RESEARCH ARTICLE

Bioaugmentation with *Pseudomonas* sp. strain MHP41 promotes simazine attenuation and bacterial community changes in agricultural soils

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Abstract

Bioremediation is an important technology for the removal of persistent organic pollutants from the environment. Bioaugmentation with the encapsulated *Pseudomonas* sp. strain MHP41 of agricultural soils contaminated with the herbicide simazine was studied. The experiments were performed in microcosm trials using two soils: soil that had never been previously exposed to *s*-triazines (NS) and soil that had > 20 years of *s*-triazine application (AS). The efficiency of the bioremediation process was assessed by monitoring simazine removal by HPLC. The simazine-degrading microbiota was estimated using an indicator for respiration combined with most-probable-number enumeration. The soil bacterial community structures and the effect of bioaugmentation on these communities were determined using 16S RNA gene clone libraries and FISH analysis. Bioaugmentation with MHP41 cells enhanced simazine degradation and increased the number of simazine-degrading microorganisms in the two soils. In highly contaminated NS soil, bioaugmentation with strain MHP41 was essential for simazine removal. Comparative analysis of 16S rRNA gene clone libraries from NS and AS soils revealed high bacterial diversity. Bioaugmentation with strain MHP41 promoted soil bacterial community shifts. FISH analysis revealed that bioaugmentation increased the relative abundances of two phylogenetic groups (*Acidobacteria* and *Planctomycetes*) in both soils. Although members of the Archaea were metabolically active in these soils, their relative abundance was not altered by bioaugmentation.

Introduction

Simazine [2-chloro-4,6-bis(ethylamine)-*s*-triazine] is a member of the *s*-triazine herbicides and has been used worldwide for the control of broadleaved weeds in agriculture, forestry and noncrop soils (i.e. roadsides, railways). Simazine and related *s*-triazines, such as atrazine, may persist in the environment for years, depending on climatic and edaphic factors such as temperature, soil moisture and organic carbon content, and the degradation activity of the soil native microbiota (Gunasekara *et al.*, 2007). As a consequence of its widespread application, this pesticide is frequently detected as a pollutant in both surface and groundwater (Rousseaux *et al.*, 2003). *s*-Triazines are potential human carcinogens and endocrine disrupters (Birnbaum & Fenton, 2003; Hayes *et al.*, 2006), and recently, their use has been banned in EU member states (European Union, 2004). However, in America, these herbicides are still commonly used for the control of annual weeds in fruit plantations such as avocado, citruses and vineyards, and in forestry (Flores *et al.*, 2009).

Microorganisms play an important role in the removal of persistent organic pollutants (POPs) from the environment (Philp *et al.*, 2005). Natural microbial degradation of *s*-triazines has been reported in soils that have a history of

s-triazine herbicide application (Ostrofsky *et al.*, 1997; Yassir *et al.*, 1999; Rousseaux *et al.*, 2003; Morán *et al.*, 2006). However, the decontamination of previously unexposed soils requires the enhancement of the natural degradation (Rousseaux *et al.*, 2003). Bioaugmentation has been proposed as an effective and low-cost bioremediation technology to remove POPs, such as simazine and atrazine, and to minimize their dispersion to nonagricultural environments. In this respect, *s*-triazine-degrading bacteria have been isolated from soil, but only a few of these strains have been used for bioaugmentation (Struthers *et al.*, 1998; Newcombe & Crowley, 1999; Topp, 2001; Rousseaux *et al.*, 2003). Selection of the bacterial strain is critical for the success of bioaugmentation. A good candidate for bioaugmentation is *Pseudomonas* sp. strain MHP41, which is one of the most efficient *s*-triazine-degrading bacterial strains described (Hernández *et al.*, 2008). Bioaugmentation is often not successful (Gentry *et al.*, 2004; Thompson *et al.*, 2005), a main problem being the survival of cells added in the soil. To protect the inoculated bacteria and to improve bioremediation, encapsulation of cells has been proposed (Bouchez *et al.*, 2000; Vogel & Walter, 2002; Gentry *et al.*, 2004).

Microbial communities involved in the natural restoration of polluted environments have been analysed using culture-independent techniques (Nogales *et al.*, 1999; Paul *et al.*, 2006). Approaches such as 16S rRNA gene clone library construction and *in situ* hybridization with specific oligonucleotide probes (FISH) have proved useful for assessing the extent of soil microbial diversity and its dynamics (Nogales *et al.*, 2001; Barra-Caracciolo *et al.*, 2005). Although bioremediation of contaminated sites has been applied as an attractive, low-cost alternative to physical remediation techniques, the changes occurring in native microbial communities as a response to environmental perturbation or the impact of microorganisms introduced remain poorly known. A study of the structure of the autochthonous microbial communities and their shifts is required to understand the effects of exogenously added microorganisms on the existing microbial community composition.

In the present study, *Pseudomonas* sp. strain MHP41 was used to evaluate the bioaugmentation processes in agricultural soils contaminated with simazine. Strain MHP41 efficiently degrades simazine and is capable of growing rapidly using simazine as the sole nitrogen source, yielding a high biomass (Hernández *et al.*, 2008). This strain possesses the *s*-triazine catabolic *atzA*, *atzB*, *atzC*, *atzD*, *atzE* and *atzF* genes. Experiments were carried out in microcosms using two soils with different histories of *s*-triazine application, and strain MHP41 was introduced as an encapsulated biocatalyst. Simazine attenuation was followed in parallel by monitoring the bacterial community structure by 16S rRNA gene clone libraries and their changes by FISH analysis. Archaea were also quantified by FISH in the two soils.

Materials and methods

Chemicals

Commercial simazine (Gesatop 90WG, 90% pure) was purchased from Syngenta (Greensboro, NC). Simazine (99% pure) and analytical standard simazine (> 99% pure) were purchased from Atanor S.A. (Buenos Aires, Argentina) and Dr Ehrensdorfer-Schäfers GmbH (Augsburg, Germany), respectively.

Bacterial growth and encapsulation procedure

Pseudomonas sp. strain MHP41 was grown in minimal medium using 100 mg L^{-1} of simazine as the sole nitrogen source (Hernández *et al.*, 2008). The minimal medium (pH 7.0) contained (L^{-1}): 10 mL of phosphate-buffered saline (PBS) stock solution (70 g $Na_2HPO_4 \cdot 2H_2O$, 28 g K_2HPO_4, 5 g NaCl), 2.95 g L^{-1} sodium succinate as the sole carbon source and 1 mL of trace element stock solution (5 g $MgSO_4 \cdot 7H_2O$, 0.5 g $FeSO_4 \cdot 7H_2O$, 0.25 g $MnSO_4 \cdot H_2O$, 0.32 g $ZnCl_2$, 0.033 g $CaCl_2 \cdot 2H_2O$, 0.018 g $CuSO_4 \cdot 5H_2O$, 0.015 g $CoCl_2 \cdot 6H_2O$, 0.325 g H_3BO_3, 0.5 g EDTA, 7.3 mL HCl 37%). Cultures were incubated at 28 °C with agitation (180 r.p.m.). Bacterial growth was monitored by measuring the turbidity at 600 nm ($turbidity_{600\,nm}$). Viability was determined by counting CFUs on trypticase soy agar (Difco, MD). Encapsulation of strain MHP41 was performed using an alginate matrix. Cells of strain MHP41, grown until $turbidity_{600\,nm} \sim 0.8$ (1.52×10^8 CFU mL^{-1}), were harvested by centrifugation (10 000 ***g*** for 20 min at 4 °C) and washed twice with 20 mL of saline buffer solution (50 mM sodium phosphate, pH 7.0; 0.5 g L^{-1} NaCl). Finally, the cells ($turbidity_{600\,nm} \sim 0.8$) were resuspended in 1% w/v sterile sodium alginate solution (Loba Chemie Ltd, Mumbai, India). The mixture was added drop by drop into 400 mL of 50 mM $CaCl_2$ sterile solution using a 25-mL sterile syringe. Alginate beads containing cells of the strain MHP41 were hardened in the $CaCl_2$ solution for 30 min. Beads were recovered and washed with sterile water before addition to soil.

Soil characterization and bioaugmentation experiments in microcosms

Soil samples were collected from the surface stratum (0–20 cm depth) of two agricultural fields located in central Chile (Quillota province, Aconcagua valley). Both soils have been characterized by Flores *et al.* (2009). A soil was collected next to a flowering plant (*Dianthus caryophyllus*) plantation from an orchard without a history of *s*-triazine application (NS soil). This is a neutral (pH 7.2) and clay–loam soil, with a nitrogen content of 1.8 g kg^{-1}, a C/N

ratio of 11.5 and an organic matter (OM) content of 3.5%. A second soil was collected from an avocado (*Persea americana*) plantation that has been annually treated for >20 years with simazine (3.5 kg simazine ha^{-1}), as a pre-emergent herbicide (AS soil). AS soil is a slightly acidic (pH 6.4) and loam soil with a nitrogen content of 2.0 g kg^{-1} soil, a C/N ratio of 12.8 and a higher OM content (8.5%). At the time of collection, residual simazine was not detected either in NS or in AS soil. For microcosms, each soil was homogeneously treated with commercial simazine to obtain different initial concentrations of 3.5 kg simazine ha^{-1} ($\sim$10 mg kg^{-1}, low level), according to standard farming practices, and 35 kg simazine ha^{-1} ($\sim$100 mg kg^{-1}, high level). Each microcosm contained 150 g of soil in a 500-mL sterile flask. Microcosms were bioaugmented by inoculating encapsulated strain MHP41 (0.5 g of alginate beads containing cells of strain MHP41 were added to 150 g soil to reach a final concentration of 1×10^8 cells g^{-1} dry soil) every 3–4 days. The soils and the alginate beads were thoroughly mixed using a sterilized spatula. In order to reduce contamination risks due to manipulation, all flasks were covered with a polyethylene foil and sampled under aseptic conditions. Microcosms were incubated at room temperature for 28 days. In control microcosms, soil was inoculated with sterile alginate beads. All treatments were performed in triplicate. Soil moisture was periodically controlled using an infrared absorption mass balance (Sartorius MH30) and maintained by sprinkling sterile water at 40–50% of the water-holding capacity. For microbial and chemical analysis, soil samples (50 g) were maintained at 4 °C. For bacterial community analysis, soil samples (10 g) were collected in sterile tubes and kept frozen at -20 °C. Statistical analysis was performed using two-way ANOVA with treatment and incubation time as factors (SYSTAT 6.1 for Windows). Differences were considered to be significant at $P \leq 0.05$.

Herbicide quantification in soil

Simazine in soil was quantified using HPLC (Agulló *et al.*, 2007; Martínez *et al.*, 2007). For simazine extraction from soil samples, 10 g of each soil sample was mixed with 20 mL of methanol solution (80% v/v, pH 2) and incubated for 2 h at room temperature. The soil particles were then separated by centrifugation (10 000 ***g*** for 1 min) and the supernatant (20 μL) was analysed for simazine quantification using a System Gold chromatograph (Beckman, Germany) equipped with a diode array detector and an RP-C18/Lichrospher 5-μm column (Supelco). Simazine was identified and quantified by comparison with an authentic standard (Flores *et al.*, 2009). The simazine removal data in soil were fitted to mathematical models using SIGMAPLOT 10.0 software (Science Software GmbH, Germany).

Enumeration of simazine-degrading microorganisms

The aerobic cultivable *s*-triazine catabolic potential of soil microbiota was determined using the respiration indicator 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) and the most-probable-number (MPN) enumeration in microtitre plates, as described previously (Dinamarca *et al.*, 2007), but with some modifications. Briefly, 1 g of soil sample was added to 9 mL of mineral medium (without simazine) and the tubes were vigorously shaken for 2 h at room temperature. Tenfold serial dilutions to extinction were prepared (in triplicate), and 100-μL aliquots of each dilution were used to inoculate the MPN microplates. *Pseudomonas* sp. strain MHP41 was used as a positive control. Cells of strain MHP41 previously killed by heating at 100 °C for 10 min were used as a negative control. The microtitre plates were incubated at 30 °C without shaking for 5 days. TTC (20 μL of a 1% w/v, TTC solution) was then added to each well. After 24 h of incubation at 30 °C, positive wells were determined based on the visualization of TTC formazan production (red colour). The number of cells per gram of dry soil was calculated using an MPN calculator program as described (Dinamarca et al., 2007).

DNA extraction from soils

Community DNA was obtained from bulk soil (10 g of soil was collected in sterile tubes and kept frozen at -20 °C). DNA was extracted from 1 g of a thawed and homogenized soil sample, as described previously (Nogales *et al.*, 2001), but with some modifications. Briefly, cells were disrupted by grinding the soil sample embedded in liquid nitrogen in a ceramic mortar. Subsequently, DNA was purified using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Extracted DNA (concentrations of 70–90 μg μL^{-1}) was frozen at -20 °C until PCR amplification. Aliquots of purified DNA were analysed by electrophoresis on a 0.8% (w/v) agarose gel and compared with mass standards.

Amplification, cloning and sequence analysis of 16S rRNA genes

For library construction, the universal primer pair for Bacteria GM3 (5′-AGAGTTTGATCMTGGC-3′) and GM4 (5′-TACCTTGTTACGACTT-3′) was used. DNA was diluted (1 : 50) and three independent PCR reactions were carried out as reported previously (Zaballos *et al.*, 2005), decreasing the annealing temperature to 44 °C. Reconditioning reactions of 16S rRNA gene amplicons were performed as described (Thompson *et al.*, 2002). PCR products were mixed and purified using a Qiaquick PCR purification kit (Qiagen). Clone libraries were constructed using the pGEM-

T Easy Vectors System (Promega, MD) according to the manufacturer's recommendations. The plasmids of positive clones were extracted directly from a colony and amplified using the primer pair pUC/M13F and pUC/M13R provided with the kit. Finally, positive PCR products were purified (Qiaquick PCR purification kit, Qiagen) and sequenced using the primer pair GM3, 518F (5′-CCAGCAGCCGCGG TAAT-3′) and GM4, according to BigDye Terminator v3.1 Cycle Sequencing Kit procedures (Applied Biosystems, CA). Ninety-nine 16S rRNA gene clones (51 from NS soil and 48 from AS soil) were partially sequenced (500–800 bp). Twenty-one almost-complete 16S rRNA gene sequences were obtained (1200–1500 bp). The sequences were submitted to the GenBank Nucleotide Sequence Database. Sequencing was performed by the 'Servicios Científico-Técnicos' of the Balearic Islands University (Mallorca, Spain) using an ABI 310 Capillary DNA Sequencer (Applied Biosystems).

Sequence analyses

Sequences were revised and corrected using SEQUENCHER 4.8 software (Gene Codes Corp., MI). The RDP CHIMERA-CHECK program (Cole *et al.*, 2003) was used to predict chimera clones. Sequences were automatically aligned and manually corrected using the ARB software package (Ludwig *et al.*, 2004). The aligned sequence dataset of the ARB-SILVA project (Pruesse *et al.*, 2007) was used as a reference. Partial sequences were inserted using the ARB parsimony tool into a previously reconstructed neighbour-joining tree of representative almost-complete 16S rRNA gene sequences. Sequences were grouped into operational taxonomic units (OTUs), assuming that one OTU includes sequences with similarity values $\geq$97% (Zaballos *et al.*, 2005).

FISH analysis and quantification of total microorganisms

From each soil microcosm, 10 g of soil was fixed in 1 × PBS / formaldehyde 4% for 2–6 h at 4 °C. In order to eliminate formaldehyde, the suspension was washed twice with 1 × PBS by repeated centrifugation (6000 ***g*** for 5 min). The pellet was resuspended in 1 × PBS/ethanol 1 : 1 and kept at − 20 °C, until used for 4′,6-diamidino-2-phenylindole (DAPI) counts and hybridization experiments. Microorganisms were detached from soil particles by sonication using a Soniprep 150 (MSE Scientific Instruments, Fisons Limited, UK) operating at 20 kHz and applying five pulses of 10 s each. Then, samples were diluted in 20 mL of 1 × PBS for homogenization, and subsequently filtered throughout a 0.22-μm pore size, 16-mm-diameter filter (Isopore GTTP, Millipore, MA). For FISH analysis, a piece of filter was cut (usually one-quarter) and hybridization with oligonucleotide probes (Table 1) was performed as described (Zarda *et al.*, 1997). Briefly, filters were incubated in 18 μL of the hybridization buffer [0.9 M NaCl, 20 mM Tris-HCl, 0.01% sodium dodecyl sulphate (SDS)] in the presence of 5.5 ng μL^{-1} of each probe and the probe-specific formamide concentration (Table 1) at 46 °C for 2 h. After hybridization, the filters were washed in 50 mL of buffer solution containing 20 mM Tris-HCl, 5 mM EDTA, 0.01% SDS and either 900, 450 or 80 mM NaCl depending on the formamide concentration during hybridization (0–5%, 10% and 35%, respectively), for 15 min at 48 °C, subsequently rinsed with distilled water and air-dried. Probes, competitor probes and formamide concentrations are specified in Table 1. Probe specificities were analysed using the PROBE MATCH tool of the RDP project II (http://rdp.cme.msu.edu/), and probe hybridization conditions were verified via the ProbeBase database (http://www.microbial-ecology.net/probebase). For quantification of total microbial abundances, a piece of filter was cut and stained with DAPI (1 μg mL^{-1}) for 90 s and further washed with distilled water (5 s). Filters were finally immersed in absolute ethanol (5 s) in order to remove unspecific DAPI binding to avoid background colour. The filters were mounted onto microscope slides and a 4 : 1 mixture of Citifluor (Citifluor Ltd, UK) and Vecta Shield solution (Vector Laboratories, CA) was added. The preparations were stored at − 20 °C until visualization. An epifluorescence microscope (Zeiss Axiophot AX10, Germany) was used for counting. Values are reported as means calculated from 15 to 20 randomly chosen microscopic fields corresponding to 600–800 DAPI-stained cells and expressed as cells g^{-1} dry soil.

Results and discussion

To remediate POP-contaminated soils, bioaugmentation may be based on the introduction of either autochthonous or allochthonous microorganisms. These microorganisms can be used as biocatalysts for the degradation of the pollutants (Vogel, 1996; Gentry *et al.*, 2004). However, the success of a bioremediation process is restricted by various factors that often result in low efficiencies. Abiotic factors such as pH, extremes in temperature, water content and nutrient availability may influence the survival of introduced microorganisms. In addition, predation and competition by indigenous microbiota could decrease the added organisms (Bouchez *et al.*, 2000; Gentry *et al.*, 2004; Thompson *et al.*, 2005; Manzano *et al.*, 2007). In this report the effectiveness of *Pseudomonas* sp. strain MHP41 as a biocatalyst for the removal of simazine in two agricultural soils was studied. To prevent stress or predation of bacterial inocula, cells of strain MHP41 were encapsulated in alginate beads (Bouchez *et al.*, 2000; Gentry *et al.*, 2004; Vancov *et al.*, 2007). In fact, in one of these soils (AS soil), predation of added bacteria by protozoa was reported previously

Table 1. List of oligonucleotide probes and hybridization conditions used for FISH

Probe	Sequence (5′–3′)*	Target RNA gene†	Specificity	Competitor	Formamide (% v/v)	Reference	Accession no.‡
EUB (I)	GCTGCCTCCCGTAGGAGT	16S	Most bacteria	No	35	Daims *et al.* (1999)	pB-00159
EUB (II)	GCAGCCACCCGTAGGTGT		Supplement to EUB 338: *Planctomycetales*				pB-00160
EUB (III)	GCTGCCACCCGTAGGTGT		Supplement to EUB 338: *Verrucomicrobiales*				pB-00161
ALF1B	CGTTCGYTCTGAGCCAG	16S	*Alphaproteobacteria*, some other *Proteobacteria* and *Verrucomicrobiales*	No	10	Manz *et al.* (1992)	pB-00017
BET42a	GCCTTCCCACTTCGTTT	23S	*Betaproteobacteria*	GAM42a	35	Manz *et al.* (1992)	pB-00034
GAM42a	GCCTTCCCACATCGTTT	23S	*Gammaproteobacteria*	BET42a	35	Manz *et al.* (1992)	pB-00174
PLA886	GCCTTGCGACCATACTCCC	16S	*Planctomycetales*	cPLA886	35	Neef *et al.* (1998)	pB-00284
HoAc1402	CTTTCGTGATGTGACGGG	16S	*Acidobacteria*	cHoAc1402	0–5	Juretschko *et al.* (2002)	pB-00183
PS56a	GCTGGCCTAGCCTTC	23S	*Pseudomonadales*	No	0–5	Schönduve *et al.* (1996)	pB-00289
ARCH915	GTGCTCCCCCGCCAATTCCT	16S	Archaea	No	10	Stahl & Amann (1991)	pB-00027

*All probes were marked with the Cy3 fluorochrome (Thermo Electron Corporation, Germany).
†16S or 23S rRNA genes of *Escherichia coli*.
‡Accession numbers were obtained from the ProbeBase database (http://www.microbial-ecology.net/probebase/).

(Manzano *et al.*, 2007). Bioaugmentation was studied in agricultural soils with different physical properties and a history of herbicide application. NS soil is a clay–loam soil with a 3.5% OM content and that not have been treated with *s*-triazine herbicides, whereas AS soil is a loam soil with an 8.5% OM content and a long history of *s*-triazine application. Clay minerals and OM are involved in the retention of pesticides in soil, influencing their bioavailability and biological degradation (Flores *et al.*, 2009). In both soils, sorption of simazine was moderate and reversible (Flores *et al.*, 2009), and therefore simazine was available for microbial degradation.

Effect of bioaugmentation with strain MHP41 on simazine removal in soils

In a first approach, bioaugmentation trials were carried out in microcosms using NS and AS soils spiked with $\sim$10 mg kg^{-1} simazine, according to the standard farming practices for weed control. Figure 1a shows the dynamics of simazine attenuation in NS soil contaminated with $\sim$10 mg kg^{-1} simazine and the improvement in removal due to bioaugmentation. In the control treatment, the simazine concentration decreased $\sim$55% within 28 days at a degradation rate of 0.20 mg kg^{-1} day^{-1}. Bioaugmentation with *Pseudomonas* sp. MHP41 increased simazine removal to 89%. Addition of strain MHP41 significantly reduced the half-life of simazine and increased the degradation rate twofold (0.40 mg kg^{-1} day^{-1}) ($P \leq 0.05$). The numbers of aerobic cultivable simazine-degrading microorganisms in these soil microcosms are shown in Fig. 1b. Application of a low level of simazine ($\sim$10 mg kg^{-1}) did not increase the number of cultivable simazine-degrading cells in NS soil during the first 3 weeks of the experiment. Only after 4 weeks were cultivable simazine-degrading cells detected. Of note, bioaugmentation with encapsulated strain MHP41 significantly increased ($P \leq 0.05$) the number of cultivable simazine-degrading microbial cells (4.5×10^2 cells g^{-1}). In agreement, a low number of indigenous atrazine-degrading microorganisms was reported by Struthers *et al.* (1998) in soils nonadapted to atrazine, but these microorganisms increased after inoculation of a biocatalyst into the soil.

Figure 2a shows simazine removal and the dynamics of the cultivable simazine-degrading microorganisms in the AS soil during bioaugmentation experiments. Simazine attenuation was observed in this soil (degradation rate of 0.45 mg kg^{-1} day^{-1}). However, bioaugmentation reduced the simazine half-life from 11 to 3 days and increased the simazine removal rate to 0.80 mg kg^{-1} day^{-1} ($P \leq 0.05$). Simazine application increased the cultivable simazine-degrading microorganisms in the AS soil from undetectable (day 0) to 2.3×10^3 cells g^{-1} after 23 days (Fig. 2b). This increase of simazine-degrading microorganisms was much higher than that observed in NS soil. This might be an

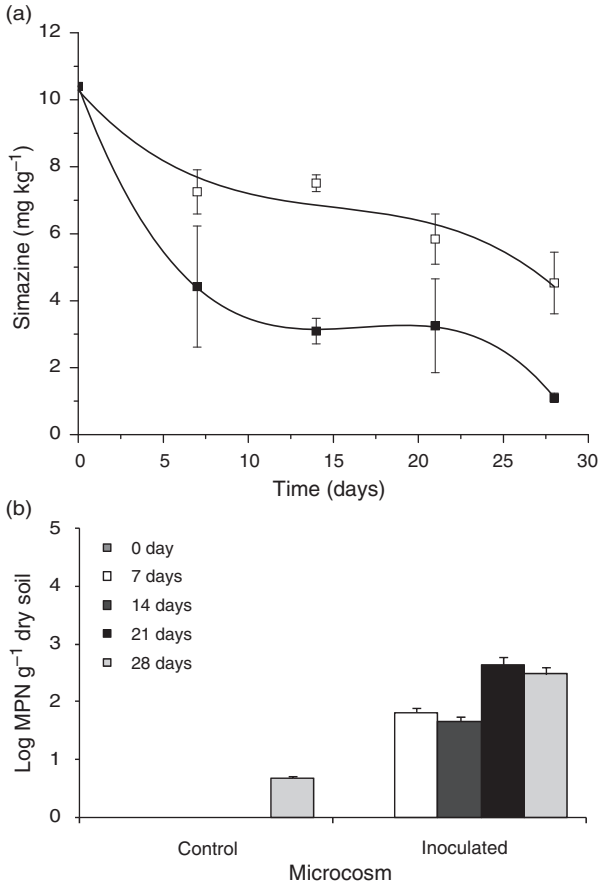

Fig. 1. Effect of bioaugmentation with *Pseudomonas* sp. strain MHP41 in a soil without a history of *s*-triazine application (NS). The soil was spiked with ~10 mg kg^{-1} simazine. (a) Simazine removal as determined by HPLC. Control treatments (□) were performed using sterile alginate beads. Inoculation treatments (■) were performed with encapsulated cells of the strain MHP41. (b) Simazine-degrading microbiota as estimated by MPN. Each point represents the average of three independent experiments. Vertical bars indicate SD.

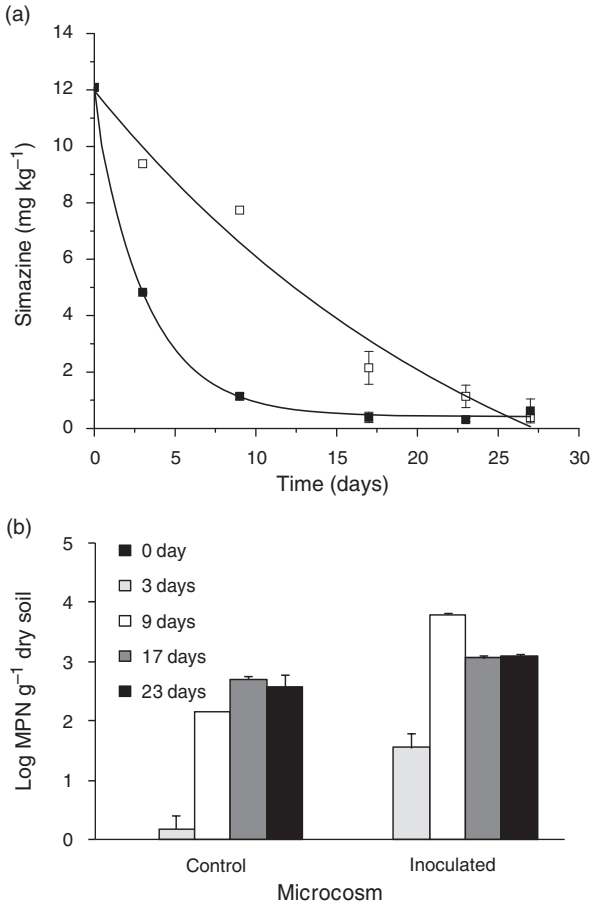

Fig. 2. Effect of bioaugmentation with *Pseudomonas* sp. strain MHP41 in a soil with a history of simazine application (AS). The soil was spiked with ~10 mg kg^{-1} simazine. (a) Simazine removal as determined by HPLC. Control treatments (□) were performed using sterile alginate beads. Inoculation treatments (■) were performed with encapsulated cells of the strain MHP41. (b) Simazine-degrading microbiota as estimated by MPN. Each point is an average of three independent experiments. Vertical bars indicate SD.

indication that the autochthonous-degrading microbiota thrives in detectable numbers in the soil with a previous treatment history, but probably remains dormant. These results are in agreement with previous reports indicating that the *s*-triazine treatment history of soils determines the intrinsic *s*-triazine-degrading potential (Yassir *et al.*, 1999; Rhine *et al.*, 2003; Morán *et al.*, 2006). Bioaugmentation with encapsulated strain MHP41 in the AS soil showed a significant increase ($P \leq 0.05$) in the cultivable simazine-degrading microorganisms, reaching values of 7.1×10^3 cells g^{-1} at the final stages of the experiment.

In agreement with our results, previous reports have demonstrated that bioaugmentation with *Pseudaminobacter* sp. strain C147, *Nocardioides* sp. C190 and *Chelatobacter heintzii* Cit1 increased atrazine removal in soils without a history of herbicide treatment (Topp, 2001; Rousseaux *et al.*, 2003). In addition, *Agrobacterium radiobacter* J14a accelerated the atrazine degradation rate in soils without and with previous herbicide application (Struthers *et al.*, 1998). Conversely, *Pseudomonas* sp. strain ADP was unable to increase atrazine or simazine degradation in *s*-triazine-polluted soil (Topp, 2001; Morán *et al.*, 2006). NS and AS soils have a nitrogen content of 1.8 and 2.0 g kg^{-1} soil, respectively. Although nitrogen sources in soils can influence simazine degradation by bacteria (García-González *et al.*, 2003; Barra-Caracciolo *et al.*, 2005), in this study, simazine removal by strain MHP41 was found in both soils.

Despite the fact that the two agricultural soils harbour autochthonous microorganisms with simazine catabolic potential, simazine removal was significantly increased after bioaugmentation with strain MHP41. Allochthonous

microorganisms that are reintroduced in soils show unsatisfactory bioremediation potential (Atlas & Unterman, 1999; Rousseaux *et al.*, 2003). However, strain MHP41 is an autochthonous bacterium (Hernández *et al.*, 2008). In this study, the use of an autochthonous bacterial strain, together with the encapsulation of cells in alginate (Vogel & Walter, 2002; Vancov *et al.*, 2005), could explain the enhanced simazine removal rates in inoculated soils. In contrast, it has been reported that bioaugmentation with free cells of the allochthonous bacterium *Pseudomonas* sp. strain ADP in AS soil was not successful (Morán *et al.*, 2006).

Effect of bioaugmentation with strain MHP41 on simazine attenuation in soil contaminated with a high concentration of simazine

As the results showed that bioaugmentation successfully removed ~10 mg kg^{-1} simazine in the two soils, investigation was further focused on the effectiveness of bioaugmentation with strain MHP41, using the nonperturbed NS soil, experimentally contaminated with a 10-fold higher simazine concentration. In this case, natural attenuation of simazine was not observed in noninoculated treatments (Fig. 3a). This is in agreement with the low number of simazine-degrading microorganisms detected after 21 days (Fig. 3b). Remarkably, bioremediation with the encapsulated strain MHP41 was successful in achieving complete simazine removal (99%) after 28 days, with a high degradation rate of 4.15 mg kg^{-1} day^{-1} (Fig. 3a). It has been reported that in highly contaminated soils, *A. radiobacter* strain J14a was able to remove almost 50% of the 200 mg kg^{-1} atrazine, whereas in control soils the natural attenuation was lower (29%). By contrast, in soils contaminated with ~160–300 mg kg^{-1} of atrazine, removal of the herbicide by *Pseudomonas* sp. strain ADP (2×10^7 cells g^{-1}) was limited (Struthers *et al.*, 1998). The present results indicate that bioaugmentation with encapsulated strain MHP41 was successful with a 10-fold higher concentration of contaminant in a soil that had an intrinsic, but low potential of simazine attenuation. Our results reinforce the use of bioaugmentation as an appropriate bioremediation strategy, especially when natural attenuation in the soil is low or undetectable.

Assessment of the bacterial community diversity and structure in agricultural soils

The bacterial communities of the two original sampled agricultural soils were characterized through 16S rRNA gene clone library analyses before the microcosm experiments. Two 16S rRNA gene clone libraries were constructed with NS and AS soil DNA. Ninety-nine 16S rRNA gene clones (51 from NS soil and 48 from AS soil) were partially sequenced (500–800 bp) (Table 2). The affiliations of clone sequences with those of the described taxa indicated the presence of mainly aerobic organisms. The degree of similarity of these sequences to their closest relatives in the database was in the range 80–99%. Sequence similarity to cultivated species was between 80% and 90% in most cases, whereas similarity to uncultured clones was often close to 99%. In addition, 21 clones of representative phylogenetic groups from the two clone libraries were fully sequenced (Table 2). Bacterial diversity coverage achieved by clone sequencing was evaluated using rarefaction analyses, assuming that one OTU is formed by the sequences that present a similarity ≥97% (Zaballos *et al.*, 2005). The analysis indicated high bacterial sequence diversity, as no asymptotic curves could be observed. Additional sequencing would be necessary to achieve

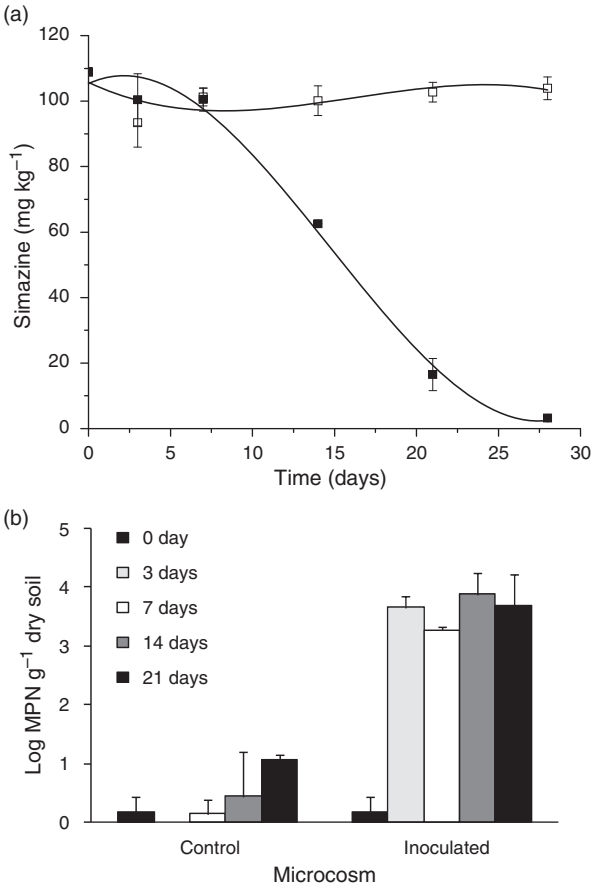

Fig. 3. Effect of bioaugmentation with *Pseudomonas* sp. strain MHP41 in NS soil contaminated with a high concentration of simazine. The soil was spiked with ~100 mg kg^{-1} simazine. (a) Simazine removal as determined by HPLC. Control treatments (□) were performed using sterile alginate beads. Inoculation treatments (■) were performed with encapsulated cells of the strain MHP41 in alginate. (b) The simazine-degrading microbiota as estimated by MPN. Each point is an average of three independent experiments. Vertical bars indicate SD.

Table 2. List of partial and nearly complete sequenced phylotypes obtained through clone library studies on 16S rRNA genes

Clone cluster (%)	Partial clones accession no.*	Percentage per soil type†	Nearly complete clone ID	Accession no.*	Percentage identity	Best match or closest related type strain	Database accession no.‡
Acidobacteria	FJ561545–FJ561554	19.5^{NS}	C003	FJ468375	94.0	*Acidobacteria bacterium* Ellin6099	AY234751
			C064	FJ468380	89.2	*Acidobacteria bacterium* Ellin7246	AM749787
	FJ561497–FJ561510	29.5^{AS}	P011	FJ468386	87.5	*Acidobacteriaceae bacterium* K22	AM749787
			P028	FJ468392	83.2	*Holophaga foetida* strain TMBS4^{T} (DSM 6591^{T})	X77215
Actinobacteria	FJ561555–FJ561560	12^{NS}					
	FJ561511–FJ561513	6.5^{AS}	P021	FJ468388	70.8	*Georgenia muralis*	EF154258
Alphaproteobacteria	FJ561561–FJ561572	23^{NS}	C063	FJ468379	90.5	*Stella vacuolata*, strain DSM 5901	AJ535711
			C078	FJ468382	93.0	*Mesorhizobium plurifarium* strain ORS10056	Y14161
	FJ561514–FJ561522	19AS					
Betaproteobacteria	FJ561573–FJ561577	10^{NS}					
	FJ561523–FJ561527	10AS	P006	FJ468384	91.9	*Burkholderia* sp. strain NK8	AB208548
Gammaproteobacteria	FJ561578–FJ561579	4^{NS}					
	FJ561528–FJ561531	8.5^{AS}	P026	FJ468390	88.2	*Legionella pneumophila*	CR628337
			P046	FJ468395	93.3	*Xanthomonas campestris* strain ATCC 33913	AE012505
Deltaproteobacteria	FJ561580	2^{NS}					
	FJ561532–FJ561534	6.5^{AS}	P007	FJ468385	81.2	*Syntrophus aciditrophicus* SB	CP000252
			P012	FJ468387	85.6	*Geobacter sulfurreducens* PCA	AE017180
			P024	FJ468389	88.6	*Haliangium tepidum*	AB062751
Bacteroidetes	FJ561581–FJ561583	6^{NS}	C022	FJ468377	81.8	*Thermonema rossianum* strain NR-27T (DSM 10300)	Y08956
	FJ561535	2AS					
Chloroflexi	FJ561584–FJ561586	6^{NS}	C053	FJ468378	82.0	*Dehalococcoides* sp. strain BHI80-15	AJ431246
Firmicutes	FJ561587–FJ561592	12^{NS}	C007	FJ468376	81.3	*Desulfosporosinus orientis*	AJ493052
Nitrospirae	FJ561593–FJ561594	4^{NS}					
	FJ561536	2AS	P045	FJ468394	95.3	*Nitrospira* sp.	AJ224039
Planctomycetes	FJ561595	2^{NS}	C074	FJ468381	94.2	*Planctomycetes* sp.	AY673410
	FJ561537–FJ561541	10AS	P027	FJ468391	87.5	*Planctomyces maris*	X62910
			P029	FJ468393	87.3	*Pirellula* sp.	X81947
Verrucomicrobia	FJ561543	2AS	P001	FJ468383	84.6	*Prosthecobacter debontii* (DSM 14044)	AJ966882
Gemmatimonadetes	FJ561542	2AS					
Unknown Bacteria	FJ561544	2AS					
Total clones	99		21				

The list provides the accession numbers of the representative clones from both soils and the closest match or type strain available in the SILVA database (http://www.arb-silva.de). Clones retrieved from agricultural soil with different history of simazine application are indicated. Accession numbers of the partial and nearly complete sequences have been listed along with accession numbers of the closest best match.

*GenBank database.

†NS, soil not previously exposed to *s*-triazines. AS, soil with a long history of *s*-triazine application.

‡SILVA-SSU database accession number.

better coverage of the diversity of the amplified 16S rRNA genes. However, it was assumed that in this case, the most abundant phylotypes thriving in the agricultural soils were identified.

Bacterial diversity and community structure changes in NS soil due to bioaugmentation

The bacterial diversity in NS soil is shown in Fig. 4a. The NS clone library was dominated by members of the phylum *Proteobacteria* (39%), most of them affiliated with the *Alphaproteobacteria* and *Betaproteobacteria*. Twenty-three per cent of the clones were affiliated with the *Alphaproteobacteria*, most of them (60%) relatives associated with the family *Rhizobiales*. The betaproteobacterial OTUs detected were dominated by members of the order *Burkorderiales*, while *Gammaproteobacteria* and *Deltaproteobacteria* were much less well represented. *Acidobacteria* comprised 19% of the library, with most of the sequences affiliating with uncultured strains (> 96% identity). Other represented groups were *Actinobacteria* (12%), *Firmicutes* (12%), *Chloroflexi* (6%), *Bacteroidetes* (6%) *Nitrospirae* (4%) and *Planctomycetes* (2%).

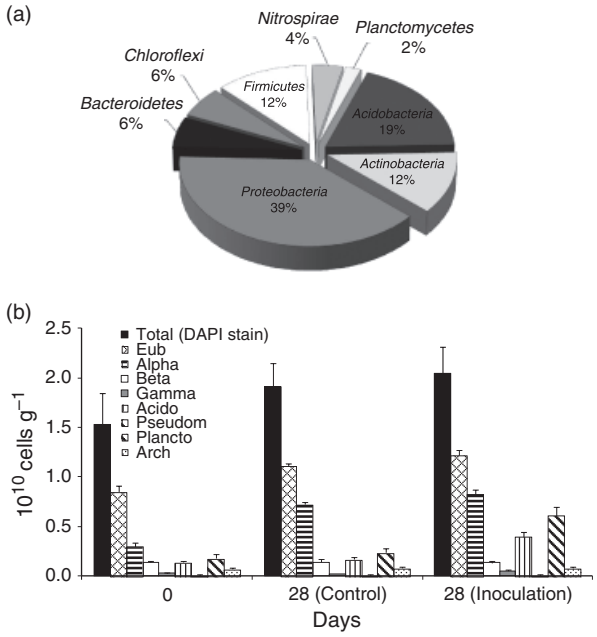

Fig. 4. Bacterial community diversity and dynamics during bioaugmentation in NS soil. (a) Representative phylogenetic groups retrieved from NS soil by the clone library. Values are shown as per cent of the total clones in each clone library. (b) FISH analysis of the microbial community from the NS soil amended with simazine (~100 mg kg^{-1}) and bioaugmented with *Pseudomonas* sp. strain MHP41. Each value is an average of three independent experiments. Vertical bars indicate SD. Total (DAPI stain), total microbial abundances; Eub, Bacteria; Alpha, *Alphaproteobacteria*; Beta, *Betaproteobacteria*; Gamma, *Gammaproteobacteria*; Acido, *Acidobacteria*; Pseudom, *Pseudomonas*; Plancto, *Planctomycetes*; Arch, Archaea.

Bacterial community structure dynamics during the bioaugmentation with encapsulated strain MHP41 was studied using FISH analysis. The selection of the phylogenetic probes for FISH was based on the major representative groups found in the clone libraries. In addition, an Archaea-specific probe (Arch915) was used. The total bacterial abundances estimated by DAPI counts were relatively high in NS soil, ranging from 1.5 to 2.1×10^{10} cells g^{-1} dry soil. At time 0 of the bioremediation experiment in NS soil, 50% of the cell abundance was assigned to the Bacteria domain (EUB338 probe mixture; Fig. 4b). This value increased by a factor of 1.5 in the bioaugmented soil after 28 days of inoculation. In the control soil, a similar increase (factor of 1.3) in the bacterial fraction was observed. Archaea (Arch915-stained cells) accounted for only 5% of the total abundance ($6–7 \times 10^8$ cells g^{-1}) in control and inoculated soils, and no changes were observed due to the bioaugmentation. Bacteria hybridizing with probes Alf1b (targeting *Alphaproteobacteria*), HoAc1402 (targeting *Acidobacteria*) and Pla886 (targeting *Planctomycetes*) were detected in large numbers in NS soils. At time 0, the *Alphaproteobacteria* dominated (10–20%), followed by *Planctomycetes* (about 11%), *Acidobacteria* (9%), *Betaproteobacteria* (9%) and *Gammaproteobacteria* (1–2%) (Fig. 4b). In these soils, pseudomonad populations (tested with probe PS56a) were detected sporadically and only in numbers that remained around the detection limit (set at 1% of the DAPI-stained cells). No increase was observed after either bioaugmentation or simazine application treatment.

The addition of simazine only produced a clear increase in the alphaproteobacterial fraction (about 2.5-fold). Bioaugmentation with strain MHP41 promoted changes in some of the most abundant groups detected by FISH (Fig. 4b), most notably the *Planctomycetes* and *Acidobacteria* (increasing almost threefold after 28 days of incubation), followed by *Gammaproteobacteria* (twofold).

These results suggest that *Acidobacteria* and *Planctomycetes* could be active simazine-degrading bacteria or may play an important role in the functionality of the bacterial community in these soils. Despite their abundance in the biosphere, knowledge of the *Planctomycetes* and *Acidobacteria* is limited by the fact that the majority of these microorganisms are uncultured (Quaiser *et al.*, 2003; Buckley *et al.*, 2006). To understand the role of *Planctomycetes* and *Acidobacteria* in agricultural soils, a novel insight into the phenotypic or the physiological characterization of these bacterial groups is desirable.

In the present study, a significant increase in pseudomonads after repeated introduction of 10^8 cells g^{-1}

of MHP41 in the soils was not observable. This is not surprising as pseudomonads do not generally account for a significant part of soil communities (Nogales *et al.*, 1999; Lloyd-Jones *et al.*, 2005; Martin-Laurent *et al.*, 2006). However, *Pseudomonas* strains have commonly been isolated from such environments (Cho & Tiedje, 2000; Lloyd-Jones *et al.*, 2005).

Bacterial diversity and community structure changes in AS soil due to bioaugmentation

In AS soil, bacterial diversity was dominated by members of the phylum *Proteobacteria*, most of them affiliated with *Alphaproteobacteria* and *Betaproteobacteria* (around 29%) as described previously for NS soil. Thus, *Gammaproteobacteria* and *Deltaproteobacteria* were not abundant. *Acidobacteria* and *Actinobacteria* were represented in the AS clone library, comprising 29% and 7% of the clones, respectively. Members of the phylum *Planctomycetes* were much more abundant in AS soil than in NS soil (10% vs. 2%). Some other minor phylogenetic groups such as *Bacteriodetes*, *Nitrospirae*, *Verrucomicrobia* and *Gemmatimonadetes* were observed. *Firmicutes* and *Chloroflexi* were not detected in soil AS (Fig. 5a). The total bacterial abundance in AS soil (DAPI counts) ranged from 2.1 to 3.0 $\times$ 10^{10} cells g^{-1} dry soil, and only 40% was assigned to the Bacteria domain at time 0 (Fig. 5b). After 28 days, Bacteria cells counts (EUB338 probe mixture) increased in bioaugmented soil by a factor of 1.8 compared with 1.3 in the control soil. The AS soil, which has a long history of simazine treatment, experienced similar changes in the bacterial community by bioaugmentation as the NS soil (Fig. 5b). After 28 days, inoculations of the strain MHP41 increased the relative abundances of *Alphaproteobacteria* (almost threefold), *Planctomycetes* (almost 2.5-fold), and *Acidobacteria* (almost 1.5-fold) whereas simazine application promoted only a slight increase in *Alphaproteobacteria* and *Planctomycetes* cells after 28 days (both $<$ 1.5-fold). The relative abundance of Archaea did not change as a result of bioaugmentation (remaining at $\sim$5% of the total).

The results indicated that despite the fact that pseudomonads (and, in this case, the inoculated *Pseudomonas* sp. strain MHP41) are not present in significant numbers in these soils, bioaugmentation with strain MHP41 promoted bacterial community shifts in both AS and NS soils. These changes at the bacterial community level could not be linked to the intrinsic agricultural soil composition or to the previous history of simazine application, but rather to the introduction of the simazine-degrading strain MHP41. To our knowledge, this is the first report showing bacterial community structure changes due to bioaugmentation in simazine-contaminated soil. In a previous report, FISH was

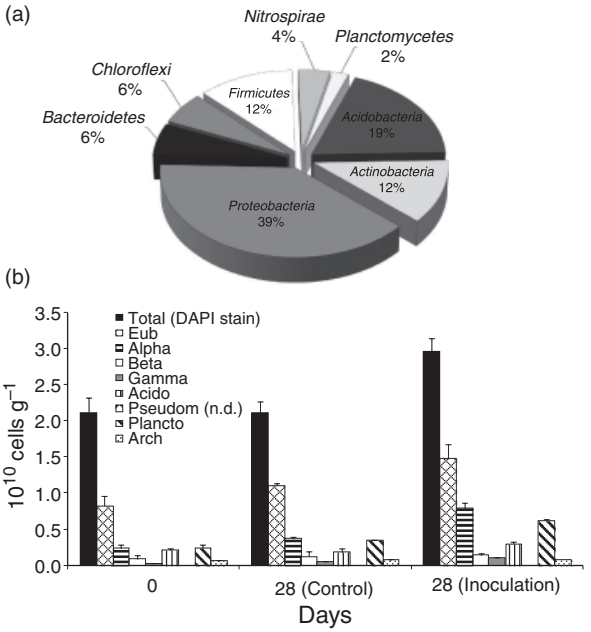

Fig. 5. Bacterial community diversity and dynamics during bioaugmentation in AS soil. (a) Representative phylogenetic groups retrieved from AS soil by the clone library. Values are shown as per cent of total clones in each clone library. (b) FISH analysis of the microbial community from AS soil spiked with simazine ($\sim$10 mg kg^{-1}) and bioaugmented with *Pseudomonas* sp. strain MHP41. Each value is an average of three independent experiments. Vertical bars indicate SD. Total (DAPI stain), total microbial abundances; Eub, Bacteria; Alpha, *Alphaproteobacteria*; Beta, *Betaproteobacteria*; Gamma, *Gammaproteobacteria*; Acido, *Acidobacteria*; Pseudom, *Pseudomonas*; Plancto, *Planctomycetes*; Arch, Archaea.

used to describe shifts in the existing community structure caused by the addition of the herbicide simazine and urea to soil (Barra-Caracciolo *et al.*, 2005). In this case, the groups most influenced were the *Alphaproteobacteria* and *Betaproteobacteria*. The fact that in our study the major changes occurred after bioaugmentation, rather than by simazine application, may indicate that the response is mainly due to the inoculated strain. In this respect, the bioavailability of nitrogen will probably be enhanced after simazine degradation into more soluble nitrogen-containing products (Cook & Hütter, 1981; Mandelbaum *et al.*, 1995; Rhine *et al.*, 2003) and may thus directly influence the community structure changes.

Conclusions

Bioremediation using *Pseudomonas* sp. strain MHP41 was successful for the removal of simazine at the scale of microcosms. Removal of simazine by bioaugmentation was independent of the previous history of soil contamination. Of note, strain MHP41 was essential for simazine removal in highly contaminated NS soil. The autochthonous bacterial

communities in the soil were also clearly influenced as a result of bioaugmentation. *Planctomycetes* and *Acidobacteria* showed detectable increased numbers via FISH as a common response to the treatment, independent of the quality and the history of the soil. However, the presence of the introduced strain could not be detected in the soils, and their numbers remained at the limits of FISH detection. The presence of archaeal lineages as metabolically active members of microbial communities in the two soils indicates the ecological significance of Archaea in agricultural soils. Together, the results indicate that bioaugmentation with strain MHP41 significantly and effectively increased the rates of bioremediation over the native autochthonous catabolic activity, and the soil bacterial community changes may respond to catalysis of the contaminant rather than to distortion due to addition of cell biomass.

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References

Agulló L, Cámara B, Martinez P, Latorre-Reyes V & Seeger M (2007) Response to (chloro)biphenyls of the PCB-degrader *Burkholderia xenovorans* LB400 involves stress proteins induced also by heat shock and oxidative stress. *FEMS Microbiol Lett* **267**: 167–175.

Atlas RM & Unterman R (1999) Bioremediation. *Manual of Industrial Microbiology and Biotechnology*, 2nd edn (Demain AL & Davies JE, eds), pp. 666–681. ASM Press, Washington, DC.

Barra-Caracciolo AB, Grenni P, Ciccoli R, Di Landa G & Cremisini C (2005) Simazine biodegradation in soil: analysis of bacterial community structure by *in situ* hybridization. *Pest Manag Sci* **61**: 863–869.

Birnbaum LS & Fenton SE (2003) Cancer and developmental exposure to endocrine disruptors. *Environ Health Persp* **111**: 389–394.

Bouchez T, Patureau D, Dabert P *et al.* (2000) Ecological study of a bioaugmentation failure. *Environ Microbiol* **2**: 179–190.

Buckley DH, Huangyutitham V, Nelson TA, Rumberger A & Thies JE (2006) Diversity of *Planctomycetes* in soil in relation to soil history and environmental heterogeneity. *Appl Environ Microb* **72**: 4522–4531.

Cho JC & Tiedje JM (2000) Biogeography and degree of endemicity of fluorescent *Pseudomonas* strains in soil. *Appl Environ Microb* **66**: 5448–5456.

Cole JR, Chai B, Marsh TL *et al.* (2003) The ribosomal database project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res* **31**: 442–443.

Cook AM & Hütter R (1981) *s*-Triazines as nitrogen source for bacteria. *J Agr Food Chem* **29**: 1135–1143.

Daims H, Bruhl A, Amann R, Schleifer KH & Wagner M (1999) The domain-specific probe EUB338 is insufficient for the detection of all *Bacteria*: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* **22**: 434–444.

Dinamarca MA, Cereceda-Balic F, Fadic X & Seeger M (2007) Analysis of *s*-triazine-degrading microbial communities in soils using most-probable-number enumeration and tetrazolium-salt detection. *Int Microbiol* **10**: 209–215.

European Union (2004) Commission Decision of 10 March 2004 concerning the non-inclusion of simazine in Annex I to Council Directive 91/414/EEC and the withdrawal of authorisations for plant protection products containing this active substance. *Official Journal of the European Union L78* 16 March 2004, pp. 50–52.

Flores C, Morgante V, González M, Navia R & Seeger M (2009) Adsorption studies of the herbicide simazine in agricultural soils of the Aconcagua valley, central Chile. *Chemosphere* **74**: 1544–1549.

García-González V, Govantes F, Shaw LJ, Burns RG & Santero E (2003) Nitrogen control of atrazine utilization in *Pseudomonas* sp. strain ADP. *Appl Environ Microb* **69**: 6987–6993.

Gentry TJ, Rensing C & Pepper IL (2004) New approaches for bioaugmentation as a remediation technology. *Crit Rev Env Sci Tec* **34**: 447–494.

Gunasekara AS, Troiano J, Goh KS & Tjeerdema RS (2007) Chemistry and fate of simazine. *Rev Environ Contam T* **189**: 1–23.

Hayes TB, Stuart AA, Mendoza M *et al.* (2006) Characterization of atrazine-induced gonadal malformations in African clawed frogs (*Xenopus laevis*) and comparisons with effects of an androgen antagonist (cyproterone acetate) and exogenous estrogen (17beta-estradiol): support for the demasculinization/feminization hypothesis. *Environ Health Persp* **114** (suppl 1): 134–141.

Hernández M, Villalobos P, Morgante V, González M, Reiff C, Moore E & Seeger M (2008) Isolation and characterization of a novel simazine-degrading bacterium from agricultural soil of central Chile, *Pseudomonas* sp. MHP41. *FEMS Microbiol Lett* **286**: 184–190.

Juretschko S, Loy A, Lehner A & Wagner M (2002) The microbial community composition of a nitrifying–denitrifying activated sludge from an industrial sewage treatment plant

analyzed by the full-cycle rRNA approach. *Syst Appl Microbiol* **25**: 84–99.

Lloyd-Jones G, Laurie AD & Tizzard AC (2005) Quantification of the *Pseudomonas* population in New Zealand soils by fluorogenic PCR assay and culturing techniques. *J Microbiol Meth* **60**: 217–224.

Ludwig W, Strunk O, Westram R *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32**: 1363–1371.

Mandelbaum RT, Allan DL & Wackett LP (1995) Isolation and characterization of a *Pseudomonas* sp. that mineralizes the *s*-triazine herbicide atrazine. *Appl Environ Microb* **61**: 1451–1457.

Manz W, Amann R, Ludwig W, Wagner M & Schleifer KH (1992) Phylogenetic oligonucleotide probes for the major subclasses of proteobacteria: problems and solutions. *Syst Appl Microbiol* **15**: 593–600.

Manzano M, Morán AC, Tesser B & González B (2007) Role of eukaryotic microbiota in soil survival and catabolic performance of the 2,4-D herbicide degrading bacteria *Cupriavidus necator* JMP134. *Antonie van Leeuwenhoek* **91**: 115–126.

Martínez P, Agulló L, Hernández M & Seeger M (2007) Chlorobenzoate inhibits growth and induces stress proteins in the PCB-degrading bacterium *Burkholderia xenovorans* LB400. *Arch Microbiol* **188**: 289–297.

Martin-Laurent F, Barrés B, Wagschal I, Piutti S, Devers M, Soulas G & Philippot L (2006) Impact of the maize rhizosphere on the genetic structure, the diversity and the atrazine-degrading gene composition of cultivable atrazine-degrading communities. *Plant Soil* **282**: 99–115.

Morán AC, Muller A, Manzano M & González B (2006) Simazine treatment history determines a significant herbicide degradation potential in soils that is not improved by bioaugmentation with *Pseudomonas* sp. ADP. *J Appl Microbiol* **101**: 26–35.

Neef A, Amann R, Schlesner H & Schleifer KH (1998) Monitoring a widespread bacterial group: *in situ* detection of *Planctomycetes* with 16S rRNA-targeted probes. *Microbiology* **144**: 3257–3266.

Newcombe DA & Crowley DE (1999) Bioremediation of atrazine-contaminated soil by repeated applications of atrazine-degrading bacteria. *Appl Environ Microb* **51**: 877–882.

Nogales B, Moore ER, Abraham WR & Timmis KN (1999) Identification of the metabolically active members of a bacterial community in a polychlorinated biphenyl-polluted moorland soil. *Environ Microbiol* **1**: 199–212.

Nogales B, Moore ER, Llobet-Brossa E, Rossello-Mora R, Amann R & Timmis KN (2001) Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. *Appl Environ Microb* **67**: 1874–1884.

Ostrofsky EB, Traina SJ & Tuovinen OH (1997) Variation in atrazine mineralization rates in relation to agricultural management practice. *J Environ Qual* **26**: 647–657.

Paul D, Pandey G, Meier C, van der Meer JR & Jain RK (2006) Bacterial community structure of a pesticide-contaminated site and assessment of changes induced in community structure during bioremediation. *FEMS Microbiol Ecol* **57**: 116–127.

Philp JC, Bamforth SM, Singleton I & Atlas RM (2005) Environmental pollution and restoration: a role for bioremediation. *Bioremediation: Applied Microbial Solutions for Real-World Environmental Cleanup* (Atlas RM & Philp JC, eds), pp. 1–48. ASM Press, Washington, DC.

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J & Glockner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* **35**: 7188–7196.

Quaiser A, Ochsenreiter T, Lanz C, Schuster SC, Treusch AH, Eck J & Schleper C (2003) *Acidobacteria* form a coherent but highly diverse group within the bacterial domain: evidence from environmental genomics. *Mol Microbiol* **50**: 563–575.

Rhine ED, Fuhrmann JJ & Radosevich M (2003) Microbial community responses to atrazine exposure and nutrient availability: linking degradation capacity to community structure. *Microb Ecol* **46**: 145–160.

Rousseaux S, Hartmann A, Lagacherie B, Piutti S, Andreux F & Soulas G (2003) Inoculation of an atrazine-degrading strain, *Chelatobacter heintzii* Cit1, in four different soils: effects of different inoculum densities. *Chemosphere* **51**: 569–576.

Schönduve P, Sara M & Friedl A (1996) Influence of physiologically relevant parameters on biomass formation in a trickle-bed bioreactor used for waste gas cleaning. *Appl Microbiol Biot* **45**: 286–292.

Stahl DA & Amann R (1991) Development and application of nucleic acid probes. *Nucleic Acid Techniques in Bacterial Systematics* (Stackebrandt E & Goodfellow M, eds), pp. 205–248. John Wiley & Sons Ltd, Chichester, UK.

Struthers JK, Jayachandran K & Moorman TB (1998) Biodegradation of atrazine by *Agrobacterium radiobacter* J14a and use of this strain in bioremediation of contaminated soil. *Appl Environ Microb* **64**: 3368–3375.

Thompson IP, van der Gast CJ, Ciric L & Singer AC (2005) Bioaugmentation for bioremediation: the challenge of strain selection. *Environ Microbiol* **7**: 909–915.

Thompson JR, Marcelino LA & Polz MF (2002) Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by reconditioning PCR. *Nucleic Acids Res* **30**: 2083–2088.

Topp E (2001) A comparison of three atrazine-degrading bacteria for soil bioremediation. *Biol Fert Soils* **33**: 529–534.

Vancov T, Jury K & Van Zwieten L (2005) Atrazine degradation by encapsulated *Rhodococcus erythropolis* NI86/21. *J Appl Microbiol* **99**: 767–775.

Vancov T, Jury K, Rice N, Van Zwieten L & Morris S (2007) Enhancing cell survival of atrazine degrading *Rhodococcus erythropolis* NI86/21 cells encapsulated in alginate beads. *J Appl Microbiol* **102**: 212–220.

Vogel TM (1996) Bioaugmentation as a soil bioremediation approach. *Curr Opin Biotech* **7**: 311–316.

Vogel TM & Walter MV (2002) Bioaugmentation. *Manual of Environmental Microbiology* (Hurst CJ, Crawford RL, Knudsen GR, McInerney MJ & Stetzenbach LD, eds), pp. 952–971. ASM Press, Washington, DC.

Yassir A, Lagacherie B, Houot S & Soulas G (1999) Microbial aspects of atrazine biodegradation in relation to history of soil treatment. *Pestic Sci* **55**: 799–809.

Zaballos M, López-López A, Ovreas L *et al.* (2005) Comparison of prokaryotic diversity at offshore oceanic locations reveals a different microbiota in the Mediterranean Sea. *FEMS Microbiol Ecol* **56**: 389–405.

Zarda B, Hahn D, Chatzinotas A, Schönhuber W, Neef A, Amann R & Zeyer J (1997) Analysis of bacterial community structure in bulk soil by *in situ* hybridization. *Arch Microbiol* **168**: 185–192.