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 γ-HCH degraders. The γ-HCH-degrading microbes were attached in mass to corn seeds sown in soil with γ-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 γ-HCH and inoculation of corn seeds were repeated. We then isolated bacteria on M9 minimal medium with γ-HCH from root tips. We were able to isolate four Sphingomonas strains, all of which degraded α-, β-, γ- and δ-HCH. Two of the strains were particularly good colonizers of corn roots, reaching high cell density in vegetated soil and partly removing γ-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 [γ-hexachlorocyclohexane (HCH)] and its non-insecticidal isomers (α-, β-, δ-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 γ-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 α-, β-, γ- and δ-HCH) which differ in their toxicity, water solubility (and thus mobility and bioavailability) and recalcitrance (Mohn et al., 2006). The γ-isomer has insecticidal effects, whereas β-HCH is less harmful but extremely recalcitrant to aerobic degradation. Several groups have found that the δ-isomer inhibits bacterial growth, thus possibly limiting bioremediation (Dogra et al., 2004; Mohn et al., 2006; our unpublished results).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
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 colleagues (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 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°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 $\mu$g ml$^{-1}$) (Oehrle et al., 2000) at 30°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 vermiculite. Around 5 $\mu$l of bacterial culture containing about 10$^6$ colony-forming units (cfu) ml$^{-1}$ was used to inoculate the seeds. After incubation for 1 h without shaking at 30°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    | α, β, γ, δ           | Sphingomonas herbicidovorans      | 1383/1386 (99%) |
| Chemnitz-Schweizerthal 'Old factory', Germany | Bulk soil          | OF-178A    | α, β, γ, δ           | Sphingomonas sp. DS3-1            | 1016/1022 (99%) |
| Chemnitz-Schweizerthal 'Old factory', Germany | Rhizosphere soil from grasses | GOF-203    | α, β, γ, δ           | Sphingomonas taejonensis           | 1016/1022 (99%) |
| Bilbao, Ansio site, Spain          | Rhizosphere soil from Plantago lanceolata | Ans-PL0  | α, β, γ, δ           | Sphingomonas sp. D12              | 519/530 (97%) |
| Bilbao, Ansio site, Spain          | Bulk soil          | Ans-PL2    | α, β, γ, δ           | Sphingomonas sp. D12              | 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.

pot 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 γ-HCH. These bath cultures were used to inoculate corn seeds that were sown in vermiculite with HCH to favor 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 γ-HCH degradation by gas chromatography–mass spectrometry (GC-MS) as described before (Böttner 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 α1-2, α4-2 and α4-5 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 μg ml⁻¹ α-, β-, γ- and γ-HCH within 96 h in 25 ml of batch culture assays carried out as described earlier by Böttner and colleagues (2005) (Table 1 and Fig. 3). β-HCH and δ-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 γ-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; Böttner et al., 2005). Polymerase chain reaction with primers described previously (Böttner
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 γ-HCH degradation in S. paucimobilis UT26 has been well characterized (Nagata et al., 1999).
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 × 0.25 mm × 0.25 μm). The injector and detector temperatures were 260°C and 280°C, respectively, and the carrier gas (helium) flow rate was 1.3 ml min⁻¹. The temperature programme started at 50°C, then temperature was increased at a rate of 8°C min⁻¹ 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 μg ml⁻¹ 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 α (grey bars), β (black bars), γ (dotted bars) and δ (open bars) respectively.

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 10⁴−10⁵ 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 ± 1 × 10⁶ 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 10⁹ per gram of root, whereas in the upper root segments the number of cfu ranged between 8 × 10⁸ and 1.5 × 10⁹ 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% CaCO₃ content. We found that GOF-203 and Ans-PL0 also colonize the root of corn plants, reaching almost 10⁶ cfu per gram of root. Then we performed a series of assays in which we spiked the soil to about 0.5 mg of γ-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 10⁷ 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 10³, 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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