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A PCR-based toolbox for the culture-independent quantification of total bacterial abundances in plant environments

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ABSTRACT

A major obstacle in the culture-independent estimation of the abundance of bacteria associated with plants is contamination with plant organelles, which precludes the use of universal rRNA bacterial primers in quantitative PCR applications. We present here a PCR-based method that allows *a priori* determination of the degree of chloroplast and mitochondrial contamination in DNA samples from plant environments. It is based on differential digestibility of chloroplast, mitochondrial and bacterial small subunit rRNA gene amplicons with the restriction enzymes *Afe*I and *Bbv*CI. Using this method, we demonstrated for field-grown lettuce plants that even a gentle washing protocol, designed to recover the microbial community and its metagenome from the leaf surface, resulted in substantial contamination with chloroplast DNA. This finding cautions against the use of universal primer pairs that do not exclude chloroplast DNA from amplification, because they risk overestimation of bacterial population sizes. In contrast, contamination with mitochondrial 18S rRNA was minor in the lettuce phyllosphere. These findings were confirmed by real-time PCR using primer sets specific for small subunit rRNA genes from bacteria, chloroplasts, and mitochondria. Based on these results, we propose two primer pairs (534f/783r and mito1345f/mito1430r) which between them offer an indirect means of faithfully estimating bacterial abundances on plants, by deduction of the mito1345f/mito1430r-based mitochondrial count from that obtained with 534f/783r, which amplifies both bacterial and mitochondrial DNA but excludes chloroplast. In this manner, we estimated the number of total bacteria on most leaves of field-grown lettuce to be between 10^5 and $10^6\,g^{-1}$ of leaf, which was 1–3 orders of magnitudes higher than the number of colony-forming units that were retrieved from the same leaf surfaces on agar plates.

1. Introduction

In recent years, there has been a dramatic increase in the number of culture-independent approaches to the study of plant-associated bacterial communities (Chelius and Triplett 2001; Yang et al., 2001; Lambais et al., 2006; Jackson et al., 2006; Sun et al., 2008; Redford and Fierer, 2009; Redford et al., 2010). Irrespective of the method employed to isolate DNA from these communities, the extracted microbial DNA is generally contaminated with DNA of plant origin, in particular chloroplasts and mitochondria (Chelius and Triplett 2001; Sakai et al., 2004; Sun et al., 2008). Since the chloroplast 16S and mitochondrial 18S rRNA genes share high sequence similarity with bacterial 16S rRNA sequences (Sakai et al., 2004), contamination with plant DNA poses a serious challenge for the application of PCR-based methods to profile and quantify bacterial populations in plant environments.

Several primer pairs have been reported which amplify bacterial 16S rRNA genes to the exclusion of chloroplast sequences (Chelius and Triplett, 2001; Sakai et al., 2004; Edwards et al., 2007). One of these, 799f/1492r, was originally used to analyze bacterial endophytes of maize roots (Chelius and Triplett, 2001). While primer 1492r (Lane 1991) is considered universal (*i.e.* it targets bacterial, chloroplast, as well as mitochondrial rRNA genes), 799f features several mismatches with chloroplast 16S rRNA gene sequences that prevent the latter from being amplified. In the original paper, evidence for this exclusion was the absence of chloroplast DNA sequences in a clone library that was derived from non-sterile maize roots using the 799f/1492r pair (Chelius and Triplett, 2001). This primer set has since been used widely for the construction of 16S rRNA gene clone libraries from below- and above-ground plant environments (Kadivar and Stapleton, 2003; Sun et al., 2008; Sagaram et al., 2009; Redford and Fierer, 2009).

Sakai et al. (2004) reported an alternative to 799f which allows it to be used as a reverse primer. Primer mix 783r-abc consists of three primers that are reverse complementary to 799f, have several nucleotides added or removed at the 5′ and 3′ end, and between them offer a slightly broader target range than 799f by

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increased nucleotide ambiguity. Primer mix 783r-abc was used together with universal primer 63f (Sakai et al., 2004) for the study of bacterial communities of wheat and spinach roots by terminal restriction fragment length polymorphism (T-RFLP) analysis. No fragments representing amplified chloroplast sequences were found in the T-RFLP profiles of these communities (Sakai et al., 2004), which offers convincing evidence for the exclusive nature of 799f also in its reverse orientation.

Primer pairs 799f/1492r and 63f/783r-abc are not recommended for the culture-independent, PCR-based quantification of total abundances of bacteria associated with plants. The sizes of their respective amplicons exceed what is considered optimal for real-time PCR purposes (Smith and Osborn 2009). Edwards et al. (2007) conceived of producing an amplicon with a smaller size, by pairing 799R2, a reverse derivative of 799f having its degenerate bases removed, with 520f, which is a truncated derivative of universal primer 518r (Muyzer et al., 1993). This primer pair was successfully used to quantify by real-time PCR bacterial population sizes on perennial ryegrass (Edwards et al., 2007).

One feature that bacterial primers 779R2, 799f and 783r-abc share with each other is that they are not exclusive of mitochondrial 18S rRNA gene sequences. For the construction of clone libraries involving primer pairs such as 799f/1492r, this is not an issue, because the mitochondrial product with these primers is larger than that of bacteria, which allows exclusion of the former by size-separation on an agarose gel (Chelius and Triplett, 2001). In T-RFLP analyses using primer pairs such as 63f/783r-abc, mitochondrial DNA typically renders a specific peak that can be similarly excluded from the analysis (Sakai et al., 2004). However, in quantitative, real-time PCR, mitochondrial amplicons cannot be distinguished from their bacterial counterparts by size or other properties, and depending on the contamination with mitochondrial DNA, this may lead, just as is true for primers that do not exclude chloroplasts, to overestimation of bacterial abundances by real-time PCR.

Methods that are specifically designed for the qualitative and quantitative estimation of mitochondrial and chloroplast contamination in DNA preparations from plant-associated bacterial communities are currently not available, yet would be very useful for assessing the risk of overestimating bacterial population sizes due to contaminating plant organelle DNA. Here, we describe a PCR-based toolbox that allows a faithful estimation of the degree of chloroplast and mitochondrial contamination in DNA isolated from plant microbiota. Part of the toolbox involves the exploitation of restriction enzymes that uniquely recognize chloroplast and mitochondrial ribosomal RNA gene amplicons. The toolbox also offers a novel set of primer pairs for the accurate estimation of bacterial abundances in plant environments, by using chloroplast-exclusive primers and by subtracting mitochondrial estimates.

2. Materials and methods

2.1. DNA extraction from phyllosphere microbial communities, Escherichia coli, and aseptically grown lettuce seedlings

Field-grown Romaine lettuce (*Lactuca sativa*) plants were harvested from production regions in Imperial (CA) and Yuma (AZ) at different times during the 2009/2010 growing season. Per field, two samples were taken just prior to harvest. Each sample contained four lettuce heads, and from each lettuce head, two outer most and two inner leaves from the fourth leaf circle were picked. Microorganisms were recovered from leaf surfaces as described by Redford and Fierer (2009). Briefly, the 16 leaves from each sample were pooled and washed for 15 min in 3 L of wash solution (20 mM Tris–HCl, 10 mM EDTA, and 0.024% Triton) by gentle swirling with a glass pipette. The leaf wash solution was filtered through a double layer of cheesecloth to remove debris. Fifty-μL aliquots were plated using an Eddy Jet spiral plater (Neu-Tec Group Inc., Farmingdale NY) on 0.1× Tryptic Soy Agar (TSA) for colony-forming unit (CFU) counts, while the rest of the filtrate was centrifuged for 10 min at 4000 rpm and 4 °C. From the resulting pellet, total DNA was extracted using a PowerSoil® DNA Isolation Kit (MO-BIO Laboratories Inc., Carlsbad CA). To extract DNA from *Escherichia coli* DH10B cultures that were grown overnight in Luria Bertani broth, we used the UltraClean® Microbial DNA Isolation kit (MO-BIO Carlsbad CA). To obtain total DNA from aseptically grown lettuce seedlings, seeds of Romaine lettuce cultivar Valmaine were surface-sterilized in 5% sodium hypochlorite for 5 min, washed thoroughly in sterile water, and grown on Murashige and Skoog agar medium (Murashige and Skoog, 1962). After 7 days, total DNA was extracted from seedlings as described by Sambrook and Russell (2001).

2.2. Primers, PCR amplification and amplicon analysis

Fig. 1A shows primers used in this study for the amplification of small subunit rRNA (SSU rRNA) gene fragments. We derived primer 534f (5′-CCAGCAGCCGCGGTAAT-3′) by reverse complementation of universal primer Eub518r (Muyzer et al., 1993). Similarly, primer 783r (5′-ACCMGGGTATCTAATCCKG-3′) is reverse complementary to 799f (Chelius and Triplett, 2001), but with two nucleotides removed from the 5′-end and two added to the 3′-end (Fig. 1A). Universal bacterial primer sequences 27f-YM (Frank et al., 2008) and Eub338f (Lane, 1991) were obtained from the literature, as was chloroplast-specific primer set CYA361f/CYA785r (Mühling et al., 2008) which targets plant chloroplast 16S rRNA genes. For the design of a primer set that amplifies lettuce mitochondrial 18S rRNA gene sequences, we PCR-amplified DNA from aseptically grown lettuce seedlings using primer pairs 799f/1492r and 27f-YM/783r, cloned the amplicons in pCR 2.1-TOPO vector (Invitrogen, Carlsbad CA), and sequenced them using primer M13F. The resulting lettuce mitochondrial 18S rRNA gene sequences were submitted to GenBank (accession numbers HM047292 and HM047290). ClustalW alignments revealed that the 1160–1540 region of the mitochondrial 18S rRNA gene sequence was missing from the *E. coli* 16S rRNA sequence. This 380-bp region was further analyzed by PrimerSelect (Lasergene, DNASTAR, Inc. WI) to design a mitochondrial 18S rRNA-specific primer pair for real-time PCR. The resulting primers mito1345f (5′-AGTTTTTGGCCTTATCTTG-3′) and mito1430r (5′-AAACCCCACTACGTACCACACCAC-3′) produced a 104-bp amplicon from lettuce seedling DNA.

All routine PCRs were carried out in a 25 μL volume containing 12.5 μL GoTaq® Green Master Mix (Promega, WI), 1–2 μL of template DNA, and 1 μL of each forward and reverse primer from 12.5 μM stocks. PCR conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 53 °C for 60 s, and 72 °C for 60 s, and a final elongation at 72 °C for 10 min. Where applicable, the 27f-YM/Eub518r and 27f-YM/783r amplicons from phyllosphere DNA, aseptically grown lettuce seedling DNA, and *E. coli* genomic DNA were digested with *Bbv*CI or *Afe*I. *Bbv*CI has a recognition site in chloroplast but not mitochondrial DNA , while the reverse is true for *Afe*I (Fig. 1A). Restriction digestion reactions (total volume 20 μL) were incubated with the appropriate buffer at 37 °C for a period of 5 h, after which 10 μL of digested products were electrophoresed on 1.5% agarose gel.

2.3. Real-time PCR for determining bacterial, mitochondrial, and chloroplast abundances in phyllosphere DNA

Real-time PCRs were carried out in a Bio-Rad CFX96 real-time PCR system (Bio-Rad Laboratories, Hercules CA). The 10-μL reaction volume contained 5 μL of SsoFast™ EvaGreen® Supermix (Bio-Rad), 1 μL of each primer (12.5 μM stock), and 5–10 ng of DNA template. Real-time PCR conditions included an initial 3-min enzyme activation at 95 °C, followed by 40 cycles of 5 s denaturation at 95 °C and 5 s

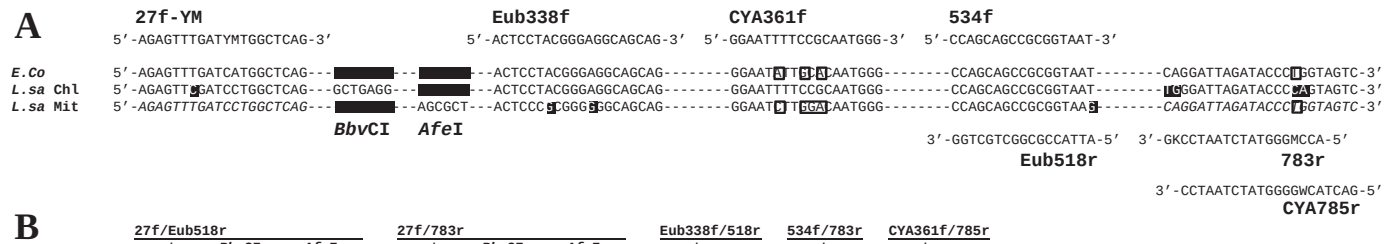

Fig. 1. (A) Alignment of the primers used in this study to relevant parts of the SSU rRNA gene sequences of *E. coli* (J01695), *L. sativa* chloroplast (NC_007578), and *L. sativa* mitochondria (this study). Not shown are mitochondrial-specific primers mito1345f and mito1430r (see Materials and methods) and universal primer 1492r: their target sites fall outside of the alignment presented here. Mismatches of ribosomal sequences to the chloroplast-specific CYA primers are marked with an open box; all other mismatches are marked with a filled box. A recognition site for *Bbv*CI is present only in the chloroplast sequence, and *Afe*I cuts only in the mitochondrial sequence. The lettuce mitochondrial DNA sequence is not known for the 27f-YM and 783r parts of the alignment, because 27f-YM and 783r were used to amplify the mitochondrial DNA from lettuce DNA; shown instead, in italic, are the corresponding parts of the mitochondrial sequence from another member of the *Asteraceae* family, *Artemisia annua* (accession number EZ230745). (B) Predicted amplicon sizes and *Bbv*CI or *Afe*I digestion products with different combinations of forward and reverse primers. Numbers in italic and parentheses represent bands that are not expected to show up on the gel, because no PCR product is predicted based on at least 3 mismatches between one of the primers and its target.

elongation at 53 °C (534f/783r and Eub338f/Eub518r), 8s at 59 °C (CYA361f/CYA785r), or 5s at 60 °C (mito1345f/mito1430r). For standard preparations, PCR products or cloned PCR products were purified by gel electrophoresis and quantified in a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc. Wilmington, DE). Target copy numbers in the unknown samples were estimated using standard curves that were generated from 10-fold dilutions of PCR products or cloned PCR products in triplicate. The absolute copy numbers in the standards were calculated based on DNA concentrations and size of PCR or cloned PCR products and the 660 g mol^{-1} average molecular mass of a double-stranded DNA molecule (Fierer et al., 2005). All real-time PCR reactions on unknown samples were performed in duplicate and mean values were estimated for each DNA sample. Melt-curve analysis of the PCR products was performed at the end of each real-time run. No-DNA template controls were included in every run.

3. Results

3.1. Assessment of plant DNA contamination by digestion of SSU rRNA amplicons

Analysis of the 16S rRNA gene sequence from lettuce (*Lactuca sativa*) chloroplasts (accession number NC_007578) revealed a recognition site for the restriction enzyme *Bbv*CI (5′-GCTGAGG-3′) which was not shared with the mitochondrial sequence (Fig. 1A). Conversely, the lettuce mitochondrial 18S rRNA gene featured a recognition site for *Afe*I (5′-AGCGCT-3′) which was absent from the chloroplast sequence (Fig. 1A). We exploited this difference to estimate plant organelle contamination in DNA isolated from the lettuce phyllosphere and to demonstrate that a newly developed primer pair 534f/783r (Fig. 1A) does not amplify lettuce chloroplast 16S rRNA gene sequences and has potential as a real-time PCR primer for culture-independent estimation of plant-associated bacterial population sizes.

Bacterial primers 27f-YM and Eub518r do not discriminate between bacterial, chloroplast, and mitochondrial SSU rRNA gene sequences (Fig. 1A). With DNA isolated from aseptically grown lettuce seedlings, primer pair 27f-YM/Eub518r produced a single PCR fragment (Fig. 2A, lane 11) with a size of approximately 475 bp. Based on the expected sizes of PCR amplicons (Fig. 1B), this would suggest that the 27f-YM/Eub518r amplification product from lettuce seedling DNA consisted mostly of chloroplast, not mitochondrial, sequences. Indeed, digestion with *Bbv*CI (Fig. 2A, lane 5) generated two fragments with sizes that were predicted for chloroplasts (Fig. 1B). With a second primer set, 27f-YM/783r, which differs only in the reverse primer, lettuce seedling DNA produced a single amplicon that was similar in size as was predicted for the mitochondrial 18S rRNA gene. This lettuce PCR product did not cut with *Bbv*CI (Fig. 2A, lane 1), but instead generated bands with *Afe*I (Fig. 2A, lane 3), suggesting that indeed it mostly consisted of mitochondrial, not chloroplast, sequences. This finding indicated that the 4 mismatches of 783r to the chloroplast sequence, 2 of which occurred at the critical 3′ end of the primer (Fig. 1A), were sufficient to prevent binding and amplification of chloroplast DNA. Amplification of mitochondrial DNA was expected with primer pair 27f-YM/783r, based on the perfect match of 783r to the mitochondrial 18S rRNA gene sequence (Fig. 1B). Consistent with this finding was our analysis of 20 cloned 27f-YM/783r amplicons from lettuce seedling DNA: they all matched lettuce mitochondrial, not chloroplast, SSU rRNA sequences.

We used primer sets 27f-YM/Eub518r and 27f-YM/783r also on DNA that was isolated from leaf surfaces of field-grown lettuce. With both primer sets, we found excellent amplification (Fig. 2B and C, lanes 7–9). Digestion of the 27 f-YM/Eub518r amplicons with *Bbv*CI (Fig. 2C, lanes 1, 3, and 5) revealed a banding pattern that indicated a high abundance of chloroplast 16S rRNA gene sequences in the PCR products. In contrast, the 27f-YM/783r amplicons showed no digestion with *Bbv*CI (Fig. 2B, lanes 1, 3, and 5), suggesting the absence of chloroplast sequences. The faint banding pattern resulting from digestion of the 27f-YM/783r amplicons with *Afe*I indicated minor amounts of mitochondrial DNA. The 0.8-kb band in the 27f-YM/783r digestions is neither of mitochondrial nor of chloroplast origin, and so must represent 16S rRNA gene sequences of bacteria that were naturally associated with leaf surfaces of the field-grown lettuce. Primer 783r matched perfectly not only to the 16S rRNA gene sequence of *E. coli* (Fig. 1A), but also to that of 83.8% of all near full-length bacterial sequences in the RDP database (http://rdp.cme.msu.edu).

3.2. A real-time PCR assay for quantifying total bacteria in plant environments and for estimating contamination with plant chloroplast and mitochondrial DNA

We tested 783r as a real-time PCR primer for the estimation of bacterial population sizes on field-grown lettuce leaves. As forward primer, we chose 534f, which is the exact reverse complement of universal primer Eub518r. With *E. coli* genomic DNA as template, we found a linear relationship between the log of the number of 16S rRNA gene copies in the reaction and the C_T threshold cycle ($R^2 = 1$), with an amplification efficiency of >95% (Fig. 3A). Using this 534f/783r primer pair on DNA isolated from 31 different pools of leaf surfaces of field-grown lettuce, the average log [SSU rRNA gene copies g^{-1} of leaf] was 5.64 ± 0.46. Given the inability of 534f/783r to distinguish

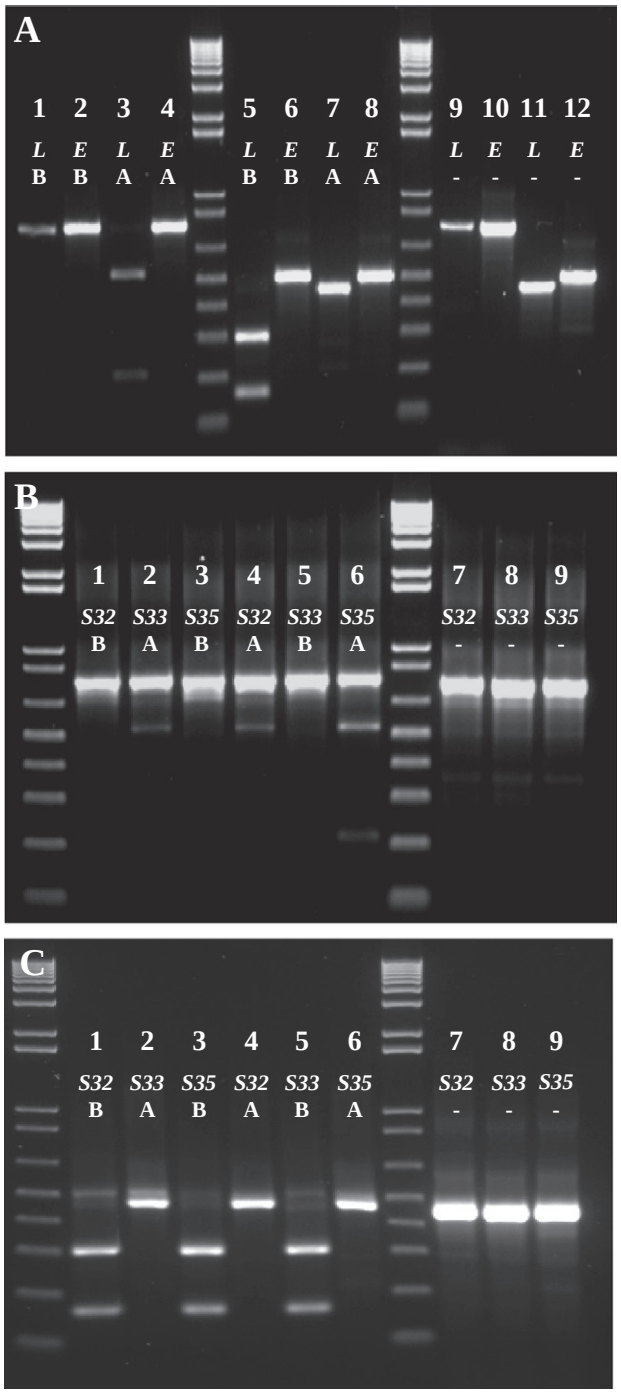

Fig. 2. PCR amplification and digestion of SSU rRNA gene fragments from lettuce seedling DNA (lanes labeled *L* in panel A), *E. coli* DNA (lanes labeled *E* in panel A), or DNA isolated from 3 representative phyllosphere samples (*S32*, *S33* and *S35* in panels B and C). Primer pairs used were 27f-YM/783r (panel B and lanes 1–4, 9 and 10 in panel A) and 27f-YM/518r (panel C and lanes 5–8, 11 and 12 in panel A). Lanes labeled 'B' represent PCR products digested with *Bbv*CI, 'A' denotes digestion with *Afe*I, and '–' means undigested. The molecular weight marker used was a 1 kb Plus DNA Ladder (Invitrogen).

between bacterial and plant mitochondrial DNA (Fig. 1A), we can assume that this number represents the sum of bacterial and plant mitochondrial copies in the samples. Using real-time PCR with primer pair mito1345f/mito1430r (Fig. 3B), which only amplifies mitochondrial 18S rRNA gene sequences, mitochondrial DNA contamination was estimated to represent on average less than 1% of the number obtained with primer pair 534f/783r, i.e. the average log[mitochondrial SSU rRNA gene copies g^{-1} leaf] value was 3.34 ± 0.27 (Fig. 4A). In other words, mitochondrial contamination in DNA extracted from the phyllosphere of field-grown lettuce was minimal, and values obtained with 534f/783r closely approximated the number of bacterial copies g^{-1} of leaf.

Using chloroplast-specific primer pair CYA361f/CYA785r (Fig. 1A), we verified the relatively high number of chloroplast 16S rRNA gene copies in the lettuce phyllosphere samples. The average log[chloroplast 16S rRNA gene copies g^{-1} leaf] value was 5.55 ± 0.37. This not only confirmed our interpretation of the results from the 27f-YM/Eub518r-*Bbv*CI method (Fig. 2C, lanes 1, 3, and 5), but it also was consistent with our finding that universal bacterial primer pair Eub338f/Eub518r, which does not exclude chloroplast 16S rRNA gene sequences (Fig. 1A), always gave higher values than 534f/783r, with an average log[copies g^{-1} leaf] of 6.23 ± 0.33 (Fig. 4A).

For each lettuce phyllosphere sample, we compared the number of bacterial 16S rRNA gene copies (estimated by subtraction of the mito1345f/mito1430r-determined copy number from the copy number obtained with 534f/783r) to the number of colony-forming units that appeared on 0.1× Tryptic Soy Agar (TSA). From this, we estimated that between 0.1 and 8.4% of the total bacterial population associated with lettuce leaves could be labeled culturable (Fig. 4B).

4. Discussion

We presented several methods that should be of interest to those who study plant-associated bacterial communities in a culture-independent, PCR-based manner. The first allows a quick assessment of the degree to which samples are contaminated with chloroplast DNA, which is a common problem in these types of study. The method is based on the *Bbv*CI digestion of PCR products resulting from amplification with the commonly used universal bacterial primers 27f-YM and Eub518r. We demonstrated proof of principle with field-grown lettuce samples, but given that many plant species share the chloroplast *Bbv*CI recognition site (Supplementary Table S1), the same method may have broad applicability in the bacterial community analysis of other plants. Notable exceptions are spinach and star anise: both lack a *Bbv*CI recognition site in their chloroplast 16S rRNA gene sequence. Of similar interest are the *Afe*I digestion of 27f-YM/Eub518r amplicons and the mito1345f/1430r primer pair as tools for the qualitative and quantitative, respectively, estimation of the number of mitochondrial 18S rRNA gene copies in plant-derived DNA samples. Both tools independently allowed us to conclude that contamination with mitochondrial DNA was relatively low compared to the total number of bacteria present. Whether this is true for lettuce under other circumstances or for other plants in general, we do not know.

The high degree of contamination of leaf surface washings with chloroplasts DNA reported here and in other studies (Edwards et al., 2007; Sagaram et al., 2009) is worthy of closer consideration. For several plant species, data are available on the numbers of chloroplasts per plant cell in leaves, the number of genomes per chloroplast, and the number of ribosomal gene copies per genome. In wheat, for example, a single plant cell may contain as many as 100,000 copies of the chloroplast 16S rRNA genes (Bendich, 1987). Strikingly, this number is close to our estimates for bacterial abundance and chloroplast contamination g^{-1} of field-grown lettuce leaf. In this study, we took great care during the washing of lettuce leaves to minimize damage to the leaf surface and avoid the release of plant cells and their organelles (chloroplasts and mitochondria) into the wash solution. Other methods for the release of bacteria from the phyllosphere, such as stomachers and blenders, involve much harsher treatments of the leaves with much higher amounts of chloroplast DNA released into the wash solution. This realization further underscores the practical worth of primer pairs that amplify bacterial sequences to the exclusion of chloroplast DNA.

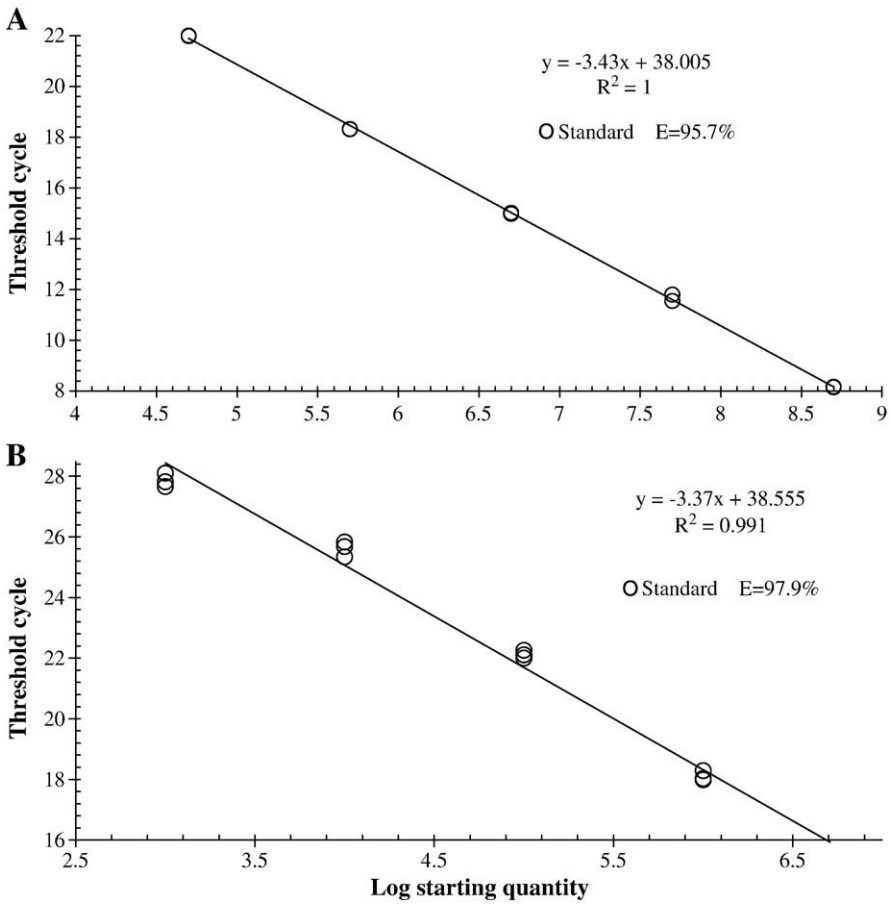

Fig. 3. Representative standard curves generated by real-time PCR using primer pair 534f/783r on *E. coli* DNA (A) and mito1345f/mito1430r on lettuce DNA (B). Standard curves were constructed using 10-fold serial dilutions of PCR product or cloned PCR product (see Materials and methods).

One such primer pair, 534f/783r, we used in a method presented here that allows the culture-independent estimation of bacterial population sizes in plant environments. The counter-selectivity of the primer pair resides with primer 783r, which does not allow amplification of chloroplast DNA but still targets close to 84% of all near full-length bacterial sequences in the RDP database. This includes 94.4% of the members of the phylum *Bacteroidetes*, 89.9% of the *Firmicutes*, 88.9%, *Proteobacteria*, and 88.8% *Actinobacteria*. Of the phyla with the most representatives, notably under-targeted by 783r are the *Cyanobacteria* (0.3%), *Planctomycetes* (9.8%), *Chloroflexi* (15.2%), and *Verrucomicrobia* (18.1%). *Cyanobacteria* share a common ancestor with chloroplasts (Giovannoni et al., 1988), which is why they are not targeted by primer 783r. Several studies have reported the presence of cyanobacteria on leaf surfaces including lettuce (Fürnkranz et al., 2008; Zwielehner et al., 2008), but if, as in those studies, cyanobacteria do not dominate the bacterial community, it is unlikely that primer 783r will greatly underestimate the total number of bacteria. A similar argument can be made for leaf-associated representatives from the *Verrucomicrobia*. Typically, these are bacteria associated with soil, and while soil particles are often found on the leaf surfaces of outdoor grown plants (Monier and Lindow, 2004), including lettuce, they might not contribute large enough numbers of *Verrucomicrobia* to the total bacterial count on leaf surfaces. Representatives of the other two phyla that are under-targeted by primer 783r, i.e. *Planctomycetes* and *Chloroflexi*, are typically found in aquatic environments (Neef et al., 1998; Morris et al., 2004) and are unlikely to add greatly to the number of total bacteria associated with terrestrial plants.

The compromise between loss of bacterial targets and chloroplast exclusion is a characteristic that 783r shares with similar primers including 799f, 783r-abc, and 799R2, and appears to be an accepted caveat by those in the field of plant microbiology who use these primers. Underestimation of total bacterial population size with any of these primers is likely to be smaller than the overestimation with primers that do not exclude chloroplasts. For example, on the basis of data presented here, it is likely that universal bacterial primer pair Eub338f/Eub518r overestimates bacterial abundances in real-time PCR assays on plant samples, and that the results from studies that have used this primer pair on plant-associated bacterial communities (Fierer et al., 2005; Lambais et al., 2006; Redford and Fierer, 2009) should be interpreted with corresponding caution.

Primer pair 534f/783r provided us with culture-independent estimates of bacterial population sizes on field-grown lettuce leaves that were 10 to 1000 times higher than the number of CFUs we counted on 0.1× TSA plates. The latter is considered a non-selective medium that is often used to estimate the culturable fraction of bacterial populations (Palumbo et al., 2007). The 0.1–10% culturability range is typical for many environmental habitats (Alain and Querellou 2009), although to the best of our knowledge it is the first such estimate reported for the plant leaf surface, or phyllosphere, habitat.

In summary, our study offers a set of practical solutions towards the faithful estimation of bacterial abundances in plant-associated environments. The *Afe*I and *Bbv*CI approach to differentiate among bacterial, chloroplast, and mitochondrial rRNA amplicons is novel and represents a semi-quantitative tool for the verification of plant DNA contamination in total DNA extracted from plant-associated microbial communities. Having tested the specificity of the 534f/783r primer pair on aseptically grown lettuce plants, in combination with *Afe*I and *Bbv*CI restriction analyses of 27f-YM/Eub518r and 27f-YM/783r amplicons to confirm the chloroplast-exclusivity of 783r, makes the 534f/783r primer pair one of the better characterized among those

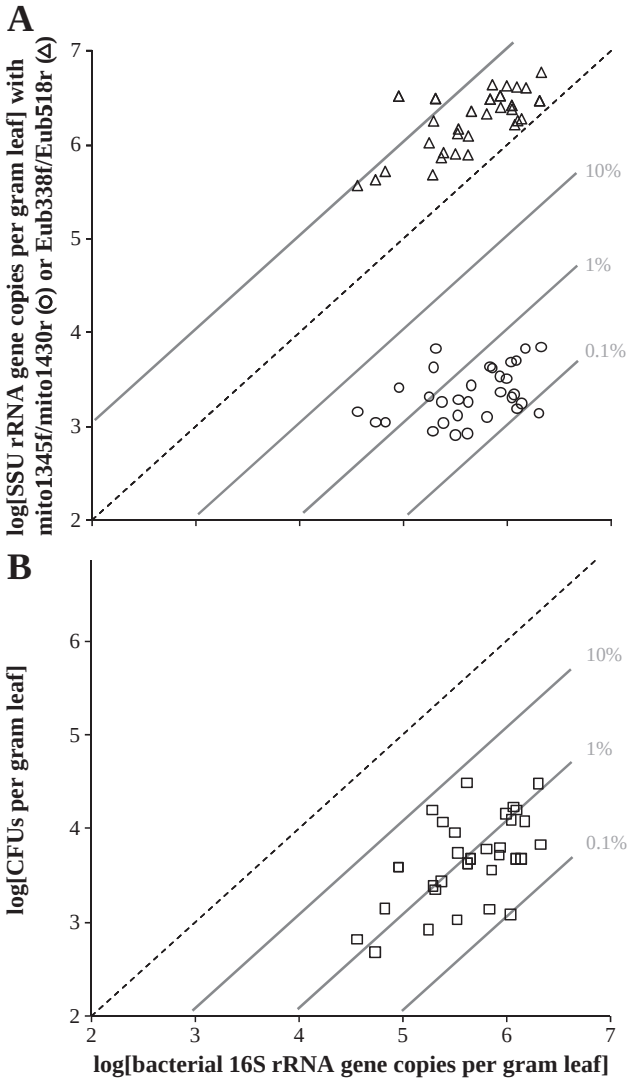

Fig. 4. Scatter plot showing for 31 lettuce phyllosphere samples (A) the number of estimated SSU rRNA gene copies based on primer pair mito1345f/mito1430r (circles; mitochondria only), or Eub338f/Eub518r (triangles; mitochondria, chloroplasts and bacteria), or (B) the number of colony-forming units on 0.1× TSA, as a function of the number of bacterial 16S rRNA copies, as deduced from 534f/783r (mitochondria and bacteria) minus mito1345f/mito1430r (mitochondria only). In panel A, if for a particular sample the mito1345f/mito1430r or Eub338f/Eub518r value would have been the same as the 534f/783r value, the corresponding data point would have lain on the broken line. Grey lines indicate 10-fold steps of over- or underestimation.

available to date for quantitative PCR purposes. Another innovation that we presented in this paper is the use of a plant mitochondrial-specific primer set (mito1345f/mito1430r) to compensate by subtraction any overestimation of bacterial abundance due to mitochondrial contamination by primer pair 534f/783r; this aspect is often neglected in the design of primers in the field of plant microbiology, where most focus tends to be on the chloroplast-exclusivity of such primers. In short, we believe that the PCR-based toolbox proposed here presents a substantial innovation to be of general use for those interested in applying culture-independent approaches in plant microbial ecology.

Supplementary materials related to this article can be found online at doi: 10.1016/j.mimet.2010.08.006.

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