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Restructuring of Endophytic Bacterial Communities in Grapevine Yellows-Diseased and Recovered *Vitis vinifera* L. Plants[▽]

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Length heterogeneity-PCR assays, combined with statistical analyses, highlighted that the endophytic bacterial community associated with healthy grapevines was characterized by a greater diversity than that present in diseased and recovered plants. The findings suggest that phytoplasmas can restructure the bacterial community by selecting endophytic strains that could elicit a plant defense response.

Flavescence dorée (FD) and Bois noir (BN), two main phytoplasma diseases of grapevine yellows complex (GY), have been seriously damaging wine production worldwide (1, 2). Until now, no grapevine cultivars have been found that are resistant to phytoplasma infection (12). Moreover, no effective control measures are available to direct control of phytoplasmas. Intriguingly, both FD- and BN-diseased plants may spontaneously recover (15). Physiological mechanisms and possible biological actors involved in this phenomenon are not yet understood. Recent studies highlighted a significant increase in reactive oxygen species (ROS), pathogen-related (PR) proteins, and H_2O_2 in recovered plants (15, 16). Other research demonstrated that bacterial endophytes may prevent the deleterious effects of plant pathogens (10, 14). Here, we described the microbial diversity in healthy, GY-diseased, and recovered grapevines in order to investigate the possible relationship between recovery and endophytic bacteria.

On the basis of previous GY surveys carried out since 2000, leaf samples were collected in September 2007 from 20 asymptomatic, 20 symptomatic (desiccation of inflorescences, berry shrivel, leaf reddening), and 20 recovered (no GY symptoms for at least 2 years after the remission) Barbera clone plants in northwestern Italy. The influence of environmental factors was reduced by collecting samples from the same vineyard and on the same day. In order to achieve further information on possible changes in the microbial community due to different sampling periods, four healthy, four diseased, and four recovered grapevines were also collected in October 2007 and in September 2008. Total plant DNAs, extracted by the DNeasy plant mini kit (Qiagen, Germany), were used as templates for phytoplasma detection by nested PCR analysis carried out using primer pairs P1/P7 (5) and R16F2n/R16R2 (8). Amplicons were digested by using the restriction enzyme MseI (New England Biolabs, Ipswich, MA) (13).

Total DNA was extracted from 20 g of leaves and analyzed by length heterogeneity-PCR (LH-PCR), as described by Bulgari et al. (3), to study the endophytic bacterial diversity in the grapevines examined. For each extracted DNA, PCR analysis was done three times, and each obtained amplicon was run three times to confirm the LH-PCR peak sizing. The repeatability of LH-PCR analyses was tested by contingency table analysis. LH-PCR results were analyzed by univariate (Jaccard similarity index) and multivariate (correspondence analysis [CA]) statistical analyses to evaluate the possible relationship between endophytic bacteria diversity and the sanitary status of grapevine plants. Bacterial richness was calculated as the presence of single peaks in the total plants analyzed. Jaccard analysis was performed by NTSYSpc statistical software (numerical taxonomy system, version 2.01; Applied Biostatistics, Inc.), while CA and contingency table analysis were calculated using JMP software (JMP version 7; SAS Institute, Inc., Cary, NC).

Cultivable bacteria associated with representative plants were characterized by cultivation-dependent methods, as previously described (3). Each bacterium, isolated on the basis of its phenotypic traits (color, shape, size) was identified by comparing its 16S rRNA gene partial sequence using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Sequences of cultivable endophytic bacteria were clustered in a neighbor-joining phylogenetic dendrogram bootstrapped 1,000 times with MEGA4 (18). Moreover, isolated bacteria were tested for catalase activity by laying a drop of 3% hydrogen peroxide directly onto the colonies.

Lengths of the LH-PCR fragments of the isolated bacteria were used as a reference to tentatively attribute the single peaks in the LH-PCR profiles of the whole bacterial grapevine communities to the identified bacterial species.

On the basis of MseI enzymatic restriction patterns, FD phytoplasmas (16SrV-C/-D) and BN phytoplasmas (16SrXII-A) were identified in the symptomatic grapevine plants (data not shown). No phytoplasmas were detected in asymptomatic (healthy and recovered) grapevines.

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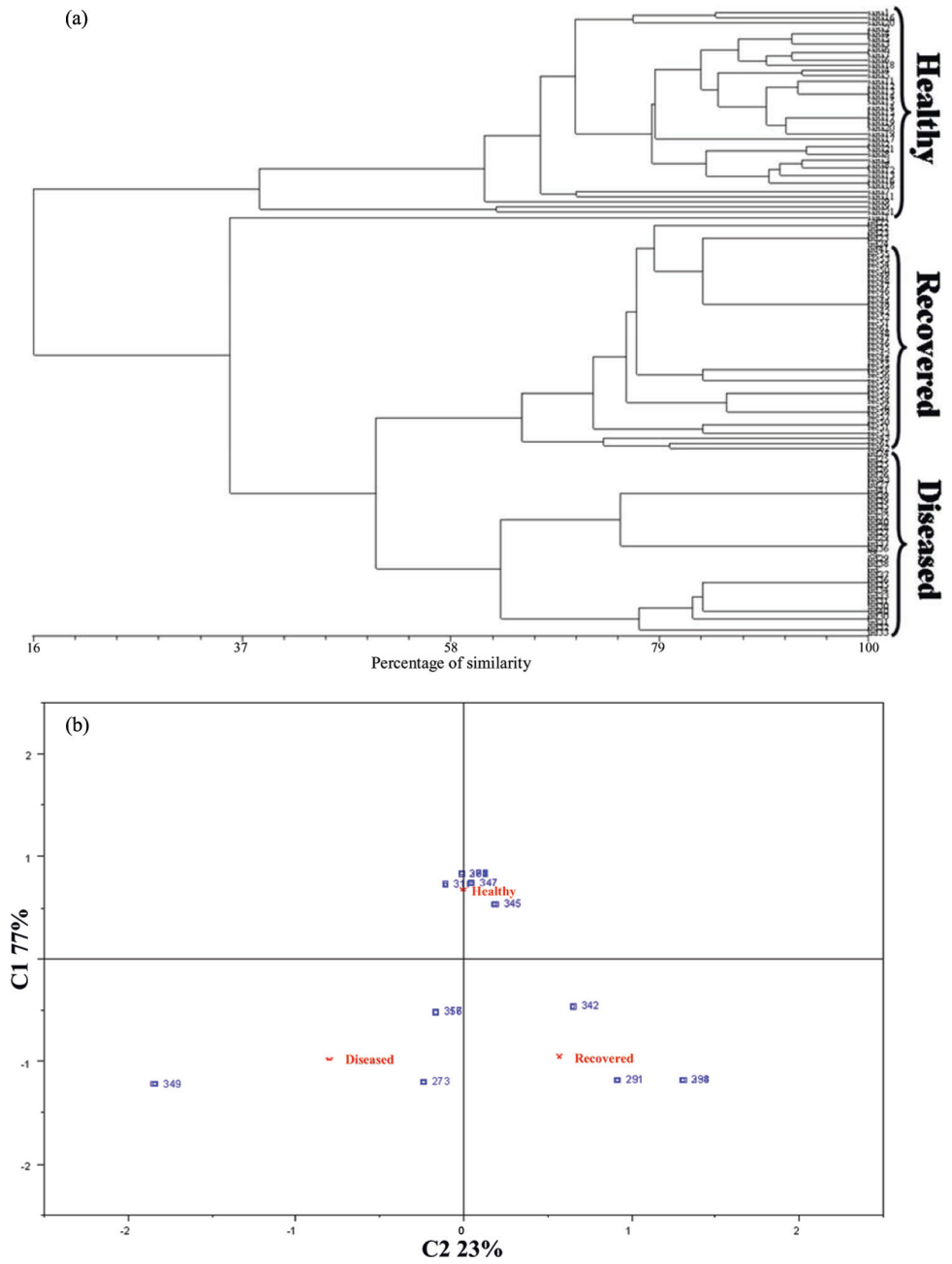

FIG. 1. LH-PCR profiles statistically analyzed by Jaccard index ($r = 0.97$) (a) and correspondence analysis (b). (b) Percent variation in species data is represented by the two ordination axes (77.25% and 22.75%, respectively). Three different clusters of samples are clearly shown in the CA diagram. Results for healthy grapevine plants are clustered at the top of diagram (C1, 0.68; C2, 0); results for diseased plants, both coordinates negative, are at the left part of the plot; results for recovered grapevines, negative for C1 and positive for C2, are at the right part of the plot.

Endophytic bacterial diversity in healthy, diseased, and recovered grapevines. Endophytic bacterial diversity in examined plants was analyzed by LH-PCR. Contingency table analysis (χ^2 of 0.045 at an α value of 0.05) evidenced the repeatability of two of the three PCRs. Jaccard analysis indicated significant differences ($r = 0.97$) among endophytic bacterial communities associated with healthy, diseased, and recovered grapevines collected in September 2007 (Fig. 1a), October 2007, and September 2008 (data not shown). This evidence suggests that the presence/absence of the phytoplasmas can influence microbial community composition more than environmental factors. Three different clusters of samples are also shown in the CA diagram: healthy (Fig. 1b, top), diseased (Fig. 1b, left), and recovered (Fig. 1b, right) grapevines. The LH-PCR peaks that determine the main separation on C1 (ordination axis 1) are 291 bp, 294 bp, and 338 bp (associated with diseased and recovered plants) and 278 bp, 280 bp, 285 bp, 287 bp, 303 bp, 306 bp, 331 bp, 334 bp, and 350 bp (associated with healthy plants). On the other hand, the separation between recovered and diseased plants on C2 (ordination axis 2) was determined

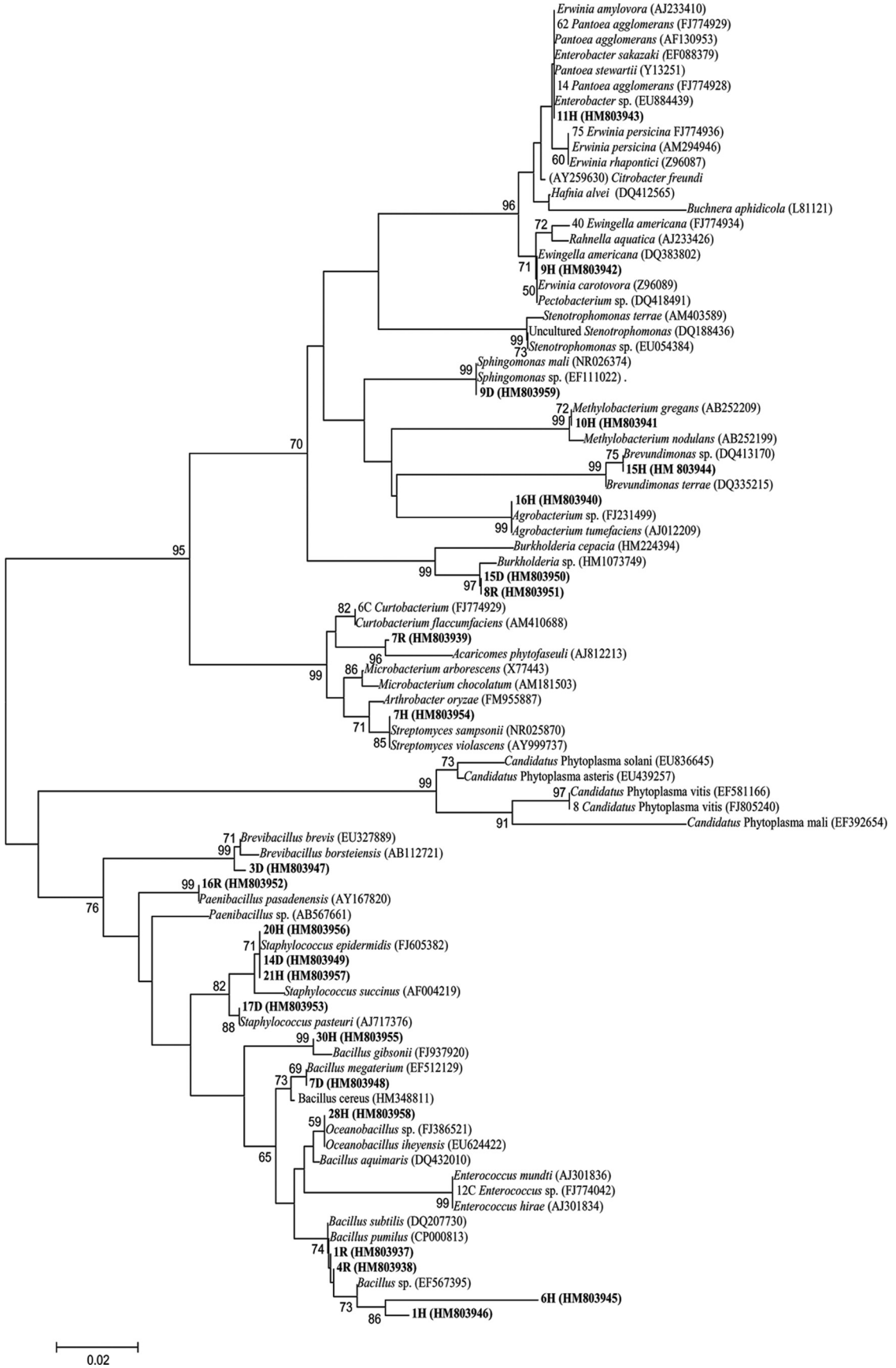

FIG. 2. Phylogenetic relationships based on partial 16S rRNA gene sequences obtained from the endophytic bacteria associated with grapevine tissues (this work) and closely related sequences retrieved from GenBank. Bootstrap values are displayed at tree nodes. GenBank accession numbers of nucleotide sequences are shown along with the name of the bacterial species.

TABLE 1. Length heterogeneity-PCR fragment database representing bacteria isolated from healthy (H), diseased (D), and recovered (R) grapevines

ID	Accession no. of isolate	GenBank closest relative	Accession no. of closest relative	% Match	Fragment length (bp)[a]	Plant
1R	HM803937	*Bacillus pumilus*	FJ263042	100	304 (350)	R
14I	HM803949	*Sphingomonadaceae bacterium*	DQ520832	99	310 (314)	D
16S	HM803940	*Agrobacterium* sp.	AM269517	99	312	H
10S	HM803941	*Methylobacterium gregans*	AB252209	98	314	H
9I	HM803959	*Sphingomonas* sp.	EF111022	98	314	D
15I	HM803950	*Burkholderia fungorum*	HM113360	99	317	D
8R	HM803951	*Burkholderia* sp.	EU723189	99	338	R
16R	HM803952	*Paenibacillus pasadenensis*	AY167820	99	338	R
7S	HM803954	*Streptomyces violascens*	EU273550	95	340	H
30S	HM803955	*Bacillus gibsonii*	EU373538	99	341	H
9S	HM803942	*Pectobacterium* sp.	DQ418491	99	342	H
3I	HM803947	*Brevibacillus brevis*	FJ598016	98	345	D
11S	HM803943	*Enterobacter* sp.	EU884439	99	345	H
7R	HM803939	*Acaricomes phytoseiuli*	AJ812213	99	348 (350)	H/R
15S	HM803944	*Brevundimonas* sp.	DQ413170	97	349 (351)	H
17I	HM803953	*Staphylococcus pasteuri*	GQ503327	99	350 (352)	D
1S	HM803946	*Bacillus subtilis*	FJ263034	99	351	H/D
4R	HM803938	*Bacillus subtilis*	GQ150489	99	351	R
6S	HM803945	*Bacillus* sp. ZW2531	EF567395	97	352	H
20S	HM803956	*Staphylococcus epidermidis*	FJ613565	96	352	H/D
21S	HM803957	*Staphylococcus* sp.	GU003862	99	352	H
7I	HM803948	*Bacillus megaterium*	FJ460474	99	352 (355)	D
28S	HM803958	*Oceanobacillus* sp.	FJ386521	99	367	H

[a] The lengths of the additional LH-PCR peaks, indicating 16S rRNA gene interoperonic length polymorphisms, are reported in parentheses.

by the peaks at 349 bp, 294 bp, and 338 bp. Moreover, LH-PCR electropherogram analysis allowed the determination of bacterial diversity, which was higher (a major number of LH-PCR peaks) in healthy plants than in phytoplasma-infected and recovered plants. However, bacterial diversity in diseased and recovered plants was quite similar (comparable number of LH-PCR peaks). The lower bacterial diversity in diseased plants could be the result of (i) a direct interaction between phytoplasmas and endophytic bacteria or (ii) a phytoplasma-mediated plant response that restructured the endophytic bacterial community. In grapevines, phytoplasmas could compete with endophytic bacteria for carbon sources and to inhabit a favorable niche, leading to reduction of bacterial richness in infected plants. Studies reported an accumulation of H_2O_2 in phytoplasma-infected plants (16). H_2O_2 in combination with other reactive oxygen species is an essential component in the signal transduction cascade(s) leading to defense reactions, such as the hypersensitive response and salicylic acid-mediated defense pathway (7). Recent research showed that salicylic acid-dependent and -independent pathways reduced endophytic bacterial diversity and the ability of bacteria to colonize plants (11). In agreement with the data reported by Trivedi et al. (19), regarding "*Candidatus* Liberibacter asiaticus," phytoplasma infection could restructure the endophytic community, selecting only a few bacterial strains that possibly overcome ROS stress generated by the plant response following infection.

Identification of endophytic bacteria and possible biological implications. A total of 26 LH-PCR peaks were detected within 272 to 356 bp in the whole bacterial community. Twenty LH-PCR peaks were found in healthy grapevine plants (13 peaks specifically associated), 8 in diseased grapevines (1 peak specifically associated), and 10 in recovered grapevines (3 peaks specifically associated). To identify bacterial species associated with LH-PCR peaks, phenotypically diverse endophytic bacteria isolated from grapevine leaf tissues were identified by sequencing 16S rRNA genes. Sequences were closed related (sequence identity, ≥97%) to (i) the *Gammaproteobacteria* genera *Enterobacter*, *Pectobacterium*, *Sphingomonas*, and *Stenotrophomonas*; (ii) the *Alphaproteobacteria* genera *Methylobacterium*, *Brevundimonas*, and *Agrobacterium*; (iii) the *Betaproteobacteria* genus *Burkholderia*; (iv) the *Firmicutes* genera *Brevibacillus*, *Staphylococcus*, *Paenibacillus*, *Oceanobacillus*, and *Bacillus*; and (v) the *Actinobacteria* genera *Acaricomes* and *Streptomyces* (Fig. 2 and Table 1).

Burkholderia sp. (peak at 338 bp), *Bacillus pumilus* (peaks at 304 and 350 bp), *Paenibacillus pasadenensis* (peak at 338 bp), and uncultured *Bacillus* sp. (peak at 351 bp), here identified only in recovered plants, are known as resistance inducers. *Burkholderia* species are capable of controlling several plant pathogens due their ability to produce antifungal compounds (6). Moreover, *Bacillus pumilus* and other *Bacillus* species, like *Bacillus subtilis* (isolated here), *Bacillus cereus*, *Bacillus pasteuri*, *Bacillus sphaericus*, and other *Bacillus* spp. elicited a significant reduction of disease severity on different hosts by inducing induced systemic resistance (ISR) (4), a process that protects a plant from pathogens for a considerable part of its lifetime. It can result as a decrease in plant susceptibility and disease severity or as a reduction in the number of diseased plants (20). In the case of GY diseases, it was observed that recovered plants maintained their sanitary condition for at least 2 years and were not easily reinfected by phytoplasmas. The evidence that recovery in grapevines is stable and the presence of ISR-inducing bacteria in recovered grapevines could indicate a possible involvement of endophytes in recovery from GY.

In conclusion, alterations induced by phytoplasmas in the grapevine endophytic bacterial community select bacterial strains that are more resistant to ROS and able to elicit plant defense responses, including ROS as well; these bacteria could ultimately lead to recovery. This view is supported by previously reported findings showing that recovered grapevine plants have higher levels of ROS than those of diseased and healthy plants (16). In order to verify this hypothesis, future studies will focus on determining the relative abundance of putative recovery inducers within microbial communities living in grapevines.

Nucleotide sequence accession numbers. The partial sequences of the 16S rRNA genes were deposited in GenBank (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/) under the accession numbers HM803937 to HM803959.

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