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Endophytic Bacterial Diversity in Grapevine (*Vitis vinifera* L.) Leaves Described by 16S rRNA Gene Sequence Analysis and Length Heterogeneity-PCR

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Diversity of bacterial endophytes associated with grapevine leaf tissues was analyzed by cultivation and cultivation-independent methods. In order to identify bacterial endophytes directly from metagenome, a protocol for bacteria enrichment and DNA extraction was optimized. Sequence analysis of 16S rRNA gene libraries underscored five diverse Operational Taxonomic Units (OTUs), showing best sequence matches with γ-Proteobacteria, family *Enterobacteriaceae*, with a dominance of the genus *Pantoea*. Bacteria isolation through cultivation revealed the presence of six OTUs, showing best sequence matches with Actinobacteria, genus *Curtobacterium*, and with Firmicutes genera *Bacillus* and *Enterococcus*. Length Heterogeneity-PCR (LH-PCR) electrophoretic peaks from single bacterial clones were used to setup a database representing the bacterial endophytes identified in association with grapevine tissues. Analysis of healthy and phytoplasma-infected grapevine plants showed that LH-PCR could be a useful complementary tool for examining the diversity of bacterial endophytes especially for diversity survey on a large number of samples.

***Keywords*:** *Vitis vinifera* L., plant endophytes, 16S rRNA gene, length heterogeneity-PCR, bacterial enrichment

Bacteria residing in plant tissues without inducing symptoms of diseases are defined as endophytes (Wilson, 1995; Hallman *et al*., 1997). They are assumed to enter plant tissues from the adjacent rhizosphere by passive diffusion or by active selection. Bacterial endophytes have been associated to different parts of plants, such as roots, tubers, stem, and leaves (Hallmann, 2001; Gray and Smith, 2005), where they mainly inhabit the vascular system, the intercellular spaces, and/or the cell cytoplasm. Bacterial plant pathogens were detected in the same plant tissues where endophytes grew (Hurek *et al*., 1994; Berg *et al*., 2005). Even if bacterial endophytes are commonly associated with plants, the implication of their presence in host tissues is basically unknown. Although most of them exhibit no detectable impact on their hosts (Kado, 1992), it was demonstrated that bacterial endophytes can produce beneficial effects on host plants, such as growth promotion (Kaul *et al*., 2008), or prevent the deleterious effects of plant pathogens (Taechowisan and Lumyong, 2003).

To date, analyses of endophytic bacterial species have been mainly performed through cultivation-dependent approaches in a large range of monocotyledonous and dicotyledonous plant species (among others, Jacobs *et al*., 1985; Whitesides and Spotts, 1991; Brooks *et a*l., 1994; Gutierrez-Zamora and Martínez-Romero, 2001). The development of cultivation-independent fingerprinting molecular methods based on 16S rRNA gene automated analysis allowed obtaining a more specific, replicable, and detailed description of the diversity in complex bacterial communities. Length Heterogeneity (LH)-PCR can be successfully used to describe the bacterial communities (Brusetti *et al*., 2006) and it has been previously applied for characterization of endophytic community in potatoes (Sessitsch *et al*., 2001; Sessitsch *et al*., 2002) and in sugar beet (Dent *et al.*, 2004). In this study, the endophytic bacterial communities associated with healthy and phytoplasma-infected grapevine leaves have been characterized by LH-PCR and sequencing of 16S rRNA gene libraries from the total leaf DNA and by bacteria cultivation.

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Materials and Methods

Grapevine collection, sample preparation, DNA extraction, and preliminary PCR tests

In September 2004 and 2006, leaf samples were collected from each of eight grapevine plants of Barbera variety in a vineyard in Lombardy region (north-western Italy) (Table 2). Plants were chosen among asymptomatic (four plants) and symptomatic (four plants) for grapevine yellows (GY) disease.

Grapevine leaves were sterilized by washing with sterile water, treating with ethanol 70% for 3 min, sodium hypochlorite 2% for 5 min, and ethanol 70% for 30 sec, and

Table 1. Primers used in this study for PCR and sequencing

Name	Primer sequence (5'-3')	Gene target	Taxon target	Reference
trnL5'	CGAAATCGGTAGACGCTACG	*trn*	Plant plastid	Taberlet *et al.* (1991)
trnF	ATTTGAACTGGTGACACGAG	*trn*	Plant plastid	Taberlet *et al.* (1991)
G1	GAAGTCGTAACAAGG	16S rRNA	Bacteria	Jensen *et al.* (1993)
L1	CAAGGCATCCACCGT	23S rRNA	Bacteria	Jensen *et al.* (1993)
P1	AAGAGTTTGATCCTGGCTCAGGATT	16S rRNA	Bacteria	Deng and Hiruki (1991)
P7	CGTCCTTCATCGGCTCTT	23S rRNA	Bacteria	Schneider *et al.* (1995)
R16F2n (F2n)	ACGACTGCTGCTAAGACTGG	16S rRNA	Phytoplasmas	Lee *et al.* (1998)
R16R2 (R2)	TGACGGGCGGTGTGTACAAACCCCG	16S rRNA	Phytoplasmas	Lee *et al.* (1998)
16S-27F	AGAGTTTGATCCTGGCTCAG	16S rRNA	Bacteria	Lane (1991)
16S-1495R	CTACGGCTACCTTGTTACGA	16S rRNA	Bacteria	Lane (1991)
8F-I	GGATCCAGACTTTGATYMTGGCTAGI	16S rRNA	Bacteria	Ben-Dov *et al.* (2006)
907R-I	CCGTCAATTCMTTTGAGTTTI	16S rRNA	Bacteria	Ben-Dov *et al.* (2006)
338R	GCTGCCTCCCGTAGGAGT	16S rRNA	Bacteria	Ritchie *et al.* (2000)

DNaseZap (Applied Biosystems, Italy), and fivefold washing with sterile water. Aliquots of sterile water, used in the final rinse step, were set on Tryptic Soya Agar medium (TSA; Sigma, Italy). The TSA plates were examined for bacterial growth after 5 days of incubation at 30°C, and the grapevine leaves not contaminated were used for further analysis (Ferreira *et al.*, 2008; Sun *et al*., 2008). Bacterial DNA from grapevine leaf samples was extracted with two strategies: with and without microbe enrichment (Jiao *et al.*, 2006). Bacterial enrichment was done from leaf veins (2 g or 20 g), separated with a sterilized blade, or from whole leaves (40 g). Plant tissues were sterilized, grounded in liquid nitrogen, and aseptically incubated at 28°C for 12 h in gentle agitation in an enzymatic solution (0.1% macerozyme, 1% cellulase, 0.7 M mannitol, 5 mM N-morpholinoethanesulfonic acid, 9 mM $CaCl_2$, and 65 μM KH_2PO_4). After incubation, differential centrifugations (200×g for 5 min; 3,000×g for 20 min) were carried out for separating plant protoplasts from bacterial cells.

Three methods for DNA extraction from microbe enriched pellets were used: (i) DNeasy Plant Mini kit (QIAGEN, Germany), according to the manufacturer's instructions; (ii) method described by Prince *et al.* (1993), here modified by the addition of lysozyme (3 mg/ml), L-lysine (0.15 mol/L), EGTA (6 mmol/L, pH 8.0), and by the incubation at 37°C for 30 min, before the lysis step; (iii) method described by Haiwen *et al.* (2001). Total DNA from non enriched pellets were extracted with the method (i).

The quality of the extracted DNA was tested through PCR separately performed with the primers sets *trnL-trnF*, G1-L1, and R16F2n/R16R2 (F2n/R2) respectively specific for plastids, bacteria, and phytoplasmas (Table 1). PCR conditions were as previously described (Taberlet *et al.*, 1991; Jensen *et al.*, 1993; Lee *et al*., 1998). Moreover, a second

Table 2. PCR-amplification assays for evaluating the efficacy of bacteria enrichment and DNA extraction methods

Sample ID	Sanitary condition	Tissue	Enrichment	Extraction method	PCR-amplification primer sets		
					*trn*L-F	G1-L1	F2n-R2
139	Symptomatic *Ca.* P. vitis	2 g veins	+	i	+	-	-
139	Symptomatic *Ca.* P. vitis	2 g veins	-	i	nd	nd	+
141	Symptomatic *Ca.* P. vitis	2 g veins	+	ii	+	-	-
141	Symptomatic *Ca.* P. vitis	2 g veins	-	i	nd	nd	+
100	Healthy	20 g veins	+	iii	-	-	-
100	Healthy	2 g veins	-	i	nd	nd	-
101	Healthy	20 g veins	+	iii	-	-	-
101	Healthy	2 g veins	-	i	nd	nd	-
102	Symptomatic *Ca.* P. vitis	20 g veins	+	iii	-	-	-
102	Symptomatic *Ca.* P. vitis	2 g veins	-	i	nd	nd	+
269	Symptomatic *Ca.* P. vitis	40 g leaves	+	ii	+	-	-
269	Symptomatic *Ca.* P. vitis	2 g veins	-	i	nd	nd	+
270	Healthy	40 g leaves	+	ii	+	+	-
270	Healthy	2 g veins	-	i	nd	nd	-
271	Healthy	40 g leaves	+	ii	+	+	-
271	Healthy	2 g veins	-	i	nd	nd	-

nd, Not determined

phytoplasma amplification was done on the DNA extracted without enrichment with a nested-PCR of the 16S rRNA gene with primers P1/P7 and F2n/R2 (Table 1; Lee *et al.*, 1998). PCR products (5 µl) were separated by 1% agarose gel. Restriction fragment length polymorphysm (RFLP) analysis was performed for identifying the '*Ca.* Phytoplasma' species, as previously described (Davis and Dally, 2001).

Establishment of a collection of bacterial endophyte operational taxonomic units (OTUs) by cultivation and cultivation independent approaches

The bacterial 16S rRNA genes of plant n. 271 (Table 2) were amplified through two distinct PCR with primers 27F/1495R and 8F-I/907R-I (Table 1). Template DNA was obtained from the extraction method (ii). For the 27F/1495R PCR, reaction mixture (50 µl) contained 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 µM of each primer, and 1.25 U of AccuPrime™ *Taq* DNA polymerase High Fidelity (Invitrogen, Italy). Initial denaturation of 2 min at 95°C was followed by 35 cycles of 1 min at 95°C, 2 min at 50°C and 3 min at 72°C, and a final extension of 7 min at 72°C. For the 8F-I/907R-I PCR, reaction mixture (50 µl) contained 1× PCR buffer, 2 mM $MgCl_2$, 1.5 mM dNTPs, 0.2 µM of each primer, 0.5 µg/ml BSA, and 1.25 U of AccuPrime™ *Taq* DNA polymerase High Fidelity (Invitrogen). Initial denaturation of 4 min at 95°C was followed by 30 cycles of 30 sec at 95°C, 2 min at 50°C, and 3 min at 72°C, and a final extension of 7 min at 72°C.

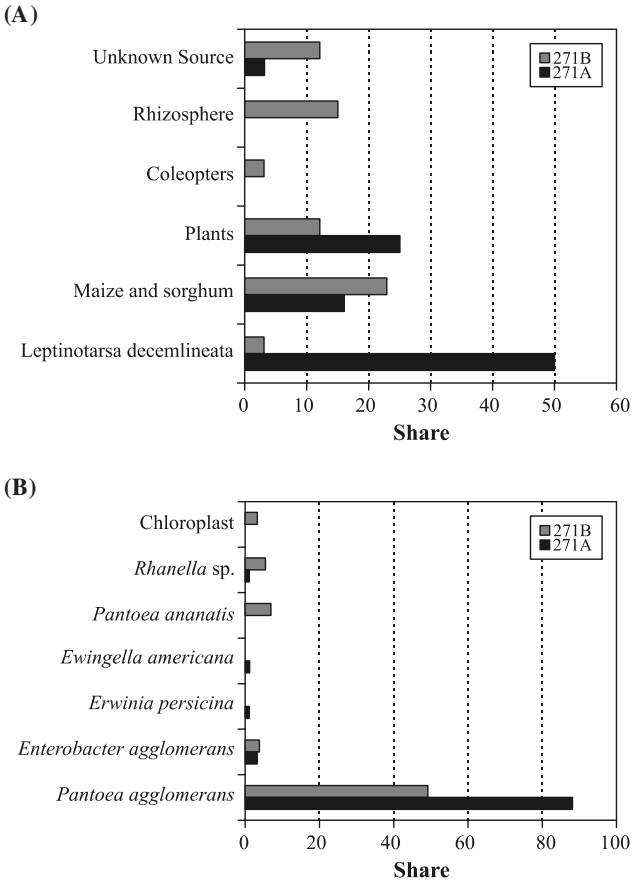

Fig. 1. Bar charts showing the sequence similarity of clones from libraries 271-A and 271-B with bacteria from (A) environmental sources of isolation and (B) public GenBank sequence database.

The PCR products from both the amplifications were separated in 1% agarose gels. The bands were excised and purified with the QIAEX II kit (QIAGEN) according to manufacturer's instructions. Purified products were cloned in the plasmid vector pCR2.1-TOPO (Invitrogen) and propagated in *Escherichia coli* as described (Shuman, 1994). The plasmid DNA was extracted from *E. coli* colonies with the QIAGEN Plasmid Mini kit (QIAGEN), and sequenced with an ABI 3730 sequencer (Primm, Italy). Sequences were identified by comparison with the GenBank sequence database with the BLAST software (http://www.ncbi.nlm.nih.gov/BLAST). Rarefaction analysis was done using PAST 1.88 software from http://folk.uio.no/ohammer/past/website.

The characterization of the cultivable microorganisms associated with the grapevine plant n. 271 was performed as described elsewhere (Bell *et al.*, 1994). Grapevine leaves (2 g) were sterilized by washing with sterile water, treating with ethanol 70% for 3 min, sodium hypochlorite 2% for 5 min, and ethanol 70% for 30 sec, and fivefold washing with sterile water. Sterilized leaves were ground in a pre-cooled mortar and mixed with a 'Ringer solution' (Oxoid, Italy). Partial volume (100 µl) of homogenates, serially diluted, was incubated on Tryptic Soya Agar medium (TSA; Sigma, Italy) at 30°C for 5 days. Bacterial colonies were selected on the basis of phenotypic traits and isolated. 16S rRNA gene PCR products, amplified with the primers 27F and 1495R, were cloned and sequenced as described. Sequence identification was done with BLAST.

All the 16S rRNA gene sequences obtained from cloning and cultivation processes were clustered in Operational Taxonomic Units (OTUs) with the software Bioedit 7.0.0 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Nucleotide sequences sharing more than 97% identity were clustered in the same OTU. One representative clone sequence for each OTU was clustered in a Neighbour-Joining phylogenetic dendrogram bootstrapped 1,000 times with the software MEGA4 (Kumar *et al.*, 2008). Frequency of OTU-representative clone sequences (number of clone sequences in each OTU) was reported in the tree (Fig. 2) and in Table 3.

Development of a LH-PCR database and LH-PCR of the endophytic bacterial community

LH-PCR analyses were carried out on grapevine DNA extracted with the method (ii) after bacterial enrichment step, and on the 16S rRNA genes from both representative bacterial isolated strains and clones. An additional LH-PCR amplification was also performed in nested PCR from the previously obtained phytoplasma amplicons, to obtain a characteristic peak for '*Ca.* Phytoplasma vitis' related to the Flavescence dorée disease.

The LH-PCR reaction was done with the primers 27F labelled at its 5' end with the phosphoramidite dye (6-FAM) and 338R (Table 1) as previously explained (Brusetti *et al.*, 2006). Quantified PCR products (100 ng) were added to 0.8 µl of 500 ROX-labelled internal size standard (Applied Biosystems, Italy) and 15 µl of deionized formamide. Samples

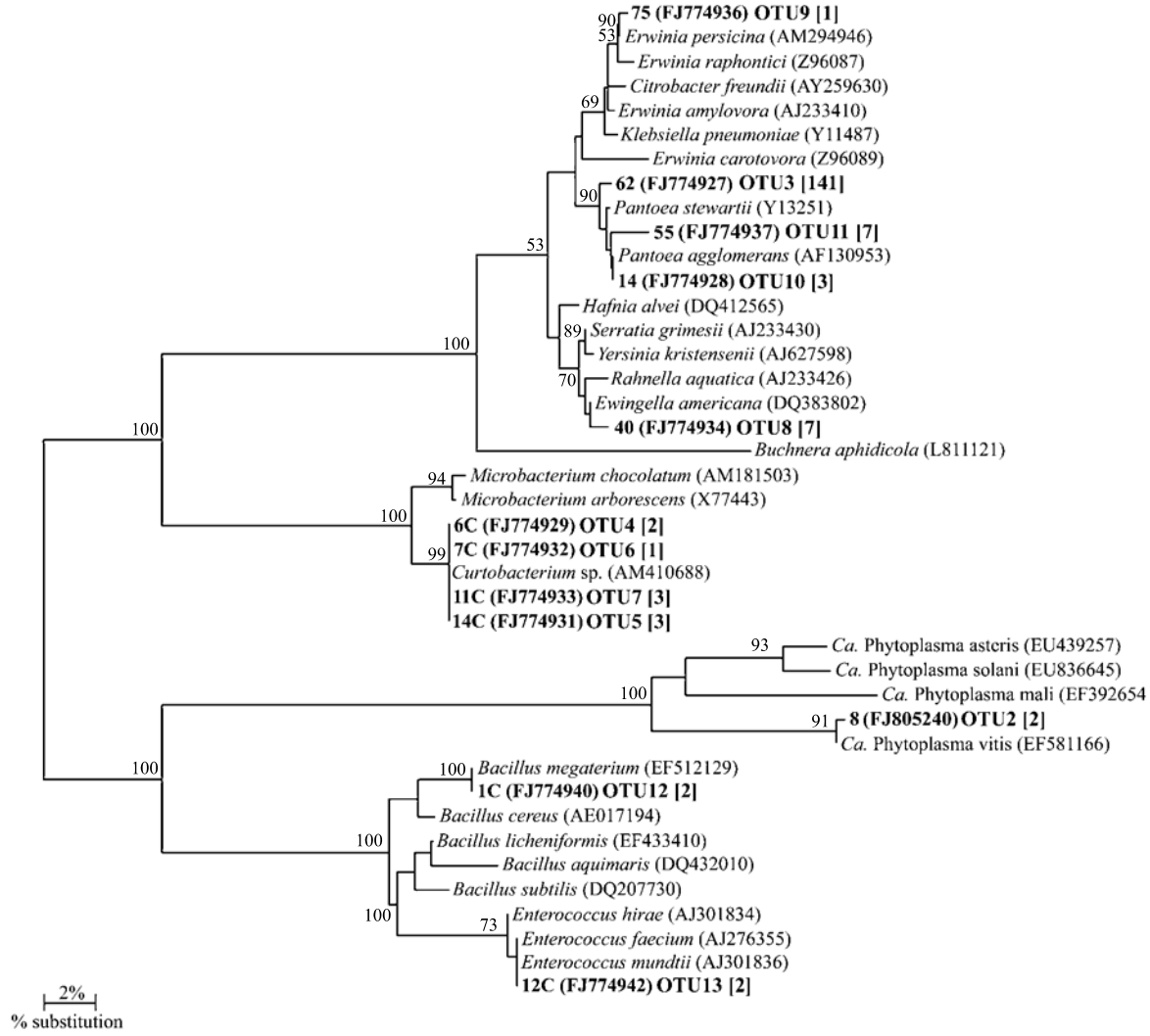

Fig. 2. Phylogenetic relationships based on partial 16S rRNA gene sequences obtained from the endophytic bacterial associated with grapevine tissues (this work) and closely related sequences, retrieved from GenBank. Bootstrap values higher than 50% are displayed at tree nodes. GenBank accession numbers of nucleotide sequences are shown along with the name of the bacterial species. Frequency of OTU-representative clone sequences (number of clone sequences in each OTU) was reported in the tree.

were denatured at 95°C per 8 min, rapidly put into ice for 5 min, and loaded on the ABI Prism 310 as shown in Brusetti *et al.* (2006). LH-PCR data were analysed with Genescan 3.1.2 software (Applied Biosystems), and a threshold of 50 fluorescent units was used. Peak sizing and peak matrix were done with the Genescan 3.1.2 software. The position of all peaks was carefully checked by eye. For all the bacterial strains and clones the same PCR amplification was run three times and three separate PCR were also run to confirm the LH-PCR peak sizing through different PCR reactions.

The length of the LH-PCR fragments of the bacterial clones and isolates were used as reference to tentatively attribute the single peaks in the LH-PCR profiles of the whole bacterial grapevine communities to the identified bacterial species.

Nucleotide sequences accession numbers

16S rRNA gene partial sequences were deposited in NCBI/EMBL/DDBJ public sequence database under the accession numbers from FJ774923 to FJ774942 and FJ805240 and FJ805241.

Results and Discussion

PCR assays for evaluating the efficacy of bacteria enrichment and DNA extraction

PCR assays were done with primers specific for plastids (*trnL-trnF*), bacteria (G1-L1) and phytoplasmas (F2n-R2; Table 1) on DNA obtained with different extraction procedures and different bacteria enrichment strategies (Table 2). Despite bacterial enrichment, DNA extracted using method (iii) did not give PCR products with all primer sets. DNA

Table 3. OTU description and length heterogeneity-PCR fragment database representing bacteria isolated from grapevine leaves

OTU	Total clones	Sequence ID	Accession no.	GeneBank closest relative	Accession no.	% match	Fragment length bp[a]
1[b]	1	20	FJ774923	*V. vinifera* chloroplast	DQ424856	98	317
1[b]	2	37	FJ774924	*V. vinifera* chloroplast	DQ424856	99	317
2	1	8	FJ805240	*Ca.* Phytoplasma vitis	FJ611961	99	318 (347)
2	1	10	FJ805241	*Ca.* Phytoplasma vitis	FJ611961	99	318 (347)
3[c]	1	23	FJ774925	*Pantoea agglomerans*	EF050810	97	345
3[b/c]	51/89	62	FJ774927	*Pantoea agglomerans*	AB004757	99	345
4[d]	1	6C	FJ774929	*Curtobacterium* sp.	DQ205304	98	345
4[d]	1	5C	FJ774930	*Curtobacterium* sp.	DQ205304	99	345
5[d]	3	14C	FJ774931	*Curtobacterium* sp.	DQ205304	99	345
6[d]	1	7C	FJ774932	*Curtobacterium* sp	DQ205304	98	345
7[d]	3	11C	FJ774933	*Curtobacterium* sp.	DQ205304	97	345
8[c]	2	40	FJ774934	*Ewingella americana*	AM167519	99	346
8[b]	5	6	FJ774935	*Ewingella americana*	AM167520	97	346
9[c]	1	75	FJ774936	*Erwinia persicina*	AM294946	98	346
10[c]	1	14	FJ774928	*Pantoea agglomerans*	EF050808	99	347
10[b]	2	50	FJ774926	*Pantoea agglomerans*	AF157694	99	347
11[b]	4	55	FJ774937	*Pantoea ananatis*	DQ133545	98	347
11[b]	3	57	FJ774938	*Pantoea ananatis*	DQ133546	99	347
12[d]	1	2C	FJ774939	*Bacillus* sp.	EF377309	99	355
12[d]	1	1C	FJ774940	*Bacillus* sp.	EF377308	99	355
13[d]	1	9C	FJ774941	*Enterococcus* sp.	DQ462329	97	363
13[d]	1	12C	FJ774042	*Enterococcus* sp.	DQ462330	99	363

[a] The fragment length is reported with an approximation of ± 0.5 bp. Secondary peaks are reported by brackets
[b] Indicates the library 271-B containing the PCR fragment 8F-I/907R-I
[c] Indicates the library 271-A containing the PCR fragment 27F-1495R
[d] Indicates the bacterial strains isolated in TSA medium

extracted with the method (ii) was always positive in the amplification of the plastidial DNA but gave also positive amplifications with bacterial-specific primers, but never with phytoplasmas-specific primers. Method (i) was applied on DNA extracted from two-grams veins and without any bacterial enrichment step. Although no amplifications were obtained with bacterial-specific primers, this strategy was the only able to detect phytoplasmas in tissues of symptomatic plants. The data showed that the DNA extraction procedure is a key point for 16S rRNA gene-based cultivation-independent studies of bacterial diversity associated with plant tissues. Despite preparative step of plant tissues for enriching bacterial cells would be useful for reducing amplification of plant plastids, the present data show that some particular bacterial strains, such as phytoplasmas could not be detected. It should be considered that wall-less phytoplasma cells are highly sensitive to osmotic pressure variations, and the rupture of the cellular membranes could determine a diverse migration and/or lysis of the phytoplasma cells during the centrifugation steps causing decreases of the total phytoplasma DNA in the final preparation. According to these data the DNA for the bacterial 16S rRNA gene libraries was extracted with method (ii) with the enrichment step.

Characterization of the bacterial community associated with grapevine leaf tissues by cultivation-independent and cultivation-dependent analyses

The diversity of the microbiota associated with the healthy grapevine plant n. 271 was investigated by sequence analysis of two 16S rRNA gene libraries, based on the amplification with bacterial primer pairs 27F/1495R (library 271-A) and 8F-I/907R-I (library 271-B). We employed two different bacterial primer sets to minimize biases related to the use of a single primer set (Curtis *et al.*, 2002). A total of 162 clones (94 from the library 271-A, and 68 from the library 271-B) were sequenced. The coverage of each single library was 99% and 93% respectively for library 271-A and 271-B; the coverage of the pooled libraries was 98%. Clones from both libraries showed high sequence similarity with cultivated bacteria, previously isolated from plant tissues (mainly maize and sorghum), rhyzosphere, and gut of phytophagous insects such as the grape phylloxera *Daktulosphaira vitifoliae* (Fitch) (Hemiptera: Phylloxeridae), and the Colorado potato beetle *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) (Fig. 1). Sequences close to isolates from *Leptinotarsa decemlineata* (Fitch) and maize plants were prevalent in the libraries 271-A (50/91), and 271-B (23/59), respectively. Sequences close to isolates from Coleoptera and plant rhizosphere were identified only in the library 271-B (Fig. 1A).

Ninety eight percent of the 159 clones yielded best matches with bacteria of the family *Enterobacteriaceae* within γ-Proteobacteria that has been previously reported as a prevalent division associated to plant tissues (Chelius *et al*., 2001; Kaiser *et al*., 2001; Idris *et al.*, 2004). Among these, 137 sequences (86%) were more than 97% identical to *Pantoea agglomerans* that was the dominant bacterium in both the libraries. This microorganism has been associated to plants and insects. It was previously isolated as endophyte from

grapevine xylem (Bell *et al*., 1994), and from *D. vitifoliae* (Vorwerk *et al*., 2007). Previous studies emphasized the potential of *P. agglomerans* as biocontrol agent against a range of plant pathogens through the secretion of antibacterial molecules (Ishimaru *et al*., 1988) and/or by the activation of plant Induced Systemic Resistance (ISR) (Ortmann *et al.*, 2006). Recently, *P. agglomerans* was successfully used for the control of the fire blight bacterial disease of apple, pear, and other plants of the family Rosaceae caused by *Erwinia amylovora* (Wright *et al*., 2001; Pusey *et al.*, 2008), and of *Rhizopus stolonifer* and *Monilinia laxa* in fruit post-harvest storage of apricots and nectarines (Bonaterra *et al*., 2002). Other bacterial species found in the libraries were *Ewingella americana* retrieved in both libraries, and *Erwinia persicina* and *Pantoea ananatis*, found in libraries 271-A and 271-B, respectively (Fig. 1). *V. vinifera* chloroplast sequence was found only in the library 271-B. The two diverse primer sets increased the amount of information on the plant bacterial community confirming that diverse bacterial primer sets give different diversity pictures from the same DNA template (Ben-Dov *et al.*, 2006; Frank *et al.*, 2008).

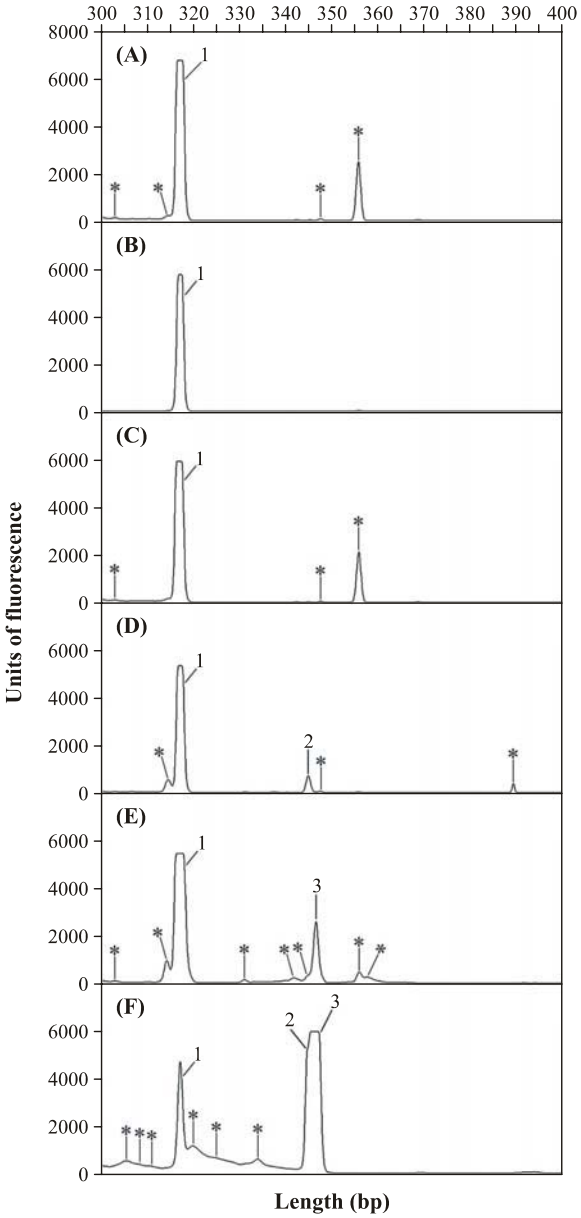

Fig. 3. Length heterogeneity (LH)-PCR electropherograms showing the profile obtained by analysing the microbial populations of (A) grapevine plant n. 100, (B) grapevine plant n. 101, (C) grapevine plant n. 102, (D) grapevine plant n. 269, (E) grapevine plant n. 270, and (F) grapevine plant n. 271. Numbers correspond to the peaks detected by Genescan software according to the LH-PCR database shown in Table 3. On the basis of the LH-PCR database, tentative attribution of LH-PCR peaks of grapevine plants is as follows: 1, peak of grapevine chloroplast; 2 and 3 peaks of *Pantoea agglomerans.* Non-attributed peaks are indicated by *.

Bacterial isolation was performed on leaf tissues of the same grapevine n. 271 and confirmed that cultivation-independent and -dependent approaches may identify diverse ranges of bacteria and should be coupled to increase the range of diversity explored in a sample (Araujo *et al*., 2002). Eleven colony types with different morphology were identified. Sequences of the 16S rRNA gene identified (sequence identity ≥97%) Firmicutes of the genera *Bacillus* and *Enterococcus* (three colonies each), and Actinobacteria of the genus *Curtobacterium* (five colonies) (Fig. 2). *Curtobacterium*-related strains were isolated as endophytes from sweet-orange, coffee, grapevine, and poplar (Bell *et al*., 1994; Araujo *et al.*, 2002; Vega *et al*., 2005; Ulrich *et al.*, 2008). Some *Curtobacterium*-related bacteria were reported like etiological agents of plant diseases (Vidaver, 1982), while *C. flaccumfaciens* protected cucumber plants from pathogens (Raupach and Kloepper, 2000) and induced ISR in other plant hosts (Raupach and Kloepper, 1998).

As expected from previous studies (Araujo *et al*., 2002; Conn and Franco, 2004; Cankar *et al.*, 2005), cultivation-independent analyses should allow to describe more accurately the microbial diversity in environmental sources rather than cultivation methods. By contrast, in the present study, cultivation as well as culture-independent methods revealed differences among bacterial endophytes in terms of diversity and abundance. In fact, the culturable component of the bacterial community associated with grapevine was different from that obtained by clone analysis. However, the selectivity of cultivation as well as a preferential amplification of certain bacterial groups with universal primer could also cause the different abundance. A disparity in the representation of different bacterial classes, genera and species between isolate collection and clone library had also been observed in several other studies (Dunbar *et al*., 1999; Hengstmann *et al*., 1999; Chelsius and Triplett, 2001; Idris *et al*., 2004). Therefore, the combination of culturing methods and cloning analysis is needed for the study of endophytic community associated with plants.

Set-up of a LH-PCR database of the bacterial diversity associated with grapevine leaf tissues

The LH-PCR is an automated analysis tool based the 16S rRNA gene sequence polymorphism that gives a faster, replicable and detailed description of the diversity in complex bacterial communities. A cultivation-dependent identification of bacterial species through 16S rRNA gene sequencing fol-

lowed by the set up of a LH-PCR database was reported for monitoring lactic acid bacteria succession during maize ensiling (Brusetti *et al.*, 2006). LH-PCR was also used to characterize the bacterial community associated with the leafhopper *Scaphoideus titanus* (Cicadellidae), the insect vector of the Flavescence dorée of grapevine (Marzorati *et al.*, 2006).

An experimental LH-PCR database representing bacterial species in the grapevine leaf tissues was established starting from the 16S rRNA gene clones and the bacterial isolates from plant 271 (Table 3). LH-PCR fragments of the 18 bacterial endophytic species analyzed were in the range of 345~363 bp, while '*Ca.* Phytoplasma vitis' (identified through PCR-RFLP assays) presented two fragments of 318 and 347 bp. The grapevine chloroplast fragment was of 317 bp. *Enterobacteriaceae* showed a main fragment of 347 bp (*P. agglomerans* OTU 10 and *P. ananatis* OTU 11). A secondary fragment of 345 bp was detected only in *P. agglomerans* OTU 3. *Curtobacterium* sp. species, grouped in OTU 4, 5, 6, and 7 shared a peak at 345 bp. *Ew. americana* (OTU 8), *Er. persicina* (OTU 9), *Bacillus* sp. (OTU 12), and *Enterococcus* sp. (OTU 13) presented single peaks. LH-PCR database allowed distinguishing the peaks of all the identified bacteria, except for closely related organisms such as *Ew. americana* and *Er. persicina* that shared a peak at 346 bp (Table 3).

Diversity of the grapevine endophytic bacteria by LH-PCR

In order to investigate the bacterial diversity in grapevine leaves, a LH-PCR study was performed on eight grapevine plants and the peak profiles were compared with those of the LH-PCR database (Fig. 3). LH-PCR patterns presented from one to nine DNA fragments. Three were identified through the comparison with the LH-PCR database. Although the use of the bacterial enrichment step, the chloroplast peak was present in all samples. *Enterobacteriaceae* peaks were recognized in three plants (n. 269, 270, and 271). In the leaves of plant n. 271, LH-PCR detected only a peak attributable to *Enterobacteriaceae*, associated with *P. agglomerans*, while 16S rRNA gene libraries revealed the presence of *P. agglomerans*, *Ew. americana*, *Er. persicina*, and *P. ananatis*. This suggests that *P. agglomerans* is more represented than the other *Enterobacteriaceae* on the leaves and is more easily amplified in a direct LH-PCR assay than the other bacteria. A similar consideration can be made for *Bacillus*, *Enterococcus*, and *Curtobacterium*, identified by cultivation that were not detected by LH-PCR assays. Also *Ca.* Phytoplasma vitis, infecting the grapevine plants n. 139, 141, 102, and 269 (Table 2), was not identified by LH-PCR. The titre of *Ca.* Phytoplasma vitis in grapevine tissues is always very low (Faoro, 2005). It should be noted that 16S rRNA gene amplification with universal bacterial primers is less sensitive than PCR with specific primers targeting a given taxon. For example it has been shown that universal bacterial primers used in another community PCR-fingerprinting method, denaturing gradient gel electrophoresis detects only those sequences represented over 1% of the total sequences in the community (Muyzer *et al*., 1996). Furthermore, presence of additional LH-PCR peaks of grapevine n. 271 in comparison with peaks of cloning-based LH-PCR database highlighted the bias inherent to any molecular technique that uses the PCR-based 16S rRNA gene sequence analysis for microbial community description (Mills *et al.*, 2007). In fact, the choice of universal bacterial primer pairs and PCR cycling conditions strongly influence the microbial community description (Curtis *et al*., 2002; Huws *et al.*, 2007). For that reason, in order to minimize the PCR-based bias, future studies of grapevine-associated bacterial endophytes will be carried out by using same primer pair for 16S rRNA gene library and LH-PCR analyses.

Our data registered several non-conserved peaks in the grapevine plants examined, indicating that the diversity of endophytic bacteria in grapevine leaves is higher than that described here. However the approach described here can be useful for comparing bacterial diversity among different grapevine cultivars or from different geographic areas. *P. agglomerans* and *Curtobacterium* sp., two cultivable bacteria represent interesting candidates for developing biocontrol strategies of phytoplasmas infecting grapevine.

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