000) Genetic analysis of functions involved in adhesion of *Pseudomonas putida* to seeds. *J Bacteriol* **182**: 2363–2369.
- Green, S.J., Inbar, E., Michel, F.C., Jr, Hadar, Y., and Minz, D. (2006) Succession of bacterial community during early plant development: transition from seed to root and effect of compost amendment. *Appl Environ Microbiol* **72**: 3975–3983.
- Jaeger, C.H., Lindow, S.E., Miller, W., Clark, E., and Firestone, M.K. (1999) Mapping of sugar and amino acid availability in soil around roots with bacterial sensors of sucrose and tryptophan. *Appl Environ Microbiol* **6**: 2685–2690.
- Jagnow, G., Haider, K., and Ellwardt, P.-C. (1977) Anaerobic dechlorination and degradation of hexachlorocyclohexane isomers by anaerobic and facultative anaerobic bacteria. *Arch Microbiol* **115**: 285–292.
- Kuiper, I., Bloemberg, G.V., and Lugtenberg, B.J. (2001) Selection of a plant–bacterium pair as a novel tool for rhizostimulation of polycyclic aromatic hydrocarbon-degrading bacteria. *Mol Plant Microb Interact* **4**: 1197–1205.
- Kuiper, I., Lagendijk, E.L., Bloemberg, G.V., and Lugtenberg, B.J. (2004a) Rhizoremediation: a beneficial plant–microbe interaction. *Mol Plant Microbe Interact Rev* **7**: 6–15.
- Kuiper, I., Lagendijk, E.L., Pickford, R., Derrick, J.P., Lamers, G.E., Thomas-Oates, J.E., et al. (2004b) Characterization of two *Pseudomonas putida* lipopeptide biosurfactants, putisolvin I and II, which inhibit biofilm formation and break down existing biofilms. *Mol Microbiol* **51**: 97–113.
- Kumari, R., Subudhi, S., Suar, M., Dhingra, G., Raina, V., and Dogra, C., et al. (2002) Cloning and characterization of *lin* genes responsible for the degradation of hexachlorocyclohexane isomers by *Sphingomonas paucimobilis* strain B90. *Appl Environ Microbiol* **68**: 6021–6028.
- Loper, J.E., and Henkels, M.D. (1997) Availability of iron to *Pseudomonas fluorescens* in rhizosphere and bulk soil evaluated with an ice nucleation reporter gene. *Appl Environ Microbiol* **63**: 99–105.
- MacCrae, I.C., Raghu, K., and Bautista, E.M. (1969) Anaerobic degradation of the insecticide lindane by *Clostridium* sp. *Nature* **221**: 859–860.
- Marschner, P., Neumann, G., Kania, A., Weiskopf, L., and Lieberei, R. (2002) Spatial and temporal dynamics of the microbial community structure in the rhizosphere of cluster roots of white lupin (*Lupinus albus* L.). *Plant Soil* **246**: 167–174.
- Mohn, W.W., Mertens, B., Neufeld, J.D., Verstraete, W., and de Lorenzo, V. (2006) Distribution and phylogeny of hexachlorocyclohexane degrading bacteria in soils from Spain. *Environ Microbiol* **8**: 60–68.
- Molina, L., Ramos, C., Duque, E., Ronchel, M.C., García, J.M., Wyke, L., and Ramos, J.L. (2000) Survival of *Pseudomonas putida* KT2440 in soil and in the rhizosphere of plants under greenhouse and environmental conditions. *Soil Biol Biochem* **32**: 315–321.
- Nagata, U., Miyauchi, K., and Takagi, M. (1999) Complete analysis of genes and enzymes for  $\gamma$ -hexachlorocyclohexane degradation in *Sphingomonas paucimobilis* UT26. *J Indust Microbiol Biotech* **23**: 380–390.
- Neufeld, J.D., Mohn, W.W., and de Lorenzo, V. (2006) Composition of microbial communities in hexachlorocyclohexane (HCH) contaminated soils from Spain revealed with a habitat-specific microarray. *Environ Microbiol* **8**: 126–140.
- Oehle, N.W., Kaar, D.B., Kremer, R.J., and Emerich, D.W. (2000) Enhanced attachment of *Bradyrhizobium japonicum* to soybean through reduced root colonization of internally seedborne microorganisms. *Can J Microbiol* **46**: 600–606.
- Ramos-González, M.I., Campos, M.J., and Ramos, J.L.

- (2005) Analysis of *Pseudomonas putida* KT2440 gene expression in the maize rhizosphere: *in vitro* expression technology capture and identification of root-activated promoters. *J. Bacteriol* **187**: 4033–4041.
- Revelles, O., Espinosa-Urgel, M., Molin, S., and Ramos, J.L. (2004) The *davDT* operon of *Pseudomonas putida*, involved in lysine metabolism, is induced in response to the pathway intermediate  $\delta$ -aminovaleric acid. *J Bacteriol* **186**: 3439–3446.
- Simonich, S.L., and Hites, R.A. (1995) Global distribution of persistent organochlorine compounds. *Science* **269**: 1851–1854.
- Tu, C.M. (1976) Utilization and degradation of lindane by soil microorganisms. *Arch Microbiol* **108**: 259–263.
- Van Diepeningen, A.D., de Vos, O.J., Zelenev, V.V., Semenov, A.M., and Van Bruggen, A.H. (2005) DGGE fragments oscillate with or counter to fluctuations in cultivable bacteria along wheat roots. *Microbiol Ecol* **50**: 506–517.
- Van Dillewijn, P., Caballero, A., Paz, J.A., González-Pérez, M.M., Oliva, J.M., and Ramos, J.L. (2007) Bioremediation of 2,4,6-trinitrotoluene under field conditions. *Environ Sci Technol* **41**: 1378–1383.
- Vilchez, S., Molina, L., Ramos, C., and Ramos, J.L. (2000) Proline catabolism by *Pseudomonas putida*: cloning, characterization, and expression of the *put* genes in the presence of root exudates. *J Bacteriol* **182**: 91–99.
- Wada, H., Senoo, K., and Takai, Y. (1989) Rapid degradation of gamma-HCH in planted soil after multiple applications. *Soil Sci Plant Nutr* **35**: 71–77.
- Yang, C.H., and Crowley, D.E. (2000) Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. *Appl Environ Microbiol* **66**: 345–351.