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Isolation and characterization of fenamiphos degrading bacteria

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Abstract The biological factors responsible for the microbial breakdown of the organophosphorus nematicide fenamiphos were investigated. Microorganisms responsible for the enhanced degradation of fenamiphos were isolated from soil that had a long application history of this nematicide. Bacteria proved to be the most important group of microbes responsible for the fenamiphos biodegradation process. Seventeen bacterial isolates utilized the pure active ingredient fenamiphos as a carbon source. Sixteen isolates rapidly degraded the active ingredient in Nemacur 5GR. Most of the fenamiphos degrading bacteria were *Microbacterium* species, although *Sinorhizobium, Brevundimonas, Ralstonia* and *Cupriavidus* were also identified. This array of gram positive and gram negative fenamiphos degrading bacteria appeared to be pesticide-specific, since cross-degradation toward fosthiazate, another organophosphorus pesticide used for nematode control, did not occur. It was established that the phylogenetical relationship among nematicide degrading bacteria is closer than that to non-degrading isolates.

Keywords Biodegradation · Fenamiphos · Fosthiazate · Soil bacteria · Nematicide efficacy

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Introduction

Fenamiphos (Ethyl 4-methylthio-*m*-tolyl isopropylphosphoramidate) is a broad spectrum, non-fumigant systemic organophosphorus (OP) nematicide, present as the active substance of the commercial formulation Nemacur GR. This nematicide is extensively used to control plant parasitic nematodes in agricultural production systems. The activity of fenamiphos tends to be nematostatic rather than nematotoxic, inhibiting movement of nematodes and delaying egg hatching (van Gundy and Mckenry 1977). The targeted amount of fenamiphos applied in the field ranges between 10 and 45 mg kg^{-1} soil, which results in the use of 6–10 kg ha^{-1} under practical conditions depending on the crop. The half-life of fenamiphos has been reported to be 63 days (Ou and Rao 1986), although degradation of this nematicide in 14 days or less has been observed in soils with a history of repeated exposure (Ou et al. 1994; Davis et al. 1993).

The enhanced degradation of OP compounds can be biologically mediated (Singh 2009). There are several OP pesticides that have been reported to undergo an enhanced biodegradation process, and different microorganisms that have been identified to play a major role (Singh and Walker 2006). The accumulation of microbes capable to degrade fenamiphos at accelerated rates has been described in a variety of agricultural soils. For example, in banana fields of Ivory Coast a step-by-step build up of microbial populations that were responsible for the accelerated degradation of fenamiphos was found (Anderson and Lafuerza 1992). In tomato growing soils of Australia a similar observation has been reported (Stirling et al. 1992). Biodegradation of fenamiphos in *Musa* cultivation from Costa Rica has been previously described (Moens et al. 2004). Enhanced fenamiphos biodegradation has similarly been observed in sweet corn-sweet potato-vetch rotation systems in USA (Johnson 1998). However, none of these studies has identified the microorganism responsible of the biodegradation process, perhaps because the isolation of these specific microbes is a complex process (Ou 1991; Stirling et al. 1992).

To date, there is little information available about the identification of fenamiphos degrading microorganisms, especially of those that can perform the degradation process individually. Initial studies suggested that fenamiphos degradation could only be achieved by a bacterial consortium (Ou and Thomas 1994; Singh et al. 2003). More recently, it was reported that individual bacterial isolates were also capable of degrading fenamiphos. Two bacteria isolated from soil in Australia, *Brevibacterium* sp. and *Microbacterium esteraromaticum*, were reported to individually hydrolyze both fenamiphos and its toxic oxidation products (Cáceres et al. 2009; Megharaj et al. 2003). However, it is not clear whether the enhanced biodegradation of fenamiphos can be individually performed exclusively by these two microorganisms or whether there are other microbes that could also perform the process.

A current agricultural practice to manage enhanced biodegradation of OPs is the rotation of nematicides (Karpouzas and Giannakou 2002). However, crossed biodegradation may occur when microorganisms have frequently been exposed to one nematicide and rapidly adapt to degrade other pesticides. The cross-enhancement of selected pesticides in soil can be dependent on the structural similarity of the compounds (Singh et al. 2005). Nemathorin 10WG contains fosthiazate, another organophosphate molecule, as active ingredient. The half-life of fosthiazate in soil has been reported to be between 0.5 to 1.5 months (Qin et al. 2004). However, it is not known if this relatively new nematicide may experience cross-degradation by the same microorganisms able to degrade fenamiphos. Moreover, there is little information whether there is a genetic relationship between fenamiphos degrading microorganisms and other nematicide degrading microbes. This information could broaden the understanding of fenamiphos biological mediated degradation and could be used to manage the rapid microbial breakdown of the nematicidal activity for crop protection.

This study reports the isolation and identification of several fenamiphos degrading microorganisms found in a German soil. We analyzed the fenamiphos degrading capability of the individual purified bacterial isolates and determined whether the additional compounds present in the commercial formulation, Nemacur GR, enhanced or interfered with this degradation process. The ability of fenamiphos degrading isolates to degrade fosthiazate was also examined. The phylogenetic relationship between the fenamiphos degrading microorganisms isolated in this study and other bacteria previously reported that degraded carbofuran, ethoprophos, cadusafos, or fenamiphos was investigated.

Materials and methods

Soil

Soil was collected from the 20 cm top layer in a field of Burscheid, North Rhein Westfalia, Germany. This soil was a silty loam (16.2% clay, 78.4% silt and 5.4% sand), pH of 6.17 (measured in water) and 0.98% organic matter content. Turf grass was grown on this soil which had received 25 applications of Nemacur GR, containing fenamiphos as the active component, in 12 years. The last Nemacur GR application was performed about 1 month before the soil was collected. This soil was previously reported to have enhanced biodegradation of fenamiphos (Cabrera et al. 2009). The soil was transferred

under moist conditions at 20 ± 2°C to the laboratory and then stored for 1 week in closed plastic containers at 8°C in the dark.

Nematicides and media

Analytical grade fenamiphos (Sigma–Aldrich), commercial granular formulation Nemacur 5GR (Bayer CropScience, Germany), containing 5% (w/w) fenamiphos, and granular formulation Nemathorin 10WG (Syngenta Crop Protection, Switzerland), containing 10% (w/w) fosthiazate, were dissolved in methanol (HPLC grade) and filter sterilized (0.4 μm) to obtain stock solutions. Supplemented tryptone soya agar (TSA^+) was prepared by adding 9.6 g agar (Agar Bacteriology grade, AppliChem) and 24 g tryptone soya broth (Oxoid) to 800 ml distilled water. After autoclaving and cooling, the medium was amended with a filter-sterilized stock solution of Cycloheximide (Sigma) to a final concentration of 100 μg ml^{-1}. Soil extract liquid medium (SELM), pH 6.0, was prepared as described by Karpouzas et al. (2000b). This medium was used because it contains no carbon. Soil extract agar medium (SEAM) was prepared as the SELM and supplemented with 20 g l^{-1} Bacto Agar (Difco) prior to autoclaving. The SEAM plates were 5 cm Petri dishes containing 5 ml medium each.

Degradation assay with all soil microorganisms

To extract the microbial population, 10 g of soil was transferred to 100 ml sterile water and incubated in the dark for 16 h at 28°C in an orbital shaker set at 120×*g*. This suspension was prepared in triplicate. From this suspension, 1 ml was subsequently transferred to 19 ml SELM and supplemented with a stock solution of analytical grade fenamiphos to achieve a final concentration of 100 μg ml^{-1} in a 50 ml sterile plastic tube (Sarstedt). Two treatments were further supplemented with either 150 μg ml^{-1} cycloheximide or 150 μg ml^{-1} streptomycin sulfate and 150 μg ml^{-1} chloramphenicol, and another treatment remained without supplements. The SELM medium amended with fenamiphos that did not receive microorganisms inoculation served as control. Every treatment had 3 replicates. The SELM suspensions were incubated on an orbital shaker set at 60×*g* in the dark at 28°C. From each treatment a 400 μl sample was taken at 0, 3, 6, 9, 12, 15 and 20 days after incubation and added to 1600 μl of methanol. This mixture was filter sterilized (0.4 μm) and transferred to HPLC vials. To determine the fenamiphos concentration, 20 μl of each sample was analyzed by Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) using a Hewlett Packard (HP) 1050 system with a Merck LiChrosphere100 C18 reversed phase column (250 × 4.0 mm, 5 μm), preceded by a LiChrosphere C18 reversed phase guard column (4.0 × 4.0 mm, 5 μm) as described by Cabrera et al. (2009). Fenamiphos in the SELM was detected by retention time (23 min) and spectral pattern. The amount of fenamiphos was calculated using the standard curve obtained with a dilution range of the pure compound and compared to the remaining fenamiphos amounts in the control without the addition of microorganisms.

Degradation assay for individual isolates

From the previous assay, 50 μl of the inoculated SELM amended with fenamiphos, in the presence and absence of cycloheximide, was individually plated on TSA^+ in 10 cm diameter Petri dishes. After 48 h incubation at 28°C in the dark, 20 well separated single colonies were collected from each plate and transferred to fresh TSA^+. After further incubation for 48 h at 28°C, one loop full of bacterial cells, was taken from each colony and transferred to a new plate containing SEAM, supplemented with 100 μg ml^{-1} fenamiphos. After incubation for 7 and 14 days at 28°C, an agar block about 1 cm from the bacterial colony was transferred to a reaction tube. For every 0.1 g of agar 100 μl of methanol was added and incubated over night at room temperature. The tubes containing the agar-methanol mixture were centrifuged for 5 min at 14000×*g* at 24°C and 200 μl of the solution was transferred to HPLC vials. 20 μl was analyzed by RP-HPLC as described above.

To determine whether the commercial formulation interferes with fenamiphos degradation, the single isolates that were able to metabolize the analytical grade fenamiphos were re-cultured on TSA^+. These bacteria were used in a similar plate assay as described above, although the SEAM was supplemented with Nemacur 5 GR to a final concentration of 100 μg ml^{-1} active substance (fenamiphos). To study the cross-degradation activity of the fenamiphos degrading bacteria, the assay was repeated with

SEAM supplemented with stock solution of Nemathorin 10WG to a final concentration of 100 μg ml^{-1} active substance (fosthiazate).

Molecular characterization and identification

The bacteria that were able to degrade fenamiphos were grown on TSA^{+} under dark conditions for 48 h at 28°C. To characterize these bacteria, the 16S small subunit (SSU) coding region was partially amplified by PCR. Fresh bacterial cells were transferred to microfuge vials and 50 μl of PCR master mix was added. This master mix contained 10 μl 5× Green GoTaq Flexi Buffer (Promega), 1.5 mM $MgCl_2$, 200 μM of each dNTP (Promega), 0.2 μM primer 27f (5′-AGAGTTTGATCCTGGCTCAG-3′, Sigma), 0.2 μM primer 9rev (5′-AAGGAGGTGATCCAGCC-3′, Sigma), and 0.63 units Taq polymerase (Promega). The DNA was amplified in a 'T Gradient' thermo cycler (Biometra), by an initial denaturation step at 95°C for 4 min, followed by 35 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 1 min and a final extensions step of 72°C for 5 min. The amplification was verified by gel electrophoresis using a 1% (w/v) agarose gel in Tris–Acetate EDTA-Buffer (TAE, AppliChem) supplemented with 0.1 μg ml^{-1} ethidium bromide (Sambrook and Russell 2001). Restriction Fragment Length Polymorphism (RFLP) analysis was performed by digesting 15 μl of PCR product with the enzyme *Cfo*1 (Promega) for 3 h at 37°C followed by agarose gel electrophoresis (Sambrook and Russell 2001).

The amplified DNA fragments were purified using the GFX PCR DNA and Gel Band Purification Kit (Illustra, General Electric Health Biosciences) according to the manufacturer's protocol. Purified SSU DNA was sequenced at *GATC Biotech AG* (Konstanz, Germany). Sequences were edited with BioEdit Sequence Alignment Editor (Hall 1999) and identified by Basic Local Alignment Search Tool (BLAST analysis) using the National Center for Biotechnology Information (NCBI) database.

Phylogenetic analysis

To analyze the relationship between the fenamiphos degrading microorganisms isolated in this study and other nematicide degrading bacteria, sequences from other nematicide degrading microorganisms were obtained from the NCBI Genebank (Table 1). In addition, sequences of bacteria isolated from two soils with no fenamiphos degrading microorganisms reported by Cabrera et al. (2009) were included. The sequence of *Staphylococcus aureus* (FJ609418) was used as outgroup. Assembling of all sequences and transformation into fasta format was performed in Vector NTI Advance 10.0 (Invitrogen). Aligning, editing and converting of fasta files into nexus format was performed in Clustal X.

To identify the best fitting model for nucleotide substitution of 16S rRNA datasets MrModeltest was used. According to AIC ($-\ln L = 4693.9512$; AIC = 9405.9023) implemented in MrModeltest general time reversible model of nucleotide substitution with gamma rates (GTR + G) and equal base frequencies was selected for the Bayesian inference which started from a random tree defining *S. aureus* as for outgroup. Burn in period started after 25% of the 1,000,000 cycles performed, discarding all trees generated prior burn in. Paup started from a random tree using *S. aureus* as outgroup. Convergence among chains was followed by log-likelihood values using tracer v.1.3. 50% majority-rule consensus tree was generated with TreeEdit (Rambaut and Charleston 2001).

Results and discussion

Fenamiphos degradation by the total culturable microbial soil population

The bioassay showed that in the absence of the antibiotics and fungicide, the biodegradation of fenamiphos started 3 days post microorganisms inoculation (dpi) and was completed at 12 dpi (Fig. 1). In the presence of the fungicide cycloheximide, the biodegradation of fenamiphos started 12 dpi and was almost completed 20 dpi. In the presence of the antibiotics there was no significant biodegradation of fenamiphos after 20 days of incubation. These results indicated that the degradation of fenamiphos was primarily mediated through soil bacteria. However, the combination of bacteria and fungi did accelerate the biodegradation process. A similar observation was reported concerning the non-fumigant organophosphate nematicide ethoprophos (Karpouzas et al. 1999). Similarly, to our findings, the nematicide degradation was inhibited in the presence of

Table 1 Organophosphate nematicides with their respective degrading bacteria inferred from the NCBI Genedatabank which sequence was used in the phylogenetic analysis

Nematicide	Bacteria	Accession number	Reference
Carbofuran	*Bordetella bronchiseptica*	X57026	Karpouzas et al. (2000b)
Carbofuran	*Pseudomonas veronii*	AF064460	Karpouzas et al. (2000b)
Carbofuran	*Pseudomonas caricapapayae*	D84010	Karpouzas et al. (2000b)
Carbofuran	*Pseudomonas asplenii*	Z76655	Karpouzas et al. (2000b)
Carbofuran	*Pseudomonas putida*	Z76667	Karpouzas et al. (2000b)
Carbofuran	*Moraxella osloensis*	X95304	Karpouzas et al. (2000b)
Carbofuran	*Flexibacter canadensis*	M62793	Karpouzas et al. (2000b)
Carbofuran	*Pedobacter heparinus*	M11657	Karpouzas et al. (2000b)
Carbofuran	*Chryseobacterium indologenes*	M58773	Karpouzas et al. (2000b)
Carbofuran	*Microbacterium liquefaciens*	X77444	Karpouzas et al. (2000b)
Carbofuran	*Paenibacillus aliginolyticus*	D78465	Karpouzas et al. (2000b)
Ethoprophos	*Pseudomonas putida*	AF131103	Karpouzas et al. (2000a)
Ethoprophos	*Pseudomonas putida*	L28676	Karpouzas et al. (2000a)
Ethoprophos	*Pseudomonas putida*	L37365	Karpouzas et al. (2000a)
Cadusafos	*Sphingomonas paucimobilis*	AJ698833	Karpouzas et al. (2005)
Cadusafos	*Flavobacterium sp.*	AJ698832	Karpouzas et al. (2005)
Fenamiphos	*Pseudomonas putida*	AF447394	Singh et al. (2003)
Fenamiphos	*Pseudomonas putida*	AF307868	Singh et al. (2003)
Fenamiphos	*Microbacterium esteraromaticum*	AB355700[a]	Cáceres et al. (2009)
Fenamiphos	*Microbacterium esteraromaticum*	Y17231[a]	Cáceres et al. (2009)
Fenamiphos	*Microbacterium arabinogalactanolyticum*	AB004715	Cáceres et al. (2009)

[a] The sequence of these bacteria was used instead of the original fenamiphos degrading *Microbacterium* sp. (EU151861) since the sequence of this bacterium was not available at the time of the analysis, however, both are reported to be close relatives on the same study

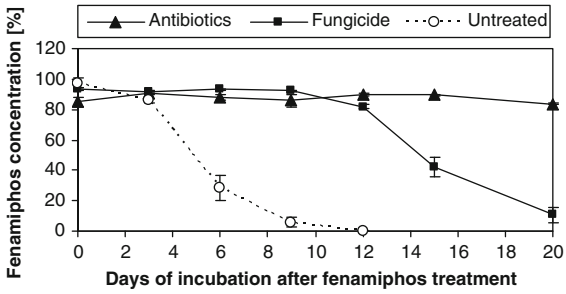

Fig. 1 Biodegradation of fenamiphos, applied at 100 μg ml^{-1}, by soil microorganisms at 0, 3, 6, 9, 12, 15 and 20 days after nematicide application to un-amended soil extract liquid media (untreated), and media amended with the antibiotics streptomycin and chloramphenicol or the fungicide cycloheximide. $N = 3$. *Bars* indicate the standard error of the mean

cycloheximide, although this degradation was still stronger than in the presence of chloramphenicol. Thus, the bacteria in the tested soil are the main group responsible for the degradation of fenamiphos. Other studies also have reported bacteria as primary factors for the enhanced fenamiphos biodegradation in soil (Megharaj et al. 2003; Singh et al. 2003) and, more generally, bacteria are capable of degrading other organophosphate pesticides such as parathion, fensulfothion and diazinon (Serdar et al. 1982; Sethunthan and Yoshida 1973; Sheela and Pai 1983). Our work thus emphasizes that bacteria are the principal microorganisms involved in the biodegradation process of fenamiphos in soil.

Fenamiphos degradation by individual culturable bacteria

From the culture filtrates in the previous assay purified individual colonies were randomly selected and analyzed for their fenamiphos degrading ability. Bacterial isolate numbers 1–20 were isolated from the cycloheximide treated culture medium, whereas

isolates number 21–40 were isolated from the untreated culture medium amended with fenamiphos. RP-HPLC quantification of the residual fenamiphos showed that of these 40 bacterial single colonies, 17 isolates, with the numbers 1, 2, 3, 4, 6, 10, 12, 15, 17, 20, 22, 23, 24, 32, 34, 35 and 38, were able to almost completely degrade fenamiphos and use it as sole carbon source (Fig. 2). Within 7 days, all these isolates had degraded fenamiphos by more than 50%. The other isolates with the numbers 5, 7, 8, 9, 11, 13, 14, 15, 18, 19, 21, 25, 26, 27, 28, 29, 30, 31, 33, 36, 37, 39 and 40 were not able to degrade fenamiphos thus not use it as sole carbon source.

The 17 bacterial isolates that were capable of degrading fenamiphos were further tested for their ability to degrade fenamiphos when formulated as Nemacur 5GR. Sixteen isolates were able to degrade the active ingredient of Nemacur GR, although not as efficiently when compared to the pure fenamiphos (Table 2). Remarkably, isolates number 3 and 38 degraded significantly less the amount of fenamiphos after 7 and 14 days of incubation compared to the other isolates. In particular, isolate number 3 was the only bacteria not capable of degrading fenamiphos in the commercial formulation. Nemacur 5GR contains 5% of the active substance fenamiphos, and 95% other unspecified compounds. Our result suggest that these additives can directly affect bacteria, by interfering with the degradation process. Generally, the isolated soil bacteria that are able to degrade pure fenamiphos can also degrade the fenamiphos in the commercial formulation. The breakdown of organophosphate pesticides is most likely caused by the synthesis of an enzyme or enzyme complex, which supports the organism in using the organophosphates as source of phosphorus or carbon for growth and development (Rosenberg and Alexander 1979). Since there was no carbon in our soil extract liquid medium, this also may be very well the case for our fenamiphos degrading bacteria. Further research is required to determine if the same bacteria that degraded fenamiphos in our liquid and agar cultures are also involved in the biodegradation process in soil. However, it may very well be the case since it has been previously demonstrated with other OP compounds and nematicides that microorganisms that can degrade in culture conditions also degrade in soil (Cycon et al. 2009; Karpouzas et al. 2000a). Furthermore it should be investigated whether our fenamiphos degrading isolates can also degrade the total fenamiphos residues. The data currently available suggest that this is likely to occur since it has been previously shown that the microorganisms that can degrade fenamiphos parent compound can also degrade its degradation products with no exception (Cáceres et al. 2009; Megharaj et al. 2003; Singh et al. 2003).

Cross-degradation of fosthiazate

The RP-HPLC analysis demonstrated that the 17 fenamiphos degrading bacteria were not able to initiate fosthiazate degradation throughout the 14 days incubation period (Table 2). Thus, there is no indication of an immediate risk for cross-degradation events. Racke and Coats (1988) isolated

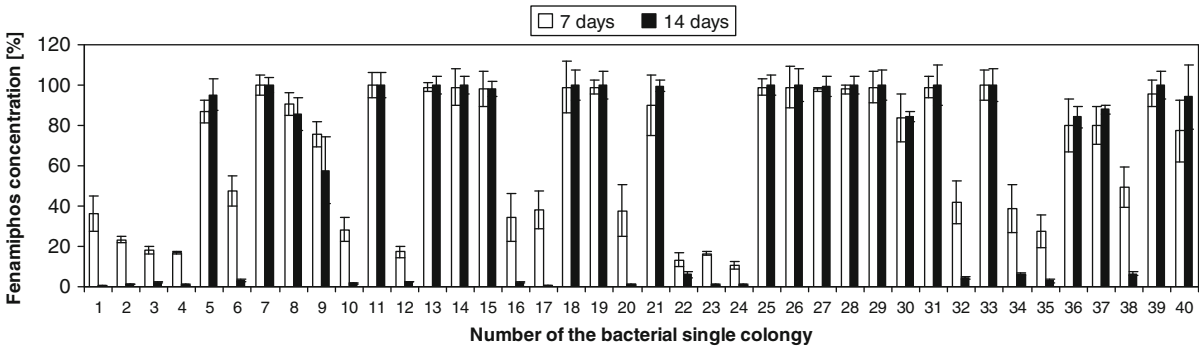

Fig. 2 Biodegradation of analytical grade fenamiphos in soil extract agar medium, initially applied at a concentration of 100 μg ml^{-1}, by 40 different randomly selected bacterial isolates after 7 or 14 days of incubation. $N = 3$. The error *bars* indicate the standard error of the mean

Table 2 Biodegradation of fenamiphos or fosthiazate as active ingredient of Nemacur 5 GR or Nemathorin 10WG, respectively, originally applied at 100 μg a.i. ml^{-1} in soil extract agar medium by 17 different pure fenamiphos degrading isolates

Bacterial code	Fenamiphos concentration (%)		Fosthiazate concentration (%)	
	7 Dpbi	14 Dpbi	7 Dpbi	14 Dpbi
1	35 ± 5	4 ± 1	100 ± 0	100 ± 0
2	62 ± 1	10 ± 1	99 ± 3	98 ± 1
3	100 ± 0	100 ± 0	100 ± 0	100 ± 0
4	39 ± 4	16 ± 1	98 ± 1	97 ± 1
6	68 ± 2	13 ± 2	100 ± 0	100 ± 0
10	38 ± 2	14 ± 1	99 ± 0	99 ± 1
12	48 ± 3	10 ± 1	100 ± 0	100 ± 0
16	56 ± 1	12 ± 2	100 ± 0	100 ± 0
17	61 ± 6	22 ± 3	98 ± 1	98 ± 1
20	59 ± 2	26 ± 2	100 ± 0	100 ± 0
22	29 ± 1	19 ± 1	100 ± 0	100 ± 0
23	37 ± 1	10 ± 1	99 ± 1	99 ± 1
24	53 ± 1	12 ± 1	100 ± 0	100 ± 0
32	58 ± 2	14 ± 2	98 ± 2	98 ± 1
34	74 ± 3	17 ± 3	100 ± 0	100 ± 0
35	47 ± 2	8 ± 1	99 ± 1	97 ± 1
38	100 ± 0	46 ± 2	100 ± 0	100 ± 0

$N = 3$. "±" indicate the standard error of the mean

Dpbi days post bacterium inoculation

Arthrobacter spp. from a soil with large history of isofenphos use. This bacterium was capable to degrade isofenphos at accelerated rates but could not degrade chlorpyrifos, fonofos, ethoprop, terbufos and phorate. In soils with large ethoprophos history, this nematicide was rapidly biodegraded but degradation of fenamiphos, fonofos, cadusafos, isazofos, aldicarb and oxamyl did not occur (Karpouzas and Walker 2000). This data supports the hypothesis that bacteria sometimes adapt to only one specific compound. Each organophosphate may very well need a specific enzymatic activity for degradation, making the degradation of organophosphates in general difficult and more complex which determines the species occurring within the soil population (Cabrera et al. 2009). Our study suggests that a rotation of fenamiphos with fosthiazate may be a tool for managing biodegradation for nematode control since their biodegrading microbes appear to be different. However, further research is required to analyze whether the fosthiazate degrading microorganisms can also degrade fenamiphos.

Molecular characterization and identification

The fenamiphos degrading bacteria produced a variety of 16S rRNA RFLP profiles. An initial clustering of strains divided into two groups with three or more members within the same DNA profile. The first group of isolates (type A) comprised eleven members; 1, 3, 4, 10, 12, 22, 23, 24, 34, 35 and 38 (Table 3). All these isolates showed high homology to the internal fragment of the 16S rRNA gene. A second group (type B) was composed by three isolates; 16, 17 and 20. There were three individual isolates with unique fragmentation patterns, been number 2 (type C), 6 (type D) and 32 (type E). The sequencing of the amplified 16S SSU DNA fragments of all degrading bacterial strains resulted in sequences ranging from 1080 to 1397 nucleotides in length. BLAST analysis revealed that all isolates from RFLP type A had high similarities, ranging from 95 to 99% identity match, with the sequences of two *Microbacterium* sp., acc. no. EU037292 and AY040877 present in the NCBI database. From the RFLP pattern type B, the isolates

Table 3 RFLP patterns and identification of 17 fenamiphos degrading (Fd) bacterial isolates according to their partial sequence of the 16S rRNA gene

Isolate[a]	RFLP[b]	New code[c]	Genus	Species	Genebank No.[d]	Identities (%)
1	A	Fd1	*Microbacterium*	n.d.	EU037292	96
2	C	Fd2	*Sinorhizobium*	n.d.	EU399910	93
3	A	Fd3	*Microbacterium*	n.d.	AY040877	95
4	A	Fd4	*Microbacterium*	n.d.	AY040877	95
6	D	Fd5	*Brevundimonas*	*mediterranea*	AJ244706	95
10	A	Fd6	*Microbacterium*	n.d.	AY040877	97
12	A	Fd7	*Microbacterium*	n.d.	EU037292	96
16	B	Fd8	*Ralstonia*	n.d.	AB167214	95
17	B	Fd9	*Cupriavidus*	n.d.	AB266612	95
20	B	Fd10	*Ralstonia*	n.d.	AB167214	96
22	A	Fd11	*Microbacterium*	n.d.	EU037292	96
23	A	Fd12	*Microbacterium*	n.d.	EU037292	97
24	A	Fd13	*Microbacterium*	n.d.	EU037292	96
32	E	Fd14	*Cupriavidus*	*necator*	AB167205	92
34	A	Fd15	*Microbacterium*	n.d.	EU037292	99
35	A	Fd16	*Microbacterium*	n.d.	EU037292	99
38	A	Fd17	*Microbacterium*	n.d.	EU037292	99

n.d. not defined species at the identification library

[a] Number of the isolate in the previous fenamiphos and fosthiazate degradation assays

[b] Restriction Fragment Length Polymorphism group to which every isolate belong according to its DNA pattern in agarose gel

[c] New code FD indicates fenamiphos degrading

[d] Genebank number according to the partial sequence of the 16S rRNA gene at the NCBI database

16 and 20 had an identity match of 95 and 96%, respectively, with the sequence of *Ralstonia* sp., acc. no. AB167214. The sequence of isolate number 2 was similar to *Sinorhizobium* spp., acc. no. EU399910 with 93% identity match. The sequence similarity between isolate 6 and *Brevundimonas mediterranea*, acc. no. AJ244706 was of 95%. Isolate numbers 17 (RFLP pattern type B) and 32 (RFLP pattern type E) had similar 16S SSU sequences as *Cupriavidus* sp. acc. no. AB266612, and *C. necator* acc. no. AB167205, with 95 and 92% identity matches, respectively. *Ralstonia eutropha* is currently considered synonymous with *Cupriavidus necator*, thus emphasizing the sequence similarities.

These results show that when culturable bacteria were randomly selected from a German soil with an intensive application history of Nemacur GR, eleven of the seventeen fenamiphos degrading bacterial isolates, i.e. 64.7%, belonged to *Microbacterium* spp. In an Australian soil a *M. esteraromaticum* isolate was found to be an effective fenamiphos degrading bacterium (Cáceres et al. 2009). Based on the two observations in different geographical areas it seems that the genus *Microbacterium* plays an important role in the degradation of fenamiphos. Isolates from this genus were also reported to degrade the non-fumigant carbamate nematicide carbofuran and the herbicide isoxaben at an accelerated rate (Arrault et al. 2002; Karpouzas et al. 2000b). Thus, the genus *Microbacterium* appears to play a major role in the biodegradation of an array of xenobiotics, including fenamiphos. Next to the genus *Microbacterium*, *Ralstonia* and *Cupriavidus* spp. appear also to be important in the fenamiphos biodegradation process. In previous research, isolates from both genera were shown to degrade the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (Huong et al. 2007; Martineza et al. 2008). *B. mediterranea* could also use fenamiphos as sole carbon source. Bacteria from this genus were previously found to degrade other organophosphate pesticides, such as the insecticide chlorpyrifos

and the herbicide trifluralin stressing its importance in the OPs biodegradation (Bellinaso et al. 2003; Li et al. 2008). *Sinorhizobium* spp. has been reported to degrade the polycyclic aromatic hydrocarbon phenanthrene (Seo et al. 2007) and can also mineralize the herbicide atrazine (Devers et al. 2007). The nematicide degradation ability, in particular the capacity to biodegrade fenamiphos, of most of these gram positive and gram negative bacteria had previously not been reported. Furthermore, we characterized and increased the microbial array that can perform the fenamiphos degradation process individually.

Phylogenetic analysis

The phylogenetic analysis included sequences of the fenamiphos degrading bacteria isolated in this study, and sequences from bacteria degrading carbofuran, cadusafos, ethoprophos or fenamiphos, as well as sequences of bacteria from soils with no fenamiphos degradation capability, reported in other studies. The phylogenetic analysis resulted in a fully resolved tree and showed sister lineage between sequences of fenamiphos degrading bacterial isolates and the sequences derived from the NCBI database (Fig. 3). The tree topology is strongly supported by general high clade credibility values (Posterior Probabilities). The phylogram showed that the bacteria with capacity to degrade nematicides are more closely related than those without degrading capability. This corresponds to phylogenetic studies reporting that organophosphate degrading bacteria able to degrade a specific pesticide are more closely related than bacteria able to degrade other pesticides (Singh

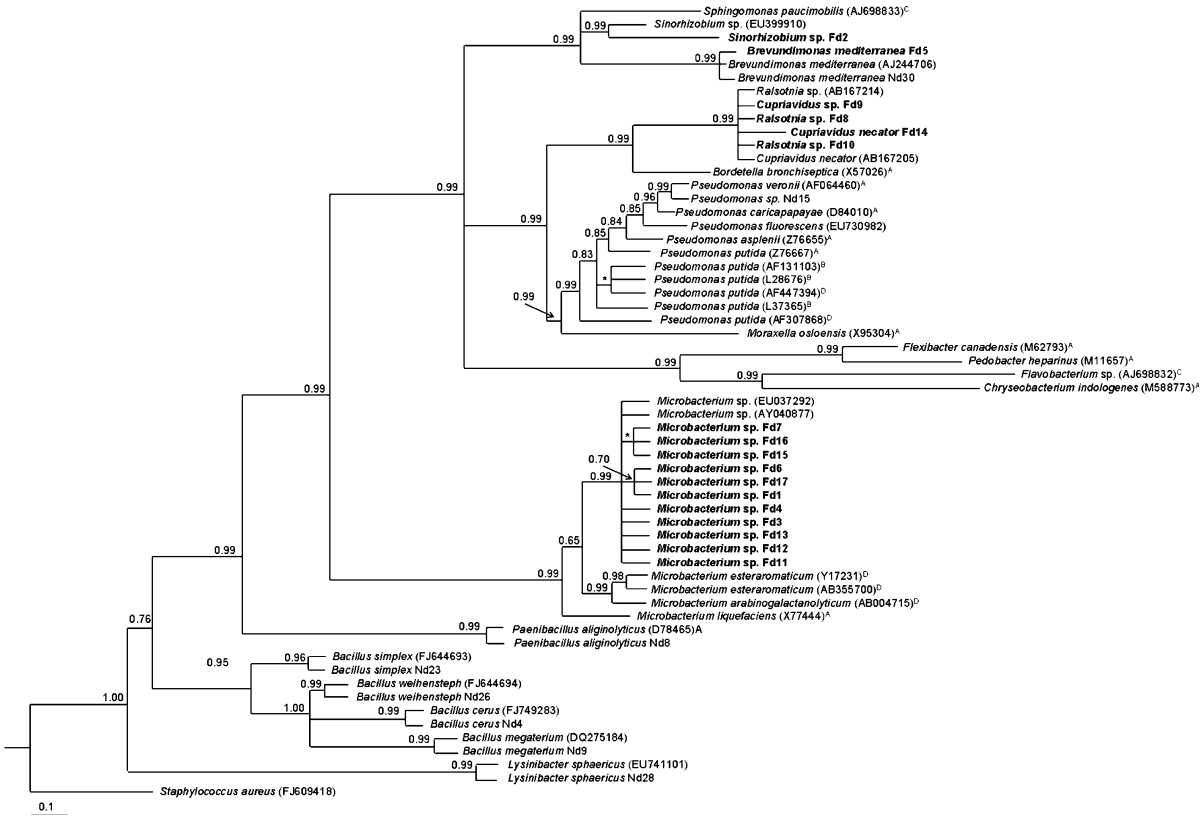

Fig. 3 Phylogenetic analysis of the partial 16S rRNA gene of fenamiphos degrading (Fd) and closely related bacteria, using *Staphylococcus aureus* (FJ609418) as outgroup. 50% majority rule consensus tree as phylogram. Model of nucleotide substitution was GTR + G. Values annotated to the nodes indicate: Posterior Probabilities. *Asterisk* (*): PP < 0.65. *Bars* indicate the relative number of substitutions per site. The accession number of the 16S rRNA gene sequences obtained from NCBI database is shown in parenthesis. Bacterial names in *bold* followed by the Fd codes are the fenamiphos degrading bacteria isolated in this study. *Nd* sequences of bacteria from soil with no fenamiphos enhanced degradation capability. Sequences of bacteria degrading carbofuran ([A]), ethoprophos ([B]), cadusafos ([C]), and fenamiphos ([D]) reported in other studies

2009). This suggests that the nematicide degrading bacteria are grouped within a common microbial population which can be of great significance for the soil ecology. If pesticides are applied to soil containing these microorganisms could result in the rapid development of microbial communities capable of enhanced pesticide degradation. In particular for fenamiphos, in a soil containing high numbers of *Microbacterium* bacteria the efficacy of the nematicide against plant parasitic nematodes could be reduced. A continuous fenamiphos application could select these adapted microorganisms in soil, especially in areas where the soil pH is optimum for the growth of *Microbacterium* species. Other studies have demonstrated the importance of soil pH in the development of microbial enhanced degradation of non-fumigant and fumigant pesticides (Singh et al. 2003; Warton and Matthiessen 2005). Furthermore, this study demonstrated that for predicting the efficacy of a nematicide not only the soil physical and chemical parameters are uniquely important factors. There are also bacteria which play a major role for the effectiveness in nematode control and persistence of a pesticide in the environment, in particular where only one nematicide is applied over an extended time period. The soil chemical and physical conditions that the bacteria require to adapt to a specific compound may be just a time regulator, giving the conditions for selecting the organisms present in the soil, determining whether the degradation process will start after one, or after repeated nematicide applications (Cabrera et al. 2009).

Conclusions

This study demonstrated that there was a wide array of gram-positive and gram-negative bacteria that are actively involved in the fenamiphos biodegradation process. These bacteria can utilize fenamiphos as carbon source and trigger the degradation process in the commercial nematicide formulation. The fenamiphos degrading bacteria were not able to cross degrade fosthiazate, even though both are organophosphate compounds, showing some pesticide specificity. The phylogenetic analysis showed that nematicide degrading bacteria are more closely related when compared to non-degrading isolates.

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