Plant Soil (2018) 422:51–66
DOI 10.1007/s11104-017-3176-2

REGULAR ARTICLE

Community structure and diversity of endophytic bacteria in seeds of three consecutive generations of *Crotalaria pumila* growing on metal mine residues

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Received: 14 August 2016 / Accepted: 2 January 2017 / Published online: 9 March 2017

Abstract
Aims We investigated the possible transgenerational transfer of bacterial seed endophytes across three consecutive seed generations of *Crotalaria pumila* growing on a metal mining site in Mexico.
Methods Seeds were collected during three successive years in the semi-arid region of Zimapan, Mexico. Total communities of seed endophytes were investigated using DNA extraction from surface sterilized seeds and 454 pyrosequencing of the V5-V7 hypervariable regions of the 16S rRNA gene.
Results The communities consisted of an average of 75 operational taxonomic units (OTUs); richness and diversity did not change across years. *Methylobacterium, Staphylococcus, Corynebacterium, Propionibacterium* and eight other OTUs constituted >60% of the community in each generation. The microbiome was dominated by *Methylobacterium* (present in >80% of samples). Functions associated with the microbiome were C and N fixation, oxidative phosphorylation and photosynthesis activity.
Conclusions The bacterial endophytic communities were similar across three consecutive seed generations. Among the core microbiome *Methylobacterium* strains were the most abundant and they can contribute to nutrient acquisition, plant growth promotion and stress resilience to their host in metal contaminated mine residues. Identification of the seed microbiome of *C. pumila* may lead to novel and more efficient inoculants for microbe-assisted phytoremediation.

Keywords Bacterial seed endophytes · Trace metals · Phytoremediation · Pyrosequencing

Responsible Editor: Eric B. Nelson.

Electronic supplementary material The online version of this article (doi:10.1007/s11104-017-3176-2) contains supplementary material, which is available to authorized users.

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Introduction

Soil contamination with trace metals has increased, posing risks for the environment and public health (Jaishankar et al. 2014; Udeigwe et al. 2015). Phytoremediation can be a suitable technology for removal of trace metals from moderately contaminated soils, or stabilizing the metals in situ in case of high levels of contamination. Through the establishment of a vegetation cover, phytoremediation aims to reduce environmental risks, by removing or immobilizing contaminants (Mench et al. 2010; Vangronsveld et al. 2009). In addition, phytoremediation is cost-effective,

sun-powered, with long-term applicability and aesthetic advantages. In order to optimize phytoremediation, several strategies have been proposed, one among them being the application of beneficial plant-associated microorganisms, especially bacteria (Kidd et al. 2015; Rajkumar et al. 2009). Due to the close interactions between plants and endophytic bacteria, the functions and potential use of such microorganisms on effective metal phytoremediation have been comprehensively addressed in recent years (Wani et al. 2015; Weyens et al. 2009). However, most of the research about endophytes in metal polluted environments focused mainly on root and/or shoot endophytes.

Since many seed endophytic bacteria provide to their host benefits similar to those described for plant growth-promoting rhizobacteria (PGPR) (Hardoim et al. 2008) and they can increase their host's tolerance to metals (Mastretta et al. 2009; Weyens et al. 2009), it is postulated that the establishment of plants in hostile environments can be attributed to the presence and transmission of seed endophytic bacteria during germination and seedling development (Truyens et al. 2013, 2014, 2016; Johnston-Monje and Raizada 2011; Hardoim et al. 2012). Moreover, seed endophytes, particularly those that are transmitted across different seed generations, can play an important role in the adaptation process of plants to metal polluted environments (Truyens et al. 2013, 2016).

An interesting case to investigate seed endophytic communities in a contaminated environment is *Crotalaria pumila* (low rattlebox), a pioneer plant species that was observed colonizing metal containing mine residues in the semi-arid mining region of Zimapan (central Mexico). This annual plant species completes its full life cycle on this contaminated substrate and produces viable seeds, hence increasing the numbers of plants colonizing the bare mine tailings year after year (Sánchez-López et al. 2015). A better understanding of the dynamics and beneficial traits of seed endophytes of a pioneer plant colonizing metal polluted substrates will allow to develop more successful phytoremediation strategies. Hence, the objectives of this study were: (i) to investigate the composition of the total endophytic bacterial communities of three consecutive generations of *C. pumila* seeds from plants colonizing multi-metal contaminated mine residues using 454 pyrosequencing; (ii) to assess eventual changes of endophytic communities across seed generations; (iii) to identify seed bacterial endophytes maintained trans-generationally, and (iv) to identify the most important functions associated with the seed endophytes.

Materials and methods

Seed material

Three consecutive generations of *Crotalaria pumila* seeds were collected from the Santa Maria mine residues, in Zimapan, central Mexico (20°44′8.89″N, 99°23′56.07″W) in October 2011, 2012, and 2013. The climate in the study area is semi-arid, with an average temperature ranging from 12 to 20 °C and an annual precipitation of approximately 700 mm (INEGI 2009).

The plants produce seeds once per year after a life cycle of 5 to 7 months depending on environmental conditions (Rzedowsky and Rzedowsky 1985). To avoid harvesting unripe seeds, dry pods attached to the *C. pumila* plants were taken. In each year (2011, 2012 and 2013), pods were removed from at least 40 different individual plants spread over the mine residues, and kept together in paper bags at room temperature till processing.

The mine residues where seeds were collected are characterized by a pH of 7.6 and an electrical conductivity of 0.7 dS cm^{-1}; total concentrations of metals were 4546 mg Zn kg^{-1}, 120 mg Cd kg^{-1}, 4183 mg Pb kg^{-1}, 1764 mg Cu kg^{-1}, and 1112 mg Ni kg^{-1} (Sánchez-López et al. 2015).

Surface sterilization

Seeds were surface sterilized as follows: they were washed with phosphorus free detergent and tap water, immersed in NaClO 0.1% solution supplemented with 0.1% Tween 80 for 10 s, and finally rinsed in sterile deionized water (8 × 100 mL). Sterilization was confirmed by plating 100 μL of the last rinsing water on 869 solid medium (Mergeay et al. 1985), and by running a PCR on the last rinsing water (Truyens et al. 2016). Briefly, each PCR reaction contained 1 μL of the last rinsing water, 1X high fidelity PCR buffer, 2 mM $MgSO_4$, 200 μm of each dNTP, 200 nM of bacterial specific primers 1392R (ACGGGCGGTGTGTRC) and 26F (AGAGTTTGATCCTGGCTCAG), 1 U high fidelity Taq polymerase and RNase-free water to get a total

volume of 50 μL. PCR conditions were as follows: 5 min at 95 °C, 35 cycles of 1 min at 94 °C, 30 s at 52 °C, 3 min at 72 °C and 10 min at 72 °C. If no bacterial colonies were observed on plates nor DNA was detected after PCR the seed surface sterilization was considered effective.

Total DNA extraction and sequencing

Total genomic DNA was extracted from 150 mg surface sterilized seeds using an Invisorb® Spin Plant Mini Kit, and then subjected to two PCR reactions. In the first one, primers 799F (AACMGGATTAGATACCCKG) and 1391R (ACGTCATCCCCACCTTCC) were used to amplify the V5-V7 hypervariable region of the 16S rRNA gene, to avoid non-target rRNA and retrieve bacterial operational taxonomic units (OTUs) (Beckers et al. 2016). A 592 bp band was obtained and extracted from 1.5% agarose gel with the Qiaquick Gel Extraction Kit (Qiagen). For multiplex DNA, in the second PCR the forward primer was extended with a sample-specific 10 bp multiplex identifier (MID). The master mix for each reaction consisted of 1.8 mM high-fidelity reaction buffer, 1.8 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM forward primer, 0.4 μM reverse primer, and 1.25 U high-fidelity Taq polymerase (Invitrogen) (Beckers et al. 2016). One microliter of the first PCR products was used for the second PCR. Cycling conditions were initial denaturation at 95 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, at 53 °C for 60 s, and at 72 °C for 1 min, and final elongation of 10 min at 72 °C (Techne TC 5000 PCR Thermal Cycler). Subsequently, PCR MID-tagged amplicons were purified with the QIAquick PCR Purification Kit (Qiagen). The concentration of DNA in each sample was determined with the Picogreen dsDNA Quantitation assay (Life Technologies); equimolar concentrations of the barcoded amplicons were pooled and pyrosequencing was carried out by Macrogen (Seoul, South-Korea) on a 454 Genome Sequencer FLX Titanium.

Analysis of obtained pyrosequencing data

In total 12 seed samples were analyzed, representing four composed samples of each seed generation: 2011, 2012, and 2013. Raw sequence reads were processed using QIIME software (Caporaso et al. 2010a). First, sequences were denoised using AmpliconNoise (Quince et al. 2009); then, libraries were split according to barcodes and trimmed to remove barcodes, primers, homopolymers longer than 6 bases and sequences shorter than 200 bases. Additional chimeric sequences formed de novo were detected and removed from the data set, including contaminants, according to USEARCH61 algorithm (Edgar 2010). The remaining sequences were grouped into OTUs based on a 97% similarity level and following Pynast alignment (Caporaso et al. 2010b) sequences were queried against Greengenes (DeSantis et al. 2006). Sequences matching with mitochondria, chloroplasts and Archaea were removed. The standard flowgram format (SFF) files were deposited in the NCBI Sequence Read Archive (SRA) under the accession number SRP080874.

Due to the different amounts of reads per sample observed after processing sequences, the numbers of reads per sample were rarified to 430. Rarefaction curves, numbers of different observed OTUs, Good's coverage, Simpson diversity index and Chao1 richness estimator were calculated for the pooled data. Good's coverage was calculated as $G = 1- n/N$; where n is the number of singleton phylotypes and N is the total number of individuals in the sample.

Identification of the core microbiome, defined as the OTUs that are present in at least 50% of the samples, was carried out in QIIME. Metataxonomic prediction using Gene prediction with Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was applied to the dataset of the core microbiome. Using marker gene data and database of reference genomes, PICRUSt applies an extended reconstruction algorithm to predict the presence and abundance of gene families in the metagenome of host-associated and environmental communities (Langille et al. 2013). Function predictions were categorized on the Kyoto Encyclopedia of Genes and Genomes (KEGG) classification at level 3.

Statistical analysis

Numbers of different observed OTUs, Good's coverage, Simpson diversity index and Chao diversity estimator were compared between seeds generations using *t* tests. To test the hypothesis of no difference of seed endophytic bacterial community across *C. pumila* generations, Principal Components Analysis (PCA) was performed using both, weighted and unweighted UniFrac distances (Lozupone and Knight 2005). Significant changes of seed endophytic communities were tested

using Analysis of Similarities (ANOSIM) and Permutational Multivariate Analysis of Variance Using Distance Matrices (ADONIS). To complete these tests Similarity Percentages (SIMPER) analysis was also applied on the basis of Bray-Curtis similarity. Both analysis, ANOSIM and SIMPER were performed using PAST software (Hammer et al. 2001). Additionally, OTUs or clades most likely to explain differences among the three seed endophytic communities were determined using Linear discriminant analysis Effect Size (LEfSe), it couples biological consistency and effect size estimation (Segata et al. 2011).

Results

Analysis of pyrosequencing data

Using 454 pyrosequencing we assessed richness and diversity of seed endophytic bacterial communities in three consecutive generations of *C. pumila* seeds collected from metal-containing mine residues. After filtering low quality reads, adapters, barcodes and primers, there were about 15,341 effective reads for the 12 seed samples. Rarefaction curves after removing samples are presented in Fig. 1; most of the samples of the 3 seed generations started to level up at the subsampling depth that we used.

Richness and diversity of seed endophytic communities

The numbers of different observed OTUs, Good's coverage, Simpson diversity index and Chao 1 richness estimator are shown in Table 1. The lowest number of observed OTUs was registered in seeds from 2012 (57.9), seeds collected in 2011 and 2013 showed higher numbers of observed OTUs, (82.3 and 84.1, respectively). However, no significant differences were detected among the 3 seed generations. Good's coverage values in 2011 and 2013 were around 99, while in 2012 this value was 98.1. Like in the case of observed OTUs no significant differences were detected (Table 1).

Simpson diversity indices calculated for consecutive generations were respectively 0.83, 0.71 and 0.83, in 2011, 2012 and 2013; these values were statistically similar (Table 1). Likewise, no significant differences were detected for the Chao1 richness estimator across seed generations (Table 1); calculated values were 129.2 in 2011, 98.3 in 2012 and 126.4 in 2013.

Description of the endophytic communities across generations

The taxonomy of the sequences is described first at the phylum level (Fig. 2). Due to the abundance of Proteobacteria in almost all samples, this phylum is presented at class level: Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria and Gamma-

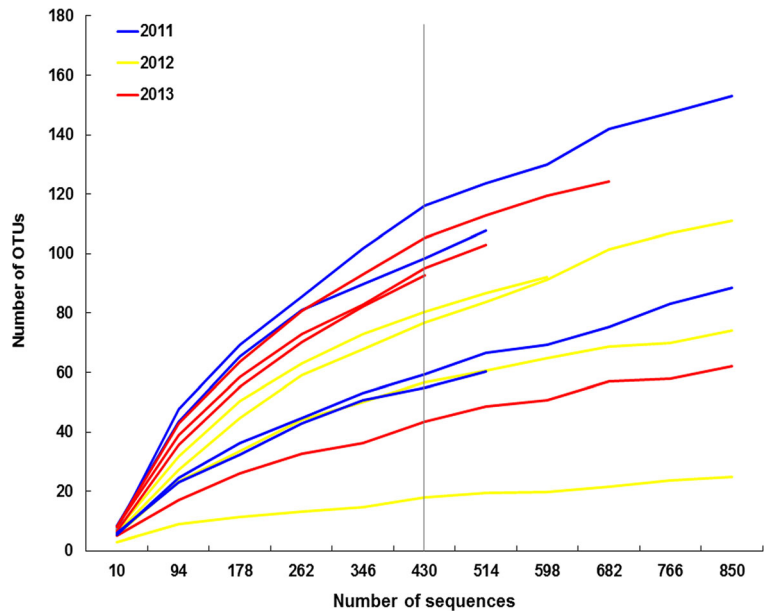

Fig. 1 Rarefaction curves for endophytes samples of three consecutive seed generations (2011, 2012 and 2013) of *Crotalaria pumila* colonizing multi-metal contaminated mine residues. The *vertical line* indicates rarefaction depth (430 reads)

Table 1 Observed OTUs, Good's coverage, Simpson diversity index and Chao1 richness estimator of endophytic bacteria of *Crotalaria pumila* seeds collected across three continuous generations from mine residues

Seed generation	Observed OTUs (genus level)	Good's coverage	Simpson	Chao1
2011	82.3 ± 25.8^{ns}	99.7 ± 0.23^{ns}	0.83 ± 0.13^{ns}	129.2 ± 36.9^{ns}
2012	57.9 ± 20.8^{ns}	98.1 ± 0.22^{ns}	0.70 ± 0.16^{ns}	98.3 ± 20.6^{ns}
2013	84.1 ± 23.9^{ns}	99.5 ± 0.51^{ns}	0.83 ± 0.11^{ns}	126.4 ± 26.6^{ns}

Values in each cell represent average ± standard deviation ($n = 4$); no significant (ns) difference between seed generations according to *t* test

proteobacteria. Alphaproteobacteria was the most abundant class in all samples of 2011 (from 21.4% to 77.7% of the total communities); in two samples of both 2012 and 2013, Alphaproteobacteria constituted around 70% and 50% of the community, respectively. In 2012 Gammaproteobacteria were the most abundant in two samples, with abundances of 93.8% and 57.5%. Deltaproteobacteria were identified only in seeds collected in 2012 representing 0.5% of the bacterial community. The dominance of the Alphaproteobacteria in the seed endophytic community in 2013 was observed in two samples, while Firmicutes and Thermi were the predominant phyla in the other samples, representing respectively 32.3% and 81.3% of the total endophytic community. Some phylogenetic groups were present only in one batch of seeds. Chlamydiae, for instance, was found only in the seed generation from 2011, representing 2.2% of the endophytic community (Fig. 2).

In total 97 different OTUs were identified, 68 were identified up to genus level, 29 OTUs were identified at family or class level only (Table S1). The results are

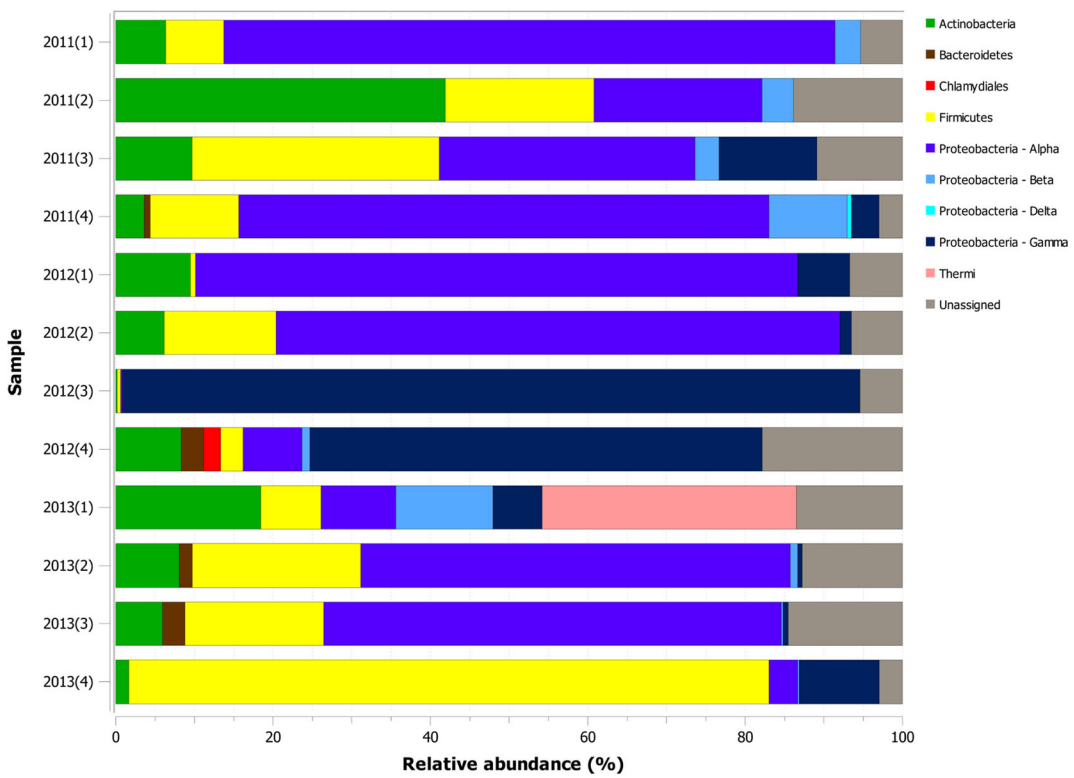

Fig. 2 Compositions at phylum level of the total endophytic community of three consecutive seed generations (2011, 2012 and 2013) of *Crotalaria pumila* colonizing multi-metal contaminated mine residues. Phylum Proteobacteria is presented as classes Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria and Gammaproteobacteria

described based on the most representative OTUs (identified at genus, relative abundance higher than 0.1%), presented in Table 2.

The endophytic bacterial community of seeds collected in 2011 consisted of 57 different OTUs; the majority (in total 70.8%) of the seed endophytic bacterial community belonged to *Methylobacterium* (47.1%), *Staphylococcus* (9.5%), *Corynebacterium* (6.1%), *Propionibacterium* (3.7%), *Peptoniphilus* (2.0%) and an OTU corresponding to a member of the Comamonadaceae family (2.4%). Other genera present at abundances higher than 1% of the total seed endophytic community in 2011 were *Rhodococcus* (1.2%), *Streptococcus* (1.1%), *Variovorax* (1.4%), *Gluconacetobacter* (1.2%) and *Pseudomonas* (1.9%) (Table 2).

In the subsequent seed generation collected in 2012, 53 different OTUs were found in total. The endophytic community of 2012 contained two dominating OTUs: *Methylobacterium* (36.7%) and the Enterobacteriaceae family (33.7%). Further, *Corynebacterium* (1.9%), *Staphylococcus* (1.7%) and *Propionibacterium* (1.2%) were present at more than 1%. The sum of the abundances of the five mentioned OTUs represented 75.2% of the seed endophytic bacterial community in seeds collected in 2012 (Table 2).

In total, 56 different OTUs were observed in the third seed generation (2013) that we investigated. Like in the previous two seed generations, also in 2013 *Methylobacterium* was the most abundant OTU, representing 28.6% of the bacterial community, followed by *Staphylococcus* (23.6%). Other OTUs observed in abundances higher than 1% were *Meiothermus* (8.1%), *Burkholderia* (2.8%), *Bacillus* (2.6%), *Corynebacterium* (2.2%) and an OTU belonging to the Planococcaceae family (3.2%). These seven OTUs formed 71.1% of the total bacterial endophyte community of *C. pumila* seeds collected in 2013 (Table 2).

Distinct and shared bacterial genera across seed generations

Some OTUs were identified only in one or two seed generations, while others were found in seeds of the 3 consecutive years under investigation. The presence of the most representative OTUs throughout the 3 seed generations is shown in Fig. 3; the entire communities, including order and family OTUs, and those occurring at low relative abundances are presented in Fig. S1. Concerning the seed generation of 2011, 16 OTUs out of the total number of 57 were found exclusively in this batch of seeds (Fig. S1), representing 7.5% of the total community. The most distinctive OTUs were *Peptoniphilus* (2.0%), *Variovorax* (1.4%) and *Gluconacetobacter* (1.2%) (Fig. 3, Table 2). Fourteen OTUs out of 53 were isolated only from seeds collected in 2012, representing 4.4% of the total seed endophytic community. The most important different OTUs were *Anoxybacillus* (1.5%), *Rabdochlamydia* (0.5%), and an OTU belonging to the Nocardiopsaceae family (0.8%). In seeds collected in 2013, 15 out of 56 OTUs (12.0% of the endophytic community) were identified only in this seed generation, among these OTUs were *Meiothermus* (8.1%), *Psychrobacter* (1.8%) and an OTU of the Nocardioidaceae family (0.5%) (Fig. 3; Table 2).

Twenty-one bacterial OTUs were found in 2 of the 3 consecutive generations of *C. pumila* seeds analyzed; most of them were common between the 2011 and 2013 generation. These were: *Brachybacterium, Burkholderia, Marinobacterium, Rhodococcus, Streptococcus, Kocuria* and *Mycoplana* (Fig. 3). The following OTUs were found in the seed generations of 2011 and 2012: *Pseudomonas, Erwinia, Acinetobacter, Actynomices* and *Leuconostoc*. While, *Desemzia, Enhydrobacter, Aerocuccus, Ochrobactrum, Carnobacterium, Clavibacter* and *Streptomyces* were common in the endophytic communities of seeds collected in 2012 and 2013 (Fig. 3).

Despite the fact that some OTUs were identified in only one or two seed generations of *C. pumila,* in total 21 OTUs, 10 at genus level (Fig. 3), eight at family level and three identified at order level (Fig. S1) were part of the total community in the three consecutive seed generations. These OTUs are: *Methylobacterium, Propionibacterium*, *Sediminibacterium, Corynebacterium*, *Staphylococcus Bacillus, Kineococcus, Brevibacterium, Pantoea, Sphingomonas, Micrococcus, Jeotgalicoccus* and OUT of Enterobacteriaceae family.

Are bacterial endophytic communities similar across seed generations?

Principal Components Analysis (PCA) was conducted to evaluate similarities of the different seed generation samples using weighted and unweighted UniFrac. When the analysis was conducted on basis of presence or absence of OTUs (unweighted UniFrac) the distribution of samples did not show any grouping (Fig. S2).

Table 2 Composition, relative abundance (%) of endophytic bacteria across three consecutive generations of *Crotalaria pumila* seeds collected from mine residues

OTU	2011	2012	2013	Previous reports*	
Actynomices	0.9	0.2	0.0		
Varibaculum	0.0	0.0	0.2		
Brevibacterium	0.1	0.04	0.3	Roots: *Prosopis laegivata*	Román-Ponce et al. (2015)
Corynebacterium	6.1	1.9	2.2	Seeds: *A. thaliana*	Truyens et al. (2016)
Brachybacterium	0.2	0.0	0.05		
Dermacoccus	0.0	0.3	0.0		
Kineococcus	0.8	0.1	0.5		
Clavibacter	0.0	0.04	0.2	Stem: *Lupinus luteus*	Weyens et al. (2014)
Microbacterium	0.0	0.3	0.0	Seeds: *A. thaliana*	Truyens et al. (2016)
Kocuria	0.4	0.0	0.3	Root: *Noccaeae carulescens*	Visioli et al. (2014)
Micrococcus	0.4	0.1	1.2	Root, stems: *Spartina maritima*	Mesa et al. (2015)
Mycobacterium	0.0	0.02	0.0	Seeds: *C. pumila, A. thaliana*	Sánchez-López (2015); Truyens et al. (2013, 2016)
Rhodococcus	1.2	0.0	0.2	Root: *Thlaspi goesingense*	Idris et al. (2004)
Aeromicrobium	0.0	0.2	0.0		
Propionibacterium	3.7	1.2	1.3		
Pseudonocardia	0.0	0.0	0.1	Seeds: *A. thaliana*	Truyens et al. (2016)
Streptomyces	0.0	0.5	0.3		
Sediminibacterium	0.2	0.7	1.0	Seeds: *A. thaliana*	Truyens et al. (2016)
Rhabdochlamydia	0.0	0.5	0.0		
Anoxybacillus	0.0	1.5	0.0		
Bacillus	0.3	0.6	2.6	Seeds: *C. pumila*, *A. thaliana*, *Agrostis capillaris*	Sánchez-López (2015); Truyens et al. (2013, 2014, 2016)
Jeotgalicoccus	0.04	0.1	0.1		
Staphylococcus	9.5	1.7	23.6	Seeds: *C. pumila, A. thaliana*	Sánchez-López (2015); Truyens et al. (2013, 2016)
Planifilum	0.4	0.0	0.0		
Aerococcus	0.0	0.03	0.9	Seeds: *A. thaliana*. Roots: *Prosopis junciflora*	Truyens et al. (2016); Khan et al. (2015)
Facklamia	0.6	0.0	0.0		
Carnobacterium	0.0	0.04	0.05		
Desemzia	0.0	0.2	0.05		
Enterococcus	0.0	0.0	0.3		
Leuconostoc	0.9	0.2	0.0		
Peptoniphilus	2.0	0.0	0.0		
Streptococcus	1.1	0.0	0.4		
Mycoplana	0.1	0.0	0.05		
Ochrobactrum	0.0	0.04	0.7	Roots: *Juncus acutus*	Dimitroula et al. (2015)
Methylobacterium	47.1	36.7	28.6	Seeds: *C. pumila, A. thaliana*	Sánchez-López (2015); Truyens et al. (2013, 2016)
Sphingomonas	0.8	0.1	0.1	Seeds: *C. pumila*, *A. thaliana*	Sánchez-López (2015); Truyens et al. (2013, 2016)
Burkholderia	0.6	0.0	2.8		
Limnohabitans	0.3	0.0	0.0		

Table 2 (continued)

OTU	2011	2012	2013	Previous reports*	
Variovorax	1.4	0.0	0.0	Seeds: *C. pumila*, *A. thaliana*	Sánchez-López (2015); Truyens et al. (2013, 2016)
Ralstonia	0.7	0.0	0.0	Roots: *Juncus acutus*	Dimitroula et al. (2015)
Enterobacteriaceae	0.8	33.7	1.4		
Erwinia	0.03	1.7	0.0		
Gluconacetobacter	1.2	0.0	0.0		
Pantoea	0.04	2.1	0.02	Seeds: *A. capillaris*	Truyens et al. (2014)
Marinobacterium	0.04	0.0	0.1		
Haemophilus	0.0	0.2	0.0		
Acinetobacter	0.1	0.04	0.0	Root, shoot: *Solanum nigrum, Commelina communis*	Chen et al. (2010); Zhang et al. (2011)
Enhydrobacter	0.0	0.6	0.7		
Psychrobacter	0.0	0.0	1.8		
Pseudomonas	1.9	1.1	0.0	Seeds: *Nicotiana tabacum, A. thaliana*	Mastretta et al. (2009); Truyens et al. (2016)
Meiothermus	0.0	0.0	8.1		
Other	8.8	4.2	8.9		
Unassigned	8.3	9.1	10.9		

This table shows the most representative OTUs identified in *C. pumila* seeds; OTUs observed in only one generation at abundance of 0.1% or less, and those not identified till genus level are not included. All identified OTUs are shown in Table S1. Values in each cell represent the average relative abundance in seeds collected in each generation ($n = 4$)

*Previous reports of bacterial genus as endophytes of seeds and other plant organs exposed to trace metals

However, the PCA plot resulting from differences in relative abundance of taxa (weighted UniFrac), rather than on which taxa are present, showed some grouping of samples (Fig. 4). 83.52% of the variance was explained by the two first coordinates. On one hand the average percentage of similarity was calculated by SIMPER (Table 3); this analysis showed 56.2% of overall average similarity among seed generations. The OTUs that explains transgenerational similarities in the three endophytic communities, considering their relative abundance, were *Methylobacterium, Staphylococcus*, *Propionibacterium, Corynebacterium* and an OTU of Enterobacteriaceae. On the other hand, when comparing the 3 seed generations LEfSe analysis showed differences between the 2011 and 2013 endophytic communities. These differences were most likely explained by *Staphylococcus, Bacillus, Enhydrobacter, Rhodococcus,* order Burkholderiales and phylum Betaproteobacteria (Fig. 5). Nevertheless, statistical parameters, either those obtained by ANOSIM ($R = 0.0347$, $p = 0.3940$) or ADONIS ($R^2 = 0.0801$, $p = 0.832$) showed that the grouping of endophytic communities in the consecutive seed generations of *C. pumila* (Fig. 4) was not significant.

Core microbiome and predicted functional traits

The analysis of the core microbiome, OTUs that are present in at least 50% of samples and taking into account the three successive generations of *C. pumila* seeds, revealed the occurrence of *Methylobacterium*, *Corynebacterium, Propionibacterium* and *Staphylococcus* as consistent members of the seed endophytic community across time (Fig. 6). It is interesting to observe *Methylobacterium* as the dominating OTU; this genus constitutes more than 80% of the core microbiome.

PICRUSt shows that taking into account the core microbiome across the three seed generations, metabolic functions were the most important, being 47% (Fig. 7). Within these functions, carbohydrates (20.6%), amino acids (20.2%) and energy metabolism (11.5%) were dominating. At KEGG level 3, abundant metabolic functions were related to methane metabolism (23.7%), oxidative phosphorylation (22.5%) and C

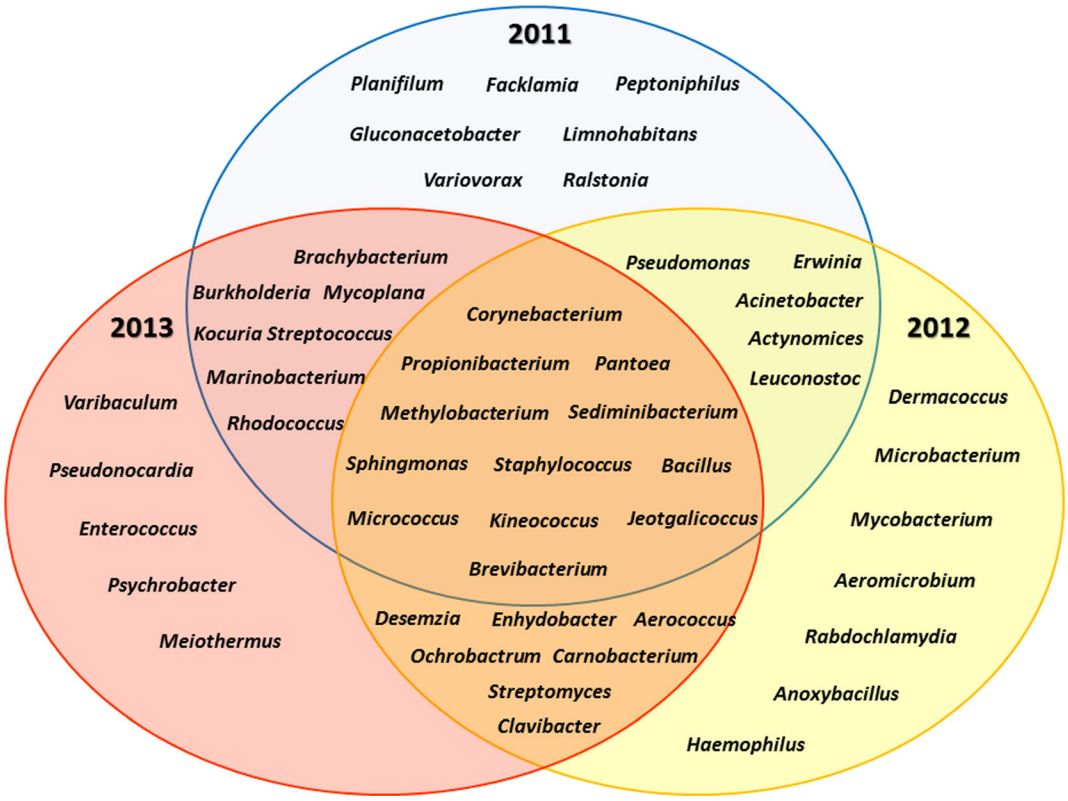

Fig. 3 Shared and distinct OTUs endophytes across three consecutive seed generations (2011, 2012 and 2013) of *Crotalaria pumila* colonizing multi-metal contaminated mine residues. This figure includes only OTUs identified at genus level; those identified till class, order, or family and those present in only one generation at relative abundance of 0.1% or less are not included

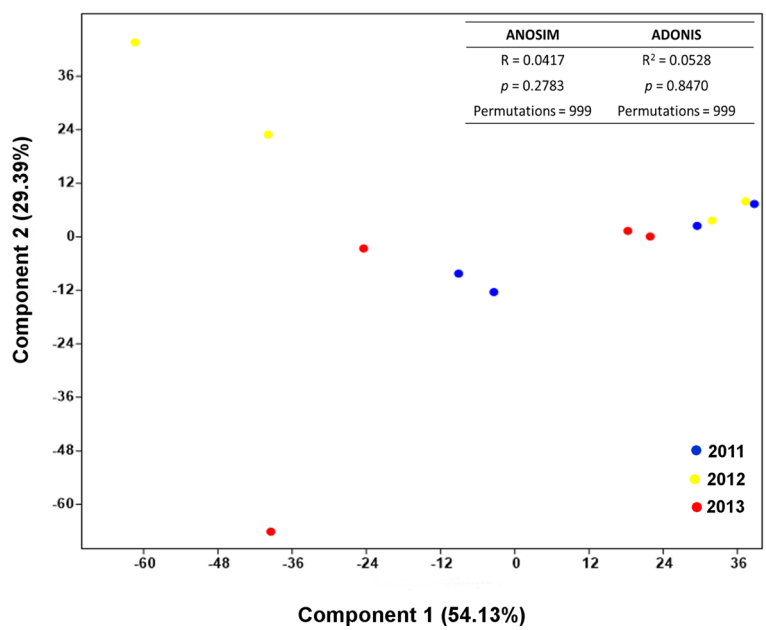

Fig. 4 Principal components analysis of total seed endophytic communities across three consecutive generations (2011, 2012 and 2013) of *Crotalaria pumila* colonizing multi-metal contaminated mine residues

Table 3 Similarity of endophytic bacteria communities across three consecutive generations of *C. pumila* seeds collected in mine residues

Comparison	Average similarity *	Principal OTUs contributing to similarity *	R**	*p***
3 generations	56.2%	*Methylobacterium* (15.1%) Entereobacteriaceae (13.5%) *Staphylococcus* (10.7%) *Propionibacterium* (4.3%) *Corynebacterium* (2.6%)	0.0417	0.2783

*Average dissimilarity and contribution to dissimilarity of each OTU calculated by SIMPER

**R-value and *p*-value in each comparison calculated by ANOSIM; $n = 4$

fixation (25.6%). Interestingly, functions in photosynthesis (12.4%) and N fixation (1.4%) were predicted too. Previously, a collection of bacterial isolates obtained from the same plants, showed tolerance to metals (Cd, Zn, Cu) in vitro, as well as multiple PGP-properties, see Table S2 (Sánchez-López 2015).

Discussion

The bacterial endophytic communities of three consecutive generations of *C. pumila* seeds collected from metal mine residues were investigated and compared in this study. Firstly, our results showed that the richness (Chao 1 estimator) and diversity (Simpson index) of the seed endophytic bacterial community did not change significantly across the three generations (Table 1). These results are contrasting with those obtained by Truyens et al. (2016) and Hardoim et al. (2012) who found decreases in the numbers of bacterial genera across generations of Cd-exposed *Arabidopsis thaliana* and rice (*Oryza sativa*) seeds, correspondingly. Moreover, we did not observe significant differences in the numbers of observed OTUs (Table 1). Truyens et al. (2016) reported that the numbers of OTUs decreased across consecutive generations of *A. thaliana* seeds, suggesting that only a minor part of the seed bacterial endophytes is transferred to consecutive generations. However, these differences can be explained by the conditions under which plants were grown and sampled. Truyens et al. (2016) harvested seeds and sowed them again under greenhouse conditions for up to 14 seed generations. The selection pressure from the environment (contaminants, climate, etc.) on the mine tailings and also the soil microbiome (Johnston-Monje et al. 2016) definitely influence the seed microbiome, and are difficult to compare to the controlled conditions from the study by Truyens et al. (2016). Moreover, there is also an obvious difference in duration of the selection pressure; therefore, only those bacteria able to cope with the harsh environments of the mine tailings year after year are expected to shape the final seed endophytic community.

Our results showed that for most of the *C. pumila* seed samples, endophytes belong to the Proteobacteria, more precisely to the Alphaproteobacteria followed by the Gammaproteobacteria (Fig. 2). These two classes were also observed in seeds of *A. thaliana* that were exposed during several generations to Cd (Truyens et al. 2013, 2016). Actinobacteria and Firmicutes were found in all samples (Fig. 2); these two phyla have also been identified as seed endophytes of different plant species (Truyens et al. 2015), and specifically after metal exposure of plants as endophytes in seeds of tobacco

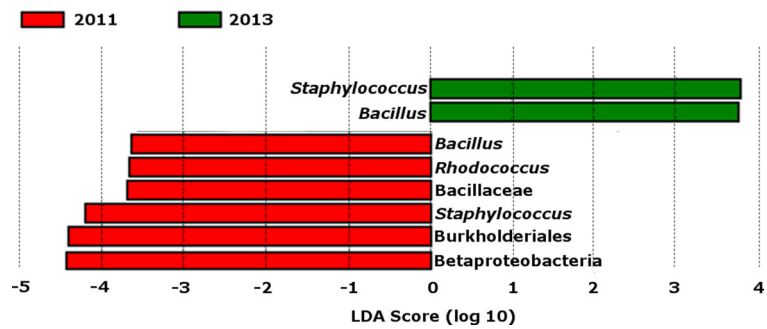

Fig. 5 Linear discriminant analysis (LDA) scores of organisms that are differential among seed endophytic communities of *Crotalaria pumila* colonizing multi-metal contaminated mine residues. Scores were obtained through LEfSe analysis

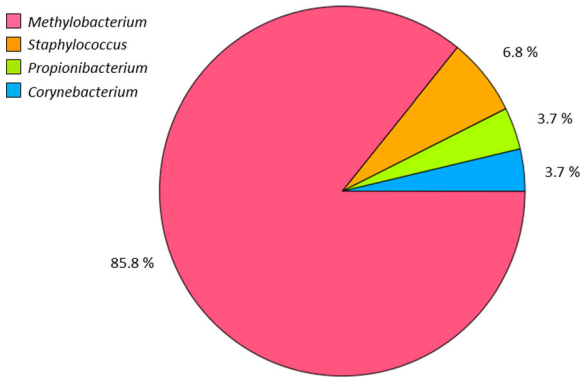

Fig. 6 Composition of the core microbiome of three consecutive generations of *Crotalaria pumila* seeds colonizing multi-metal contaminated mine residues

(Mastretta et al. 2009), *A. thaliana* (Truyens et al. 2013, 2016) and *Agrostis capillaris* (Truyens et al. 2014). The phylum Thermi has not commonly been reported as a plant endophyte; many representatives of this phylum are associated to extreme environments and are known for their resistance to severe stresses including radiation, oxidation, desiccation and high temperature (Griffiths and Gupta 2007). This phylum was reported as endophyte of Arctic and Antarctic lichens (Lee et al. 2014), as endophyte in rice roots (Sun et al. 2008), and as a member of the phyllospheric community of spinach plants (Lopez-Velasco et al. 2013). To our knowledge this is the first report of the phylum Thermi as a seed endophyte.

It has to be mentioned that the predominance of a phylum was driven by the high abundance of one OTU at genus level identified in each seed generation. For instance, the high abundance of *Methylobacterium* explains the high values observed for Alphaproteobacteria, as *Meiothermus* for Thermi, and *Staphylococcus* for Firmicutes (Fig. 2). This trend was earlier observed by Bodenhausen et al. (2013) when analyzing bacterial communities in leaves and roots of *A. thaliana;* the abundance of a certain phylum was determined by the most abundant OTU in each plant compartment.

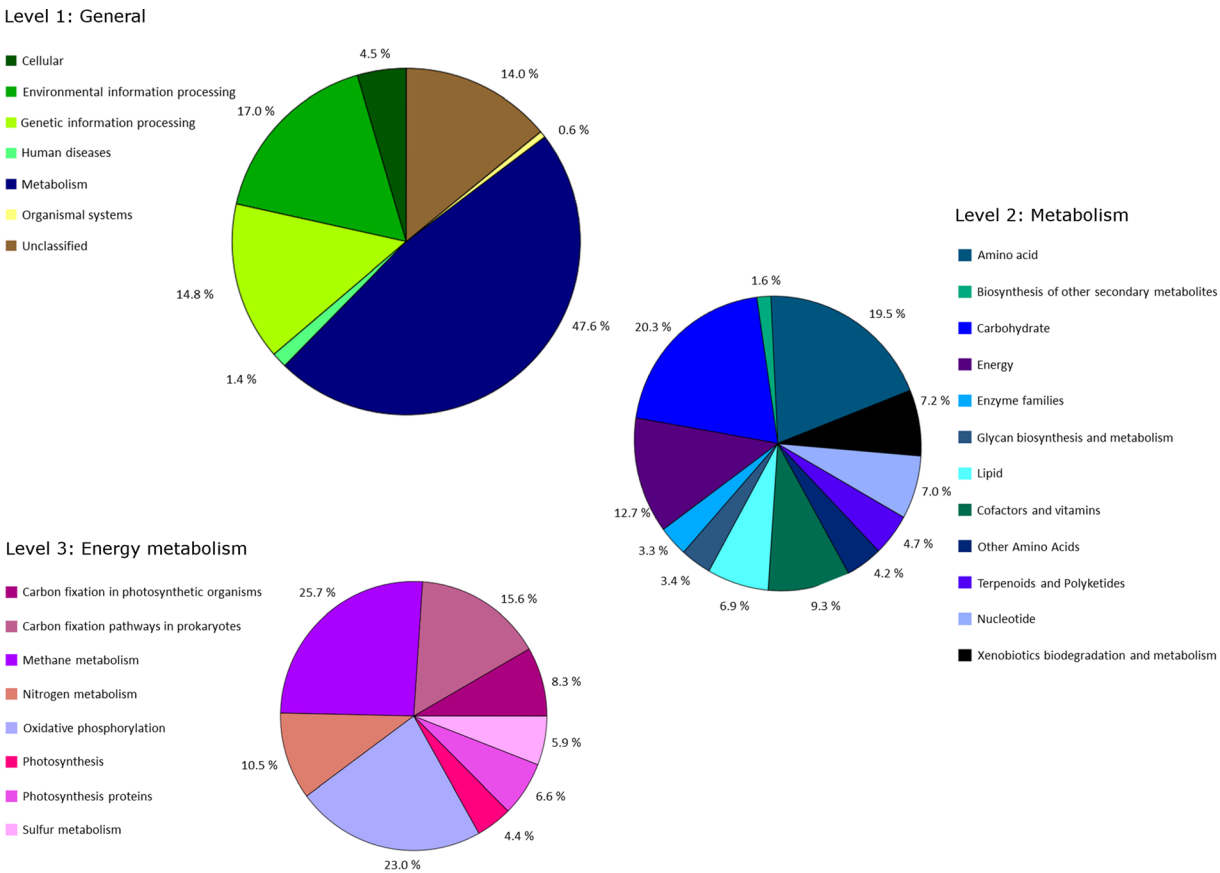

Fig. 7 Metataxonomic functions prediction of core microbiome of three consecutive seed generations of *Crotalaria pumila* seeds colonizing multi-metal contaminated mine residues. Function predictions were categorized on KEGG classification at level 3

The cultivation-dependent analyses of the endophytic bacteria of the same *C. pumila* colonizing mine residues over three generations performed previously (Sánchez-López 2015), lead to the identification of a total of 13 bacterial genera, 11 of them were also detected in this work using 454 pyrosequencing: *Methylobacterium*, *Staphylococcus, Sphingomonas, Kocuria, Bacillus, Mycobacterium, Streptomyces, Brachybacterium, Variovorax, Burkholderia* and *Arthrobacter* (Table 2, Table S1). This leads to the conclusion that several representative seed endophytes are well culturable, which has also been observed for leaf epiphytic bacteria recently (Burch et al. 2016).

It is also interesting to notice that, taking into account the few existing reports concerning seed endophytes of metal-exposed plants, there are some bacterial genera that are found commonly, and these were also identified in our study (Table 2). These genera are *Bacillus, Methylobacterium, Mycobacterium, Staphylococcus, Sphingomonas, Variovorax*, *Pseudomonas, Corynebacterium, Microbacterium, Aeromicrobium, Pseudonocardia, Sediminibacterium* and *Pantoea*. Therefore, it is conceivable that these 13 bacterial genera are important during seed germination and establishment of seedlings of their host plant in metal contaminated environments. To our knowledge, the following bacterial genera have not been reported as endophytes before: *Varibaculum, Rhabdochlamydia, Jeotgalicoccus, Planifilum, Facklamia, Desemzia, Carnobacterium, Limnohabitans, Meiothermus* (Table 2), *Luteococcus, Kaistobacter* and *Flavisolibacter* (Table S1). *Kaistobacter* (Xiao et al. 2016), *Flavisolibacter* (Hong et al. 2015) and *Meiothermus* (Pereira et al. 2015) were formerly identified in a substrate heavily contaminated with metals and metalloids. Thus, these bacterial genera present in seeds of *C. pumila* growing on metal-containing mine residues might originate from the substrate in which the plants were growing (Bulgarelli et al. 2013; Hardoim et al. 2008). To determine the source of the seed-endophytes, in a subsequent study also the bacterial community in the rhizosphere and surrounding bulk soil of the plants can be investigated.

The varying presence of certain seed endophytes across generations (Fig. 3), suggests that the composition of the seed endophytic community is a dynamic process through generations. Nevertheless, it has been shown that some members of seed-associated microbiomes can be conserved across plant generations (Hardoim et al. 2012; Truyens et al. 2016) and even through evolutionary and human selection boundaries (Johnston-Monje and Raizada 2011). In the present work the variations in presence/absence of certain OTUs represented only a limited fraction (from 4.4 to 12.0%) of the total seed endophytic community in the three seed generations of *C. pumila*.

Despite the variations in the composition of the seed endophytic community, 12 OTUs at genus level were consistently present in the three consecutive generations of *C. pumila* seeds growing on the metal containing mine residues: *Corynebacterium, Propionibacterium, Sediminibacterium, Bacillus, Kineococcus, Jeotgalicoccus, Brevibacterium, Staphylococcus, Methylobacterium, Pantoea, Sphingomonas* and *Micrococcus* (Fig. 3). Culturable strains of *Methylobacterium* and *Sphingomonas* were also isolated from the same three types of *C. pumila* seeds (Table S2; Sánchez-López 2015). This information suggests that the endophytic communities of seeds of *C. pumila* growing on mine residues maintain the core members of the bacterial endophytes.

Studies concerning bacterial endophytic community composition across different seed generations are scarce. However, Hardoim et al. (2012) mentioned that using PCR-based denaturing gradient gel electrophoresis of rice seed-extracted DNA, approximately 45% of the bacterial community from the first seed generation was found in the second generation as well. In a study of the total community using 454 pyrosequencing of successive generations of *A. thaliana* seeds exposed to Cd, it was found that only one OTU, corresponding to *Rhizobium*, was transferred from one generation to the next one (Truyens et al. 2016).

It is mentioned that both plants and microbiome sustain the functionality and fitness of each other (Berg et al. 2015), especially during acclimatization to local environmental conditions (Johnston-Monje and Raizada 2011; Vandenkoornhuyse et al. 2015). Microbes that are beneficial for the host plant are expected to be transferred to successive generations and eventually become permanently settled in the host population (Bibian et al. 2016). Thus, it is conceivable that bacteria transgenerationally preserved can contribute to the establishment and further growth of new generations of plants. Previously, we isolated different bacteria from the same *C. pumila* plants and these strains exhibited various functional traits corresponding to PGP properties (Table S2). For instance, strains of *Methylobacterium, Sphingomonas, Staphylococcus,*

and *Bacillus*, were found in three seed generations in this study (Fig. 3); the majority possesses tolerance to Zn, Cd, Cu, and also presented organic acid production, phosphate solubilization capacity, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Table S2). Previously, also one of the dominating seed endophytes of the same *C. pumila* plants, a *Methylobacterium* sp. Cp3 (MNAO01000000) was genome sequenced. The *Methylobacterium* sp. Cp3 genome encodes multiple P-type ATP metal transporters which can be involved in Cu and Zn resistance, and homeostasis. *Methylobacterium* sp. strain Cp3 also displayed genes involved in phytohormone production that can improve plant fitness on extreme soils (Sanchez-Lopez et al., submitted). Thus, previously isolated (Table S2) and genome sequenced *C. pumila* seed endophytes (Sanchez-Lopez et al., submitted) were identified also in this work using 454 pyrosequencing (Fig. 3), and these endophytes are metal tolerant and have functional traits to improve nutrient acquisition (phosphate solubilization, phytate mineralization, siderophores production) or reduce levels of the stress hormone ethylene by ACC-deaminase activity. Certainly, these characteristics are helpful for the establishment of *C. pumila* in a restrictive metal-contaminated environment like mine residues in Zimapan. Moreover PICRUSt results showed the core microbiome of *C. pumila* seeds presents genes related to N and C fixation, photosynthesis, oxidative phosphorylation and methanol metabolism (Fig. 7). The ability to use 1-C compounds as energy source (methanol metabolism) represents an advantage over other endophytes (Dourado et al. 2015); this characteristic can result in a more efficient endophytic colonization (Sy et al. 2005). The presence of genes involved in N metabolism suggests that the core microbiome favors acquisition of this element (de Voogd et al. 2015) for its host plant. Wang et al. (2015) observed that the oxidative phosphorylation pathways of radish were altered when it was exposed to Cd. Since in the present work functions related to oxidative phosphorylation were predicted, it is feasible that the seed microbiome is assisting *C. pumila* to overcome trace metal stress. These observations together with its importance for photosynthesis and C fixation (Fig. 7) suggest that the seed microbiome might participate in trace metal stress alleviation, and promote plant growth and biomass production of its host plant.

Furthermore, other OTUs that were not part of the core microbiome have been found as *C. pumila* seed endophytes that can bring benefits to their host plant in metal contaminated environments (Sánchez-López 2015). *Brachybacterium, Kocuria, Streptomyces, Burkholderia, Variovorax* and *Arthrobacter* were observed in one or two seed generations of *C. pumila* seeds in the present study (Fig. 3, Fig. S1), and were previously tested for PGP properties and metal tolerance giving positive results (Table S2). Moreover, other bacterial genera identified in *C. pumila* seeds were found as beneficial endophytes of different plant species in unfavorable environmental conditions; for instance *Micrococcus* (Qin et al. 2015), *Jeotgalicoccus* (Yadav et al. 2015), *Microbacterium* (Chimwamurombe et al. 2016), *Acinetobacter* (Joe et al. 2016), *Gluconacetobacter* (Bertalan et al. 2009).

The final shape and functions of the seed microbiome is a complex process that can be affected by several factors, including environmental ones and those inherent to plant host genotype characteristics. Nevertheless, our results showed that the dominating seed endophytes may provide their host plants with many benefits that can well cover the initial requirements of germinating seeds and establishing seedlings (Hardoim et al. 2008; Johnston-Monje and Raizada 2011; Truyens et al. 2014). Undoubtedly, improving photosynthesis and plant nutrition, and stress alleviation (Arshad et al. 2007; Hardoim et al. 2008) are important factors for plant establishment in limiting or stressful conditions. The aforementioned traits of the core microbiome of the three seed generations, eventually complemented with the functional characteristics of the other seed endophytes, might explain the success of *C. pumila* to complete its full life cycle on a multi-metal contaminated substrate, tolerating high metal concentrations and the intrinsic environmental restrictions of the mine residues in the semi-arid climate in Zimapan.

The results obtained in the present work demonstrated the presence of seed endophytes that can be beneficial for the host plant. Culture-collections showed that the gene families are active (Sánchez-López 2015) and seed bacterial endophytes can be important for host plant survival and establishment on metal-polluted soil. Consequently, bacterial seed endophytes of *C. pumila* colonizing mine residues represent an alternative for future field applications of endophyte-assisted phytoremediation. For some bacterial seed endophytes, functional traits that are beneficial for plants have been demonstrated in in vitro conditions. For many yet-to-be-cultured strains application of gene profiling or gene

expression with metagenomic and metatranscriptomics approaches can help to elucidate evidences about their possible roles (Guttman et al. 2014; Xu et al. 2015).

In this study we only investigated the seed endophytic bacteria; undoubtedly also other endophytes, e.g. fungi contribute to shape the endophytic community and plant survival in harsh conditions. For a better understanding of endophytes and their relation with their host plants in metal contaminated environments, future work should also explore interactions between endophytes and the dynamics of these communities along the plant life cycle. This knowledge will allow to optimize microorganism-assisted phytoremediation of metal contaminated soils.

Conclusions

The composition of the total endophytic communities of three consecutive generations of pioneer plant species *C. pumila* seeds growing on multi-metal contaminated mine residues was studied. We found that the composition of the total bacterial endophytic community in seeds did not alter significantly across successive generations. *Methylobacterium, Staphylococcus, Corynebacterium, Propionibacterium, Sediminibacterium, Bacillus, Sphingomonas, Kineococcus, Brevibacterium, Pantoea, Micrococcus, Jeotgalicoccus* and one OTU of the Enterobacteriaceae family were found as common taxa in the three generations that were investigated. These endophytes constituted more than the 60% of the total community from each sampling year. Moreover, our results indicate the existence of a core microbiome occurring in *C. pumila* populations across three successive seed generations. The most representative endophyte of the core microbiome was *Methylobacterium,* which possesses plant growth promotion traits and is metal tolerant. Our results revealed that the existing bacterial genera present in seeds and preserved across generations, possess functional traits which can result in beneficial outcomes for their host plant when growing in restrictive environments, like metal polluted mine residues. The information presented here highlights the relevance and drives future avenues in using seed-endophytes occurring in natural systems for a more efficient assisted phytoremediation of metal polluted sites.

Acknowledgements This research was supported by a BOF-BILA grant from Hasselt University, the Hasselt University Methusalem project 08M03VGRJ and was also part of the CONACYT project PDCPN2013-1-215241.

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