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SELECTIVE ELIMINATION OF CHLOROPLASTIDIAL DNA FOR METAGENOMICS OF BACTERIA ASSOCIATED WITH THE GREEN ALGA *CAULERPA TAXIFOLIA* (BRYOPSIDOPHYCEAE)[1]

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Molecular analyses of bacteria associated with photosynthetic organisms are often confounded by coamplification of the chloroplastidial 16S rDNA with the targeted bacterial 16S rDNA. This major problem has hampered progress in the characterization of bacterial communities associated to photosynthetic organisms and has limited the full realization of the potential offered by the last generation of metagenomics approaches. A simple and inexpensive method is presented, based on ethanol and bleach treatments prior to extraction, to efficiently discard a great part of chloroplastidial DNA without affecting the characterization of bacterial communities through pyrosequencing. Its effectiveness for the description of bacterial lineages associated to the green alga *Caulerpa taxifolia* (M. Vahl) C. Agardh was much higher than that of the preexisting enrichment protocols proposed for plants. Furthermore, this new technique requires a very small amount of biological material compared to the other current protocols, making it more realistic for systematic use in ecological and phylogenetic studies and opening promising prospects for metagenomics of green algae, as shown by our data.

Key index words: **16S; chloroplasts; elimination; green algae; metagenomics**

Abbreviations: **16S rRNA, 16S ribosomal RNA; BE, bleach-ethanol pretreatment; CF, chloroplast filtration protocol; CTAB, cetyl trimethylammonium bromide; DAPI, 4',6-diamidino-2-phenylindole; DGGE, denaturing gradient gel electrophoresis; DIC, differential interference contrast microscopy; OTU, operational taxonomic unit; RDP, Ribosomal Database Project; SK, Spin Kit for Soil; TGGE, temperature gradient gel electrophoresis; TRFLP, terminal restriction fragment length polymorphism**

The exploration of microbial diversity has been hindered until recently by the fact that ~ 99% of all microorganisms in almost every environment cannot be cultured, particularly symbiotic bacteria due to highly specialized relationships with their hosts (Amann et al. 1990, Handelsman 2004). Metagenomics is expected to become the main technique used to overcome the culture bottleneck and to unveil the diversity of bacterial communities associated with a wide range of environmental niches (Venter et al. 2004, Daniel 2005, Edwards et al. 2006, Martín-Cuadrado et al. 2007, Wang et al. 2009). The exploration of prokaryote diversity living inside other eukaryotes has not, however, progressed at the same pace as its exploration in other, nonliving habitats.

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Bacterial communities living associated to plants and algae have been reported across a wide range of aquatic and terrestrial niches, such as root nodules (Sun et al. 2008), galls (Ashen and Goff 1996, 1998), or inside other plant tissues (Meusnier et al. 2001, Koopman et al. 2010). Their identity, role, and function are essential information for the understanding of the metabolism and dynamics of macroscopic primary producers and the ecosystems they support. The study of bacterial communities in association with macroalgae progressed significantly during the last decade, due to the use of several culture-independent molecular approaches such as community fingerprinting (denaturing gradient gel electrophoresis [DGGE], temperature gradient gel electrophoresis [TGGE], terminal restriction fragment length polymorphism [TRFLP]) and clone libraries, allowing an increase in the number and complexity of studies (Goecke et al. 2010). Several recent studies on 36 macroalgal species brought to light 56 new bacterial species (Goecke et al. 2010), and others also demonstrated a highly specific association of bacterial communities with marine macroalgae (Staufenberger et al. 2008, Lachnit et al. 2009, Wiese et al. 2009). A major stumbling block for the application of metagenomic analyses to investigate plant microbiota is the cyanobacterial origin of all chloroplast lineages, whether derived from primary or secondary endosymbiosis (Whatley et al. 1979), which will interfere with the 16S rRNA characterization of bacteria through massive coamplification of the host's chloroplastidial DNA with the conserved 16S rRNA primers typically used (Chelius and Triplett 2001, Sakai et al. 2004, Green and Minz 2005, Wang et al. 2008). The construction of metagenomic libraries for bacteria associated with plants or algae is therefore technically challenging, as in the absence of a previous elimination of chloroplastidial DNA from extraction products a high amount of plant/algae-derived DNA is likely to mask the microbial contribution. Enrichment procedures based on enzymatic hydrolysis and/or subsequent differential centrifugation have recently been proposed to solve the problem of chloroplastidial contamination (Jiao et al. 2006, Wang et al. 2008) when characterizing bacterial communities associated with seeds and leaves. Yet this requires a huge amount of tissue (''in kilogram quantities''; Wang et al. 2008), which is not feasible or manageable for a variety of plants or algae or for specific purposes where single individuals are required. The alternative method (Jiao et al. 2006) involves a long enzymatic step at 28°C, which may bias the bacterial community structure owing to microbial propagation (Wang et al. 2008).

Herein we examined different approaches to remove ''contamination'' by plastidial DNA in studies aiming at characterizing bacteria associated to photosynthetic organisms. The methods under comparison were enzymatic hydrolysis and differential centrifugation (Jiao et al. 2006), and a simpler/inexpensive method based on preliminary disinfection, previously described but aimed only at surface sterilizing wheat roots (Coombs and Franco 2003). We conducted these tests on the siphonous green alga *C. taxifolia*, within which a diverse community of endophytic bacteria has been described (Chisholm et al. 1996, Meusnier et al. 2001, Delbridge et al. 2004). Comparisons across methods were based on pyrosequencing estimates of ratios of chloroplastidial versus bacterial DNA sequences to select the method revealing the most complete diversity of the associated bacterial communities. Finally, the best method selected for the green algal samples was applied to leaves and roots of a seagrass (*Posidonia oceanica*) to test for its usefulness for plants.

MATERIALS AND METHODS

Sampling. Samples of *C. taxifolia* were collected in Villefranche-sur-mer (France) (43°42′2.93″ N/7°19′0.32″ E) in March 2009 and were kept at −80°C until DNA extraction. Sample size consisted of two individuals per treatment, each individual containing 100 mg of thalli including fronds, stolons, and rhizoids. Sediment or macroscopic living organisms encrusted on the thallus surface were removed with sterile seawater and a toothbrush, identically for all control and treatment samples.

To test on plants the usefulness of the protocol here estimated as being the best for an alga, two individuals of the seagrass *P. oceanica* were collected in Marseille, France (43°16′47.44″ N, 5°20′56.27″ E). These were subdivided into samples of leaves and of the corresponding roots and were later processed using the treatment defined as optimal for *C. taxifolia* (see Results).

Removal of chloroplasts and external bacteria by a bleach-ethanol pretreatment. To remove chloroplasts and external bacteria, in order to get 16S DNA from just the endophytic bacteria, a ''bleach-ethanol pretreatment'' developed by Coombs and Franco (2003) as a surface-sterilization method, was applied prior to DNA extraction. This consisted of three simple steps with modifications developed to avoid damaging the algal material: (i) the material was placed in a bath of 99% ethanol during 1 min, (ii) the sample was subsequently transferred for 5 min to a 3% bleach (diluted in seawater) solution, and (iii) the sample was immersed for 30 s in a 99% ethanol solution and rinsed with sterile seawater.

To evaluate the efficiency of this method in removing epiphytic (i.e., external) bacteria, samples were stained for 5 min with 0.1 $mg \cdot mL^{-1}$ 4′6-diamidino-2-phenylindole (DAPI) and subsequently viewed under a confocal and fluorescence microscope with an ApoTome.2 (Zeiss, Germany) to determine whether or not the outer surface bacteria were effectively eliminated by the sterilization protocol applied. Differential interference contrast microscopy (DIC) (Axiovert 200 MAT Microscope; Zeiss, Ontario, NY, USA) was also performed.

This treatment was compared with a previously described chloroplast removal method, based on a filtration step, the method II of Jiao et al. (2006).

Bacterial DNA extraction. For DNA extraction, two types of methods were tested, the FastDNA Spin Kit for Soil (MP Biomedicals LLC, Solon, OH, USA, Catalog # 6540-600) hereafter named SK, and the cetyl trimethylammonium bromide (CTAB) plant DNA extraction procedure of Doyle and Doyle (1987).

Experimental treatments. The bleach-ethanol (BE) pretreatment combined with/without chloroplast filtration (CF) and with two bacterial DNA extraction methods (SK and CTAB),

TABLE 1. Treatments applied to obtain bacterial DNA associated to *Caulerpa taxifolia* (A–E) and *Posidonia oceanica* (individual 1/individual 2), and summary of sequencing results and statistical parameters calculated for each protocol. The % of coverage represents the number of operational taxonomic units (OTUs) in each sample divided by Chao factor.

Experimental treatments	BE	CF	DNA extract	No. sequences obtained	No. sequences after quality control[1]	OTU	Chao1	Coverage (%)	No. OTUs standardized for the lowest	% of chloroplastidial sequences
A (control)	–	+	SK	1,370	1,328	158	248.621	63.55	158	77.64
B	+	+	SK	1,522	1,496	403	439.457	91.41	392	0.54
C	+	–	SK	1,555	1,533	391	435.536	89.77	378	0.26
D	+	+	CTAB	1,839	1,800	505	597.764	84.48	455	0.22
E	+	–	CTAB	1,488	1,466	235	308.244	76.23	227	0.55
P. oceanica leaves	+	–	SK	6,361/8,078	6,361/8,078	349/466	768.45/906.917	45,41/51.38	–	97.30/94.41
P. oceanica roots	+	–	SK	9,512/12,073	9,462/12,073	942/578	2,654.349/1,334.276	35.48/43.32	–	80.44/90.01

BE, ''bleach-ethanol pretreatment''; CF, chloroplast filtration step (Jiao et al. 2006); SK, FastDNA® SPIN Kit for Soil DNA extraction; CTAB, DNA extraction following Doyle and Doyle (1987). [1]Removal of chimeras and sequences with <100 bp or >2 undetermined nucleotides (Schloss et al. 2009).

plus a non-pre-treated control resulted in five treatments (Table 1): (A) no BE pretreatment, chloroplast removal by filtration (CF), SK DNA extraction (the control for the performance of the BE pretreatment); (B) BE pretreatment, CF, SK DNA extraction; (C) BE pretreatment, no CF, SK DNA extraction; (D) BE pretreatment, CF, CTAB DNA extraction; (E) BE pretreatment, no CF, CTAB DNA extraction.

DNA sequencing. The DNA concentration in each sample was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) to verify that samples had concentrations in excess of 50 $ng \cdot \mu L^{-1}$, the value required for metagenomic analyses.

DNA was submitted to BIOCANT (Cantanhede, Portugal) for tag-pyrosequencing. Samples were amplified with modified primers for region V4 of 16S rRNA (Wang et al. 2007), and the amplicons were sequenced using 454 GS FLX (Roche-454 Life Sciences, Branford, CT, USA) with Titanium chemistry. The quality criteria imposed to consider sequences in the analysis were: (i) sequences with <100 nucleotides were discarded and (ii) sequences with two or more undetermined nucleotides were discarded. Sequences with differences below 3% were grouped and considered the same operational taxonomic unit (OTU).

Statistical analyses. The RDP (Ribosomal Database Project) pyrosequencing pipeline (Cole et al. 2009) was used to align the 454 sequence data and assign identities with 80% confidence. The software MOTHUR version 1.4.1 (Schloss et al. 2009) was used to construct distance matrices (dist.seq function), assign sequences to OTUs (97% similarity), calculate OTU richness using the nonparametric estimator Chao 1 (Chao 1987), calculate coverage (%), and to construct rarefaction curves. The Chao parameter predicts total diversity (OTU richness) of a community, providing an indicator of the coverage of the bacterial community allowed by the sequencing effort (Handelsman 2004). Chao estimation was based on (Chao 1984):

$$S_{\text{Chao1}} = S_{\text{obs}} + \frac{n_1(n_1 - 1)}{n_2(n_2 - 1)} \quad (1)$$

where S_{obs} is the number of observed OTUs; n_1 is the number of OTUs observed once (singletons); and n_2 is the number of OTUs observed twice (doubletons).

RESULTS

The bleach-ethanol sterilization was efficient in reducing external bacteria to very few that could only be found after screening several images, contrary to the control treatment, as revealed by using DAPI-stained bacterial DNA fluorescence on treated and control samples (Fig. S1 in the supplementary material).

In *C. taxifolia*, most organisms present in each sample were assigned to, at least, the class level (Table 2). About 98% of all sequences passed the quality control and were used in further statistical analyses (Table 1). There were major, 4-fold, differences in the number of sequences derived using different protocols (Table 1). Samples prepared without the ''bleach-ethanol pretreatment'' (control treatment A) yielded the lowest results in all statistical parameters measured (Table 1). Treatment D yielded the highest number of OTUs (505 upon 1,800 sequences, 455 OTUs when standardized for the lowest number of sequences, 1,328) and

TABLE 2. Distribution (in %) of each operational taxonomic unit (OTU) class by the different treatments.

OTU class	Treatment A	Treatment B	Treatment C	Treatment D	Treatment E
Deltaproteobacteria	11.11	44.35	30.17	39.65	31.94
Gammaproteobacteria	31.50	29.03	31.03	28.40	33.33
Alphaproteobacteria	44.44	10.49	11.21	8.88	16.67
Clostridia	0.00	2.42	8.62	7.10	5.56
Betaproteobacteria	1.85	3.23	3.45	5.33	0.00
Cyanophyceae	3.70	4.83	5.17	2.96	8.33
Bacilli	1.85	2.42	6.04	2.37	1.39
Deferribacter	0.00	1.61	0.00	1.18	0.00
Bacteroidea	0.00	0.81	1.72	1.18	0.00
Verrucomicrobiae	0.00	0.00	0.00	0.59	0.00
Nitrospira	0.00	0.00	0.00	0.59	0.00
Mollicutes	0.00	0.00	0.00	0.59	0.00
Chlamydiae	0.00	0.00	0.00	0.59	0.00
''*Chlorobi* group''	0.00	0.00	0.00	0.59	0.00
Spirochetes	0.00	0.00	2.59	0.00	0.00
Flavobacteria	3.70	0.00	0.00	0.00	0.00
Deinoccoci	0.00	0.00	0.00	0.00	1.39
Sphingobacteria	1.85	0.81	0.00	0.00	1.39

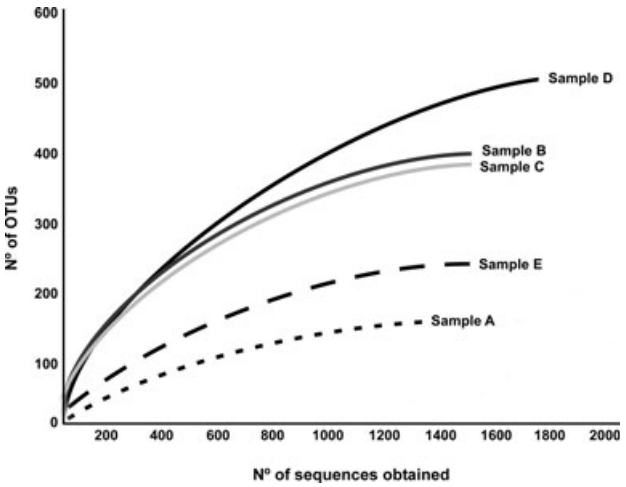

FIG. 1. Rarefaction curves derived for samples processed using the different protocols (A–E, cf. Table 1). OTU, operational taxonomic unit.

treatment A (used as control) yielded the lowest (158 upon 1,328 sequences) (Table 1 and Fig. 1). The number of OTUs varied among treatments but all seemed close to reach the plateau along the rarefaction curve (Fig. 1). Although the number of OTUs was beneath the expected Chao 1 index in all treatments, coverage was satisfactory for most samples (~80%–90%) except for the nondisinfected one (A, with ~60%) due to dominance of chloroplastidial sequences.

Comparison of the no bleach-ethanol treatment (control A) with its equivalent pretreated (B) reveals that the sequences of the former are dominated by the kingdom Viridiplantae—that is, chloroplastial sequences (77.64%; Fig. 2)—whereas treatment B resulted in only 0.54% of chloroplasts sequences (Table 1). Comparing pair-wise treatments that differ only in the use or not of the chloroplast filtration step (B vs. C and D vs. E), showed no consistent differences in the number of chloroplast sequences, which was always negligible (i.e., respectively 0.54% vs. 0.26% and 0.22% vs. 0.55%; Table 1) in all these cases pretreated with bleach-ethanol.

As for the diversity of OTUs, the no ethanol-bleach treatment A showed much fewer bacterial OTUs compared to all others, mostly due to dominance by chloroplastidial sequences (Fig. 2). The difference in diversity between filtered versus nonfiltered is negligible for the treatments with the Soil Kit extraction (B and C) (392 and 378 OTUs, respectively, for 1,328 sequences; Table 1, Fig. 1). For CTAB extractions, a larger difference is observed between the filtered (D) and nonfiltered (E) treatments (1,800 vs. 1,466 for the number of sequences and 505 vs. 235 for the number of OTUs identified; 455 vs. 227 OTUs once standardized for the lowest amount of sequences that passed the quality control, 1,328 in treatment A; Table 1, Fig. 1). In summary, our results showed that adding the filtration step to the bleach-ethanol pretreatment did not result in dramatic diversity changes with any of the two extraction methods used (CTAB and SK), and that the SK extraction seems to deliver more diversity and more consistent results (coverage 90%/91% for SK vs. 76/85% for CTAB; Table 1).

The Delta, Gamma, and Alfa subdivisions of *Proteobacteria* were the most abundant OTUs in all treatments (Fig. 3, Table 2), independently of the chloroplast removal or DNA extraction protocol. The relative proportions of the main bacteria classes were similar among samples, except for sample A where the number of classes identified was much smaller and *Clostridia* (one of the most abundant in other treatments) was not present (Fig. 3, Table 2).

Detailed results on sequences obtained in these treatments are not presented since the aim of this study was not to fully describe the bacterial community associated with *C. taxifolia*.

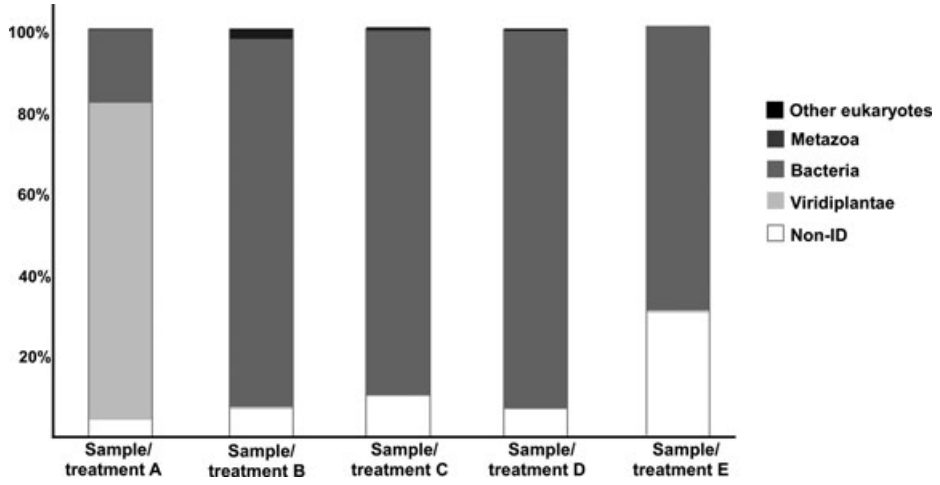

FIG. 2. Distribution of operational taxonomic units per kingdom in samples derived using the different treatments (A–E, cf. Table 1). Non-ID sequences were those that had >3% difference from the best BLAST hit (and then rejected) or sequences in which BLAST result was ''uncultured.''

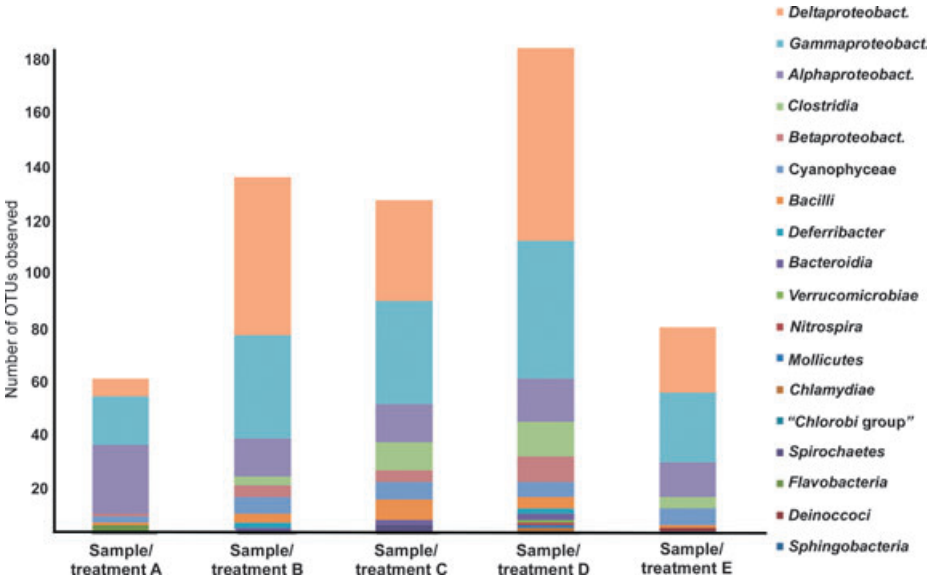

FIG. 3. Distribution of operational taxonomic units (OTUs) into bacteria classes in samples derived using the different treatments (A–E, cf. Table 1).

Application to leaves and roots of a plant host (*P. oceanica*) of the treatment that resulted in the best bacterial diversity description for the alga (bleach-ethanol followed by soil kit DNA extraction) was not effective in removing chloroplasts. This resulted in >90% of chloroplastidial sequences in all samples except the roots of individual 1 with 80% (Table 1). In both root samples (individuals 1 and 2) many sequences (17.70% and 9.38%, respectively) were unassigned (RDP Classifier automatically bins unclassified data <80% confidence) and taxonomic assignment of bacteria was low, respectively 0.11%, 0.15%, 0.77%, and 1.04% for leaves and roots of individuals 1 and 2. Coverage was below 50% in almost all cases.

DISCUSSION

This study discovered an effective way of obtaining representative bacterial 16S DNA sequences associated to a green alga without interference from the dominant chloroplast 16S DNA. This is a significant methodological step forward toward progress in the large-scale characterization of microbial communities associated to primary producers. A large amount of information and data on microbial ecology have been obtained during the last 5 years through metagenomics approaches (Galperin 2004, Edwards and Rohwer 2005, Poinar et al. 2006, Wilhelm et al. 2007, Thurber et al. 2008, Lazarevic

et al. 2009, Kielak et al. 2010). Although some cryptic habitats have already been explored and their hidden microbial life unveiled (Venter et al. 2004, Edwards et al. 2006), the study of microbes associated with plants and algae remains a major challenge for metagenomics. Predominant cross amplification of chloroplasts masking bacterial 16S rDNA is likely the major factor explaining the paucity of studies taking advantage of the last metagenomics advances to characterize bacteria associated with algae and plants.

Another important result was the low performance, on milligrams of *C. taxifolia* tissue, of the first protocol proposed to discard chloroplast sequences (chloroplast filtration), which had been demonstrated on grams to kilograms of leaves and/or seeds of tree species (Jiao et al. 2006). Only about 23% of the DNA sequences obtained using chloroplast filtration alone did correspond to bacterial strains compared to ~90%–97% reported by Jiao et al. (2006). The differential centrifugations of this procedure were previously shown as not efficient in eliminating chloroplasts (Wang et al. 2008). Two main hypotheses might explain this unexpected difference. Either the structural difference in algal versus trees tissues resulted in a different success of the filtration protocol, or the enormous difference in the amount of material used, milligrams for *C. taxifolia* versus kilograms in the previous studies, influenced the success of filtration.

The alternative method used here to remove chloroplasts, based on a ''bleach-ethanol pretreatment'' that was previously described for surface disinfection (Coombs and Franco 2003), proved to be a simple method to discard a great part of the chloroplasts for optimization of metagenomics analyses of endophytic bacteria. Less than 1% of chloroplast sequences remained in the samples pretreated with bleach-ethanol, independently of whether the chloroplast filtration step was added or not, revealing that this step caused no improvement in the ratio of bacterial versus chloroplastidial sequences. An overwhelming difference between the no bleach-ethanol pretreatment and the corresponding pretreated one shows the efficiency of this pre-treatment in eliminating chloroplasts.

This effectiveness of the bleach-ethanol pretreatment may be expected on a wider range of photosynthetic organisms and in particular on a diversity of green algae on which elimination of chloroplasts with bleach is consistently used in taxonomy and histology (Habib Langar, pers. com.). However, Hollants et al. (2010) developed a surface disinfection method for the green coenocytic alga *Bryopsis* sp. based on a combination of CTAB buffer, proteinase K and the bactericidal cleanser Umonium Master and compared it to ethanol and bleach surface disinfection. Ethanol cleaning was ineffective both in eliminating epiphytes and for bacterial 16S rDNA amplification. Instant bleach in this study caused an apparent elimination of the endosymbionts, suggesting that *Bryopsis* cell structures may be more fragile than those of *Caulerpa* causing higher bleaching susceptibility.

As the first step in the disinfection protocol is an ethanol bath for 1 min, this alcohol may act as a detergent/solvent, breaking down the phospholipid bilayer and opening holes in the membrane, making it permeable and, in this case, promoting the invasion of the bleach (Baker et al. 1979) that further damages and apparently eliminates the chloroplasts. One concern might be that bleach would also selectively damage and eliminate some bacterial lineages inside the alga, even though bacteria have more complex and robust membranes and cell walls compared to chloroplasts. Yet, despite the much more limited number of bacterial sequences obtained for the nondisinfected sample, the bleach-ethanol disinfection did not seem to lead to any significant loss of strains according to overall list of OTUs recognized in each sample (i.e., no major strains appeared in the nondisinfected treatment A that were not in the other treatments). Considering class the taxonomic level of our analysis, the only strain that appears exclusively in the nondisinfected (not discarding the possibility of being present in the other samples, although only classified to phylum) sample, was Flavobacteria, from the genera *Aquimarina* and *Tenacibaculum*. These strains are known to be present as free-living groups in marine waters (Nedashkovskaya et al. 2006) in the first case, and to be fixed to the surface of marine organisms (Suzuki et al. 2001) in the second. *Tenacibaculum* genus is thought to be one of the strains responsible for inducing morphogenesis in algae (Hanzawa et al. 1998, Matsuo et al. 2003). Their exclusive presence in the nondisinfected protocol may therefore be explained by an external occurrence, explaining their absence on the surface of samples disinfected precisely to improve the external cleaning (i.e., removing external bacteria). Furthermore, coverage was relatively high and satisfying in all disinfected samples, particularly those obtained from the FastDNA® SPIN Kit for Soil (about 90% with or without filtration), whereas the nondisinfected sample reached only 64% coverage. It is therefore likely that dominant OTUs are well characterized, although some poorly represented OTUs could still escape detection.

Contrary to previous methods proposed to eliminate chloroplasts for metagenomics purposes, the ''bleach-ethanol pretreatment'' surface-disinfection is fast and inexpensive, requiring a limited amount of material and solving the serious problem of excess chloroplast DNA when estimating bacterial diversity, although at the same time selectively removing epiphytic bacteria. It seems therefore more accurate for ecological or phylogenetic studies of associated bacteria that may require a protocol adapted to a lower amount of plant or algal tissues and allowing a higher throughput (i.e., a much

larger number of specimens to be analyzed). Besides, it showed superior performance in these conditions for *C. taxifolia*. The results presented herein also suggest a slightly better performance of the FastDNA® SPIN Kit for Soil over the classical CTAB method for the *C. taxifolia* samples studied herein.

The additional tests made with *P. oceanica* suggest that this disinfection protocol is not as effective for plants, as the chloroplast sequences remained on the order of 90%. Among the 10% to 20% remaining OTUs, the low rate of identified bacterial and high number of unassigned (<80% confidence) sequences did not give very encouraging results. Besides, the RDP classifier is a database for bacteria and, despite effectively identifying chloroplast-derived sequences, there is evidence that it is not that effective in identifying plant mitochondrial sequences (Kretzer et al. 2009). Unassigned sequences may therefore be either cryptic bacterial lineages absent from the database or mitochondrial sequences from *P. oceanica*.

The results presented in this study open promising perspectives to unlock the characterization of diversity of bacterial communities associated to algae, although other methods still have to be explored for plants.

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Supplementary Material

The following supplementary material is available for this article:

Figure S1. Fluorescence microscopy images of sterilized (a) and untreated (b) *Caulerpa taxifolia* stained with DAPI (a1 and b1), differential interference contrast (DIC) microscopy (a2 and b2), and with both methods superimposed (a3 and b3). Scale bars, 5 μm.

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