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Metagenomics of mine tailing rhizospheric communities and its selection for plant establishment towards bioremediation

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A B S T R A C T

Mining operations often generate tailing dams that contain toxic residues and are a source of contamination when left unconfined. The establishment of a plant community over the tailings has been proposed as a containment strategy known as phytostabilization. Previously, we described naturally occurring mine tailing colonizing plants such as *Acacia farnesiana*, *Brickellia coulteri*, *Baccharis sarothroides*, and *Gnaphalium leucocephalum* without finding local adaptation. We explored the rhizosphere microbes as contributors in plant establishment and described both the culturable and *in situ* diversity of rhizospheric bacteria using the 16S rRNA gene and metagenomic shotgun sequencing. We built a synthetic community (SC) of culturable rhizosphere bacteria from the mine tailings. The SC was then the foundation for a serial passes experiment grown in plant-derived nutrient sources, selecting for heavy metals tolerance, community cooperation, and competition. The outcome of the serial passes was named the 'final synthetic community' (FSC). Overall, diversity decreased from *in situ* uncultivable microbes from roots (399 bacteria genera) to the cultivated communities (291 genera), the SC (94 genera), and the lowest diversity was in the FSC (43 genera). Metagenomic diversity clustered into 94,245 protein families, where we found plant growth promotion-related genes such as the *csgBAC* and *entCEBAH*, coded in a metagenome-assembled genome named *Kosakonia* sp. Nacozari. Finally, we used the FSC to inoculate mine tailing colonizing plants in a greenhouse experiment. The plants with the FSC inocula observed higher relative plant growth rates in sterile substrates. The FSC presents promising features that might make it useful for phytostabilization tailored strategies.

1. Introduction

Unconfined mine tailings (MTs) are harsh environments for plant colonization due to their extreme conditions, such as pH, heavy metals, and reduced water retention capacity. Additionally, the presence of autotrophic bacteria contributes to the lowering of the pH and increases the bioavailability of heavy metals (Schippers et al., 2000). Dispersion of toxic waste derived from MTs through hydric or eolic erosion in desertic areas is associated with human diseases, while plants can reduce MT erosion (Meza-Figueroa et al., 2009; Nriagu, 1988). Mine reclamation should be the ideal scenario at the end of the mine operations; however, there is no regulation for old abandoned mines in multiple developing countries (Favas et al., 2018). Open mining reclamation strategies include phytostabilization, which aims to reduce pollutant motility by plant coverage.

Phytostabilization is an *in situ* bioremediation approach that

Abbreviations: SC, synthetic community; FSC, final synthetic community.

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prevents the exposure of MTs to the environment using local plants and their associated microbiota to prevent erosion through MT root consolidation (Mendez and Maier, 2008). Moreover, this bioremediation strategy looks for plant communities to immobilize the metals in their roots with low shoot translocation while recruiting heterotrophic bacteria and stabilizing the wasteland (Ali et al., 2013). Additionally, the plant roots microbiota and plant roots can precipitate heavy metals through the biofilm polysaccharides and exudates, respectively (Salt et al., 1999). A plant species is a candidate for phytostabilization of heavy metals if they are not translocated to shoots, keeping most of them precipitated in the soil or root-accumulated, thus preventing incorporation of heavy metals into trophic networks (*e.g.,* through cattle foraging; Santos et al., 2017). The plant colonization of MTs increases some functional groups, *e.g.*, some trees and shrubs, increasing the soil fertility and bacterial diversity, which might promote plant growth (Colin et al., 2019).

Experimental evolution demonstrated that it is possible to select bacteria for a desired phenotypic trait. A paramount example is the long-term evolution experiment (LTEE) that started with 12 *Escherichia coli* cell lines. LTEE cells were cultured in a liquid glucose-limited medium and put through a bottleneck of 1 % of the whole population in each serial pass into a fresh medium every 24 h. After the first 2000 generations, the populations presented a higher fitness than the original cell lines growing in glucose-limited conditions (Lenski and Travisano, 1994). The experiment is now over 60,000 generations, with the most dramatic fitness effects described in the first generations (Good et al., 2017). Bacterial communities harbor different interactions, such as cooperation, inhibition, and competition. Nevertheless, some community members might remain neutral and avoid all interactions, resulting in community-intrinsic properties, or properties of bacteria that are shown only at the community level (Madsen et al., 2018). A new member introduced into the community will likely be sensitive to the antagonism effectors of the original members (Pérez-Gutiérrez et al., 2013). Building synthetic communities (SCs) involves scaling up the experimental evolution from one- or two-species interactions by integrating the emergent members and molecular interactions of complex model systems. These communities must also be culturable to allow experimental testing: from genome interactions to phenotype outputs (Cairns et al., 2018; Zomorrodi and Segrè, 2016).

Plant-microbe interactions are critical for plant establishment, nutrient acquisition, and microbe interactions, including parasitic or mutualistic interactions affecting the plant's health (Bulgarelli et al., 2013). The plant root-microbe interactions are essential for establishing the plant's microbiome; some community members are plant growth-promoting bacteria (PGPB). PGPB benefits plants through multiple strategies, such as nitrogen fixation, phosphate acquisition, and plant-hormone production (Liu et al., 1992; Rodriguez et al., 1999; Spaepen et al., 2007; Glick et al., 1998; de Zélicourt et al., 2018). Bacteria can also immobilize heavy metals through biosorption and dissimilatory reduction while modulating their micronutrient concentration homeostasis through cation efflux pumps (Valls and De Lorenzo, 2002; Nies, 1999). Biofertilizers have been widely proposed as a sustainable and eco-friendly approach to promote plant growth (Bhardwaj et al., 2014). However, single species biofertilizers lack the community interactions described previously, and even from simple communities ($\leq$ 3 bacteria species), synergistic effects could lead to effective bio-augmentation and improvements in plant growth (Mansotra et al., 2015). Likewise, PGPB are challenged by the receiving rhizospheric ecosystem upon inoculation (Martínez-Viveros et al., 2010). Therefore, the design of biofertilizer inocula should try to include communities (3 bacteria species), which have shown higher stress protection on the plant host than single strain treatments even 45 days post-inoculation (Molina-Romero et al., 2017). The designing of synthetic bacterial communities is a newly developing field in the study of plant-microbe interactions, with a goal of influencing the interacting host phenotypes (Herrera Paredes et al., 2018).

Previously, we have reported spontaneous plant colonization of an abandoned (*ca.* 1949) copper MT deposit in Nacozari de García, Sonora, Mexico (Nacozari MT; Santos et al., 2017). Vegetal growth in MTs is a goal for phytostabilization strategies and we identified multiple plant species capable of bioremediation uses in the Nacozari MT: *Acacia farnesiana, Brickellia coulteri, Baccharis sarothroides,* and *Gnaphalium leucocephalum* (Santos et al., 2017), some of them belonging to plant genera such as *Baccharis* that have been found colonizing other MTs in Mexico (Salas-Luévano et al., 2017). The MT deposit lies adjacent to the residential zone (Supporting Information Fig. S1) and contains quartz (SiO_2), gypsum ($CaSO_4H_2O$), lepidocrocite (FeO[OH]), and copper sulfate ($CuSO_4$, Romero et al., 2008; Meza-Figueroa et al., 2009). The MT mean pH is 3.8 ± 0.3 and its average electrical conductivity is 340.1 ± 2 μS/cm (Meza-Figueroa et al., 2009). These substrates contain several heavy metals at phytotoxic concentrations as defined by the Environmental Protection Agency of the United States: Ni 69.5 ± 15.5 mg kg^{-1}; Cu 400.5 ± 15.8 mg kg^{-1}; Mo 58.3 ± 3.5 mg kg^{-1}; Ag 47.9 ± 2.3 mg kg^{-1}; Ba 423.1 ± 140 mg kg^{-1} (Meza-Figueroa et al., 2009).

Similar observations of mine tailing pioneer plants with phytoremediation potential, including species of the *Acacia* and *Baccharis* genera, have been previously found in the United States and Australia (Haque et al., 2008; Kabas et al., 2017). Other plant taxa have also been found colonizing mine tailings, such as *Miscanthus sinensis* in south east Asia, which present an increased metal accumulation in their root tissue and a rhizospheric community with a reduced alpha diversity in comparison with control plants (Sun et al., 2021). There are reports of MT colonizing plants observed in semi-arid regions of Mexico (Salas-Luévano et al., 2017), and their rhizospheric bacteria have been tested for heavy metal tolerance (Román-Ponce et al., 2016) and reported as PGPB (Navarro-Noya et al., 2012; Román-Ponce et al., 2017). Since assays on the pioneer plants on the Nacozari de García MT showed no differences in both substrate physicochemical composition nor local adaptation (Santos et al., 2017), a viable explanation of the establishment of these plants on the MT substrate is the effect of their associated microbiota. Until now, the taxonomic and functional diversity of microorganisms in the Nacozari MT have been kept unknown, and an assay of these communities could lead to an enhancement of a phytostabilization based reclamation strategy on the site.

In this work, we explore the microbial community role in plant establishment in this location. We describe both culturable and culture-free microbiomes from Nacozari MT rhizosphere associated communities through 16S rRNA gene and shotgun metagenomic sequencing. Furthermore, we have assembled a collection of rhizospheric culturable isolates into a SC. The SC was grown in serial passages (22 passes), looking for a culturable microbial community capable of surviving with MT conditions, depending on carbon sources derived from roots, and interacting with the other bacteria community members (Fig. 1). After serial passes, the resulting outcome was the final synthetic community (FSC), and this was used as an inoculum in greenhouse experiments with sprouted *A. farnesiana* plants to test the FSC's effects on plant development.

2. Material and methods

2.1. Sampling

The town of Nacozari de García, Sonora is a mining district in northern Mexico (30°22′2.4″N, 109°41′38″O). It has a semi-arid climate with an annual mean temperature of 19.6 °C and annual mean precipitation of 490 mm. Root samples were collected on December 1st, 2015, for amplicon and metagenomic DNA sequencing of the following plant species growing on MT and were recognized as candidates for phytostabilization by Santos et al. (2017): *A. farnesiana* (Afp1), *B. coulteri* (Bc), *B. sarothroides* (Bs), and *G. leucocephalum* (Gl), as well as an additional sample from an *A. farnesiana* plant growing in a secondary vegetation patch (Afp2, Supporting Information Fig. S1; Blaz Sánchez, 2017).

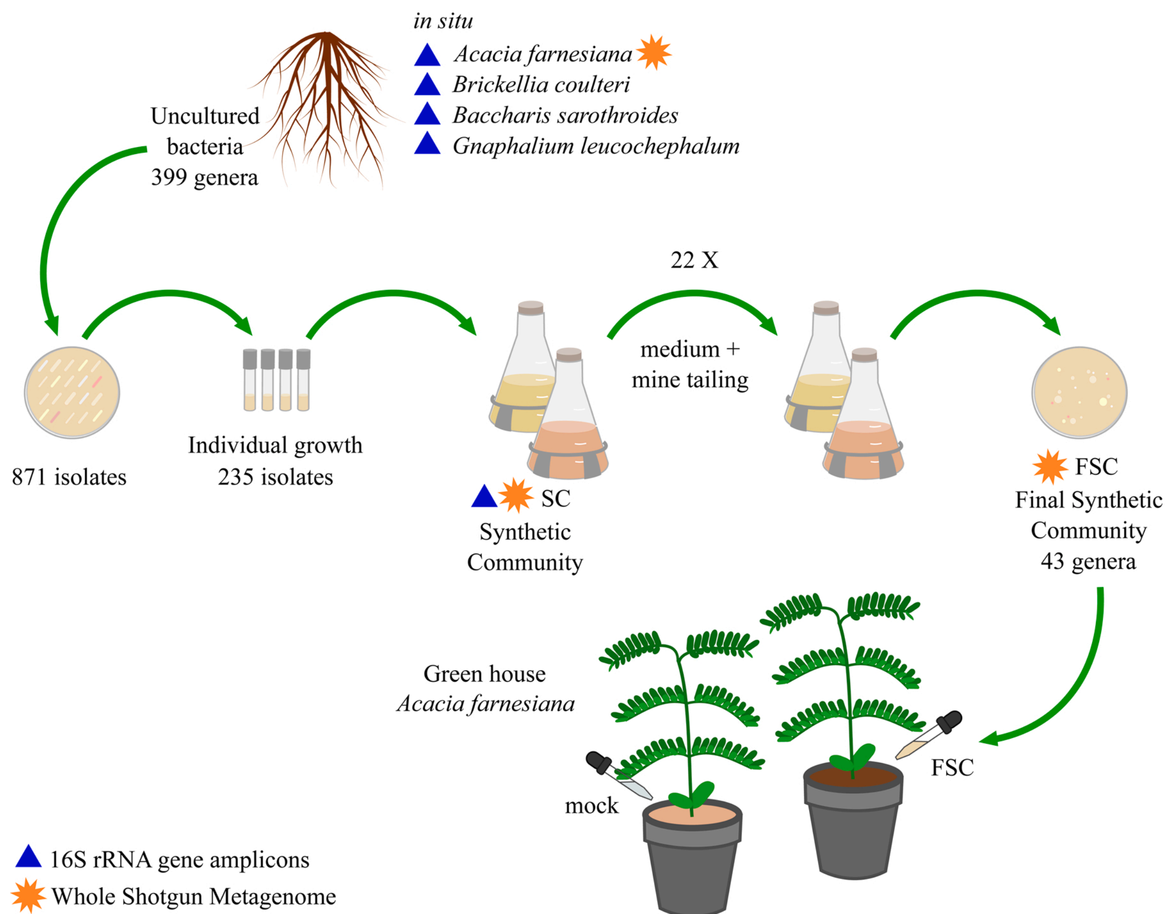

Fig. 1. Overview of this work. *In situ* biodiversity of the Nacozari mine tailings (MTs) was described both by 16S rRNA gene amplicons and metagenomic shotgun sequencing. A total of 235 rhizosphere-derived bacterial strains were selected to assemble the synthetic community (SC). The SC was cultured in plant-derived culture media or complex sugars, with an inhibiting MTs concentration for heavy metal unadapted bacteria (see Experimental Procedures). We executed 22 consecutive passes of the SC, each for 48 h, and the last serial pass was known as the final synthetic community (FSC). We predicted metabolic and taxonomic profiles from shotgun metagenomes. The FSC was used as inoculum in greenhouse experiments to evaluate its effect on plant growth.

Duplicated root tissue was *in situ* collected and approximately filled up the volume of 50 mL sterile centrifuge tubes and immediately stored in liquid nitrogen then stored in a freezer at −80 °C until metagenomic DNA extraction. Sampling for microbiological cultures was collected on March 29th, 2016. Duplicated root samples from *A. farnesiana* (Afp1−CC), *B. coulteri* (Bc−CC), *B. sarothroides* (Bs−CC), and *G. leucocephalum* (Gl−CC) from the main tailing vegetation patch, and an additional sample from an *A. farnesiana* (Afp2−CC) plant growing in a secondary vegetation patch (Supporting Information Fig. S1), were collected as described above, with the exception that the 50 mL sampling tubes contained 30 mL of sterile phosphate-buffered saline (PBS) solution and stored at 4 °C until processing.

2.1.1. Microbiological procedures

The sampled tubes from plant roots were shaken and serially diluted; dilutions 10^{-3} and 10^{-5} were cultured in solid Luria Agar (LA) and Peptone Yeast (PY) media. The Petri dishes were then incubated (28 °C). Single colonies were isolated and colony-forming units (CFU) were estimated from colony counts with averages of 10^{6} CFU. Isolated colonies were screened for the following morphological traits: form, border, elevation, surface, consistency, color, and transmitted and reflected light (Fig. S2). Results from the morphological variables were used as input to a multiple classification analysis (MCA) ordination to select representative colony diversity, resulting in the selection of 235 isolates (Supporting Information Fig. S2). Each isolate was grown in LB liquid media for 42 h. Absorbance was followed at 600 nm in a Genesys 20 spectrophotometer (Thermo Scientific). Each isolate was classified as slow, medium, and fast growing (Supporting Information Fig. S3).

2.1.2. Experimental evolution setup

The 235 representative isolates of bacterial morphological diversity were chosen as input for an experimental evolution microcosm (Supporting Information Fig. S3). We denominated this 235 isolate subset as the synthetic community (SC). Our goal was to assemble a SC capable of tolerating MT heavy metals, using plant-derived or complex carbon sources as the main building block for their metabolism, and capable of establishing as a community and thus forming relationships of mutualism and competition. Each one of the 235 SC isolates was grown until reaching an $OD_{600} \sim 0.4$, and then 100 μL of culture was transferred to fresh LB liquid media and all of the isolates were used as the source for the experimental evolution setup.

As the first selective constraint, we determined the concentration (w/v %) of the Nacozari MTs (grinded MT agate mortar, two rounds of autoclave sterilization) capable of inhibiting the growth of two type strains. *Escherichia coli* BW25113 and *Pseudomonas koreensis* were used as a growth control to discard competition from non-adapted strains. Strains were kindly donated by Dr. Luis Servín and Dr. Gloria Soberón, both from *Instituto de Investigaciones Biomédicas,* UNAM. We chose the selecting condition of 16 % (w/v) MT, as it reduces growth one-fold when compared to LB media (Supporting Information Fig. S4). The selection of plant-derived or complex carbon sources (mannitol) was made by using two independent media culture: 1) Soybean (*Glycine max*)

sprouts sterilized homogenate as a sole C, N, and P source; 2) LB media with mannitol (3% w/v) as a sole carbon source. There were two independent lines of each culture media, each one with duplicates.

The SC was inoculated into 30 mL of each experimental medium. After SC inoculation, the flask was incubated for 48 h (28 °C, 70 rpm), then 1 mL of culture was transferred to a fresh medium; this procedure was repeated three more times. Then, twenty-two serial passes were performed as described above, with the new fresh medium. After the experimental evolution setup, bacterial colonies were re-isolated. The whole set of isolates was named the final synthetic community (FSC), stored in the *Facultad de Ciencias*, UNAM and under patent licensing.

2.1.3. Metagenomic DNA extraction

Plant roots were vortex-shaken and sonicated in sterile PBS solution to get the rhizospheric pellet, as described in Lundberg et al. (2012). The metagenomic DNA was extracted from the pellets with the MoBio® PowerSoil extraction kit (MoBio Laboratories, Solana Beach, CA, USA). For the cultured microbes, individual bacterial colonies from each plant, SC, and FSC were tooth-picked into a 2 mL centrifuge tube for each community, containing sterilized water, proteinase K and lysozyme (both from Sigma-Aldrich, St. Louis Missouri, United States) for initial lysis (37 °C, 30 min). The tubes were centrifuged, and the pellets were used as the source for DNA extraction with the MoBio® PowerSoil extraction kit.

2.1.4. Amplification of the 16S rRNA gene

The PCR reactions were carried out to amplify the V3-V4 region (341 F and 805R primers; 464 bp amplicon; Klindworth et al., 2013; Blaz Sánchez, 2017) following the Illumina® MiSeq™ protocol with 5′ overhangs. We performed triplicate PCR reactions for each sample, using high fidelity *Pfx platinum* polymerase (Invitrogen, Thermo Fisher Scientific Corporation, Carlsbad, California, USA) with the following conditions: denaturation at 95 °C for 3 min; 5 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 68 °C for 30 s, 25 cycles of two-step cycling with denaturation at 94 °C for 5 s, extension at 68 °C for 30 s, and final extension at 68 °C for 5 min. Finally, amplicons from each replicate were pooled and purified with the Wizard SV Gel and PCR Cleanup System kit (Promega Corporation, Madison, Wisconsin, USA).

2.1.5. DNA sequencing

Amplicons of the 16S rRNA gene were sequenced through Illumina MiSeq 2 × 250 paired-end technology (Illumina®, San Diego, California, USA) at the Unidad de Secuenciación Masiva of the Instituto de Biotecnología, UNAM (Cuernavaca, Morelos, Mexico). The whole shotgun metagenomic DNA from the rhizosphere of *A. farnesiana* growing on MT substrate and the SC were sequenced with Illumina® HiSeq 2000 (2 × 100) at Macrogen (Korea). Finally, the shotgun metagenomic DNA of the FSC was sequenced at the Genomic Services laboratory *Unidad de Genómica Avanzada* (UGA, LANGEBIO-CINVESTAV, Mexico) using Illumina® MiSeq 2 × 300 technology.

2.1.6. Bioinformatic analyses

Quality control of the 16S rRNA gene amplicon sequences was performed with FASTX-toolkit v0.0.14 (http://hannonlab.cshl.edu/fastx_toolkit/) and paired reads were merged with PANDAseq v2.11 (Masella et al., 2012). OTUs were built clustering the merged reads at 97 % identity with *cd-hit-est* v4.6 (Fu et al., 2012). The QIIME v1.9.1 pipeline (Caporaso et al., 2010) was used to assign taxonomy using the BLAST v2.2.22 algorithm (Camacho et al., 2009) against the chimera-checked 16S rRNA Greengenes database (release 13_8; DeSantis et al., 2006) and sequences from chloroplasts and mitochondria were removed. The relative abundances of the resulting OTU table were analyzed with the phyloseq library v1.24.2 (McMurdie and Holmes, 2013) for R v3.5.1 (www.r-project.org), removing singleton OTUs.

The whole metagenome shotgun (WMS reads) were processed with Trimmomatic v0.33 (Bolger et al., 2014), keeping sequences with a minimum length of 36 nucleotides and Phred quality >15 in sliding windows of 4 nucleotides. The whole shotgun metagenomic taxonomic profile was done using high-quality reads (HQ-reads), then mapped against the NCBI-nr-protein database with the Kaiju v1.6.2 program (Menzel et al., 2016). Additionally, WMS 16S rRNA gene fragments were recovered from the HQ-reads with SSU-ALIGN v0.1.1 (Nawrocki & Eddy, 2013), and taxonomy was assigned with BLAST v2.2.22 (Camacho et al., 2009) against the Greengenes database (release 13_8; DeSantis et al., 2006).

HQ-reads of each sample were assembled separately with MEGAHIT v1.1.3 (Li et al., 2016a,b), using minimum kmer size = 21, maximum kmer size = 141, kmer increment = 12, and removing unitigs with average kmer depth <2, and the resulting contigs were used to predict ORFs and protein sequences with Prodigal v2.6.3 (Hyatt et al., 2010). Protein sequences were clustered at 90 % identity with CD-HIT v4.7 (Fu et al., 2012) and the representative sequences were annotated against the M5nr database (Wilke et al., 2012) using DIAMOND v0.9.22 (Buchfink et al., 2015). An independent annotation was also done using the BlastKOALA server (Kanehisa et al., 2016). Unannotated proteins of all samples were clustered with CD-HIT v4.7 (Fu et al., 2012) at 70 % identity to add unannotated data to the protein content comparisons. For protein content comparisons, a protein feature table was built for each metagenome, including both the abundance of proteins annotated with the M5nr database and the abundance of unannotated protein families clustered at 70 % identity. The abundance of each protein was assigned through the mapping of HQ-reads against predicted ORFs with Bowtie 2 v2.3.4.2 (Langmead, and Salzberg, 2012). The gene table was inspected using phyloseq v1.24.2 (McMurdie and Holmes, 2013) for R v3.5.1 (www.r-project.org) through the Subsystems ontology (Overbeek et al., 2005), downloaded from the MG-RAST API (Wilke et al., 2015).

Metagenomic HQ-reads of the FSC dataset were mapped against *Enterobacter* sp. SA187 genome (RefSeq accession GCF_001888805.2; Andrés-Barrao et al., 2017) with Bowtie 2 v2.3.4.2 (Langmead, and Salzberg, 2012) and read coverage along the reference genome were visualized with CIRCUS v2.7 (Naquin et al., 2014) and the CGView server (Grant and Stothard, 2008). Metagenomic contigs of all samples were classified with Kraken v0.10.6 (Wood and Salzberg, 2014) and assigned contigs from each analyzed genus was mapped to a reference genome downloaded from the NCBI Assembly database using scaffold_builder (Silva et al., 2013), Quast v5.0.0 (Gurevich et al., 2013), and Mummer v3.0 (Kurtz et al., 2004). A genomic distance dendrogram was computed, as previously described (Barajas et al., 2019) for 25 genomes of *Enterobacter*, 8 of *Kosakonia*, and 4 of *Leolottia* spp., and the genome built from metagenomic reads. In summary, the predicted protein sequences of all genomes were searched against all with the DIAMOND program, then the reciprocal best hits from each pairwise comparison were recovered and used to estimate genomic similarity scores (GSS). An inverse distance matrix (1-GSS) was used to build a neighbor-joining tree using the DendroPy library for python 3.6 (Sukumaran and Holder, 2010). Detailed bioinformatics and statistical protocols are available at https://github.com/genomica-fciencias-unam/nacozari/.

2.1.7. Greenhouse experiments

Acacia farnesiana seeds were collected in Nacozari de García, Sonora, Mexico. No special permissions were required for the sampling location. *Acacia farnesiana,* also known as *Vachellia farnesiana,* is a pantropical species, not endangered, and listed as the least concern (LC) species in the red list of the International Union for Conservation of Nature (Botanic Gardens Conservation International [BGCI] & IUCN SSC Global Tree Specialist Group et al., 2020). Previously scarificated and primed seeds of *A. farnesiana* were planted in Petri dishes with agar at 1% W/V in a growth chamber (Conviron Ltd., Winnipeg, Canada) with a temperature of 27 °C and a 12/12 h light/darkness photoperiod. Seven days after germination, the seedlings were transferred into seedbeds and

watered with Murashige & Skoog plant salt mixture (M&S medium, MP Biomedicals, LLC) at 0.1 % W/V. The M&S medium was supplemented a second time after 25 d, and then after 50 d, the seedlings were transferred to 500 mL pots with peat moss and perlite (2:1). Treatment pots included 20 % Nacozari MT. The greenhouse treatments were FSC inocula and mock LB media inocula with: 1) substrate, 2) sterilized substrate, 3) MT-substrate mix, 4) sterilized MT-substrate mix, 5) mock LB and substrate, 6) mock LB into sterilized substrate, 7) mock LB with MT-substrate mix, and 8) mock LB into sterilized MT-substrate mix. For each treatment, a total of 13 replicates were made. The inoculation volume was 1.5 mL, FSC with an estimated 1.86×10^2 CFU. The inoculation was done directly over the primary root. Inoculation procedures were repeated every third day for 10 d: with a total of 4 inoculations per plant. The inocula effects to *A. farnesiana* were measured through a classic growth analysis described previously (Chiariello et al., 2000) and using the growth parameters described by Evans (Evans, 1973) and Hunt (Hunt, 1990). Two harvests were made at the beginning and at the end of the experiment (90 d later). The fresh and dry weights of the plant

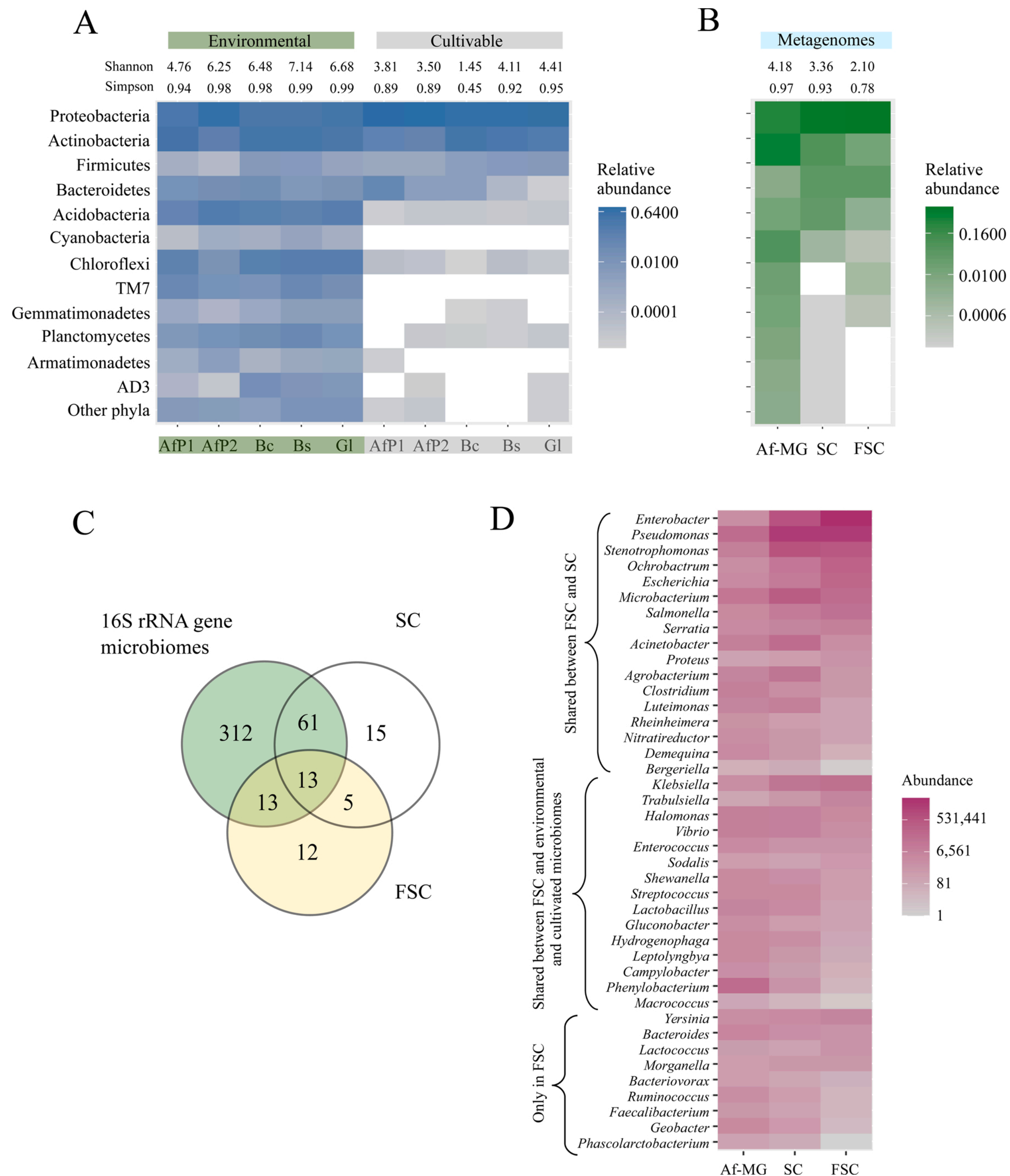

Fig. 2. Root-associated environmental and cultivable bacterial diversity of the Nacozari mine tailings. **(A)** Bacterial diversity declined in cultivated communities, as described by 16S rRNA gene amplicons and shown with Shannon and Simpson diversity indexes. Actinobacteria dominated the environmental samples and Proteobacteria dominated cultivated samples. **(B)** Taxonomic profiles derived from shotgun metagenomics 16S fragments binned from *Acacia farnesiana* roots, the synthetic community (SC), and the final synthetic community (FSC). **(C)** Genera shared between all the environmental and cultured samples. **(D)** Metagenomic binning and taxonomic placement using the NCBI's NR database for the 41 found genera reported for the FSC. Af: *Acacia farnesiana* (P1 and P2 are vegetation patches); Bc: *B. coulteri*; Bs: *B. sarothroides*; Gl: *G. leucocephalum.*

organs were recorded. The plant tissue was placed in paper bags and stored in an oven at 50 °C for 30 d for recording dry weights. A one-way ANOVA was used to analyze the plant growth indices, and a *post hoc* Tukey's HSD test was performed to define statistical differences between treatments ($p < 0.05$) with R v3.5.1 (www.r-project.org).

3. Results

3.1. Describing root-associated microbial diversity associated with mine tailing colonizing plants

Our first approach to explore the microbial rhizospheric diversity in the Nacozari MT, was the massive sequencing of V3-V4 regions of the 16S rRNA from metagenomic DNA and cultured bacteria. We sequenced a total of 2,072,065 paired-end reads (250 bp, 460 bp assembled) clustered into 32,590 operational taxonomic units (OTUs, 97 % identity) from the environmental metagenomic DNA (Table S1). The Bc roots had the largest observed richness, with 8868 OTUs, followed by Bs (8742 OTUs), AfP2 (5914 OTUs), Gl (5485 OTUs), and AfP1 with the lowest OTU count (3581).

A total of 871 isolates were recovered from the rhizospheres of the pioneer plants growing on the MT deposit at Nacozari de García (Experimental Procedures). Among them, 343 recovered from the roots of *A. farnesiana* in two vegetation patches (AfP1 and AfP2), 218 from *B. sarothroides* (Bs), 165 from *B. coulteri* (Bc), and 145 from *G. leucocephalum* (Gl). From the cultured bacterial communities (CC), we obtained 9271 OTUs, with the largest richness observed in Gl−CC (2716 OTUs), then Bs−CC (2142 OTUs), Afp1- and Afp2−CC (1848 and 1847 OTUs, respectively), and finally Bc−CC (718 OTUs).

Expected richness, expressed as Chao1 index, had results within the same magnitude of OTUs, with an average observed OTU to Chao1 ratio of 93.18 ± 0.04 %, indicating comprehensive community coverage with our sequencing depth (Table S1). Diversity, expressed as the Shannon index (H'), showed that environmentally derived sequences had larger values ($H'_{(average)} = 6.26 \pm 0.8$ SD) than the cultured bacteria ($H'_{(average)} = 3.45 \pm 1.04$), as expected (Fig. 2A). Larger dominance was also observed (with the inverse Simpson index (D)) in environmental ($D_{(average)} = 0.976 \pm 0.01$) than in culturable ($D_{(average)} = 0.82 \pm 0.18$) MT rhizosphere bacteria.

In most environmental MT samples, the dominant bacterial phylum was Actinobacteria and showed fewer sequences for Proteobacteria when compared to CCs (Fig. 2 and Table S2). The CC bacteria were dominated by Proteobacteria relative abundance (*Rel. ab.* = 80.06 %) and Actinobacteria to a lesser extent (*.ab.* = 18.47 %, Table S3).

3.2. Selection of a synthetic community

To merge the cultivated diversity into a representative community and test it for plant growth promotion, we classified the 871 isolates from all plants into 105 unique morphotypes, through a multivariate morphology analysis (Supporting Information Fig. S2). We evaluated morphological diversity and selected a subset of 235 representative isolates to assemble the synthetic community (SC, see Experimental Procedures). The SC was the input for an experimental evolution setup resulting in a total of 144 isolates representing ten morphotypes recovered from the serial passes, and these made up the FSC (see Fig. 1 and Experimental Procedures).

3.3. Shotgun metagenomic diversity of environmental and cultivated communities

To describe the overall diversity of the synthetic communities and compare them to an environmental sample, we analyzed the MT rhizospheres of *A. farnesiana* growing on vegetation patch 1 (Af-MG), the SC, and the FSC through whole metagenome shotgun sequencing. The Af-MG metagenome included 3.3×10^7 reads spanning 3.3×10^9 bp and the GC % content was 58.71 %. The SC metagenome comprised 3.6×10^7 reads adding up to 3.6×10^8 bp with a GC % of 62.02 %. The FSC metagenome had 2×10^7 reads spanning 6.1×10^9 bp with 59.5 GC% (Table S1).

With 399 bacterial genera identified in the rhizospheric communities, and a total of 88 genera found in the Af-MG and 94 were identified in the SC, using only the reads matching to the V3-V4 regions of the 16S rRNA gene, sharing 18 genera out of 43 with FSC (Fig. 2C and Table S4). From the shared genera, the most abundant ones (*Pseudomonas* and *Ralstonia)* were also found in all the MT growing plants (17.39 and 9.69 %, respectively). Another abundant genus, *Enterobacter*, was only observed in the rhizosphere of some plants, such as Bc. The SC also included 10 unique genera, which were all observed in <0.03 % of the metagenomic reads. Additionally, most FSC reads (54.34 %) were mapped to *Enterobacter* sequences (Fig. 2D and Table S5).

Non-bacterial sequences in the metagenomic samples were also found: eukaryotic reads represented 2.39 % of the Af-MG metagenome, 0.14 % of the SC metagenome, and 0.06 % of the FSC metagenome. The genera with the highest number of mapped metagenomic reads in all three metagenomes were the fungi: *Rhizophagus,* with 14,965 reads in Af-MG (and with 54 and 4 reads in the SC and FSC metagenomes, respectively); *Coniochaeta* with 2600 reads in the SC metagenome (and with 567 and 2 reads in Af-MG and FSC samples, respectively); and *Rhizoctonia* with 613 reads in the FSC metagenome (and with 2641 and 525 reads in Af-MG and SC datasets, respectively). Archaea sequences were also found, representing 0.24 %, 0.02 %, and 0.005 % of the metagenomic reads in the Af-MG, SC, and FSC samples, respectively. In all samples, the most abundant group was the phylum *Euryarchaeota* and the *Halobacteria* class (Table S5).

3.4. Predicted protein differences from environmental to selected cultivated communities

Protein function diversity of the metagenomes was annotated by homology against the M5nr database, including hypothetical and conserved hypothetical proteins. Since many protein functions are still unknown, we also analyze all protein diversity by including hypothetical proteins without M5nr matching as 70 % identity protein clusters (see Experimental Procedures). A total of 318,157 proteins were found: 225,733 annotated proteins with the M5nr database and 92,424 hypothetical protein clusters. The environmentally derived Af-MG metagenome had the highest count of proteins, followed by the SC metagenome and then the FSC metagenome (173,717; 146,928; and 23,196 proteins, respectively). Interestingly, the SC metagenome had a higher Shannon index than the Af-MG metagenome (11.04 and 11.03, respectively). The FSC metagenome presented the lowest protein richness and Shannon index (8.78), although it had the most considerable matched amount of proteins to the M5nr database (90.78 %), compared with SC (77 %), and Af-MG (63.35 %) metagenomes (Fig. 3A).

Classification of the metagenome predicted coding genes with the SEED subsystems ontology revealed enriched categories for the synthetic communities (SC and FSC), compared to the environmental metagenome (Af-MG). The SC and FSC were enriched in membrane transport, amino acid and derivatives, cell wall and capsule, and iron acquisition and metabolism-related genes (Fig. 3B). Regarding stress and motility related genes, the FSC had the largest normalized read counts, with 3.68 % and 2.65 % of mapped reads, respectively. Specifically, from the stress genes, the osmoprotectant ABC transporter YehZYXW subsystem was most abundant in the FSC, with 0.2 % of mapped reads, while the SC mapped 0.02 % and not detected in Af-MG. From the amino acid and derivatives subsystem, degradation of arginine and ornithine were enriched in the FSC, with 1 % mapped reads (against 0.84 and 0.5 % in the SC and Af-MG, respectively). The FSC was also enriched in the conjugative transfer (1.34 % of mapped reads) and ABC dipeptide (0.87 % of mapped reads) transporters, in the membrane transport subsystem, and in enterobactin biosynthesis (0.58 %). The FSC

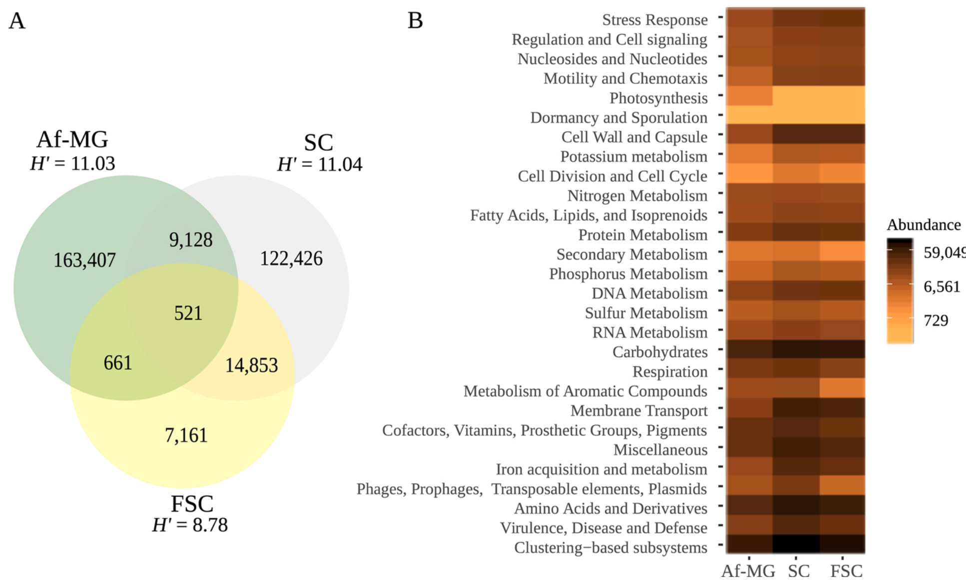

Fig. 3. The selected synthetic community reduces environmental and initial protein diversity, is enriched in subsystems associated with stress response and motility, and lacks photosynthesis genes. **(A)** Total predicted, shared, and unique proteins for wild *Acacia farnesiana* rhizosphere metagenome (Af-MG), compared to the protein numbers of the SC and FSC. Shannon's protein diversity index (*H'*) is decreased in the FSC compared to Af-MG and SC. **(B)** Metagenomic functional classification showing thousands of reads mapped to genes associated with the first level of SEED Subsystems.

had a high frequency of functions from the miscellaneous category (6.39 % of mapped reads), which includes ACC deaminase (COG2515). Some subsystems were only observed in the Af-MG metagenome, such as nitrogen fixation related proteins (including NifH, NifE, NifN, NifW, and NifO; Supporting Information Fig. S5). Some subsystems were detected in the SC but were lost after the microcosm experiment (FSC), such as the coenzyme F420 synthesis subsystem. Similarly, the indole acetamide hydrolase (involved in auxin synthesis) found in the Af-MG and SC metagenomes but not detected in the FSC metagenome (Supporting Information Fig. S5).

A core set of 521 proteins were shared between all metagenomes, including arsenic resistance proteins, metallic cation efflux systems, antibiotic resistance factors like the polymyxin resistance protein ArnT, and carbohydrate ABC transporter proteins (Supporting Information

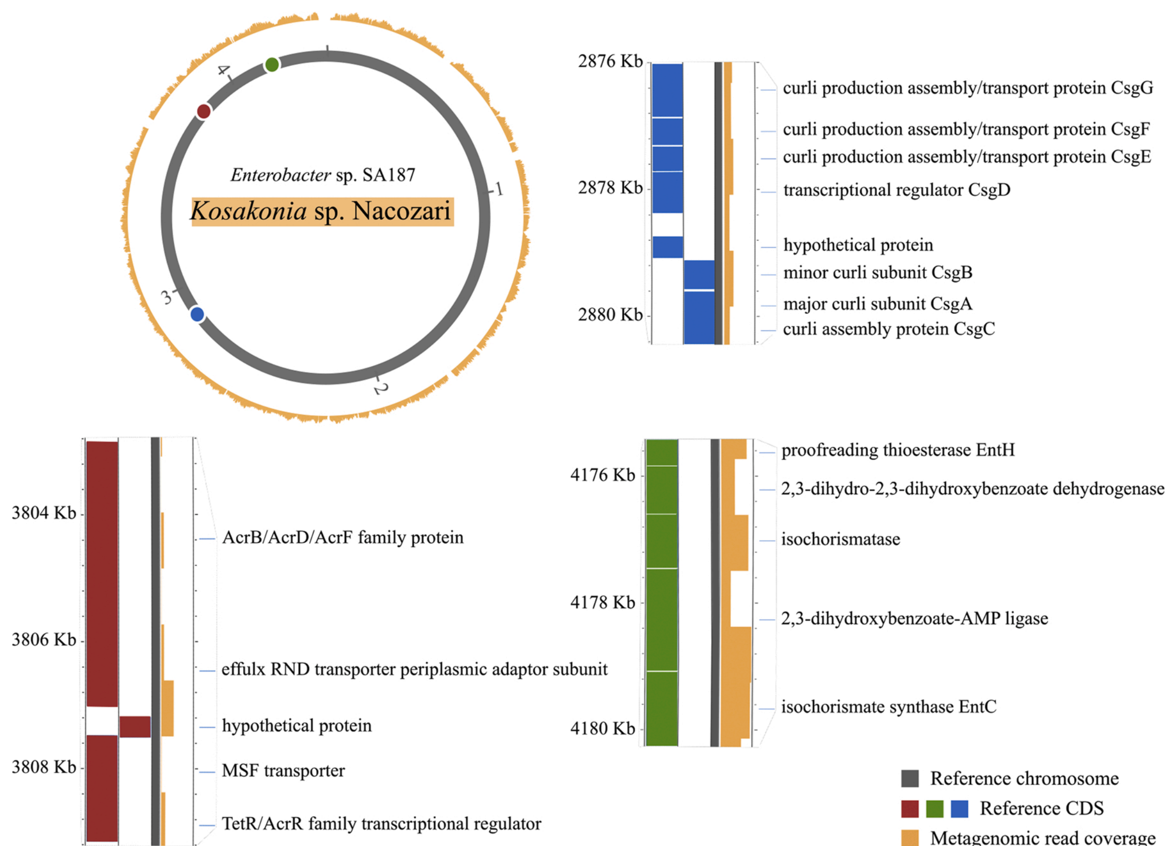

Fig. 4. Recruitment of FSC metagenomic reads against the reference genome of an already described plant-growth promoter bacteria, *Enterobacter* sp. SA187, including described genes associated with plant growth promotion. FSC metagenomic sequences (outer yellow ring) aligned to the *Enterobacter* sp. SA187 genome as a reference (scale in Megabases). Selected loci coverage involved in plant-growth promotion in *E.* sp. SA187, such as curli fiber subunits (*csgBAC)* and secretion genes (*csgDEFG)*, the multidrug efflux pump *acrAB*, and the enterobactin biosynthesis *entCEBAH*.

Fig. S6). Among these proteins, a membrane protein possibly involved in Co/Zn/Cd efflux was enriched in the FSC, with 5.95 % of mapped reads in comparison with the 3.66 % of reads in the SC metagenome (Table S6).

3.5. Metagenome assembled genome reconstruction from the final synthetic community

Since most (54.34 %) of the mapped reads of the FSC metagenome mapped against *Enterobacter* and 37.14 % specifically mapped against *Enterobacter* sp., SA187 (SA187, RefSeq accession GCF_001888805.2; Andrés-Barrao et al., 2017), we used this genome as a reference to perform comparative genomics and guide a metagenome-assembled genome (MAG). The SA187 genome recruited 7.3 million sequences from our metagenomic reads, with an average sequence identity of 99.1 % (Fig. 4A). Mapped loci included 90.71 % of the coding sequences in the reference SA187. The MAG mapped multiple plant symbiosis genes reported in the SA187 genome, including the operons *csgBAC* and *csgDEFG* (curli fibers subunits and secretion proteins, respectively, Fig. 4B), *acrAB* (multidrug efflux pump, Fig. 4C), *entCEBAH* (enterobactin biosynthesis, Fig. 4D), and the siderophore exporter protein EntS. Other plant-symbiosis related proteins such as UbiC (chorismate pyruvate-lyase), along with oxidative stress response proteins like SOD1/2 and KatE, were matched to our sequencing data. The Nacozari strain showed differences to the reference SA187 genome, such as the absence of prophage related coding genes of SA187, *e.g.*, the phage tail protein, the phage baseplate assembly protein V, and the phage repressor protein CI (Supporting Information Fig. S7).

To recover additional genes that might be present in our assembly but absent in the reference *Enterobacter* sp., SA187 metagenomic contigs of the three metagenomic samples were classified to get all the *Enterobacter* assigned sequences. The 344 contigs that were tagged as belonging to the *Enterobacter* genus represented 80.14 % of the reference genome, while the five built scaffolds covered 48.33 % of the reference genome (Supporting Information Fig. S8 and Table S7) and had a G-C content of 56.38 %. A total of 5150 proteins were predicted from these scaffolds, and annotation against the KEGG GENES database revealed that the coding sequences belonging to the PqqBCDE operon, pyrroloquinoline quinone biosynthesis proteins were found within these genome fragments (Supporting Information Fig. S9 and Table S8).

Recent works have reclassified multiple strains previously classified as *Enterobacter* into the new genus *Kosakonia* (Li et al., 2016a,b; Gu et al., 2014; Brady et al., 2013). From genomic evidence, SA187 was suggested to be a *Kosakonia* (Andrés-Barrao et al., 2017). The new MAG was named *Kosakonia* sp. Nacozari based on a genomic distance dendrogram to establish the genomic relatedness of *Kosakonia* sp. Nacozari and other related organisms (Supporting Information Fig. S10). Briefly, we used pairwise ortholog comparisons and obtained their genome similarity score (GSS) from their normalized protein alignments. We have used this metric previously to use whole-genome relatedness between compared organisms (Moreno-Hagelsieb et al., 2013; Alcaraz et al., 2010; Barajas et al., 2019). Our MAG is within a monophyletic clade with *Kosakonia* species, all of them previously classified as *Enterobacter*. Surprisingly, the Nacozari MAG showed a closer relationship with *K. cowanii*, with the SA187 as a sister clade. As an outgroup for the GSS dendrogram, we used multiple *bona fide Enterobacter* species. Our work supports the previous genomic suggestions to reclassify SA187. The Nacozari MAG is within the same clade of plant-interacting bacteria, SA187 is described as plant growth-promoting endophyte, while *K. cowanii* strain 888−76 is a human pathogen and some other strains are plant pathogens (Yang et al., 2018; Furtado et al., 2012).

3.6. Inoculation effects of the synthetic community on the plant phenotype

Our conducted selection experiments aimed to isolate native strains capable of heavy metal resistance and plant interactions. We observed differences when sterilizing substrates, watching significant RGR increases ($p < 0.05$) compared to the standard substrate or MT controls. Moreover, the effect was significant in the MT sterilized substrate, compared to all the other tested treatments, including substrate and inoculation controls (Fig. 5). A slight decrease in the RGR was observed when inoculating the FSC in mine tailing (FSC_MT) and sterilized mine tailing conditions (FSC_S_MT). Still, these RGR values were higher than the inoculation and substrate control (C). These results suggest a positive effect of FSC inoculation in plant development.

4. Discussion

Microbe cultivation only allows the recovery of a fraction of total microbes from the environment. Most bacteria from soil have not been cultivated yet, with soils representing the largest microbial reservoirs, containing up to 10^9 cells/g (Whitman et al., 1998; Delgado-Baquerizo et al., 2018). MTs are not proper soils and are inadequate for plant growth due to their poor water retention and microbial communities with low frequencies of heterotroph and diazotroph organisms. They are dominated with Fe/S oxidizing organisms such as *Acidithiobacillus ferrooxidans* (Mendez et al., 2008). Previous works on MTs in arid environments have revealed differences in community compositions between bulk MT substrate and the rhizospheres of plants growing on-site (Valentín-Vargas et al., 2018; Honeker et al., 2019). The taxa observed exclusively in rhizosphere environments included organisms from the *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes* phyla. Specifically, organisms from the *Bradyrhizobiaceae* family were associated with the rhizosphere of quailbush (Valentín-Vargas et al., 2018). Testing the reacidification of tailings amended with buffalo grass, found that organisms belonging to the *Rhizobiales* and *Sphingomonadales* orders were enriched in the rhizosphere and that even at low pH, a significantly higher content of the *nifH* gene was found in the rhizosphere in comparison to the bulk substrate (Honeker et al., 2019).

Here, we reported the root-associated microbiomes and metagenomic diversity of plants colonizing an abandoned MT deposit. As expected, the environmental culture-free diversity was higher ($H'_{(average)} = 6.26 \pm 0.8$ SD) than the cultured microbes ($H'_{(average)} =$

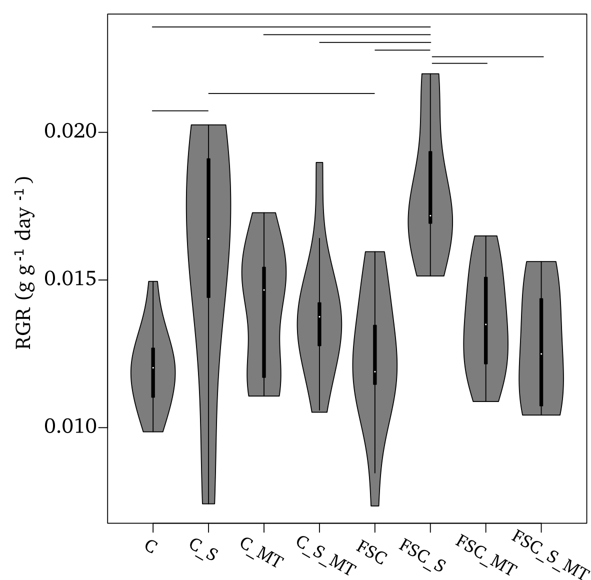

Fig. 5. The relative growth rate of *Acacia farnesiana* inoculated with the FSC. Violin plots the relative growth rate (RGR) of *A. farnesiana* seedlings. The lines over the violin plots indicate significant differences ($p < 0.05$, Tukey HSD). Control treatments (C) use peat-moss:agrolite as substrate and mock LB medium inocula. Some substrates were sterilized (S). MT represents a mixed substrate with mine tailing (20 % v/v) treatments. FSC denotes treatment *via* inoculation with 1.86×10^2 colony-forming units of the final synthetic community.

3.45 ± 1.04, Fig. 2). These cultured microbes were the starting point of a two-step selection to assemble the SC, aimed to be composed of heterotrophic microbes capable of growing on MT substrate, with plant-derived C, N, and P sources. The SC was built by combining the isolates of four plant species colonizing the Nacozari MT: *A. farnesiana*, *B. coulteri*, *B. sarothroides*, and *G. leucocephalum*. The environmental samples were dominated by Actinobacteria (37.99 % on average); this dominance has also been observed for arid soils (Crits-Christoph et al., 2013). Proteobacteria dominated the cultivated rhizospheres, SC, and FSC. Other studies have also recovered isolates belonging to *Enterobacter*, *Pseudomonas,* and *Chryseobacterium* from heavy metal contaminated microenvironments (Toribio-Jiménez et al., 2014; Matlakowska and Sklodowska, 2009). We also isolated bacteria of plant-associated genera such as *Ralstonia* and *Burkholderia* in high frequency, and some *Ralstonia* species are well-known pathogens of plants (Hayward, 1991). However, no canonical pathogenesis factors such as Egl, PehA/B, or CbhA were found in our shotgun metagenomic samples, and also *Burkholderia* had been reported as a diazotrophic symbiont (Gillis et al., 1995). *Pseudomonas putida* was the reference genome with the most mapped reads in the SC metagenome, it has been found in soil, and rhizospheric communities and recent studies comparing several strains have concluded that this bacterial taxon's core-genome lacks virulence factors and can catabolize complex carbon sources, including aromatic compounds (Udaondo et al., 2016).

Even though the inspection of an environmental microbial community on its own is valuable to understand better the underlying processes defining it, culture-based research takes us closer to applied science, such as phytostabilization. Accordingly, we cultivated microorganisms from the rhizospheric MTs and firstly selected 235 isolates to assemble the SC taking into account colony morphology. Then, SC was used as input to the second step in our selection: a microcosm experiment with serial passages. C, N, and P sources derived from plant debris and MT inhibited non-adapted bacteria growth (Supporting Information Fig. S4). The microcosm experiment decreased SC taxa diversity (H' = 3.36) to the output FSC (H' = 2.10). While 18 genera were shared between the SC and the FSC metagenomes, the relative abundances of these taxa in the FSC metagenome represented more than 90 % of the metagenomic reads (Fig. 2D). Thus, even though most genera in the FSC are either low abundance taxa in environmental samples or contamination, the major players of the FSC were consistently observed in the original SC and the environmental tailing rhizospheres. Most metagenomic reads of the FSC metagenome were mapped to the genome of *Enterobacter* sp. SA187. This bacterial strain was isolated from the root endosphere of a desert plant and showed plant growth promoting activity (Andrés-Barrao et al., 2017). Furthermore, its genome sequence revealed proteins associated with oxidative stress tolerance, antibiotic production, plant hormone regulation, and adhesion (Andrés-Barrao et al., 2017).

As expected, cultivation recovered a fraction of the microbes in the environment (Schloss and Handelsman, 2006). In the SC, we lost some microbes detected in the metagenomes and 16S libraries. We lost *Thiobacillus* and *Bradyrhizobium* through cultivating the SC. *Thiobacillus,* are acidophilic autotrophs involved in metal-sulfide oxidation that lowers the pH (Schippers et al., 2000). *Bradyrhizobium* are symbiotic bacteria that form nitrogen-fixing nodules on the roots of legumes; they have previously shown plant growth promotion in heavy metal contaminated soils (Wani et al., 2007). In the SC to FSC selection, we did not detect *Paenibacillus* or *Mesorhizobium*, which could have plant growth-promoting activity (Navarro-Noya et al., 2012; Mansotra et al., 2015).

Archaea have been observed to be tolerant to heavy metals and might be essential in helping other organisms to grow in heavy metal contaminated environments (Li et al., 2017). Even though archaeal sequences were observed at low frequencies (0.24 %, 0.02 %, and 0.005 % of the mapped metagenomic reads in the Af-MG, SC, and FSC samples, respectively), a possible explanation for the detected archaeal genes in SC and FSC could be a detection of horizontal gene transfers. Fungi were represented in the metagenomic samples (1.59 %, 0.17 %, and 0.04 % of mapped reads in the Af-MG, SC, and FSC samples, respectively), including the *Rhizophagus* genus, a well-known arbuscular mycorrhizal symbiont of plants that has been shown to stimulate plant growth in *Acacia holosericea* (Duponnois et al., 2005). *Rhizophagus* was the most abundant fungus in the Af-MG metagenome (14,965 reads, 0.09 %), but its frequency was reduced drastically in the SC (54 reads) and FSC (4 reads).

We also used the Shannon diversity index to describe metabolic diversity, analyzed through matched proteins against the M5nr database and the clustering of unmatched proteins at 70 % sequence identity. The most substantial diversity was observed in the SC (H' = 11.04), then Af-MG (H' = 11.03), followed by FSC (H' = 8.78). The slightly higher metabolic diversity in the SC metagenome, resembling the value of the culture-free Af-MG, may be because SC resulted from the combination of several cultured rhizospheric communities from four plant species, and that each one carried a specific functional profile that added up to the unexpectedly high number of unique protein features in this sample (Fig. 3A). Interestingly, the taxonomic diversity in the SC was lower (3.36) than in the Af-MG sample (4.18). This result is in opposition to previous observations, where the functional diversity strongly correlates with the taxonomic diversity (Fierer et al., 2013). It might be a result of similar taxa carrying different genes (*e.g.,* a large pan-genome), as has been observed for the *Pseudomonas* genus (Hesse et al., 2018), which was present in high frequency in the SC metagenome (17.39 % of mapped reads).

A total of 521 shared proteins were detected in the Af-MG, SC, and FSC, thus being a shared core between environmental metagenomes and cultivable ones. The core features proteins that have been reported as heavy metal resistance factors, such as the CusA P-type ATPase (Taylor et al., 1988; Gillan et al., 2015) and the As resistance protein ArsH (Chen et al., 2015; Li et al., 2014). Furthermore, within this core, we also found antibiotic resistance genes, such as beta-lactamases and the polymyxin resistance protein ArnT, which might be essential to withstand the antagonism in these microbial communities. They could also be co-selected along with the heavy metal resistance genes (Pal et al., 2015).

Exploring the coding gene features of the FSC, we found stress response genes selected in the harsh conditions of the microcosm experiment. Some relevant growth promoting proteins were lost from the Af-Mg and SC to the FSC, such as those coding for indole acetamide hydrolase, which is involved in auxin synthesis (Clark et al., 1993). Nevertheless, the FSC was able to select and hold relevant genes for MT and plant interactions. An example of a critical gene conserved in MT is *yehZ*. The YehZ protein is an osmoprotectant transporter upregulated in nutrient starvation, acidic pH, and hyperosmotic stress conditions (Kim et al., 2013). Nutrient acquisition strategies that may be relevant in a plant-host associated environment were also selected in this system, such as TonB-dependent receptors that are involved in the uptake of dissolved organic matter, siderophores, and vitamins (Tang et al., 2012). Likewise, we found peptide ABC transporters were also enriched in genomes from rhizospheric bacteria (Matilla et al., 2007) and components of bacterial secretion systems, which might be relevant in the bacterial interactions with the plant host and other bacteria (Green and Mecsas, 2016). Other proteins associated with plant-bacteria symbiosis found in the FSC are siderophore producing enzymes (Kloepper et al., 1980) and ACC deaminase, which modulates ethylene levels in the plant, thus preventing the adverse effects of this plant hormone in stressing conditions (Mayak et al., 2004). The FSC also codes for biosynthetic genes for galactoglucans, involved in biofilm formation, which might form complexes with the metal ions from the MT substrate, thus preventing their accumulation by the plant (Macaskie and Dean, 1987).

The strain *Enterobacter* sp. SA187 (SA187) has been described as an endophytic plant growth-promoting organism that could stand unfavorable abiotic conditions such as oxidative stress and that might be

more closely related to *Kosakonia* species than to organisms of the genus *Enterobacter* (Andrés-Barrao et al., 2017). Our metagenomic reads mapped to SA187 plant symbiosis proteins such as AcrAB, which forms an efflux pump involved in plant colonization (Burse et al., 2004) and CsgBAC, which codes for the curli fiber subunits that are required during late plant root colonization and might mediate the adhesion of this organism to the plant surface (Cowles et al., 2016). The enterobactin biosynthesis polycistron *entCEBAH* was recovered from the metagenomic reads of the FSC. *EntCEBAH* has been shown to promote plant growth in heavy metal contaminated soil through the provision of Fe in the presence of other metals and the decrease of oxidative stress in the plant due to the binding of metals near the roots (Dimkpa et al., 2009). Thus, *entCEBAH* might be a consistent plant growth promoting factor in MTs as siderophores. The enterobactin exporter EntS has been reported as highly expressed when SA187 was associated with the roots of *Arabidopsis thaliana* (Andrés-Barrao et al., 2017). Indirect plant growth promoting proteins were also included, such as UbiC, which is involved in the synthesis of 4-hydroxybenzoate, an antimicrobial that reduces the *Phytophthora infestans* rate of infection in *ubiC*-transformed potato (Köhle et al., 2003). Additionally, proteins relevant to MT adaptation, including superoxide dismutases (SOD1/2) and catalase (KatE) were detected, however these proteins could also be relevant in plant tissue establishment as an external plant defense mechanism against reactive oxygen species (Keppler, 1989).

The MAG *Kosakonia* sp. Nacozari has the metabolic potential to assimilate plant-derived carbohydrates such as mannose, fructose, and starch. A diverse array of monosaccharide and amino acid ABC transporters was also observed, which could be mediating the capture of plant rhizodeposits and have been found enriched in the plant-associated *Pseudomonas putida* (Matilla et al., 2007). Moreover, we identified several proteins that have been previously described as plant growth-promoting factors such as the PqqBCDE proteins that contribute to mineral phosphate solubilization in *Enterobacter intermedium* (Kim et al., 2003). The genomic distance dendrogram revealed that the genome of *Kosakonia* sp. Nacozari is similar to the genome of *K. cowanii*, a pathogen from the family *Enterobacteriaceae* that has been isolated from blood but with other strains also found in plants and soil (Yang et al., 2018). Additionally, the outgroup of this clade was the genome of SA187, but the tree distances between these genomes were equivalent to distances between genomes of enterobacteria from different genera in the same tree. This result suggests that *Kosakonia* sp. Nacozari belongs to a novel organism different from *K. cowanii* and SA187. Additionally, we support the previous proposal to reclassify SA187 into a new species (Andrés-Barrao et al., 2017). Current work in our laboratory is being undertaken to re-isolate *Kosakonia sp.* Nacozari for testing its metabolic capabilities and propose it as a new *Kosakonia* species. Furthermore, our following work is to test the plant growth-promoting activities of single isolates from the FSC.

Previous work identified the plant diversity of the Nacozari MT vegetation patches, finding plant species with potential use for phytostabilization already growing *in situ* (Santos et al., 2017). Additionally, they are the same vegetal species of the surrounding areas to the MT, so a revegetation strategy is possible. Interestingly, plants were determined to reproduce within MT, but with little evidence of local adaptation or ecotype specialization when compared to plants outside MT (Santos et al., 2017). We explored the role of root microbiota in the local adaptation to the Nacozari MT, through FSC inoculation into seedlings of the most abundant plant in the vegetation patches. A significant positive effect was observed when inoculating *A. farnesiana* seedlings with FSC, measured by their relative growth rate, but only using a sterile substrate (Fig. 5). We observed no effect of the FSC inocula in the mixed substrate with MT, although plants under sterile substrate with MT showed a slightly better RGR than the ones with the unsterilized substrate. Possible explanations include the out-competition of FSC by the microbes found in the substrate and the MT.

In this work, we tested 94 *A. farnesiana* plants for evaluating the role of FSC in their development (see Experimental Procedures). Future tests will be done using other plant models (*e.g.,* tomato) along with *A. farnesiana,* which is the right species for long term bioremediation in Nacozari MT, but its life cycle complicates experimental setups as it is a tree. The exploratory greenhouse experiments, in which we tested the inoculation of the FSC on *A. farnesiana* seedlings, suggest that under certain conditions, such as substrate sterilization, the FSC might provide a plant growth-promoting effect over its host. The incipient decrease of growth rate observed in mine tailing conditions might be an effect of the FSC capturing biomass to fuel its growth and biofilm production. Conversely, a low biomass production in the FSC couldn't be able to chelate the excess of metals and promoted a toxic metal uptake to its plant host as has been observed for some isolates of Streptomyces in symbiosis with willow (Kuffner et al., 2008). Further experiments will explore the plant output with an increased FSC inoculum, and we were conservative with the colony-forming unit (CFU) density applied to our experiment (1.86×10^2 CFU/mL). Previous works inoculating single strains into gnotobiotic plants were tested using CFU concentrations several orders of magnitude higher than the ones tested in this work (10^7–10^8 CFU/mL; Lugtenberg et al., 2001). We observed some positive plant effects of FSC inoculation using a very low CFU concentration (10^2), suggesting increased plant beneficial effects using higher inocula concentrations. Further work will test plant phenotypes under higher FSC colony-forming units.

5. Conclusions

The spontaneous plant colonization of abandoned Nacozari MT gives us valuable lessons for the phytostabilization process. Below ground microbial colonization also occurred, which seems to help establish vegetation cover in contaminated areas. The assembled synthetic communities from the MT highlighted enriched genes related to plant-interacting bacteria. The new MAG *Kosakonia sp.* Nacozari is related to plant-interacting growth bacteria like SA187, along with the FSC community members, promoting plant biomass augmentation when inoculated in a sterile substrate. Further work is looking to re-isolate and describe the bacteria physiology, molecular genetics, and regulation traits of the FSC members, along with testing efficient FSC plant inoculation to complement bioremediation strategies of mine reclamation.

Data availability

The whole metagenomic reads are available in the DDBJ/EMBL/Genbank databases under Bioproject accession: PRJNA525709; BioSample accessions:SAMN11174940, SAMN11174941, SAMN11174942, SAMN11174943, SAMN11174944, SAMN11174945, SAMN11174946, SAMN11174947, SAMN11174948, SAMN11174949, SAMN11174950, SAMN11174951, and SAMN11174952. Detailed bioinformatic and statistical protocols are available at https://github.com/genomica-fciencias-unam/nacozari/

CRediT authorship contribution statement

Miguel F. Romero: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing - original draft. **Diana Gallego:** Conceptualization, Methodology, Formal analysis, Investigation, Visualization. **Arely Lechuga-Jiménez:** Methodology, Formal analysis, Investigation. **José F. Martínez:** Methodology. **Hugo R. Barajas:** Methodology. **Corina Hayano-Kanashiro:** Resources. **Mariana Peimbert:** Resources, Visualization. **Rocío Cruz-Ortega:** Conceptualization, Funding acquisition, Resources. **Francisco E. Molina-Freaner:** Conceptualization, Funding acquisition, Resources. **Luis D. Alcaraz:** Conceptualization, Supervision, Funding acquisition, Project administration, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest, including any commercial or financial relationships.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2021.126732.

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