Bacteria associated with *Pinus sylvestris–Lactarius rufus* ectomycorrhizas and their effects on mycorrhiza formation *in vitro*

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Received: *22 January 2001*
Accepted: *10 May 2001*

Summary

• The structure and functioning of mycorrhizosphere bacterial communities from the soil organic fermentation horizon (FH) of a *Pinus sylvestris* stand was investigated.
• Bacteria were isolated from *P. sylvestris–Lactarius rufus* mycorrhizas, and their effect on ectomycorrhiza formation, localization within the mycorrhizosphere, and phenotypic and genotypic characteristics determined.
• The isolates could be divided into seven distinct phenotypic and physiological groups. Two *Paenibacillus* isolates stimulated infection of both first and second order lateral roots, resulting in a doubling of the percentage root conversion to mycorrhizas, relative to the control treatment, after 8 wk. Two *Burkholderia* isolates, and a *Rhodococcus* sp., had no effect on numbers of first order mycorrhizal lateral roots, but increased formation of secondary mycorrhizal lateral roots. There was evidence that the *Burkholderia*, but not the *Paenibacillus* or *Rhodococcus* isolates, associated preferentially with mycorrhizal roots, and that the *Burkholderia* strains were capable of more effective spread to the root tip than the other isolates.
• Ectomycorrhizosphere bacteria appear to promote mycorrhiza formation by means of a variety of mechanisms, and there are similarities in the nature of bacteria inhabiting ecto- and arbuscular mycorrhizospheres.

Key words: ectomycorrhiza, mycorrhizosphere, helper bacteria, *Lactarius rufus*, *Pinus sylvestris*, *Burkholderia*, *Paenibacillus*, *Rhodococcus*.

Introduction

Bacteria are ubiquitous colonizers of the ectomycorrhizosphere, and occur on the mycorrhizal mantle, and at inter- and intracellular locations within the mantle and Hartig net. Extensive bacterial biofilms have also been observed covering the foraging hyphal front (Nurmiaho-Lassila *et al.*, 1997). The structure of ectomycorrhizosphere bacterial communities is distinct from that of the bulk soil, and can vary between species of ectomycorrhizal fungus (Timonen *et al.*, 1998; Mogge *et al.*, 2000).

Despite numerous accounts of bacterial isolations from healthy ectomycorrhizal roots and sporocarps of a wide range of fungal species (Li & Castellano, 1987; Ali & Jackson, 1989; Danell *et al.*, 1993; Varese *et al.*, 1996), the functional significance of the bacterial associate has rarely been elucidated. Additionally, the characteristics of bacterial associates have generally been determined using phenotypic or physiological tests, and the taxonomic affiliation and relatedness of the isolates is uncertain. However, it is clear that specific bacterial isolates from the ectomycorrhizosphere can promote ectomycorrhiza formation under a variety of environmental conditions.

Garbaye & Bowen (1989), using the *Pinus radiata–Rhizopogon luteolus* symbiosis, showed that a number of bacteria isolated from inside the ectomycorrhizal mantle had stimulatory effects on the mycelial growth of *R. luteolus*, and in some cases, enhanced mycorrhiza formation. Much work on such mycorrhiza 'helper' bacteria (MHB) has subsequently

focused on the *Pseudotsuga menziesii–Laccaria bicolor* symbiosis. In this symbiosis, a number of fluorescent pseudomonads and sporulating bacilli are able to stimulate ectomycorrhiza formation (Garbaye, 1994).

MHB from the *P. menziesii–L. bicolor* symbiosis have been shown to express a degree of fungus-specificity in their activity. While *P. menziesii–L. bicolor* MHB can have neutral or stimulatory effects on mycorrhiza formation by other *Laccaria* species (Duponnois *et al.*, 1993; Dunstan *et al.*, 1998), they can inhibit mycorrhiza formation on *P. menziesii* by ectomycorrhizal fungi belonging to other genera (Duponnois *et al.*, 1993). Further, MHB associated with *L. bicolor* stimulate mycorrhiza formation with a range of gymnosperm and angiosperm species, suggesting that they are not plant-specific (Garbaye & Duponnois, 1992; Dunstan *et al.*, 1998).

Garbaye (1994) proposed a number of mechanisms by which MHB could potentially stimulate mycorrhiza formation. These include improving the receptivity of the root to mycorrhizal infection, enhancing root–fungus recognition processes, stimulating fungus growth in the rhizosphere, and altering the physico-chemical properties of the soil to facilitate mycorrhizal infection. However, there is little direct evidence to support any of these mechanisms.

While the MHB from the *P. menziesii–L. bicolor* symbiosis and their effects have been well characterized, the applicability of the findings to other ectomycorrhizal symbioses is unclear. In particular, little is known of the occurrence or characteristics of MHB in the mycorrhizosphere of symbioses involving other ectomycorrhizal fungi. Further, MHB have been isolated from highly managed forest nursery sites (Bowen & Theodorou, 1979), nursery sites supporting nonnative *P. menziesii* (Duponnois & Garbaye, 1991), or Mediterranean-type soils (Garbaye & Bowen, 1989; Dunstan *et al.*, 1998). The extent to which MHB may occur in temperate and boreal forest soils, which typically contain high organic matter contents and possess only small bacterial communities (Read, 1991), is unclear.

The aims of this study were to determine the nature of interactions between *Lactarius rufus*, an ectomycorrhizal fungus typical of temperate and boreal forest soils, and its associated ectomycorrhizosphere bacteria, and to characterize those isolates capable of stimulating ectomycorrhiza formation. Further, the population dynamics and localization of the helper bacteria were investigated in order to contribute to understanding of the mechanisms underlying the mycorrhiza helper effect.

Materials and Methods

Isolation of *Lactarius rufus*

Lactarius rufus (Scop. Ex Fr) Fr. was isolated from ectomycorrhizal roots collected from the FH soil horizon underneath sporocarps, in a mature stand (*c.* 20 yr) of *Pinus sylvestris* L. at Lady Canning's Plantation, Ringinglow, South Yorkshire, UK (OS Map no. 110, grid reference 285833). This site is an acidic peaty podzol overlying Millstone Grit. Ectomycorrhizal roots were examined under a dissection microscope, and their identity confirmed as *L. rufus* using anatomical features and ectomycorrhizal mantle colour (Agerer, 1987–93). Mycorrhizal root tips were removed using fine forceps, surface sterilized by immersion in H_2O_2 (30% v/v) according to Heinonen-Tanski & Holopainen (1991), rinsed in three changes of sterile distilled water, and individual root tips plated out on Modified Melin-Norkrans medium (MMN) (Marx, 1969). Plates were incubated at 25°C and fungal cultures maintained by subculturing every 6 wk.

Isolation of bacteria

Bacteria were isolated from the same sample of *L. rufus–P. sylvestris* ectomycorrhizal roots that was used for isolation of the fungus. Approximately 50 root tips were macerated in 1 ml of sterile distilled water using a glass tissue grinder. The macerate was diluted between 10^{-1} and 10^{-4} and 200 µl plated onto Nutrient Agar (NA, Oxoid, Basingstoke, UK), Tryptic Soy Agar (3 g l^{-1}, Oxoid), Tryptic Soy Agar (3 g l^{-1}) containing cycloheximide (75 mg l^{-1}, Sigma, Sigma Chemical Company, Dorset, UK), and King's Medium B (King *et al.*, 1954). Plates were incubated at 25°C for 4 d. Colonies were grouped according to morphology, growth rate and colour, and single representatives of the groups were picked off using a sterile plastic loop and subcultured onto fresh NA plates. Few bacterial colonies grew on the 10^{-3} or 10^{-4} dilutions, and most isolates were selected from the 10^{-1} and 10^{-2} dilutions. A total of 30 isolates were selected and maintained at −40°C in a sterile storage system (Protect, Technical Service Consultants Ltd Heywood, UK).

Phenotypic characterization of isolates

A number of phenotypic and physiological tests were performed to group the 30 isolates. Isolates were cultured on NA at 25°C for 2–4 d. A Gram test was performed by staining and by determining solubility in 3% KOH (Gregersen, 1978). Morphology and spore formation were determined using phase contrast microscopy at 400× magnification. Motility was determined using semisolid NA according to Krieg & Gerhardt (1981). Isolates were further tested for production of fluorescent pigments on King's Medium B (King *et al.*, 1954), production of catalase and cytochrome C oxidase (Kovács, 1956), and ability to oxidize carbohydrates fermentatively (Hugh & Leifson, 1953).

Screening of bacterial isolates for effects on ectomycorrhiza formation

Seeds of *Pinus sylvestris* L. were obtained from The Forestry Commission, Alice Holt, Surrey, UK. The seeds were soaked for 20 h in a 0.001% solution of chlortetracycline before

being surface-sterilized by immersion in 0.1% $AgNO_3$ for 10 min followed by 0.5% NaCl for 5 min (Speakman & Krüger, 1983). The seeds were subsequently washed by vortexing for 2 min in three changes of sterile distilled water before being placed to germinate on 1% distilled water agar (Oxoid) in 9 cm diameter Petri dishes. Five seeds were arranged in a line down the middle of each dish. Plates were incubated in a controlled-environment growth room for 3 d at 20°C in the dark until the first signs of germination were visible. Plates were then placed at an angle of 45° to the horizontal, with the line of seeds positioned horizontally, to encourage straight root growth. This assisted subsequent seedling manipulation. The plates were incubated at 20°C for a further 11 d with an alternating 16 h light–8 h dark cycle (irradiance 27 Wm^{-2}; rh 80%). Sterile seedlings were used for ectomycorrhiza synthesis when the taproot was 4.5–6.0 cm long.

Ectomycorrhizas were synthesized using a Petri dish method modified from Duddridge (1986). Irish moss peat was passed through a 3-mm sieve and added to vermiculite that had been screened through a 2-mm sieve to remove dust, in a v : v ratio of one part peat to seven parts vermiculite. To eight parts of this mixture, three parts of 1/2 MMN liquid medium (no glucose or agar) was added by volume and mixed thoroughly. After autoclaving, the mixture was packed densely into 9 cm diameter Petri dishes. A slit was cut into the side of the Petri dishes and their covers using a hot scalpel and a 2-wk-old seedling of *P. sylvestris* was laid into the slit so that the roots would remain in aseptic conditions inside the Petri dish whilst the shoot system would grow exposed to the atmosphere. The fungal inoculum consisted of 6 mm diameter discs cut from the growing margin of an MMN agar culture, which had been incubated for 2 d at 25°C on MMN agar so that active hyphal growth had commenced. Three discs were placed upside-down along each side of the root at 15 mm intervals, at a distance of 5 mm from the root.

A preliminary screen of bacteria on plant growth and mycorrhiza formation involved 21 isolates, which were selected to include representatives of each phenotypic group identified. The method of preparation and addition of the test bacterium to the screening plate was modified from that of Duponnois & Garbaye (1991). The bacterial isolates were retrieved from 'Protect' and subcultured onto NA plates. A single bacterial colony was grown in Luria-Bertani broth (Oxoid) at 26°C for 16 h on an orbital shaker. Cells were harvested by centrifugation, then washed and resuspended in sterile 0.1 M $MgSO_4.7H_2O$ buffer to an optical density of 1.8–1.9 at 600 nm. A 100 µl aliquot of bacterial cells was pipetted midway between each fungus disc and the root, providing a total volume of 600 µl. The Petri dish lid was replaced and the slit–hypocotyl junction sealed with autoclaved anhydrous lanolin (Sigma). Plates were sealed with Parafilm and incubated within propagators, with plates set upside down at an angle of approx. 70° to the horizontal so that the roots grew down against the lid. Propagators were placed in a controlled-environment room under the conditions described previously. Five replicate plates were prepared for each isolate. Parallel control plates were also prepared, in which 0.1 M $MgSO_4 \cdot 7H_2O$ buffer replaced the bacterial inoculum. Blocks containing 2–4 bacterial treatments, plus five control plates, were set up at weekly intervals. Plates were examined weekly, over a period of 8 wk, using a binocular microscope, and the numbers of leaves and first order and second order uninfected and mycorrhizal lateral roots were recorded (data not shown).

From the results of the preliminary screen, six isolates (EJP59, EJP67, EJP70, EJP73, EJP75 and EJP77), which had displayed positive effects on mycorrhiza formation during the preliminary screen, were selected for a repeat test. Blocks containing five replicate plates for each of four bacterial treatments, plus 10 control plates, were set up at weekly intervals for 3 wk. Thus, for each isolate, 10 replicate plates were set up in total, with 30 control plates. After 8 wk, a destructive harvest was carried out under aseptic conditions to determine shoot and root d. wt, the number of leaves initiated, and the numbers of first order and second order uninfected and mycorrhizal lateral roots. In addition, the numbers of bacteria at defined locations in the peat–vermiculite medium and the root system within the screening plate were determined.

Plant harvest and sampling of bacterial numbers

The shoot was removed at the point of the slit–hypocotyl junction using a sterile scalpel blade. The number of leaves was counted and the shoot was then dried at 80°C. Peat–vermiculite medium samples of approx. 0.1 g f. wt were taken from between the central fungal inoculum plug and the root (the point of inoculation), and at the edge of the plate. Root samples of 1 cm length were also taken from the main tap root at the central inoculation point, from a mycorrhizal root near to the central inoculation point, and from the root tip.

Peat–vermiculite and root samples were placed in microcentrifuge tubes containing 0.5 ml of 1/4-strength Ringer's solution (Fischer Scientific, Leicestershire, UK) and macerated using sterile 1.5 ml pellet mixers. Serial dilutions of each sample were prepared and spread plated onto NA plates. Plates were incubated at 25°C for 2–4 d, after which time the number of colony forming units (cf.u.) was counted. Medium and root samples from the control plates were sampled in the same way in order to assess whether sterility had been maintained during the screening trial.

The 90 mm section of root running down the middle of the Petri dish was examined under a binocular microscope and the numbers of first and second order uninfected lateral roots and mycorrhizal roots counted. The mycorrhizal rate, expressed as the number of mycorrhizas divided by the total number of roots (Duponnois & Garbaye, 1991), was calculated for first order roots, second order roots and total number of roots. The whole root system was washed clear of peat–vermiculite and dried at 80°C.

The 90 mm section of root was chosen for sampling of root numbers because contact between the root and the peat–vermiculite medium was optimal in this location. Subsequent root growth around the edge of the Petri dish was found to result in stunted root development, poor contact with the fungus-colonized peat–vermiculite medium, and infrequent synthesis of mycorrhizas.

Genotypic characterization of isolates

The six bacterial isolates selected for the repeat test were characterized by analysis of their fatty acid methyl ester (FAME) profiles by The Central Science Laboratory, Sand Hutton, York, UK. Additionally, the isolates were further characterized by partial 16S rRNA gene sequence analysis. Isolates were grown on NA at 25°C for 18 h, following which cells were washed off with distilled H_2O, centrifuged at 400 ***g*** for 5 min, and the pellet resuspended in 1 ml distilled H_2O. A DNA extract was prepared by freeze-thaw lysis of cells, which involved two cycles of rapid freezing in liquid N_2 followed by transfer to a boiling water bath. The 16S rRNA gene (1504 bp) of each isolate was amplified using a Hybaid Omnigene thermocycler (Ashford, UK), with 1 cycle of 95°C for 1 min, 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, with a final extension of 72°C for 5 min. The 50 µl PCR reactions contained 5 µl DNA extract, 25 pmol of the universal primer set 8f (CACGGATCCAGACTTTGATYM-TGGCTCAG) and 1512r (GTGAAGCTTACGGYTAGCT-TGTTACGACTT) (Life Technologies, Paisley, UK), 1 unit of DNA polymerase (DyNAzyme, Finnzymes, Espoo, Finland), and 200 µM each of dATP, dCTP, dGTP and dTTP (Abgene, Surrey, UK) in a 10-mM Tris-HCl buffer (pH 8.8), with 1.5 mM $MgCl_2$, 50 mM KCl and 0.1% Triton X-100 (DyNAzyme, Espoo, Finland). Amplified products were visualized on 1% agarose gels, following which the amplified DNA band was cut from the gel, and the DNA extracted and purified using an agar extraction-DNA purification kit (Qiagen Ltd, Dorking, UK). The purified DNA was amplified and sequenced using a TaqDyeDeoxy terminator cycle sequencing kit (Applied Biosystems, North Warrington, UK). The PCR reactions contained 8.8 µl DNA template and 3.2 pmol of the primers 968f (AACGCGAAGAACCTT) and 1512r (Life Technologies, Paisley, UK). Sequencing was performed on an Applied Biosystems 733 sequencer. Analysis of sequences was determined by alignment of the partial 16S rRNA gene sequences to those on the EMBL database, using the programme FASTA.

Statistical analysis

Data for the numbers of first and second order uninfected lateral roots and mycorrhizal roots, and the total number of roots were subjected to a square root transformation using the equation $y = \mathrm{sqrt}(x + 0.375)$, to satisfy the assumption of homogeneity of variance. Data for shoot and root d. wt, root : shoot ratios and mycorrhizal rates were not transformed. The results were then analysed using REML (REsidual Maximum Likelihood) using the program Genstat (Version 5.3, Lawes Agricultural Trust, Rothamsted Experimental Station, UK). This analysis allowed the combination of data from different blocks, and therefore comparison between isolates set up on different dates.

The numbers of bacterial cf.u. from the medium and root samples were analysed using one-way ANOVA with a nested treatment structure to extract information on treatment comparisons of interest. This allowed the numbers of cf.u. from the two medium samples and the three root samples to be compared, allowing the spread and colonization patterns of each isolate within the microcosm to be determined. Count data for the number of cf.u. were subjected to a log transformation before statistical analysis, using the equation $y = \ln((x/1000) + 0.375)$, to satisfy the assumption of homogeneity of variance required by ANOVA analysis. Each isolate was used in two blocks in this screen which were set up on consecutive weeks. Because the numbers of cf.u. added were not exactly the same for both weeks, the analysis for the numbers of cf.u. in each block was treated separately. Thus there are two sets of values for each bacterium, relating to the two treatment blocks.

Results

Phenotypic characterization of isolates

The phenotypic and physiological tests placed the isolates into seven groups (Table 1). Twenty of the isolates were Gram-negative, and could be divided into four groups, one of which produced fluorescent pigments on King's Medium B. The other Gram-negative groups were distinguished on the basis of motility and catalase production. The remaining 10 isolates were Gram-positive and could be divided into three groups, one of which produced characteristically pleomorphic cells, with the other two groups showing differences in their ability to produce oxidase. On the basis of their ability to stimulate mycorrhiza formation in the preliminary screen, six isolates were selected for use in a repeat test and for detailed characterization. These isolates fell into three of the phenotype groups. Isolates EJP67 and EJP77 were members of group 1, EJP75 fell within group 5, while EJP59, EJP70 and EJP73 were members of group 7.

Effect of bacteria on plant growth and mycorrhiza formation

None of the six bacterial isolates had significant effects on shoot, root or total plant d. wt after 8 wk (data not shown). However, isolate EJP77 significantly increased ($P < 0.05$) the total number of leaves initiated (46.5) relative to the control treatment (41.0). None of the other isolates had significant

Table 1 Phenotypic and physiological characterization of bacterial isolates from the mycorrhizosphere of *Lactarius rufus*

Group	Gram test	Morphology[1]	Spore formation	Motility	Fluorescence	Catalase	Oxidase	Carbohydrate utilization[2]	Number of isolates
1	–ve	R	–	–	–	+	+	O	6
2	–ve	R	–	+	–	–	+	O	6
3	–ve	R	–	+	–	+	+	O	2
4	–ve	R	–	+	+	+	+	O	6
5	+ve	P	–	–	–	+	–	O	4
6	+ve	R	+	+	–	+	–	F	2
7	+ve	R	+	+	–	+	+	F	4

[1]R, rod; P, pleomorphic. [2]O, oxidative utilization only; F, oxidative and fermentative utilization.

effects on leaf initiation (data not shown). Isolate EJP59 was found to significantly decrease ($P < 0.05$) the root : shoot ratio (0.19) compared with the control (0.23). None of the other isolates had significant effects on root : shoot ratio (data not shown).

Three isolates, EJP67, EJP70 and EJP73, significantly decreased ($P < 0.05$) the number of first order uninfected lateral roots relative to the control treatment, by 43%, 29%, and 41%, respectively (Table 2). No significant effects on the number of second order uninfected lateral roots were recorded with any of the bacterial treatments. Two of the isolates that produced significant decreases in the number of first order uninfected lateral roots, EJP70 and EJP73, produced significant increases ($P < 0.05$) in the number of first order mycorrhizal roots, which rose by 132% and 162%, respectively, relative to the control treatment. Five of the six isolates tested (EJP67, EJP70, EJP73, EJP75 and EJP77) produced significant ($P < 0.05$) increases in the number of second order mycorrhizal roots. The most effective of these isolates, EJP70, increased the number of second order mycorrhizas by 427% relative to the control. No significant effects on the total number of roots initiated were recorded with any of the bacterial treatments.

The isolates EJP70 and EJP73 both significantly increased ($P < 0.05$) the first order, second order and total mycorrhizal rates after 8 wk. EJP73 appeared to be the most efficient isolate, increasing the first order mycorrhizal rate from 21.3% in the control to 49.7%, the second order mycorrhizal rate from 28.2% in the control to 68.5%, and the total mycorrhizal rate from 25.8% in the control to 60.9%. Isolate EJP67 also significantly increased the second order and total mycorrhizal rates. No significant effects on mycorrhizal rates were recorded with isolates EJP59, EJP75 and EJP77.

Location and enumeration of bacterial isolates within the medium and along the root

The number of log cf.u. per gram d. wt of root of the six isolates from the three root sampling points are shown in Table 3. Two isolates, EJP67 and EJP77 were found in greater numbers on the mycorrhizal root compared with the main root after 8 wk. However, this difference was significant ($P < 0.05$) only for one of the two treatment blocks for each isolate. The number of cf.u. of five of the isolates was found to be significantly lower on the root tip compared with the main root near to the point of inoculation. In the case of isolates EJP70, EJP73 and EJP75, this was true for both blocks, but for EJP59 and EJP77, only a single block.

The number of cf.u. of isolate EJP73 was found to be significantly lower ($P < 0.05$) at the edge of the plate (4.79 and 4.76 log cf.u. g^{-1} d. wt medium in blocks 1 and 2, respectively) than at the inoculation point (5.75 and 5.80 log cf.u. g^{-1} d. wt medium in blocks 1 and 2, respectively) in both blocks. However, the other five isolates were found in similar numbers at both sampling points (data not shown).

Dilution plating of the medium and root samples from control plates produced no bacterial or fungal growth on NA plates after incubation at 25°C for 7 d, demonstrating that sterility had been maintained during the experiment.

Characterization of bacteria

The results of the FAME and partial 16S rRNA gene sequence characterization of the helper isolates are shown in Table 4. The FAME analysis only made possible identification to the level of genus for the isolates. Isolates EJP70 and EJP73 were found to be morphologically similar spore-forming bacilli. Cells of isolate EJP59 were slightly smaller than those of EJP70 and EJP73, with less evidence of spore-formation. EJP59, EJP70 and EJP73 showed similar FAME profiles, with anteiso-$C_{15:0}$ acids predominating, and accounting for 52–61% of the fatty acids. This is typical of *Paenibacillus* and a number of *Bacillus* groups (Shida *et al.*, 1997). Isolate EJP75 was a Gram-positive coccobacillus similar to the coryneform type of bacteria, its morphology and FAME profile being consistent with that of *Rhodococcus* spp. FAME analysis demonstrated that isolates EJP67 and EJP77 belonged to the genus *Burkholderia*.

These characterizations were confirmed by the partial 16S rRNA gene sequence analysis. Isolates EJP59, EJP70 and EJP73 were closely related to each other. Isolates EJP70 and EJP73 showed 100% sequence homology to both *Paenibacillus*

Table 2 Effect of bacterial isolates on lateral root and mycorrhiza formation of *Pinus sylvestris* grown with *Lactarius rufus* after 8 wk. Values significantly higher (+) or lower (–) than the corresponding control treatment ($P < 0.05$) are indicated. Figures in brackets give transformed data to which the relevant LSD relate. Mean of 10 replicates

Bacterial isolate	No. of first order uninfected lateral roots	No. of second order uninfected lateral roots	No. of first order mycorrhizal roots	No. of second order mycorrhizal roots	Total no. of roots	First order root mycorrhizal rate (%)	Second order root mycorrhizal rate (%)	Total mycorrhizal rate (%)
None	14.3	13.4	3.4	3.3	40.2	21.3	28.2	25.8
	(3.83)	(3.71)	(1.94)	(1.92)	(6.37)			
EJP59	15.4	12.4	2.3	2.9	35.5	14.8	27.6	18.6
	(3.97)	(3.58)	(1.62)	(1.80)	(5.99)			
EJP67	8.2–	10.6	5.2	12.9+	40.7	38.1	60.1+	48.9+
	(2.92)	(3.31)	(2.36)	(3.65)	(6.41)			
EJP70	10.1–	11.1	7.9+	17.4+	50.7	43.1+	61.7+	53.7+
	(3.23)	(3.39)	(2.87)	(4.22)	(7.14)			
EJP73	8.5–	8.6	8.9+	17.2+	46.0	49.7+	68.5+	60.9+
	(2.99)	(3.00)	(3.04)	(4.19)	(6.81)			
EJP75	13.8	14.1	5.5	10.0+	45.9	30.2	46.7	36.6
	(3.77)	(3.81)	(2.43)	(3.23)	(6.81)			
EJP77	11.9	19.9	3.6	8.8+	51.4	24.8	39.4	34.1
	(3.50)	(4.51)	(1.98)	(3.03)	(7.20)			
LSD ($P < 0.05$)	0.55	1.41	0.82	1.02	1.12	17.35	20.02	15.01

amylolyticus (PAD396), and an unidentified isolate from the *Sorghum bicolor–Glomus mosseae* mycorrhizosphere (AJ011424). EJP59 showed 98.7 and 99.7% sequence homology to isolates PAD396 and AJ011424, respectively. Isolates EJP67 and EJP77 were closely related to each other, showing greatest 16S rRNA sequence similarity to *Burkholderia phenazium* (U96936), with 97.4 and 97.9% homology, respectively. Isolate EJP75 showed 99.6% 16S rRNA gene homology to *Rhodococcus globerulus* (RG16SR) and *Rhodococcus* sp. (RS27579).

Discussion

Our results demonstrate that a number of bacterial isolates associated with *L. rufus* mycorrhizas from a seminatural temperate forest soil were capable of stimulating ectomycorrhiza formation between *L. rufus* and *P. sylvestris*. This finding extends the soil and management environments from which ectomycorrhiza helper bacteria have been isolated, and suggests that mycorrhiza helper bacteria may have a ubiquitous distribution.

Seven distinct phenotypic groups of bacteria were isolated from *P. sylvestris–L. rufus* mycorrhizas, with Gram-negative types, including *Burkholderia* and *Pseudomonas*, dominating relative to Gram-positive spore formers. Similarly, using fluorescent oligonucleotide probes to investigate the *in-situ* distribution of bacteria on mycorrhizal roots, Mogge *et al.* (2000) showed that α- and β-subclass proteobacteria were the dominant members of the bacterial community on mycorrhizal *Fagus sylvatica–Lactarius* spp. roots from natural forest soil. However, Timonen *et al.* (1998), found that Gram-positive spore-forming bacteria were more abundant on *P. sylvestris–Suillus bovinus* and – *Paxillus involutus* mycorrhizal roots from dry pine forest soil than Gram-negative bacteria such as *Burkholderia* and *Pseudomonas*. These differences between ectomycorrhizal types demonstrate that the characteristics of ectomycorrhizosphere bacterial communities vary between different ectomycorrhizospheres. This could be the result of differences in fungal species, as well as contrasting chemical and physical characteristics of the soil environment.

Previous studies of ectomycorrhiza–bacteria interactions have found that those bacterial isolates capable of stimulating fungal growth and ectomycorrhiza formation included fluorescent and nonfluorescent pseudomonads and *Bacillus* spp. (Bowen & Theodorou, 1979; Garbaye & Bowen, 1989; Garbaye & Duponnois, 1992; Varese *et al.*, 1996; Dunstan *et al.*, 1998). In our study, the most effective ectomycorrhiza stimulatory bacteria were two *Paenibacillus* isolates (EJP70 and EJP73), which increased the total mycorrhizal rate from 26% in the control treatment to 54% and 61%, respectively. However, none of the Group 4 isolates, which could include fluorescent pseudomonads, stimulated mycorrhiza formation.

Paenibacillus is a newly recognized group, into which a number of groups formerly classified as *Bacillus* have been placed (Shida *et al.*, 1997). Comparison of the *Paenibacillus* partial

Table 3 Recovery of bacteria after 8 wk (log cf.u. g^{-1} dw of root) from roots of *Pinus sylvestris* grown with *Lactarius rufus*

Bacterial isolate	Block[1]	Main root near inoculation point	Mycorrhizal root	Root tip	LSD ($P < 0.05$)
EJP59	1	6.67 (8.46)	6.75 (8.63)	5.10 (4.83)–	2.35
	2	6.79 (8.73)	6.73 (8.58)	5.78 (6.41)	2.41
EJP67	1	9.85 (15.77)	10.45 (17.15)+	9.49 (14.95)	1.24
	2	10.11 (16.38)	10.34 (16.90)	10.21 (16.60)	0.60
EJP70	1	6.49 (8.04)	5.54 (5.84)	3.05 (0.41)–	2.70
	2	6.14 (7.23)	6.36 (7.73)	5.12 (4.88)–	1.62
EJP73	1	6.77 (8.68)	6.66 (8.42)	4.71 (3.94)–	1.17
	2	6.24 (7.47)	6.29 (7.57)	4.33 (3.08)–	1.89
EJP75	1	7.10 (9.45)	7.21 (9.70)	4.94 (4.46)–	3.52
	2	7.10 (9.43)	7.29 (9.88)	5.64 (6.09)–	2.59
EJP77	1	9.70 (15.42)	10.37 (16.96)+	9.70 (15.42)	0.79
	2	10.09 (16.32)	10.37 (16.97)	9.51 (15.00)–	1.24

Figures in brackets give transformed data, to which LSD relate. Values significantly higher (+) or lower (–) than the main root near inoculation point are indicated. [1]Blocks set up on separate weeks and analysed separately.

Table 4 Characterization of mycorrhiza helper isolates by FAME-profiling and partial 16S rRNA gene sequence analysis

Bacterial isolate	Phenotypic group	EMBL accession number	Identification by FAME profiling	EMBL ribosomal database sequence similarity to next related isolates
EJP59	7	AJ302335	*Bacillus/Paenibacillus* sp.	99.7% to an unidentified mycorrhizosphere bacterium (AJ011424); 98.7% to *Paenibacillus amylolyticus* (PAD396)
EJP67	1	AJ302336	*Burkholderia* sp.	97.4% to *Burkholderia phenazium* (U96936); 97.2% to *B. glathei* (U96935)
EJP70	7	AJ302334	*Bacillus/Paenibacillus* sp.	100% to *Paenibacillus amylolyticus* (PAD396) and to an unidentified mycorrhizosphere bacterium (AJ011424)
EJP73	7	AJ302333	*Bacillus/Paenibacillus* sp.	100% to *Paenibacillus amylolyticus* (PAD396) and to an unidentified mycorrhizosphere bacterium (AJ011424)
EJP75	5	AJ302331	*Rhodococcus* sp.	99.6% to *Rhodococcus globerulus* (RG16SR) and to a *Rhodococcus* sp. (RS27579)
EJP77	1	AJ302332	*Burkholderia* sp.	97.9% to *Burkholderia phenazium* (U96936); 97.7% to *B. glathei* (U96935), *B. pyrrocinia* (U96930), *B. cepacia* (AF097533) and *B. stabilis* (AF148554)

16S rRNA gene sequences with others on the EMBL databank revealed that isolates EJP70 and EJP73 had 100% homology to an isolate from the mycorrhizosphere of *Glomus mosseae* growing with *Sorghum bicolor* (AJ011424). Further, other *Paenibacillus* isolates have been shown to promote establishment of the arbuscular mycorrhizal symbiosis (Budi *et al.*, 1999). Isolates EJP70 and EJP73 showed identical partial 16S rRNA gene sequences, and the magnitude of their stimulatory effects on mycorrhiza formation were very similar, suggesting that they could be a single strain that has been isolated twice.

Burkholderia and *Rhodococcus* have not been identified as MHB in previous studies. Our findings therefore extend the diversity of bacterial groups with strains capable of stimulating ectomycorrhiza formation. *Burkholderia* spp. have been shown to have intimate associations with arbuscular mycorrhizal fungi, and have been found within living spores of diverse species of arbuscular mycorrhizal fungi (Bianciotto *et al.*, 2000). Further, Andrade *et al.* (1997) found that *B. cepacia* was ubiquitous in the rhizosphere and hyphosphere of three arbuscular fungi tested, while other bacterial species were not as widely distributed. Bacteria can also occur at intracellular locations within the mantle and Hartig net of ectomycorrhizal roots (Nurmiaho-Lassila *et al.*, 1997), and the results of Timonen *et al.* (1998) suggest that *Burkholderia* may be a component of this community. There may therefore be similarities in the characteristics of both Gram-positive and Gram-negative inhabitants of ecto- and arbuscular mycorrhizospheres.

In our study, there were differences between the bacterial isolates with respect to their effects on the dynamics of mycorrhiza formation. None of the isolates affected the total number of roots formed by the plants. However, the *Paenibacillus*

isolates EJP70 and EJP73 stimulated formation of both first and second order mycorrhizal roots, while the *Burkholderia* and *Rhodococcus* isolates stimulated formation of only second order roots. This could indicate differences between the bacteria in terms of their capacity to promote mycorrhiza formation on older primary lateral roots relative to younger secondary roots, suggesting differences in the functioning of the bacterial isolates.

The *P. sylvestris–L. rufus* bacterial isolates stimulated the mycorrhizal rate by up to 35% compared with the control treatment. In the *Pseudotsuga menziesii–Laccaria bicolor* symbiosis, bacterial inoculation has been shown to increase mycorrhizal rate by 30–50% relative to uninoculated controls (Garbaye, 1994). Although direct comparisons with other studies are not possible due to differences in the length of the trials and the systems used, our isolates appear to provide a similar enhancement of ectomycorrhiza formation to those of other ectomycorrhizal symbioses.

The precise mechanisms of MHB activity were not elucidated in this study, although some suggestions as to possible modes of action may be made. The observed increase in the number of mycorrhizal roots was not associated with an increase in the total number of roots. This suggests that MHB had no effect on lateral root initiation, which has been proposed as a mechanism of helper activity (Garbaye, 1994). Increased mycorrhiza formation therefore could have arisen by means of bacterial effects on root receptivity to fungal infection, or by direct stimulation of fungal growth.

Pseudomonas and *Bacillus* helper bacteria from the *P. menziesii–L. bicolor* symbiosis, and uncharacterized isolates from the *P. sylvestris–Suillus luteus* symbiosis, have been found to stimulate growth of associated ectomycorrhizal fungi, and promote colonization of *P. sylvestris* roots by the fungus (Poole, 1999). However, this did not necessarily result in enhanced ectomycorrhiza formation. By contrast, Garbaye (1994) reported that the capacity of mycorrhizosphere bacterial isolates to stimulate growth of *L. bicolor* was correlated to effectiveness at promoting ectomycorrhiza formation on *P. menziesii*.

The evidence therefore suggests that there may be diverse helper mechanisms operating in the environment, with differences evident both between different ectomycorrhizal symbioses, and between different bacteria which promote the same symbiosis.

There were indications that the *Burkholderia* isolates EJP67 and EJP77 were found in higher numbers on the mycorrhizal root compared with the main root near to the point of inoculation. The other three isolates which promoted mycorrhiza formation were found in approximately equal numbers on both root sections. The stimulation of mycorrhiza formation by the bacteria was not therefore always related to an enhanced ability of the bacteria to colonize the mycorrhizal root surface. However, the results indicate that the *Burkholderia* and *Paenibacillus/Rhodococcus* isolates could differ in the degree to which they preferentially associate with mycorrhizal roots.

Although the gnotobiotic system used in this study may indicate an organism's potential ecological niche, it is not possible to draw general conclusions as to whether MHB are specifically or preferentially associated with the mycorrhizal root from the results of this screen. In a natural rhizosphere, competition from other indigenous microorganisms would affect the ability of a specific bacterial strain to colonize root surfaces. Using nonsterile soils, Frey-Klett *et al.* (1997) found that the population of a *Pseudomonas fluorescens* isolate capable of promoting *P. menziesii–L. bicolor* ectomycorrhiza formation declined at the same rate in rhizosphere and bulk soil, and was not preferentially associated with mycorrhizas of *P. menziesii–L. bicolor*.

There was no relationship between the capacity of isolates to spread through the microcosm, and motility in pure culture agar tests. Isolates EJP67 and EJP77, which were apparently nonmotile in pure culture, were capable of more effective spread to the root tip and the edge of the microcosm, than the *Paenibacillus* and *Rhodococcus* isolates which were motile in pure culture. This indicates that there were differences between the isolates with respect to their behaviour in the rhizosphere environment, which cannot be ascribed to motility.

In the 8 wk assay for effects of the isolates on mycorrhiza formation, none of the isolates stimulated root or shoot growth, although one of the *Burkholderia* isolates (EJP77) promoted leaf initiation. In general, most studies of MHB have not reported beneficial effects of the bacteria on plant growth, although the experiments typically have been of only relatively short duration, which may not be sufficient for the stimulation of mycorrhiza formation to result in enhanced plant growth. Further, the benefits to the plant from increased mycorrhiza formation may include enhanced resistance to soil borne pathogens (Schelkle & Peterson, 1996) and are unlikely to result in the direct promotion of growth in all situations. However, in a nonsterile nursery soil, Frey-Klett *et al.* (1999) reported that inoculation with a *P. fluorescens* isolate shown to enhance mycorrhiza formation *in vitro*, was able to stimulate mycorrhiza formation and growth of *P. menziesii* with *L. bicolor* after a period of 2 yr.

Acknowledgements

We thank Dr Andy Taylor for help with the identification of ectomycorrhizal roots, Andrew Mead for statistical advice, Dr Alun Morgan for useful discussion, and the Biotechnology and Biological Sciences Research Council for funding.

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