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Molecular Characterization of Cellulosolytic-Chitinolytic Bacteria Associated with Fruitbodies of the Ectomycorrhizal Fungus *Tuber borchii* Vittad.

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Abstract

This study describes a molecular characterization of fluorescent pseudomonads and spore-forming bacteria isolated from fruitbodies of the ectomycorrhizal fungus *Tuber borchii* Vittad. These strains had previously been characterized for their cellulosolytic and chitinolytic activities and studied for their putative role in facilitating ascus opening and ascospore germination within fruitbodies of *T. borchii* (Gazzanelli et al., 1999). In the present study, representative pure cultures of these bacteria were genetically characterized using amplified ribosomal 16S rRNA gene (rDNA) restriction analyses (ARDRA). Examination of the ARDRA patterns revealed two different 16S rDNA types among the pseudomonads and three 16S rDNA types among the spore-forming bacteria. Strains representative of each type were chosen for 16S rDNA sequencing. Phylogenetic analysis of the 16S rDNA sequence placed members of the pseudomonads in branches belonging to the *Pseudomonas fluorescens* and *P. syrigae* lineages of the *P. fluorescens* cluster, while phylogenetic analysis of the spore-forming bacteria placed them in branches belonging to the *Bacillus subtilis* group, *B. cereus* group and *Paenibacillus* spp. These results represent one the first attempts to make systematic molecular comparisons and carry out functional studies of the bacteria-fungus-plant symbioses within the ectomycorrhizal fungus *T. borchii* Vittad.

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1. Introduction

Truffles are ascomycetous fungi that form hypogeous fruitbodies. The truffle life cycle has been explained as a succession of different phases: spore germination, mycelium growth, symbiotic establishment of mycorrhizae within the host plant roots, and development of extraradical mycelia leading to fungal maturation and fruitbody formation (Trappe, 1979; Harley and Smith, 1993). The symbioses between the truffle mycelium and the roots of plants is affected in various ways by the microorganisms present in the mycorrhizosphere (Duponnois and Garbaye, 1992; Garbaye, 1994; Citterio et al., 1995; Varese et al., 1996). Over the last decade several bacteria have been isolated from mycorrhizal fungi and described as strains able to either positively or negatively affect mycelial growth and mycorrhizal infection (Garbaye et al., 1990; Bedini et al., 1999). Furthermore, it has been hypothesized that bacteria affect the germination of fungal propagules in the soil (Garbaye, 1994) and the germination of spores in fruitbodies of ectomycorrhizal fungi (Gazzanelli et al., 1999).

In particular, in the latter study cellulosolytic and chitinolytic pseudomonads and spore-forming bacteria were isolated from young sporocarps of *Tuber borchii* Vittad., a white truffle species of great economic value due to its organoleptic properties. The possible role of these bacterial strains in facilitating ascus opening and, perhaps, affecting the ascospore germination was examined. The potential adaptation of bacteria to this complex microbe-host association also depends on its genetic diversity.

For this reason, and to complement the previous characterization of the phenotypic traits of these cellulosolytic and chitonolytic strains (Gazzanelli et al., 1999) in the present study we carried out their genetic characterization. The recent applications of molecular techniques with PCR and rRNA-based approaches have been helpful in bacterial identification by allowing differentiation of taxa at the species and higher levels (Liesack and Stackebrandt, 1992; Amann et al., 1995; Pace, 1996; Hugenholtz et al., 1998). In particular, the use of genes coding for 16S rRNA (rDNA) as molecular marker has become a common technique for microbial ecology studies. We used the amplified ribosomal 16S rDNA restriction analysis (ARDRA), a useful method to evaluate the degree of polymorphism among strains (Liu et al., 1997). Moreover, an extensive phylogenetic analysis was carried out to determine

their relationships with the most closely related microorganisms by comparison with sequence in the databases currently available. The results of this study could be a starting point for illustrating the value of rDNA sequence analysis in the identification of bacteria compared with an analysis of phenotypic traits and in further molecular systematic and functional studies of bacteria-fungus-plant symbioses.

2. Materials and Methods

Collection of bacterial strains

As previously described (Gazzanelli et al., 1999) cellulosolytic and chitinolytic pseudomonads and spore-forming bacteria were isolated from the inner part of sporocarps of *T. borchii* Vittad. Among theses isolates twenty-one strains were randomly chosen for molecular characterization.

DNA extraction

Bacterial cultures were grown and purified in tryptic soy agar medium (TSA Oxoid). The bacterial cells were washed once in TES buffer (20 mM Tris, 50 mM EDTA, 150 mM NaCl [pH 7.9]), centrifuged for 2 min at 13,000 rpm and dissolved in 1.2 ml of TES buffer. The cells were then lysed by treatment with 300 µl of lysozyme (5 mg/ml) (Sigma) and incubated at 37°C for 60 min. The lysate was then incubated at 37°C for 30 min after the addition of 5 µl of RNase A (10 mg/ml) (Sigma). Nucleic acids were extracted using the standard phenol DNA extraction technique (Sambrook et al., 1989).

PCR conditions

Universal prokaryotic primers (UP) (Weisburg et al., 1991) specific for the ends of the 16S rRNA gene were used to amplify rRNA genes from all samples (UP-Forward: 5'-AGA GTT TGA TYM TGG C-3', *E. coli* positions 8-23 [Brosius et al., 1978]; UP-Reverse: 5'-GYT ACC TTG TTA CGA CTT-3', positions 1496-1514). The PCR procedure consisted of 35 cycles: denaturation at 94°C for 45 sec, annealing at 53°C for 45 sec, and elongation at 72°C for 2 min. PCR products were purified using the QIAquik purification kit (Qiagen GmbH) and eluted in sterile H_2O at a final volume of 30 µl. The final concentration was estimated by agarose gel electrophoresis, quantifying the ethidium bromide-stained DNA using the Gel Doc 2000 "Quantity One" software program (BioRad) and the DNA mass Ladder marker (GIBCO/BRL) to calibrate the bands.

Amplified ribosomal gene restriction analysis (ARDRA)

Chitinolytic and cellulosolytic isolates phenotypically characterized as fluorescent *Pseudomonas* spp. and spore-forming bacteria (Gazzanelli et al., 1999) were subjected to PCR-ARDRA of their 16S rDNA. Aliquots (500 ηg) of amplified 16S rDNAs of all bacterial strains were digested separately with *Taq*I (MBI fermentas), *Mbo*I, *Alu*I, *Mps*I, *Hinf*I, *Pst*I (Takara, Shuzo Co. Ltd., Japan) enzymes according to the manufacturer's instructions. These restriction endonucleases were chosen because they revealed a clear polymorphism in the 16S rDNA of fluorescent *Pseudomonas* species (Laguerre et al., 1994; Frey et al., 1997) and in several soil *Bacillus* strains (Mavingui et al., 1992). Aliquots (20 µl) of each digested product were mixed with 2 µl of 10x loading dye buffer and resolved by electrophoresis through a 3% agarose (NuSieve 3:1; Bio-Rad Laboratories, Hercules, CA). A DNA molecular weight marker VIII (Boehringer Mannheim GmbH, Germany) was run at the side of each gel. Electrophoresis was carried out at 80 V for 2 hrs. Gels were stained in an aqueous solution of ethidium bromide (1 mg/ml) and the ARDRA patterns were analyzed with the Gel Doc 2000 Quantity One program (Biorad). Similar band pattern were obtained and the presence or absence of each restriction site was visually determined and converted into binary data. The resulting matrix was analyzed with the PAUP (Swofford, 1991) and PHYLIP (Felsenstein, 1993) computer software using neighbor-joining (Saitou and Nei, 1987) algorithms and the Nei-Li (1979) genetic distances method. The restriction of 16S ribosomal DNAs (rDNAs) amplifications has been repeated three folds in order to verify the reproducible of the pattern obtained for each strains isolated from fruitbodies of *T. borchii* Vittad. Strains representative of each cluster derived from the restriction site analysis were chosen for 16S rDNA sequencing and phylogenetic analyses.

16S rDNA sequencing

Sequences of purified PCR products were determined using an ABI prism cycle-sequencing kit (dRhodamine Terminator Cycle Sequencing kit with AmpliTaq DNA polymerase FS, Perkin-Elmer). Sequencing was performed using an ABI 377 DNA sequencer (Perkin-Elmer, Applied Biosystems Div.).

Phylogenetic analysis

A phylogenetic analysis was performed to evaluate the genetic relationship between isolates from this study and a set of closely related or phylogenetically relevant sequences obtained from the Ribosomal Database Project (RDP) (Maidak et al., 2000) and from the DDBJ/EMBL/GenBank

database using the Basic Local Alignment Search Tool (BLAST, Altschul et al., 1997). The chimera check program of the RDP was used to search for chimerical sequences. Twenty-five 16S rDNA sequences from fluorescent pseudomonads and twenty from spore-forming bacteria (Table 2) were pre-aligned according to secondary structure using the RDP web site and manually adjusted using the sequence alignment editor SeqPup 0.5 (Gilbert, 1996). Sequences from the RDP database served as the alignment guideline. Phylogenetic trees were inferred with the distance matrix (DM) programs DNADIST, with FITCH as implemented in the PHYLIP software package version 3.5c (Felsenstein, 1993), and with maximum likelihood (ML) and maximum parsimony (MP) with the PAUP 3.1.1 program (phylogenetic analysis using parsimony, Swofford, 1991). Bootstrap analyses based on 100 re-samplings of the sequence alignment were performed with DNABOOT as implemented in PHYLIP.

3. Results

ARDRA analysis of the 16S rDNA target

Chitinolytic and cellulosolytic isolates phenotypically characterized as fluorescent *Pseudomonas* species and spore-forming bacteria (Gazzanelli et al., 1999) were subjected to ARDRA of their 16S rDNAs. Amplification of the 16S rDNA for each isolate yielded the expected PCR product as a band of approximately 1,500-bp corresponding to the nearly full-length 16S rRNA gene. All the amplified products were digested with each of the six restriction endonucleases described above. Depending on the restriction enzyme, three to six restriction fragments were recorded. A map of the restriction sites in the 16S rRNA genes was inferred from known 16S rRNA gene sequences of *Pseudomonas fluorescens* (DDBJ/EMBL/GenBank accession number D84013), *Bacillus subtilis* (DDBJ/EMBL/GenBank accession accession number Z99104) and *Escherichia coli* (DDBJ/EMBL/GenBank accession number J01695) using OLIGO software. A total of 78 restriction sites representing more than 150 bp were examined. Two of the restriction sites were found to be polymorphic among pseudomonads strains, 51 among the spore-forming bacteria, while the other restriction sites were conserved among the pseudomonads and in the spore-forming bacteria and absent from the *E. coli* gene sequence. Twelve other restriction sites were only present in the *E. coli* gene sequence (Table 1). Each isolate was assigned to a 16S rDNA type defined by the combination of the restriction patterns obtained with the restriction endonuclease. Among the six restriction endonucleases used, all six for the spore-forming bacteria revealed polymorphism in their 16S rDNAs, while only *Alu*I revealed polymorphism for the pseudomonad isolates. In order to evaluate the level of polymorphism among PCR-amplified 16S

Table 1. Binary matrix relative to the 16S rRNA gene restriction patterns obtained from each of the six endonucleases used: *Taq*I, *Alu*I, *Mbo*I *Hinf*I and *Pst*I. Presence and absence of a each DNA restriction site was visually determined and converted into binary data.

Strains	*Taq*I	*Alu*I	*Mbo*I	*Msp*I	*Hinf*I	*Pst*I
B2	1000000011000	001000000100101	010000010010000	001010000000100101	010000000100011	00
B4	1000000011000	001000000100101	010000010010000	001010000000100101	010000000100011	00
B20	1000000011000	001000000100101	010000010010000	001010000000100101	010000000100011	00
B24	1000000011000	001000000100101	010000010010000	001010000000100101	010000000100011	00
B22	1000000011000	001000000100101	010000010010000	001010000000100101	010000000100011	00
B23	1000000011000	001000000100101	010000010010000	001010000000100101	010000000100011	00
C1	1000000011000	100000000100101	010000010010000	001010000000100101	010000000100011	00
C18	1000000011000	010000000100101	010000010010000	001010000000100101	010000000100011	00
C38A	1000000011000	001000000100101	010000010010000	001010000000100101	010000000100011	00
P. fluorescens	1000000011000	001000000100101	010000010010000	001010000000100101	010000000100011	00
A6	0001001000001	001000001011000	001000010100010	010000100100000100	001000001001000	00
B3	0010100000001	001100000000010	100001000000000	100000001010000000	100001000000000	00
B2-0	0000010110100	000000110111000	000100100001101	000100100100010010	000010110010000	11
B2-00	0000010110100	000000110111000	000100100001101	000100100100010010	000010110010000	11
B18	0000010110100	000000110111000	000100100001101	000100100100010010	000010110010000	11
B28	0000010110100	000000110111000	000100100001101	000100100100010010	000010110010000	11
C9	0000010110100	000000110111000	000100100001101	000100100100010010	000010110010000	11
C10	0000010110100	000000110111000	000100100001101	000100100100010010	000010110010000	11
C15	0000010110100	000000110111000	000100100001101	000100100100010010	000010110010000	11
C21	0000010110100	000000110111000	000100100001101	000100100100010010	000010110010000	11
C31	0000010110100	000000110111000	000100100001101	000100100100010010	000010110010000	11
C33	0000010110100	000000110111000	000100100001101	000100100100010010	000010110010000	11
C38	0000010110100	000000110111000	000100100001101	000100100100010010	000010110010000	11
B. subtilis	0000010110100	000000110111000	000100100001101	000100100100010010	000010110010000	11
E. coli	0100000010010	000011000100000	000011001000010	000001010001001001	000100011000100	00

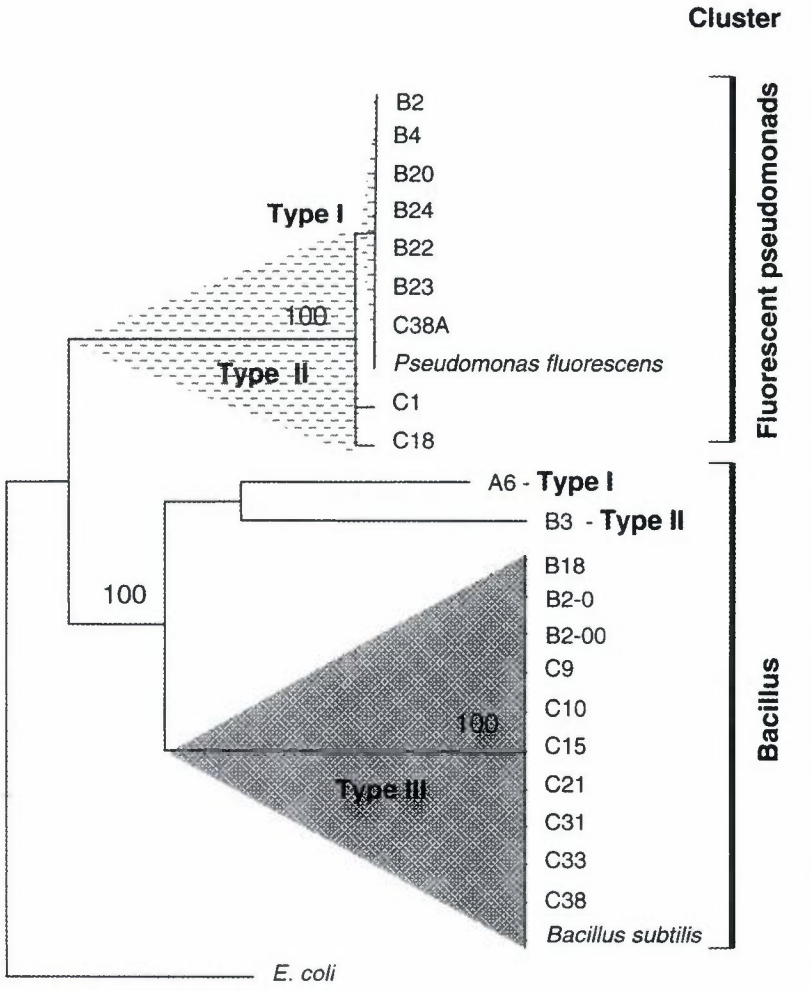

Figure 1. Dendrogram of genetic divergences among strains of fluorescent *Pseudomonas* species and spore-forming bacteria. The cluster analysis of the five types (type I and II among the pseudomonads and type I, II and III among the spore-forming bacteria) was based on the combined data for the six ARDRA profiles obtained using *Taq*I, *Mbo*I, *Alu*I, *Mps*I, *Hinf*I and *Pst*I restiction enzymes (Table 1).

rDNA fragments from different strains able to degrade chitin and cellulose within fruitbodies of *T. borchii* Vittad., restriction site differences were analyzed using genetic distance analysis. *Pseudomonas fluorescens* and *Bacillus subtilis* 16rDNA sequences were included in these analyses on the basis of their

Table 2. Phylogenetic affiliation of bacteria isolated from *Tuber borchii* fruitbodies and close relatives.

Group designation	Taxa/strain	Accession No.	Source/reference
Pseudomonas mendocina lineage	*P. mendocina* Str. LMG 1223T	Z76664	Moore, 1996
Pseudomonas syringae/PsA group	*Pseudomonas* sp. PsA	AF105379	Kuske et al., 1999
	Pseudomonas sp. strain BI*7	U81870	Whyte, 1996
	Pseudomonas sp. PsD	AF105382	Kuske et al., 1999
	Pseudomonas sp. PsJ	AF105388	Kuske et al., 1999
	P. syringae	Z76669	Moore et al., 1996
	P. chlororaphis str. LMG 5004T	Z76657	Moore et al., 1996
	B20	AF274242	this study
	B4	AF274243	this study
Pseudomonas fluorescens lineage	*P. fluorescens*	D84013	Anzai et al., 1997
	Pseudomonas sp. CRE 11	U37338	Mueller, unpublished
	P. fluorescens	AJ011331	Elo et al., 2000
	Pseudomonas azotoformans	D84009	Anzai et al., 1997
	Pseudomonas synxantha	D84025	Anzai et al., 1997
	Pseudomonas tolaasii	Z76670	Moore et al., 1996
	P. fluorescens bv. G.	AF228366	Heinaru et al., 2000
	P. fluorescens bv. C.	AF228367	Heinaru et al., 2000
	Pseudomonas rhodesiae	AF064459	Coroler et al., 1996
	C1	AF274240	this study
	C18	AF274241	this study
Pseudomonas putida lineage	Unidentified soil bacteria	D26260	Ueda, T. unpublished
	P. putida	D86003	Yamamoto et al., 1998
Pseudomonas aeruginosa lineage	*P. aeruginosa* str. LMG 1242T	Z76651	Moore et al., 1996
	P. lutescens str. ATCC 27951	M59156	Woese, C.R. unpublished
	Pseudomonas stutzeri	D84024	Anzai et al., 1997
	Pseudomonas alcaligenes	Z76653	Moore, 1996
	Pseudomonas migulae	AF074383	Verhille et al., 1999

Table 2. Continued

Group designation	Taxa/strain	Accession No.	Source/reference
Bacillus cereus group	*Bacillus cereus* Str. IAM 12605	D16266	Suzuki and Yamasato, 1994
	Bacillus thuringiensis	D16281	Suzuki and Yamasato, 1994
	Bacillus weihenstephanensis	AB021199	Goto et al., 2000
	Bacillus mycoides Str. ATCC6462	AB021192	Goto et al., 2000
	Bacillus antracis	X55059	Ash et al., 1991
	A6	AF274244	this study
	Bacillus sp.	Y15466	Margulis et al., 1998
Bacillus subtilis group	*Bacillus subtilis*	X60646	Ash, 1991
	Bacillus subtilis subsp. *spizizenii*	AF074970	Nakamura, 1999
	Bacillus mojavensis Str. IFO15718	AB021191	Goto et al., 2000
	B2-0	AF274246	this study
	B2-00	AF274247	this study
	B24	--	this study
	C15	AF274248	this study
Unidentified bacteria	Isolate B3	AJ011421	Budi et al., unpublished
	Isolate B4	AJ011422	Budi et al., unpublished
	Isolate B5	AJ011423	Budi et al., unpublished
	Isolate B7	AJ011425	Budi et al., unpublished
Paenibacillus group	*Paenibacillus* sp.	5701913	Achouak et al., 1999
	Paenibacillus sp	AJ011322	Elo, 2000
	Paenibacillus azotofixans	X60608	Ash et al., 1991
	Paenibacillus durum	X77846	Collins et al., 1994
	B3	AF274245	this study
Unidentified bacterium	Isolate B8	AJ011426	Budi et al., unpublished

predicted restriction patterns. A 16S rDNA sequence of *E. coli* was also included (Brosius et al., 1978). Clusters were reconstructed from the data matrix shown in Table 1. The 9 pseudomonad isolates studied were assigned to two different 16S rDNA types, both of which were different from the 16S rDNA types previously described by Laguerre et al. (1994) and from the 48 pseudomonads studied by Frey et al. (1997). The 12 spore-forming isolates were assigned to three different 16S rDNA types (Fig. 1).

Phylogenetic analyses

Nine different isolate types representative of the clusters among the pseudomonads and the Bacillaceae were phylogenetically analyzed from the isolates in the total collection. Sequence data encompassing approximately 600 bp (5'-3' end) were obtained from each of the individual strains representative of each type characterized by the restriction sites analysis (Fig. 1). Both forward and reverse strands of the 600 fragment were sequenced and the consensus sequence was determined and submitted to the DDBJ/EMBL/GenBank data bases. The similarity sequence analysis was performed using the Ribosomal Database Project (RDP) (Maidak et al., 2000) and the Basic Local Alignment System Tool (BLAST) (Altschul et al., 1997) of the National Center for Biotechnological Information on small subunit prokaryotic sequences deposited in the database. Phylogenetic analyses using distance matrix, parsimony and maximum likelihood criteria were performed on a minimum of 600 bp.

Twenty-three 16S rDNA sequences from environmental *Pseudomonas* populations and twenty-two from spore-forming bacteria (Table 2) were chosen from the database in the present study.

The choice has been based on relevant sequences considered to be representative of the most well-defined lineages in the *Pseudomonas fluorescens* cluster as suggested by Moore et al. (1996) and Anzai et al. (1997). In this analysis bacteria isolated from the rhizosphere habitat were added (Kuske et al., 1999), as well as the 16S rDNA of *E. coli*, which served as outgroup. In contrast, multiple sequence alignments with the most significant 16S rDNA sequence for the genus *Bacillus* (Goto et al., 2000) were chosen from the GenBank database to identify the phylogenetic relationship of the Bacillaceae strains isolated. Specific sequences of mycorrhizal associated bacteria have also been added to this analysis in order to verify potential evolutionary connections among strains which are part of the mycorrhizal symbiotic system. The topologies of the tree produced by MP and ML analyses based on this data set agreed with that of the distance presented in Figs. 2 and 3. The stability of the different clusters was verified by bootstrap analysis

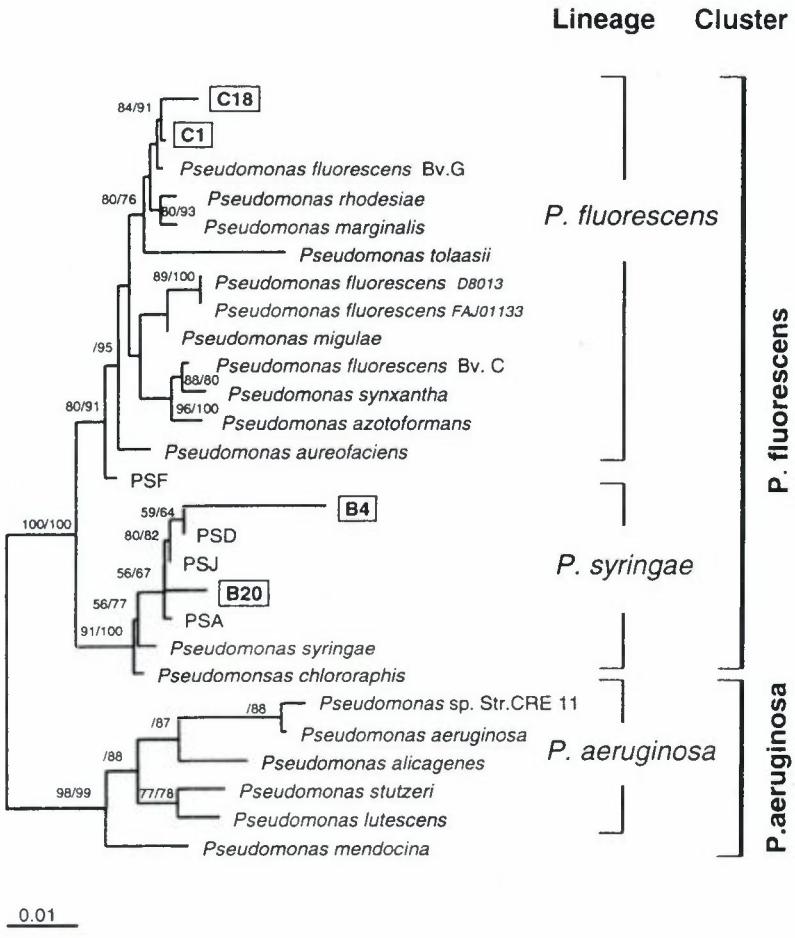

Figure 2. Phylogenetic tree for representative 16S rRNA gene sequence from chitinolytic pseudomonads isolated from ascocarps of *Tuber borchii* Vittad. based on about 600 bp (5'-end) sequences, derived from the representative of each ARDRA type defined in Fig. 1. The two numbers at each branch node are bootstrap numbers from 100 re-samplings, the first number represents the distance matrix, the second number for parsimony bootstrapping. Only numbers >50% were written. The scale bar represents a 10% difference in nucleotide sequences as determined by measuring the lengths of the horizontal lines connecting two species. Out-group *E. coli* not shown.

with a confidence level of $\geq$90% obtained with all methods. Trees with the same affiliation for pseudomonads were obtained after complete exclusion or inclusion of both insertions and deletions and/or variable regions.

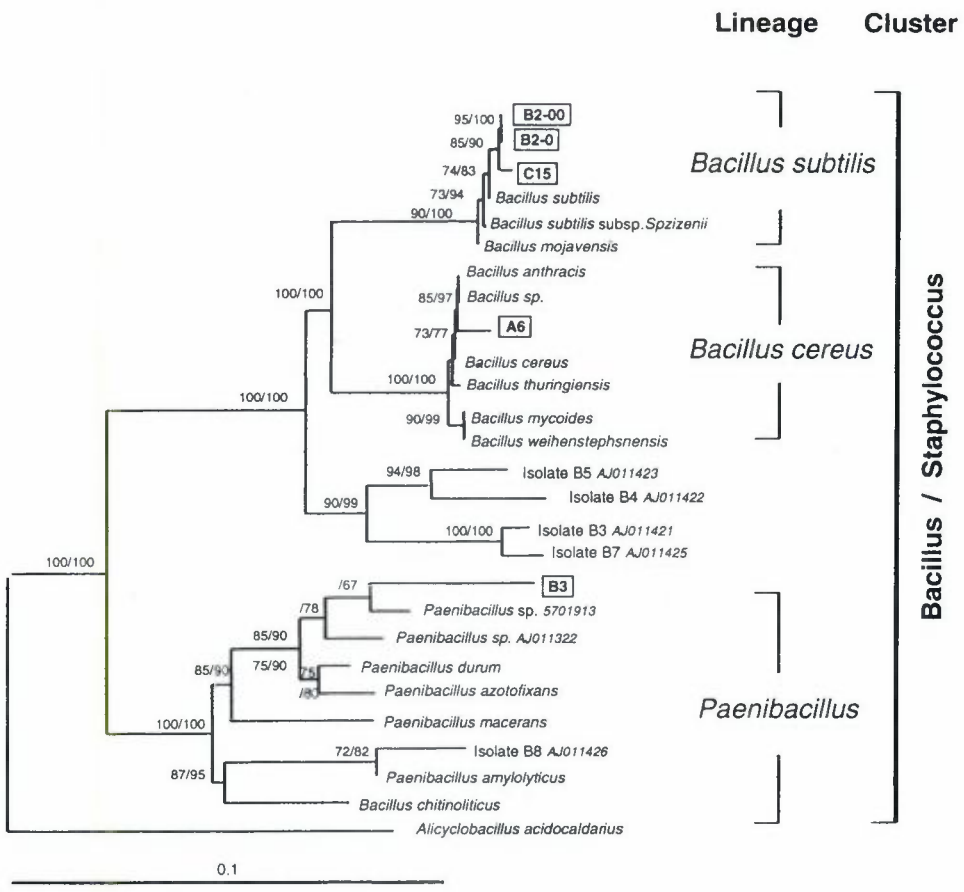

Figure 3. Phylogenetic tree for representative 16S rRNA gene sequence from chitinolytic spore-forming isolated from ascocarps of *Tuber borchii* Vittad. based on about 600 bp (5'-end) sequences, derived from the representative of each ARDRA type defined in Fig. 1. The two numbers at each branch node are bootstrap numbers from 100 re-samplings, the first number represents the distance matrix, the second number for parsimony bootstrapping. Only numbers >50% were written. The scale bar represents a 10% difference in nucleotide sequences as determined by measuring the lengths of the horizontal lines connecting two species. Out-group *Alicyclobacillus acidocaldarius* (Wisotzkey et al., 1992).

Sequences from the pseudomonad isolates were grouped among the true *Pseudomonas* spp. (Fig. 2) and fell into two distinct groups within the *P. fluorescens* cluster. The sequence of isolates B4 and B20, representative of the ARDRA type I, clustered into a novel rhizosphere group closely related to

member of the *P. syringae* lineage described by Kuske et al. (1999). The sequence of isolates C1, C18, representative of the ARDRA type II, clustered with the *P. fluorescens* lineage. All lineages were supported with >90% in bootstrap analyses in all optimal trees.

Moreover, sequences from the spore-forming bacteria fell into three well-defined clusters: most of the spore-forming bacteria were clustered within the ARDRA type III and the 16S rDNA sequence of strains B2-0, B2-00 and C15 were very closely related to *Bacillus subtilis*. In contrast, the sequence of strain A6, which is representative of the ARDRA type I, fell within the *Bacillus cereus* group closely related to *B. cereus* sp. (Fig. 3), while the sequence of strain B3, which differed from all the other ARDRA types and represent type II, fell among the species of the genus *Paenibacillus*. Concerning to the ribosomal sequences of mycrorrhizal strains recently submitted to the DDBJ/EMBL/GenBank databases, they did not reveal a significant relationship with the bacteria associated to the ectomycorrhizal fungus *T. borchii* Vittad.

DDBJ/EMBL/GenBank accession numbers

The 16S rDNA sequences of the isolates obtained in this study are available from DDBJ/EMBL/GenBank under accession numbers AF274240–AF274248: strain C1, AF274240; strain C18, AF274241; strain B20, AF274242; strain B4, AF274243; strain A6, AF274244; strain B3, AF274245; strain B2-0, AF274246; strain B2-00, AF274247; strain C15, AF274248.

4. Discussion

In this paper, we applied a current molecular method for the identification or grouping of species characterized on the basis of their chitinolytic and cellulosolytic activity. The strains isolated from different *T. borchii* fruitbodies were phenotypically characterized as pseudomonads and spore-forming bacteria (Gazzanelli et al., 1999).

Our analysis of the 16S rDNA sequences from these representative isolates was compared to the most complete analyses of *Pseudomonas* and *Bacillus* phylogeny in terms of number of rhizosphere or mycorrhizal sequences available and range of phylogenetic analysis techniques employed (Ash et al., 1991; Moore et al., 1996; Anzai et al., 1997; Lechner et al., 1998; Achouak et al., 1999; Kuske et al., 1999; Goto et al., 2000). The molecular identification of these strains represents a first attempt to quantify the level of divergence among the bacteria associated with *T. borchii*.

The increasing sequencing information of other bacteria associated with

rhizosphere or mycorrhizal system developed by the ectomycorrizal fungus *Tuber* could be useful to monitor and better understand the processes of adaptation, selection and control of these bacteria to this complex microbe-host association.

The results of this study could be a starting point for illustrating the value of rDNA sequence analysis in the identification of bacteria compared with an analysis of phenotypic traits and in further molecular systematic and functional studies of bacteria-fungus-plant symbioses.

Furthermore this study offers a starting point for systematic molecular studies of bacteria involved in the complex ectomycorrhizal symbiosis. The results obtained from this first attempt at molecular characterization of cellulosolytic-chitinolytic fluorescent and spore-forming bacteria associated to fruitbodies of *T. borchii* extend the taxonomic information available on bacteria present in the mycorrhizosphere and could be helpful in further molecular systematic comparisons and functional studies of bacteria associated with fungi-plant symbioses.

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