Ascomycete communities in the rhizosphere of field-grown wheat are not affected by introductions of genetically modified *Pseudomonas putida* WCS358r

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**Summary**

A long-term field experiment (1999–2002) was conducted to monitor effects on the indigenous microflora of *Pseudomonas putida* WCS358r and two transgenic derivatives constitutively producing phenazine-1-carboxylic acid (PCA) or 2,4-diacetylphloroglucinol (DAPG). The strains were introduced as seed coating on wheat into the same field plots each year. Rhizosphere populations of ascomycetes were analysed using denaturing gradient gel electrophoresis (DGGE). To evaluate the significance of changes caused by the genetically modified microorganisms (GMMs), they were compared with effects caused by a crop rotation from wheat to potato. In the first year, only the combination of both GMMs caused a significant shift in the ascomycete community. After the repeated introductions this effect was no longer evident. However, cropping potato significantly affected the ascomycete community. This effect persisted into the next year when wheat was grown. Clone libraries were constructed from samples taken in 1999 and 2000, and sequence analysis indicated ascomycetes of common genera to be present. Most species occurred in low frequencies, distributed almost evenly in all treatments. However, in 1999 *Microdochium* occurred in relatively high frequencies, whereas in the following year no *Microdochium* species were detected. On the other hand, *Fusarium*-like organisms were low in 1999, and increased in 2000. Both the DGGE and the sequence analysis revealed that repeated introduction of *P. putida* WCS358r had no major effects on the ascomycete community in the wheat rhizosphere, but demonstrated a persistent difference between the rhizospheres of potato and wheat.

**Introduction**

Worldwide losses of cultivated crops due to plant diseases amount to 12% (Agrios, 1997). In many cases plant pathogens can be controlled effectively by chemicals. However, not all plant diseases can be controlled chemically. Moreover, noxious pesticides will be taken from the market in the near future, e.g. methyl bromide (Martin, 2003). Therefore, development of alternative methods of crop protection is necessary. One alternative is the use of biological control agents (BCAs) to suppress plant pathogens. In this respect, fluorescent *Pseudomonas* spp. have received special attention, because of their excellent root-colonizing ability, and their potential to produce a wide variety of anti-microbial metabolites (O’Sullivan and O’Gara, 1992). Selected strains have been shown to suppress various soilborne plant pathogens, such as *Fusarium oxysporum*, *Gaeumannomyces graminis* var. *tritici*, *Phytophthora cinnamoni* and *Rhizoctonia solani* (Weller et al., 2002; Van Loon and Bakker, 2003). Despite decades of research, the commercial use of BCAs is still limited. Their commercial development is being hampered by failure or inconsistent performance of the BCAs under field conditions. Reasons for the latter include variable root colonization, loss of ecological competence and low or late production of disease-suppressing metabolites (Weller, 1988).

To circumvent these limitations, genetically modified BCAs with enhanced biocontrol activity can be constructed. Despite the potential benefits of such genetically modified microorganisms (GMMs), their use is often associated with public concern. There are several potential effects of introducing BCAs in the environment that may, or may not, be linked to an engineered trait: (i) displacement of non-target organisms, (ii) allergenicity to humans and animals, (iii) toxigenicity and (iv) pathogenicity to nontarget organisms (Cook et al., 1996). Effects of BCAs on bacterial community structure have been repeatedly found (Glandorf et al., 2001; Sigler et al., 2001; Bakker et al., 2002; Viebahn et al., 2003; Winding et al., 2004). How-
ever, reports on effects of introduced BCA on the fungal microflora are scarce.

In this study effects of genetically modified pseudomonads on rhizosphere ascomycete communities were monitored in a long-term field experiment. *Pseudomonas putida* WCS358r and two genetically modified derivatives were introduced into the rhizosphere of field-grown wheat in four consecutive years (1999–2002). The recombinant strains constitutively produced either phenazine-1-carboxylic acid (PCA), or 2,4-diacetylphloroglucinol (DAPG) (Viebahn et al., 2003), secondary metabolites of low molecular weight with a broad-spectrum activity against soilborne plant pathogens. In previous studies, a one-time field introduction of the PCA- and DAPG-producing derivatives of WCS358r significantly influenced the fungal and bacterial microflora, as detected by amplified ribosomal DNA restriction analysis (ARDRA) (Viebahn et al., 2003). Here, we focus on the group of ascomycetes, as an important member of this group, *Fusarium*, was shown to be affected by the introduction of WCS358r and its derivatives (Glandorf et al., 2001; Leeflang et al., 2002). We describe the application of denaturing gradient gel electrophoresis (DGGE) for analysis of genetic fingerprints derived from the amplification of the internal transcribed spacer (ITS) regions of rDNA with ascomycete-specific primers.

Possible effects caused by the introduction of the GMMs were compared with effects caused by crop rotation of wheat and potato. Crop rotation is commonly used in agriculture to control plant pathogens (Cook, 1992). It is well accepted that different crops establish different microbial communities in their rhizospheres (Lemanceau et al., 1995; Olsson and Alström, 2000; Oehl et al., 2003).

In addition to DGGE analysis of ITS rDNA amplicons, the same amplicons were used to construct clone libraries, which provide taxonomic information reflecting the ascomycete community composition (Kent and Triplett, 2002).

**Results**

To determine shifts in the ascomycete community upon introduction of the GMMs, DGGE was applied to rhizosphere samples of wheat plants that were grown from seeds treated with wild-type or genetically modified *P. putida* WCS358r.

A typical DGGE gel with banding pattern of ascomycete ITS1/ITS2 amplicons from differentially treated wheat rhizosphere samples (2000, day 59) is shown in Fig. 1. The dendrograms (Fig. 2) represent the percentage similarity of the cluster analysis of the patterns from rhizosphere samples taken at 13, 25, 40 and 54 days after sowing in 1999. Samples from the different treatments were grouped in either three or four clusters throughout the growing season. However, significant variability was evident, as indicated by the different clustering at different times. Moreover, differences between the samples throughout the season were high, as indicated by the low similarity indices (0–20%). At the beginning of the growing season mainly samples of the non-treated wheat rhizospheres clustered together (Fig. 2A). All other samples grouped at a similarity of 47%, with the exception of one replicate of the treatment with the combination of the
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GMMs. At day 25 the two samples from the combination treatment with both GMMs formed a distinct cluster (Fig. 2B). All other samples clustered at a similarity of 30%, with one replicate of the DAPG treatment and one replicate of the rotation plot each representing a distinct cluster. At 40 days two main clusters could be distinguished with only 14% similarity, while similarity within the clusters was around 30% (Fig. 2C). One cluster contained all replicates from the treatments with the DAPG-producing derivative of WCS358r, including those from the treatment with the combination of the GMMs, and two samples from non-treated wheat. The second cluster contained samples of the treatment with wild-type WCS358r and with the PCA producer, and the other two samples from non-treated wheat. At 54 days one main cluster was observed at a similarity level of 68% (Fig. 2D). In this cluster samples from all treatments were present. Only three samples, two from non-treated wheat and one from the treatment with the wild-type WCS358r, fell outside the cluster, but were also significantly different from each other. Thus, treatments with the combination of the PCA and the DAPG-producing derivatives clustered away from the other treatments, indicating a significant effect on the ascomycete community after a one-time introduction. Neither the treatment with the PCA producer nor that with the DAPG producer alone had an effect that exceeded the variation in the control treatments.

Similar dendrograms were constructed from samples taken at four time points in 2000 and 2001, and three time points in 2002 (data not shown). The field trial was destroyed by activists in June 2002, resulting in only three sampling dates. The overall similarity of the samples varied between 5% and 38% in 2000, between 0.3% and 9% in 2001, and between 0.4% and 4% in 2002. To visualize

Fig. 2. Dendrograms based on the genetic similarity of the ascomycete communities of field-grown wheat plants in 1999 at 13 (A), 25 (B), 40 (C), 54 (D) days after sowing. For treatments see legend to Table 1. Samples from three plots were pooled, resulting in two replicates per treatment, from the left (a) and from the right half (b) of the field. rp, samples taken from a rotation plot, in which wheat was grown in 1999. Similarities are based on DGGE patterns generated from amplified sequences of the ITS1/ITS2 regions of rDNA using Pearson’s correlation coefficient. Cluster analysis was performed with UPGMA. Significant clusters are indicated by the grey lines and were separated by the point-bisectional cut-off method. The level of similarities is shown above the dendrograms.
the overall effects in each year, we constructed composite dendrograms. Composite dendrograms represent the average of the dendrograms of all samples in 1 year. Figure 3 shows the composite dendrograms of each of the 4 years. The number of significant clusters varied between two and three. The composite dendrogram for the data obtained in 1999 at 13, 25, 40 and 54 days is presented in Fig. 3A. In agreement with the analysis shown in Fig. 2, a distinct cluster, containing the rhizosphere samples from the treatment with the combination of both GMMs, stands out (Fig. 2A). With the exception of one of the samples from non-treated wheat, all other samples grouped at a similarity of about 40%.

In the second year the effect of cropping potato instead of wheat was predominant (Fig. 3B). Both samples from the rotation plots formed one significant cluster, whereas all other samples, from wheat, formed the second significant cluster at a similarity level of 50%. The effect of growing potato in 2000 was still significant in 2001, although wheat was now again sown in the rotation plots (Fig. 3C). Again, samples from the rotation plots formed a cluster distinct from all other samples. Interestingly, samples from the left (a) and samples from the right half (b) of the field tended to form separate clusters, although differences were too small to be significant. After the fourth trial in 2002, in which again potatoes had been planted in the rotation plots, the samples from the rotation treatment clustered again separate from the samples of all other treatments (Fig. 3D). During the 4-year field trial only an effect of the combination treatment of both GMMs after the first introduction was significant. Once potato has been planted instead of wheat, the effect of this crop rotation was maintained during the following years.

**Clone library screening**

Polymerase chain reaction (PCR)-amplified ITS1/ITS2 fragments from rhizosphere samples taken in 1999 and...
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2000 were cloned and 960 clones were sequenced. All sequences revealed similarities to ascomycetes except one that was most similar to an arbuscular endomycorrhizal fungus. Technical failures (including sequences with a similarity lower than 90%) ranged from 12% to 35%. To determine whether the number of clones would sufficiently represent the ascomycete diversity in the rhizosphere soil, a rarefaction curve was constructed. In this curve the number of clones analysed (from samples treated with the wild type in 2000) was plotted against the cumulative number of ascomycete genera detected for that number of clones (Fig. 4). This relationship provides an estimate of the sampling efficiency of any given sample size. The fact that the rarefaction curve almost reached a plateau and that the range of the standard deviation decreased with increasing sample size suggested that the analysis of 55–60 clones per treatment provided reasonable coverage of the fungal diversity.

Genera that were identified from the sequence analysis comprised common ascomycetes such as *Fusarium*, *Giberella*, *Nectria*, *Penicillium*, *Trichoderma* and *Verticillium*. Most genera occurred in low frequencies and were distributed almost evenly in all treatments (Table 1). Sequences obtained in 1999 from samples collected statistically from the control treatments revealed similarity with *Microdochium*, and from the treatment with the PCA-producing GMM 50% revealed similarity with *Paecilomyces*, and 20% with a leaf litter ascomycete. In the right half of the field more than 40% of the clones from the control treatment, the treatment with the wild type, and the treatment with the combination of both GMMs were indicative of *Microdochium*. The percentage of any of the ascomycetes ranged from 2 to 13. In samples taken in 2000, the percentage of *Fusarium* species was about 20 in all treatments, including the rotation plots, compared with only 5 in 1999. The percentage of *Nectria* also increased in 2000, but this fungus was not recovered from the potato rhizosphere. Twenty-seven per cent of the sequences obtained from the control treatments revealed similarity with *Trichoderma*, compared with only 2% or 3% in samples from the other treatments. In the potato rhizosphere two ascomycetes occurred in higher numbers than in rhizosphere of wheat, namely *Plectosphaerella* and *Verticillium*.

**Discussion**

Molecular engineering to construct BCAs with enhanced biocontrol activity may lead to increased interest in commercial use of GMMs. Several studies showed that such introductions of genetically modified BCAs could have an influence on resident microbial communities. However, most of these studies were performed in microcosm and pot experiments, and do not necessarily reflect the complex situation in natural field environments. Possible long-term effects and effects of repeated introductions of GMMs should be studied preferentially in the field.

In the present study genetically modified derivatives of *P. putida* WCS358r, which produced either PCA or DAPG, were introduced into the rhizosphere of field-grown wheat for four consecutive years (1999–2002). The genetically modified derivatives expressed the inserted genes constitutively, making the production of the anti-microbial compounds independent of environmental variables. Both production of antibiotics and siderophore-mediated competition for iron have been implicated as mechanisms of suppression of plant diseases by fluorescent pseudomonads (Bakker et al., 1991). The production of PCA and DAPG was demonstrated to be important in suppression of take-all in wheat and barley by *Pseudomonas fluorescens* strains 2-79 (Thomas and Wellar, 1988) and Q2-87 (Bangera and Thomas, 1999). For *P. putida* WCS358r production of its fluorescent siderophore pseudobactin 358 is essential for suppression of *Fusarium* wilt in carnation and radish (Duijff et al., 1994; Raaijmakers et al., 1995). Combining two modes of action, either in one strain or by using combinations of different strains, can improve their efficacy (Van Loon, 1998; De Boer et al., 2003). The naturally DAPG-producing *P. fluorescens* Q8r1-96, which was genetically modified to produce PCA in addition, has been shown to be more suppressive to *Rhizoctonia* root rot than the wild-type strain (Huang et al., 2004). For the PCA-producing strain used in this study it was demonstrated that it provides improved control of take-all in wheat (Bakker et al., 2002).

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Table 1. Ascomycete genera with ITS-rDNA sequences most similar to clones isolated from the rhizosphere of field-grown wheat originated from the left and right half in 1999, and from the right half in 2000.

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Ascomy whole communities not affected by Pseudomonas putida WCS358r

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Plants were grown from seeds that were untreated (contr), treated with P. putida WCS358r (wt), its PCA- and DAPG-producing derivatives (phz, phl), and a combination of both (phz+phl). Ascomycete sequences were identified with BLASTN Search (NCBI). Numbers are the percentage out of a total of 60 clones per treatment. rP, rotation plot, in which potatoes were planted in 2000. Abundant genera are indicated in bold type.

After the first year of introducing the GMMs in 1999, an effect of seed treatment with the combination of the DAPG- and the PCA-producing derivatives of WCS358r on the ascomycete community was apparent. Application of either the wild type or the DAPG and PCA producers alone did not affect the ascomycetes as detected by DGGE analysis. Whereas one would expect enhanced non-target effects after repeated introduction of the GMMs, no such effects were detected. This lack of effects of the GMMs cannot be ascribed to insensitivity of the DGGE technique, as a significant difference between the ascomycete communities of wheat and potato was detected. The effect of crop rotation was still evident after cultivating wheat again in the rotation plots in the third year, suggesting that a single cropping of potato had a long-term effect. Also in 2002 samples from the rotation plot, in which potato has been planted, were significantly different from all other treatments.

In two previous field experiments, a single introduction of the PCA-producing derivative of WCS358r significantly influenced the fungal microflora as determined by ARDRA (Glandorf et al., 2001). Similarly, Viebahn and colleagues (2003) described a significant effect of the DAPG-producing derivative of WCS358r on the fungal community after a one-time introduction. However, after a second introduction in the same field plots this effect was no longer evident. Therefore, we conclude that the effects of the GMMs on the fungal microflora that were observed previously (Glandorf et al., 2001; Viebahn et al., 2003) cannot be explained by a major perturbation of the ascomycete community. Recently, Schouten and colleagues (2004) screened 117 Fusarium oxysporum strains isolated from various cultivated soils for their sensitivity to DAPG. Seventeen percent of the strains were highly tolerant to DAPG. The tolerance was independent of formae speciales, geographic location, genetic background, or production of fusaric acid, which is known to negatively affect antibiotic production in P. fluorescens CHA0 (Notz et al., 2002). It was suggested that deacetylation of DAPG to monoacetyl-phloroglucinol and phloroglucinol is one of the mechanisms involved in tolerance. The study focused on Fusarium, but it is quite possible that other ascomycetes possess similar defence strategies. Preliminary evidence indicates that Fusarium species isolated from the rhizosphere of wheat treated with the DAPG-producing Pseudomonas strain were able to degrade DAPG (J.M. Raaijmakers, pers. comm.). Whether these strains were also able to tolerate PCA needs to be investigated.

The activation of efflux ATP-binding cassette (ABC) transporters, which are common among eukaryotes (Higgin, 1992), has been reported to protect certain fungi from toxicants. Schoonbeek and colleagues (2001) demonstrated that Botrytis cinerea became sensitive to reser-vatrol by disruption of the ABC transporter gene BcatrB. Tolerance to, or degradation of, the antibiotic and/or expression of drug transporter genes might explain why no effect of the GMMs on the ascomycete communities was detected.

In addition to fingerprinting the ascomycete community by DGGE, we constructed clone libraries of samples taken in 1999 and 2000 to detect specific ascomycota genera. The coverage of the ascomycetes was estimated by constructing a rarefaction curve, which suggested a sample size of 60 clones per treatment to be sufficient to cover the ascomycete diversity. Similar coverage has previously been reported by Anderson and colleagues (2003). They tested different primer pairs to assess the fungal diversity in natural grassland, and found 50 clones per library to be sufficient to cover the fungal diversity.

In our study, with only one exception, all ITS1/ITS2 region sequences amplified with the ITS5/ITS4A primer pair showed high similarities to ascomycetes in public
structed by insertion of the chromosome. The DAPG-producing GMM was con-
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The bacterial strains used in this study were
P.putida WCS358r (Geels and Schippers, 1983) and two transgenic derivatives (Glandorf et al., 2001; Viebahn et al., 2003). The
PCA-producing derivative, WCS358r::phz, contained the phz
ABCDEF-G gene cluster on the disarmed mini-Tn5 transpo-
son-based vector pUTKm (Herrero et al., 1990) inserted into
the chromosome. The DAPG-producing GMM was con-
structed by insertion of the phiFACBDE genes from
P.fluorescens Q2-87 (Bangera and Thomasaw, 1996), a
naturally DAPG-producing strain, into the chromosome of
WCS358r, and designated WCS358r::phz (Viebahn et al., 2003). The wild-type strain WCS358r was grown on King's Medium B (KB) agar (King et al., 1954) supplemented with
150 µg rifampicin ml⁻¹, and the transgenic derivatives on KB
with 150 µg rifampicin ml⁻¹ and 30 µg kanamycin ml⁻¹. All
strains were routinely grown at 28°C for 2 days.

Seed treatment and experimental field
Wheat seeds (Triticum aestivum cv. Baldus) were sown in
four consecutive years (1999–2002) in an experimental field
located near the Botanical Garden of Utrecht University, the
Netherlands, as described by Viebahn and colleagues
(2003). Permission for the deliberate field release of GMMs
was granted by the Dutch Ministry of Housing, Spatial Plan-
ing and the Environment, the Netherlands (BGGO 98/10).
Wheat seeds were treated with a 1:1 mixture of washed
bacterial suspensions and 3% methylcellulose, as described
by Glandorf and colleagues (2001) The bacterial treatments
used were WCS358r, WCS358r::phz, WCS358r::phl, or a 1:1
mixture of WCS358r::phz + WCS358r::phl. For the control
treatment the bacterial suspension was replaced by 10 mM
MgSO₄. Coated seeds were air-dried overnight and sown
the next day. Coating resulted in approximately 10² colony-form-
ing units per seed, as determined from plate count enumer-
ations. An additional treatment was a rotation plot, in which
in the first (1999) and third year (2001) non-treated wheat
seeds were sown, and in the second (2000) and fourth year
(2002) non-treated potatoes (Solanum tuberosum L. cv. Mod-
esta) were planted. The bacterial strains were introduced
every year on seeds in the same plots, and rhizosphere soil
samples were taken each year at three to six different time
points.

DNA extraction and polymerase chain reaction
From each plot 3–5 g of roots with adhering soil were sam-
peld at the different time points during the growing season
and mixed with 10 ml of sodium phosphate buffer (120 mM,
pH 8). One gram of gravel was added and samples were
vortexed for 30 s. The supernatant was decanted into a new
tube. One ml of the supernatant was used to extract total
DNA with the FastDNA® SPIN Kit for Soil (Bio 101, Biogene,
Vista, CA, USA) in combination with a Ribolyser (Hybaid,
Ashford, UK) (Smit et al., 2003). The extracts were resus-
pended in 100 µl of Millipore-filtered distilled water and puri-
fied with the Wizzard® DNA Clean-Up System (Promega,
Madison, WI, USA) according to the manufacturer's protocol.
The six replicate DNA samples of each treatment were
pooled into two samples, each containing three replicates,
and DNA was amplified with the forward primer ITS5 (5'-GGA
AGT AAA AGT CGT AAC AAG G-3') (White et al., 1990) and
the reverse primer ITS4A-GC (5'-CGC CCG CCG CGC CCC
CGC CCC GCC CCG CCG CCC CCG CCC CCG CCC CCG
TGC GGC AAT CCC TG-3') (Larena et al., 1999). The primer
pair amplified a fragment of about 700 bp of the ITS1/ITS2
region of ascomycete rDNA. ITS4A-GC contained a 40 bp
GC-clamp to stabilize the melting behaviour of the DNA frag-
ments for DGGE analysis (Sheffield et al., 1989). The PCR
was performed in 10× PCR buffer 2, pH 9.2, containing
2.25 mM MgCl₂ (Roche, Diagnostics, Mannheim, Germany),
250 µM of each of the four deoxynucleoside triphosphates,
200 nM of each primer, 2.5 U Expand Long template enzyme

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Denaturing gradient gel electrophoresis (DGGE)

The PCR fragments were separated on a denaturing gradient polyacrylamide gel containing 0.5x TAE buffer (20 mM Tris-acetate, 10 mM sodium acetate, 0.5 mM EDTA). The gel consisted of 8% (w/v) polyacrylamide (acylamide/bisacrylamide in a ratio of 37.5:1) with a denaturant gradient of 20–50%. One hundred percentage stock solution contained 7 M urea and 40% formamide. After correction for concentration up to 25 µl per lane of PCR product was loaded, and gels were run for 17 h at 80 V at a constant temperature of 60°C in a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Veenendaal, the Netherlands). For comparison a reference DNA marker, consisting of 18S rDNA amplicons of seven different ascomycete species, was run in triplicate on each gel. After electrophoresis gels were stained for 30 min in 1:10 000 diluted SybrGold (Molecular Probes, Leiden, the Netherlands) and viewed on a blue-light transilluminator (Clare Chemical Research, Dolores, CO, USA). Images were digitalized using the GeneGenius Bio Imaging System (Syngene, Cambridge, UK).

Cluster analysis

The ascomycete community fingerprints of the DGGE gels were analysed with the BIONUMERICS program version 3.5 (Applied Maths, St. Marten-Latem, Belgium). After normalization and background subtraction Pearson’s correlation coefficient was used to calculate the similarity between each banding pattern. Patterns were grouped into clusters by the unweighted pair-group method using average linkages (UPGMA). Significant clusters in the dendrograms were determined by calculating the cut-off value that produced the highest point-bisectional correlation (BIONUMERICS manual, version 3.5). Briefly, a line is drawn through the dendrogram at a certain similarity level, and from the resulting number of clusters defined by that line, a new similarity matrix is created, in which all within-cluster values are 100%, all between-cluster values are 0%. Then, the correlation between this new matrix and the original matrix is calculated, which is called the point-bisectional correlation. The same is done for different cut-off similarity levels, and the level with the highest point-bisectional correlation is the one defining the significant clusters.

Cloning and sequencing

Clone libraries were constructed from samples taken in 1999 from the left and the right half of the field, and from samples taken in 2000 from the right half of the field. Two microlitres of the PCR product (see above) was ligated to the pGem® Easy Vector (Promega, Madison, WI, USA) and transformed into Escherichia coli JM109 Ultra Competent Cells (Stratagene, Cambridge, UK) according to the manufacturer’s protocol. The bacteria were plated on LB agar supplemented with 50 µg ml⁻¹ ampicillin, 80 µg ml⁻¹ X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) and 20 mM IPTG (isopropyl-1-thio-β-d-galactopyranoside). For each sample 60 white colonies were resuspended in the PCR mixture and amplified directly using the primer pair ITS5 and ITS4A under the conditions as described above. Polymerase chain reaction products of each clone were analysed on a 1% agarose gel and fragments of the correct size (approximately 700 bp) were purified using the QiAquick PCR Purification Kit (Quiagen, Venlo, the Netherlands) according to the manufacturer’s protocol. The sequencing reaction was carried out with the BigDye® Terminator v.3.1 Cycle Sequencing Kit in an ABI3700 Sequencer (Applied Biosystems, Nieuwerkerk, the Netherlands). Clones were sequenced in both directions with the reverse primer ITS5 and the forward primer ITS4A. Both sequences from each clone were aligned with the program KODON version 2.0 (Applied Maths, St. Marten-Latem, Belgium) and sequences were compared with known ascomycete sequences from GenBank with the Standard Nucleotide BLAST program (Altschul et al., 1997) provided by the National Center for Biological Information (NCBI, USA).

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References


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Ascomycete communities not affected by Pseudomonas putida WCS358r


