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Research Article

Characterization of Rhizobacteria isolated from Wild *Coffea arabica* L.

Rhizobacteria from wild Arabica coffee populations (*Coffea arabica* L.) in southwestern Ethiopia were isolated and characterized. The main purpose was to identify coffee-associated rhizobacteria and evaluate their potential in synthesizing the phytohormone indole acetic acid (IAA) and in degrading the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC). A total of 878 bacterial isolates were screened, of which 395 (45%) isolates were preliminarily characterized using metabolic identification kits (API). Both Gram-negative and Gram-positive bacteria were isolated, with the former group predominating (63% of cases). Based on pre-screening results of the biochemical tests, 51 of the isolates were subjected to PCR-RFLP (Restriction Fragment Length Polymorphism) analysis that yielded ten groups, of which 24 isolates were identified by 16S rRNA gene sequencing. The major genera identified were *Pseudomonas* (six species) and *Bacillus* (four species). Single species of *Erwinia*, *Ochrobactrum* and *Serratia* were also identified. The *Erwinia* sp., *Serratia marcescens* and many *Pseudomonas* spp. produced IAA, and some isolates (all *Pseudomonas* spp.) were also able to degrade ACC. Several of the microbes found in association with wild Arabica coffee bushes have potential agronomic importance, like e.g. *Bacillus thuringiensis*, which deserve further testing. According to these *in vitro* studies, isolates of *Erwinia, Serratia* and *Pseudomonas* are of particular interest in inoculant development due to their plant growth promoting traits.

Keywords: ACC / Coffee populations / Native rhizobacteria / Phytohormones

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1 Introduction

The current serious coffee price crisis in the international markets and the high costs of agrochemicals make cost reductions, sustainability and quality improvement the major priorities in coffee production systems and motivate organic growing of coffee. The demand for eco-friendly coffee far exceeds the present supply in many developed countries and such coffee receives a premium over mainstream coffees [1].

Thus, a rational exploitation of soil microbial activities for agricultural sustainability has gained increasing attention [2]. Rhizobacteria, in particular have been subjected to extensive research among microbiologists and agronomists because of their profound effect on the growth and/or health of plants. The beneficial free-living soil bacteria that take part in sustainable production methods are usually referred to as plant growth-promoting rhizobacteria or PGPR [3]. At present, PGPR include representatives from very diverse bacterial taxa, among which *Pseudomonas, Bacillus, Burkholderia* and *Streptomyces* spp. in particular have been exploited widely in low input production systems [4–6].

PGPR may promote plant growth by direct or indirect modes of action. Direct mechanisms involve the production of phytohormones, e.g. indole-3-acetic acid (IAA), degradation of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC), improvement of the plant nutrient status, non-symbiotic nitrogen fixation and stimulation of disease-resistance mechanisms [7–9].

Certain PGPR strains possess the enzyme ACC deaminase [10] which can cleave the plant ethylene precursor ACC and thereby lower the level of ethylene in a developing seedling or stressed plant [11]. If the level of ethylene following germination is too high, root elongation is inhibited. Glick *et al.* [10] claimed that any rhizosphere bacterium with ACC deaminase activity that can colonize plant roots or seeds in the soil should also be able to promote root elongation and should thus be considered a PGPR.

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The natural forests in southwestern Ethiopia are the primary gene center for Arabica coffee [12] where coffee still grows wild. This is a source of the Arabica coffee gene pool which is highly important for future breeding programs [13]. There is only one [14]; [this journal] published study about rhizobacteria associated with wild Arabica coffee populations in Ethiopia in terms of their distribution and/or functional characteristics toward plant growth promotion. However, an array of bacteria with plant growth promoting potential have been already found associated with *Coffea arabica* from other parts of the world, including the genera *Acetobacter, Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Paenibacillus, Pseudomonas*, *Serratia*, and *Stenotrophomonas* showing different forms of existence, i.e., as endophytes, epiphytes or rhizobacteria [15–17]. In another study, the authors found wild Arabica coffee rhizosphere samples to contain several arbuscular mycorrhizal fungi (AMF) genera of agronomic importance [18]. The greatest benefit to the plant host arises from suitable combinations of indigenous PGPR strains and AMF [19] and Pandey *et al.* [20] have demonstrated the potential biotechnological applications of such native microbes in promotion of plant growth.

Therefore, the objectives of this study are to isolate and characterize native rhizobacteria associated with wild Arabica coffee and to determine their potential for the synthesis of the phytohormone IAA and degradation of ACC.

2 Materials and methods

2.1 Sampling sites

Sampling sites were at two locations, the Bonga (Kaffa zone) district in Southern Nations and Nationalities Peoples' Regional State (S.N.N.P.R.S) and Yayu (Illubabor zone) district in Oromia Regional State (O.R.S). The sites are located between 07°28′–08°28′N and 35°50′–36°45′E, with an altitudinal range of 1376–1890 m asl. The study sites have diurnal and seasonal fluctuations in temperature (14 to 30°C), relative humidity (43 to 85%) and heavy rainfall (1000 to 2000 mm per year). Soil physical and chemical parameters of the study areas have been described previously [18, 21].

2.2 Soil sampling, rhizobacteria isolation and maintenance

Three or four young (2–4 years) coffee plants per sample were carefully uprooted along with a good amount of non-rhizosphere soil and brought intact to the laboratory in sterile plastic bags within two to three days. The samples were stored at +4°C prior to further processing. For rhizosphere bacterial isolation, the non-rhizosphere (loosely adhering) soil was removed by vigorous shaking, leaving behind only the rhizosphere soil (strongly adhering to the roots) followed by cutting of the whole roots into segments. Coffee root fragments with strongly adhering soil particles (approx. 10 g) were placed in flasks containing 90 mL sterile saline (0.85% NaCl) solution and shaken for 25 minutes on an orbital shaker at 100 rpm. The subsequent suspension was serially diluted. The appropriate serial dilution was spread on King's B (KB) medium [22] for the isolation of Gram-negative rhizobacteria. For the isolation of Gram-positive endospore-forming bacteria, the appropriate serial dilutions were heat-treated in a water bath at 80°C for at least 10 minutes. Thereafter, a 0.1 mL aliquot was spread onto nutrient agar (Oxoid). Isolates were purified and stored at −70°C in freezing medium containing K_2HPO_4, 0.82 g; KH_2PO_4, 0.18 g; $Na_3C_6H_5O_7$, 0.59 g; $MgSO_4 \times 7\ H_2O$, 0.25 g; glycerol (87%), 172 mL, and distilled water, 1000 mL for the subsequent analyses.

2.3 Identification and characterization of isolates

Morphological parameters in combination with specific staining procedures (Gram and spore stains for example) and physiological characteristics were used for the identification of the initial isolates. This preliminary characterization was complemented by API 50 CH (for *Bacillus* spp.) and API 20 NE (for Gram-negative non- Enterobacteriaceae bacteria) (BioMérieux, Marcy-L'Etoile, France) identification kits. The isolates were subjected to the relevant API kit characterization on the basis of their abundance and preliminary morphological and biochemical features.

2.4 Genomic DNA extraction

Of 395 API rhizobacteria characterized, 33 Gram-negative and 18 Gram-positive isolates were further identified using PCR-RFLP and 16S rRNA gene analyses. Selection criteria for the isolates were based on abundance of the particular group, functional characteristics from in vitro studies [14] and API identification results (>90% identification indices). Genomic DNA was extracted with the Qiagen DNeasy tissue kit (Qiagen) following the manufacturer's instructions and the approximate amount of DNA was determined on a 1% agarose gel and visualized by staining with ethidium bromide and UV illumination.

2.5 PCR amplification of 16S rDNA

Two μL of DNA (undiluted or diluted 1:10 with sterile H_2O) were used as a template for PCR amplification using a thermal cycler (Gene Amp PCR System 9700, Perkin Elmer, Norwalk CT, USA). In addition to the DNA, the PCR reaction consisted of 200 μM dNTP, 1 × PCR buffer, 2.5 units of Taq polymerase (GE Healthcare Chalfont St. Giles, UK), 10 pmol of forward primer 27f (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 10 pmol of reverse primer 1492r (5′-GGY TWC CTT GTT ACG ACT T-3′). All primers were synthesized by Invitrogen (Carlsbad, CA). The PCR program included a denaturation step of 5 min at 94°C, followed by 30 cycles of 94°C for 40 sec, 55°C for 40 sec, 72°C for 1 min, and finally an extension step of 7 min at 72°C.

2.6 Restriction enzyme digest

Six μL portions of PCR product in a final volume of 50 μL were digested for 2 h at 37°C using the combination of restriction enzymes *Hha*I and *Hae*III (GE Healthcare) according to the manufacturer's instructions. Digested fragments were separated on a 2.5% agarose gel and visualized by staining with ethidium bromide and UV illumination. RFLP patterns were grouped visually, and representatives from each group were selected for 16S rRNA gene sequences.

2.7 Cloning and sequencing

PCR products were purified with QIAquick PCR purification columns (Qiagen) according to the supplier's instructions, followed by sequencing with forward primer 27f at Macrogen Inc. (Seoul, Korea). Those PCR products that generated unreadable sequences were cloned into the pCR 4-TOPO vector (Invitrogen) and transformed into competent cells (TOP10 Chemically Competent *E. coli*) as described by the manufacturer (Invitrogen). The vector insert was sequenced using the T7 primer (5′-TAATACGACTCACTATAGGG-3′) at Macrogen Inc. (Seoul, Korea). DNA sequences were edited using the 4 Peaks program [http://mekentosj.com/4peaks/].

2.8 Phylogenetic analysis

The nearest neighbors of the 16S rRNA gene sequences were found using the tool Sequence Match at Ribosomal Database Project II (RDP), release 9.48 [http://rdp.cme.msu.edu/]; [23]. The 16S rRNA gene sequences (approximately 800 bp) were aligned with sequences of the nearest type strains, some other relevant sequences and an out group sequence using the tool myRDP at RDP. A distance matrix of the aligned sequences was calculated with the DNADIST program of the PHYLIP package [24] using the Jukes-Cantor corrected distance model and a phylogenetic tree was constructed with the NEIGHBOR program. The statistical significance of the grouping was estimated by bootstrapping (100 replicates) using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE, all of which are from the PHYLIP package. The phylogenetic tree was displayed using the program TreeViewX [25]. The sequences from type strains ($>$1200 bp) are presented in Table 2 and the strains *Burkholderia cepacia* ATCC 25416 (U96927), *Chromobacterium violaceum* ATCC 12472 (M22510), *Pseudomonas putida* DSM 291 (Z76667), *Pseudomonas fluorescens* DSM 50090 (Z76662), *Pseudomonas luteola* IAM 13000 (D84002), *Clostridium perfringens* ATCC 13124 (M59103), *Bacillus mycoides* ATCC 6462 (AB021192), *Bacillus pumilus* DSM 27 (AY456263), *Bacillus firmus* JCM 2512 (D78314), *Paenibacillus polymyxa* IAM 13419 (D16276) and *Brevibacillus brevis* JCM 2503 (D78457) were used in the analyses.

2.9 Nucleotide sequence accession numbers

Partial 16S rRNA gene sequences of isolates were submitted to the GenBank. The accession numbers are available in Table 2.

2.10 Production of IAA and utilization of ACC

Qualitative detection and quantitative estimation of rhizobacterial IAA were accomplished using the appropriate media following standard methods [26]. For the quantitative estimation of IAA, rhizobacterial isolates were propagated overnight in DF salt minimal medium [27] and 20 μL cultures were inoculated to sterile DF salt minimal medium amended with 500 μg of *L*-tryptophan. After incubation for 48 hours, the density of the culture was measured at 600 nm, and 3 mL of culture were sampled for quantitative estimation of IAA. The bacterial cells were removed by centrifugation (4500 rpm) for 20 min at 4°C. After treating 1 mL of the culture supernatant with Salkowski's reagent, IAA was quantified using a colorimetric method. The standard curve was prepared from pure IAA (Sigma-Aldrich).

The ability of rhizobacterial isolates to utilize ACC as their sole nitrogen source was assayed as described by Pentose and Glick [28] with little modification. All the test isolates were propagated overnight in 5 mL of tryptic soy broth (TSB; Oxoid), and 0.5 mL culture was inoculated to 25 mL DF salts minimal medium contained in 100 mL Erlenmeyer flasks. Cultures were allowed to grow overnight on a shaker (200 rpm) at 30°C. Sterile DF salts minimal medium was amended with filter (0.2 μm) sterilized 3 mM ACC instead of $(NH_4)_2SO_4$ as a sole nitrogen source and dispensed in 25 mL amounts into 100 mL Erlenmeyer flasks. The flasks were inoculated with appropriate, serially diluted young cultures previously propagated in DF salts minimal medium. The cultures were incubated at 30°C for 120 hours on an orbital shaker (200 rpm). Growth of rhizobacterial isolates in each flask was assessed by measuring the optical density (OD) (405 nm) at different points of time (0, 24, 48, 72, 96 and 120 hours). Uninoculated sterile growth medium incubated under the same conditions was used as a negative control.

2.11 Data analysis

ANOVA was employed to test the differences in IAA production between rhizobacterial isolates using SPSS version 10 and Tukey's HSD post hoc test to assess for mean separation ($p<0.05$). The Pearson correlation analysis was used to explore possible relationships between cell density and IAA production.

3 Results

3.1 Characterization of coffee-associated bacterial isolates

A total of 878 bacterial isolates were screened from *Coffea arabica* rhizospheres based on colony morphology, pigmentation and growth rate, of which a total of 395 (45%) isolates were characterized biochemically using API kits (see Table 1). This technique of biochemical identification indicated the presence of wide arrays of Gram-negative and Gram-positive bacterial genera. Fourteen major genera were tentatively

identified from coffee rhizosphere samples. The most commonly occurring genus was *Bacillus* ($n = 147$), accounting for about 37% of the root-associated populations. Members of the *Bacillus* species seemed to be dominated by *Bacillus cereus* (44.2%; see Table 1).

As many as 248 Gram-negative rhizobacteria were isolated and the most frequently occurring genera were *Pseudomonas* ($n = 134$), *Burkholderia* ($n = 51$), *Chrysomonas* ($n = 20$), *Aeromonas* ($n = 13$), and *Acinetobacter* ($n = 13$) (see Table 1). Other suggested bacterial genera included *Stenotrophomonas, Vibrio*, *Chromobacterium*, *Pasteurella*, *Agrobacterium* and *Chryseobacterium* spp. in order of occurrence (see Table 1). The presence of four species of *Pseudomonas* were suggested by API results in the current study (see Table 1) and the most commonly occurring species seemed to be *P. fluorescens* ($n = 87$) and *P. putida* ($n = 43$). Collectively, these two species appeared to account for 97% of the pseudomonad population. In contrast, strain identification based on 16S rRNA-gene sequencing resulted in the identification of 8 different *Pseudomonas* species (see below).

Restriction Fragment Length Polymorphism (RFLP) analysis of the 16S rRNA genes was carried out to rapidly estimate the microbial diversity of the coffee plant rhizobacteria. Of 395 API kit characterized rhizobacteria, 51 (33 Gram-negative and 18 Gram-positive) isolates were selected (cf. *Materials and Methods* section) and partially characterized by RFLP techniques with *Hha*I and *Hae*III enzymes, which resulted in 10 profiles (see Table 2). The initial clustering of the Gram-negative and Gram-positive isolates divided them into six and four groups of one or more members, respectively (see Table 2).

Sequence analysis of a significant proportion (>800 bp) of the 16S rRNA genes of representative rhizobacteria from each RFLP group ($n = 24$) was used to further identify the isolates, for which the closest database match (% homology) is presented in Table 2. Members of each RFLP group included the genera *Pseudomonas* (groups A, B and C; 29 isolates), *Bacillus* (groups G, H, I and J; 18 isolates), *Erwinia* (group E; two isolates), *Ochrobactrum* (group F; one isolate), and *Serratia* (group D; one isolate) (see Table 2).

The partial sequences of the 16S rRNA gene of both Gram-negative and Gram-positive rhizobacteria were aligned with the nearest neighbors and other relevant bacteria and the identity of coffee-associated rhizobacteria was evaluated by constructing phylogenetic trees (see Figs. 1 and 2). There were a number of mismatches between API kit identification (sometimes with >98% identification indices) and 16S rRNA gene analysis even to the genus level particularly for Gram-negative isolates (data not shown). According to the API kit characterization method, members of the pseudomonads were dominated by *P. fluorescens* but the technique with 16S rRNA gene sequencing identified none of this species (see Table 2). Gram-negative isolates presented more diverse species ($n = 9$) compared to the *Bacillus* group ($n = 4$). The isolate AUPB02 showed the same closest similarity (98.1%) to both *P. plecoglossicida* and *P. monteilii* (see Fig. 1). Similarly, the strain AUPY26 also grouped with both *P. monteilii* and *P. plecoglossicida* on 99.6% sequence similarity (see Fig. 1). Gram-negative

Table 1. API kit based overall diversity of rhizobacteria associated with *Coffea arabica* in natural coffee forests, southwestern Ethiopia with identification indices of >90%.

Identity	Gram reaction	Isolated strains per site		Total
		Bonga	Yayu	
Pseudomonas fluorescens	−	44	43	87
B. cereus	+	29	37	66
Burkholderia spp.	−	18	33	51
Pseudomonas putida	−	15	28	43
Bacillus spp.	+	12	10	22
Chryseomonas sp.	−	9	11	20
Aeromonas spp.	−	8	5	13
Acinetobacter spp.	−	4	9	13
Brevibacillus spp.	+	6	6	12
B. mycoides	+	3	7	10
B. anthracis	+	6	1	7
B. megaterium	+	4	3	7
Stenotrophomonas sp.	−	2	3	5
B. firmus	+	3	1	4
B. stearothermophilus	+	4		4
Vibrio spp.	−	1	3	4
B. subtilis	+	2	2	4
Chromobacterium sp.	−		3	3
P. aeruginosa	−		3	3
B. amyloliquefaciens	+		2	2
B. licheniformis	+	2		2
B. pumilus	+	2		2
B. spaericus	+	1	1	2
Pasteurella sp.	−		2	2
Agrobacterium sp.	−	1		1
Alcaligenes sp.	−		1	1
B. circulans	+		1	1
B. coagulans	+	1		1
Chryseobacterium sp.	−	1		1
Paenibacillus sp.	+		1	1
P. stutzeri	−	1		1
Total		179	216	395

Table 2. PCR-RFLP analysis and partial sequences (c. 800 bp) of the 16S rRNA gene.

Isolate	RFLP group	Closest neighbor (type strain)	Similarity [%]	Accession No.
AUPB01[a)b)]	A			
AUPB02	A	*P. plecoglossicida* FPC951; AB009457	98.1	EF520791
		P. monteilii CIP 104883; AF064458	98.1	
AUPB07[a)]	A			
AUPB08[a)]	A			
AUPB09[a)b)]	A			
AUPY10	A	*P. chlororaphis* ATCC 13985; AF094722	100	EF520794
AUPY11	A	*P. umsongensis* Ps 3-10; AF46450	99.3	EF520795
AUPB13[a)]	A			
AUPB16[c)]	A	*P. tremae* CFBP 6111; AJ492826	98.7	EF520797
AUPB21	A	*P. chlororaphis* ATCC 13985; AF094722	100	EF520798
AUPB22	A	*P. chlororaphis* ATCC 13985; AF094722	100	EF520799
AUPB25[a)b)]	A			
AUPY26	A	*P. monteilii* CIP 104883; AF064458	99.6	EF520800
		P. plecoglossicida FPC951; AB009457	99.6	
AUPY27[a)]	A			
AUPB49[a)b)]	A			
AUPB04[a)b)]	B	*P. koreensis* PS 9-14; AF468452	100	EF520792
AUPB06	C	*P. tremae* CFBP 6111; AJ492826	98.7	EF520793
AUSB14[a)]	D	*S. marcescens* ATCC 13880; M59160	100	EF520796
AUEY28[a)c)]	E	*Erwinia rhapontici* LMG 2688; Z96087	98.8	EF520801
AUEY29[a)]	E			
AUOY47	F	*Ochrobactrum pseudogrignonense* CCUG 30717; AM422371	100	EF520802
AUBB05	G	*B. velezensis* CR-502; AY603658	99.9	EF520803
AUBB12	G	*B. subtilis* DSM10; AJ276351	99.9	EF520804
AUBB19	G	*B. subtilis* DSM10; AJ276351	99.9	F520805
AUBB20	G	*B. velezensis* CR-502; AY603658	99.9	EF520806
AUBB37	G	*B. subtilis* DSM10; AJ276351	99.9	EF520810
AUBB52	G	*B. subtilis* DSM10;AJ276351	99.9	EF520812
AUBB34	H	*B. cereus*; ATCC14579;AF290547	100	EF520807
AUBB35	H	*B. cereus*; ATCC14579;AF290547	100	EF520808
AUBB36	H	*B. cereus*; ATCC14579; AF290547	100	EF520809
AUBB38	H	*B. cereus*; ATCC14579; AF290547	100	EF520811
AUBY54	H	*B. thuringiensis;* ATCC10792; AF290545	99.9	EF520814
AUBY53	J	*B. subtilis;* DSM10; AJ276351	99.9	EF520813

a) Isolates that produced IAA.
b) Isolates that degraded ACC.
c) Isolates for which cloning was carried out.

strains identified to 100% similarity belonged to *P. chlororaphis* (3 isolates) and one isolate each of *P. koreensis*, *Serratia marcescens* and *Ochrobactrum pseudogrignonense* (see Table 2 and Fig. 1). Gram-positive isolates belonged to *Bacillus subtilis* (5 isolates), *Bacillus cereus* (4 isolates), *B. velezensis* (2 isolates), and one isolate of *B. thuringiensis* (see Table 2; Fig. 2).

3.2 Production of Indole Acetic Acid (IAA)

Of 51 (PCR-RFLP and 16S rRNA gene) rhizobacterial isolates characterized, 18 (16 Gram-negative and 2 Gram-positive) strains belonging to different RFLP groups were assessed for the production of IAA and utilization of ACC as their sole nitrogen source. Twelve of the 18 rhizobacteria tested (all Gram-negative isolates) secreted IAA under in vitro conditions which ranged from 7.39−57.08 μg/mL (see Fig. 3). The identities (PCR-RFLP analysis and 16S rRNA gene sequence) of all the IAA producers are presented in Table 2. These rhizobacterial isolates, however, varied significantly ($p<0.05$) in their efficiency of releasing IAA into the growth medium. Isolate AUEY28 (*Erwinia* sp.) possessed the greatest potential for synthesizing this phytohormone, followed by the isolate AUSB14 (*Serratia marcescens*). The measured cell number (OD at 600 nm) was not related ($p>0.05$) to IAA production. Six isolates (two *B. subtilis* and four *Pseudomonas* spp.) failed to secrete detectable amounts of IAA (data not shown).

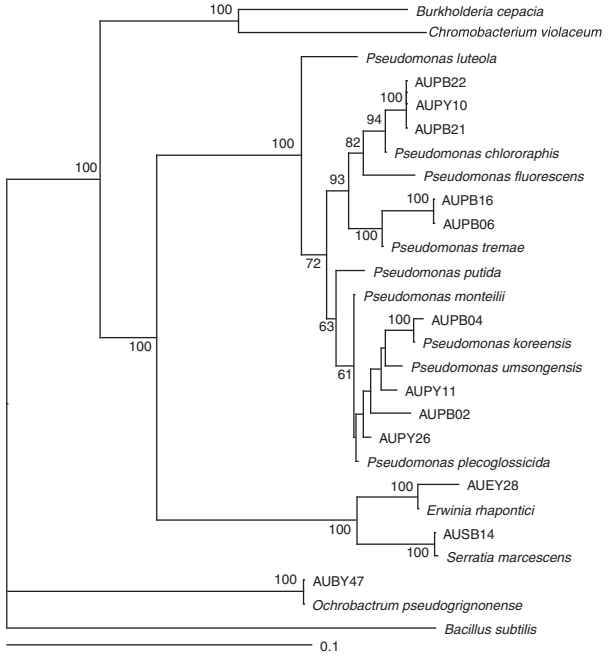

Figure 1. Unrooted phylogenetic tree derived from 16S rRNA gene sequence analysis of the Gram-negative coffee rhizobacterial isolates, showing the relationship to the nearest type strains and other relevant type strains. The sequence of *Bacillus subtilis* was used as an outgroup representative. Scale bar, 10% estimated sequence divergence. Numbers indicate bootstrap values for branch points (values < 50 not included).

Figure 2. Unrooted phylogenetic tree derived from 16S rRNA gene sequence analysis of the Gram-positive coffee rhizobacterial isolates, showing the relationship to the nearest type strains and other relevant type strains. The sequence of *Clostridium perfringens* was used as an outgroup representative. Scale bar, 10% estimated sequence divergence. Numbers indicate bootstrap values for branch points (values < 50 not included).

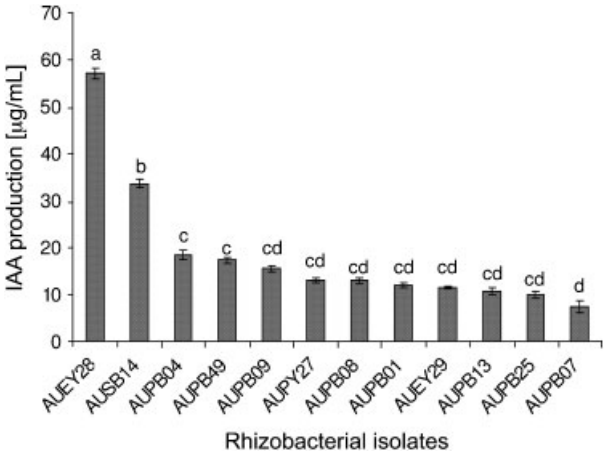

Figure 3. IAA production by rhizobacteria associated with *Coffea arabica* in natural coffee forests, south western Ethiopia. Identity of strains according to Table 2. Data are means ± SD from two replications, and values followed by different letter(s) indicate significant differences ($p < 0.05$). Note that the colorimetric reading of the negative control was 0.083 at 535 nm.

3.3 Utilization of ACC

Five of the 18 rhizobacterial isolates had the ability to grow in the minimal salt medium with ACC as the sole nitrogen source. The optical density reading of most isolates indicated a progressive increase in cell numbers until 72 hours of incubation time and a decline thereafter (see Fig. 4). The rhizobacterial isolates varied greatly in their efficiency of ACC utilization (see Fig. 4). Isolate AUPB25 (*Pseudomonas* sp.) showed the highest OD reading (405 nm) during the course of incubation followed by isolate AUPB49 (*Pseudomonas* sp.), both from RFLP group A (see Fig. 4). PCR-RFLP analysis and partial 16S rDNA sequences identified all ACC utilizers as pseudomonads and partial 16S rDNA sequence identified one strain as *P. koreensis* (see Table 2). Thirteen isolates (11 Gram-negative and two Gram-positive) were unable to degrade ACC (data not shown).

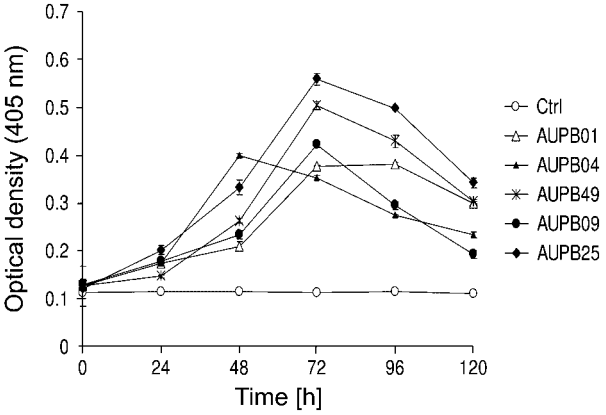

Figure 4. ACC utilization as the sole nitrogen source by rhizobacteria associated with *Coffea arabica* in natural coffee forests, southwestern Ethiopia. Identity of strains was chosen according to Table 2. The results are mean values of duplicate cultures.

4 Discussion

It should be noted that API kits have limited application in the characterization of environmental isolates compared to 16S rDNA sequences. For instance, mismatches even to the genus level were observed between these identification techniques for the isolates of *Erwinia* (group E), *Ochrobactrum* (group F) and *Serratia* (group D). Thus only the complete 16S rDNA sequences of this phylogenetic marker gene can give a good identification (cf. below).

The distribution and characteristics of the rhizobacteria found in association with *Coffea arabica* agreed with the universal observation that the rhizosphere of many plants, including *Coffea arabica*, favors Gram-negative bacteria [17].

Evidence is accumulating that numerous rhizobacteria are actively involved in the synthesis of auxins in pure culture and in soil and it has been reported that up to 80% of rhizobacteria can synthesize indole-3-acetic acid (IAA) [26, 29]. The phytohormone IAA plays a major role in the development of the host-plant-root system [30]. The majority of IAA producers in the present study were *Pseudomonas* spp.

It was found that the isolate AUEY28 (*Erwinia rhapontici*) produced exceedingly high amounts of IAA (see Fig. 3). This isolate also showed potent efficiency of in vitro phosphate

solubilization (Muleta *et al.* unpubl). Simultaneous production of hormones and phosphate solubilization has great agronomic significance [6].

The two Gram-positive isolates tested (*B. subtilis*) did not produce any IAA during the qualitative and quantitative studies (data not presented). However, de Freitas *et al.* [31] demonstrated that six *Bacillus* spp. were able to secrete small amounts of IAA-like compounds. Plant growth promotion of *Bacillus* and *Paenibacillus* spp. including the production of phytohormones has been repeatedly reported [31, 32], necessitating further screening of such rhizobacterial strains. In our previous in vitro studies [14], as also presented in Table 2, *B. subtilis* strains mainly exhibited efficient antagonistic effects against emerging deleterious Arabica coffee pathogenic *Fusarium* spp. (*F. xylarioides*, *F. stilboides* and *F. oxysporum*).

Conventional identification techniques (API kit), PCR-RFLP analysis and 16S rDNA sequences traced all ACC-utilizing isolates to members of *Pseudomonas* spp., among which *P. koreensis* was fully identified (see Table 2). At present, members of the pseudomonads, e.g. *P. chlororaphis*, have received particular attention because of their biotechnological applications in agriculture [5]. Multiple lines of evidence [10, 28] have confirmed that the utilization of ACC as a sole nitrogen source depends on the possession of an ACC deaminase trait by particular soil bacteria. Similarly, rhizobacteria that possess the ACC deaminase trait can break down plant-exuded ACC and safeguard plants from deleterious effects of ethylene accumulation caused by abiotic and biotic (pathogens) stresses [33]. Rhizobacteria possessing the gene for ACC deaminase are particularly important to dicotyledonous plants (to which coffee belongs), which are more susceptible to the effects of ethylene [34] especially under stress conditions [35]. By facilitating the formation of longer roots through the action of ACC deaminase and other useful attributes, these coffee-associated rhizobacteria may enhance the survival of plant seedlings under various abiotic and biotic stresses. This is highly relevant for forest coffee bushes, which are naturally exposed to a range of plant pathogens that could induce excess ethylene production [36] due to conducive conditions (high precipitation and humidity) in the study areas [37].

Among the Gram-positive rhizobacteria, isolate AUBY54 showed the closest match from the database with *Bacillus thuringiensis* and the isolation of this known bioinsecticidal strain could be important for wild coffee bushes exposed to a range of insect pests. Potent larvicidal strains of *B. thuringiensis* have been isolated from different agroecological zones of Ethiopia (Z. W. Tenssay, unpubl.). The 16S rRNA gene sequence analysis revealed that strains AUBB05 and AUBB20 (possibly identical) were most similar to *B. velezensis* (99.9% sequence similarity) and these strains have previously shown a remarkable inhibitory effect against noxious coffee fungal pathogens, including *Fusarium xylarioides* [14]. Very recently, Ruiz-García *et al.* [38] have shown *B. velezensis* to be a novel surfactant-producing species and Debode *et al.* [39] have demonstrated that biosurfactant production is one of the main mechanisms involved in a mode of action of biocontrol agents.

Among the isolates of interest, 16S rRNA gene sequence analysis confirmed that isolates AUPB16 and AUPB06 had close (98.7%) sequence similarity with *P. tremae* although they belonged to different RFLP groups (see Table 2). *P. tremae* has so far been reported only as a pathogen to *Trema orientalis* [40]. The presence of the normal host (*T. orientalis*) in the study areas is not known. In our case, however, the strains were isolated from apparently healthy wild coffee seedlings and were able to mobilize mineral phosphate (Muleta *et al.*, unpubl.), produce HCN and siderophores, and effectively antagonize deleterious coffee fungal pathogens [14].

The strain AUOY47 showed 100% 16S rDNA similarity with *Ochrobactrum pseudogrignonense* (see Table 2; Fig. 1). Plant associations by members of this genus have also been reported by Verma *et al.* [41] and Lebuhn *et al.* [42]. In addition, an isolate of *Ochrobactrum* has been described which possesses the complete symbiotic ability to form nitrogen-fixing nodules in *Acacia mangum* [43]. If this also applies to related tree legumes, e.g. other *Acacia*/*Albizia* species, it could have relevance for natural coffee forests in Ethiopia since they harbor such legume shade trees [18, 44]. However, it needs to be stated that many strains within the genus *Ochrobactrum* are characterized as opportunistic human pathogens. Also because of the close phylogenetic relatedness to the genus *Brucella*, which contains human pathogens, field applications should not be considered.

Altogether, in addition to phytohormone (IAA) production and ACC degradation (this study), many coffee-associated rhizobacterial isolates (mainly *Pseudomonas* spp.) mobilized insoluble phosphate (Muleta *et al.* unpubl.), produced siderophores, HCN and lytic enzymes, and some (over 10%) exhibited potent inhibitory effects against emerging coffee fungal pathogens [14]. In addition, wild *Coffea arabica* rhizosphere samples contained several members of the Glomeromycota [18]. The synergistic effect of AMF and beneficial rhizobacteria has been reported repeatedly [45, 46]. The authors suggest that the concerted microbial inputs of coffee-associated rhizobacteria and AMF may promote and secure the growth and health of wild Arabica coffee populations in the study areas. Future research work should give due attention to investigations on Arabica coffee associated endophytic bacterial communities. Very recently, Vega and co-workers [17] have successfully isolated a range of endophytic bacteria from *Coffea arabica* L. with known agronomic importance.

5 Conclusions

Although API-testing with environmental isolates is questionable, it demonstrated that diverse communities of rhizobacteria were associated with wild *Coffea arabica*, whereas PCR-RFLP and 16S rRNA gene analyses only revealed a limited number of mainly *Pseudomonas* and *Bacillus* spp. These two groups appear rather frequent, but there are other interesting isolates of minor frequency. Certainly, the dominance of *Pseudomonas* and *Bacillus* spp. was caused by the chosen isolation procedure (Kings B medium) and heat enrichment. Using for example R2A-agar or enrichment with nitrogen-free semisolid agar for microaerobic nitrogen fixing bacteria, other

types of isolates would have been obtained. Some of our rhizobacterial isolates though showed direct plant growth promotion traits such as IAA production and ACC utilization under *in vitro* conditions.

The study of bacterial diversity using a culture-dependent approach has great limitations as it excludes the majority of microorganisms, which are not able to be cultivated. Its main advantage, however, is that a number of bacteria can actually be isolated and characterized for further studies including plant growth promoting traits and inoculant development, e.g. for the promotion of organic coffee. The bacteria isolated in this study are microbes of healthy plants, and thus potentially beneficial to the growth and survival of coffee in its original habitat. Future work is needed to include tests of the ability to promote coffee plant growth under both greenhouse and field trials. In future experiments, the microbial diversity associated with Arabica coffee should also be assessed using culture-independent approaches by performing the phylogenetic analysis based on DNA extracted from rhizosphere soil or roots.

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Conflict of interest statement

The authors have declared no conflict of interest.

References

[1] H. A. M. van der Vossen, A critical analysis of the agronomic and economic sustainability of organic coffee production. *Exp. Agr.* **2005**, *41*, 449–473.

[2] J. M. Lynch, Introduction: Some consequences of microbial rhizosphere competence for plant and soil. in *The Rhizosphere* (Ed. J. M. Lynch), Wiley & Sons, Chichester, England **1990**, 1–10.

[3] J. W. Kloepper, R. Lifshitz, R. M. Zablotowicz, Free-living bacterial inocula for enhancing crop productivity, *Trends Biotechnol.* **1989**, *7*, 39–44.

[4] J. K. Vessey, Plant growth promoting rhizobacteria as biofertilizers, *Plant Soil* **2003**, *255*, 571–586.

[5] M. Lucy, E. Reed, B. R. Glick, Applications of free living plant growth-promoting rhizobacteria, *Antonie van Leeuwenhoek* **2004**, *86*, 1–25.

[6] N. Vassilev, M. Vassileva, I. Nikolaeva, Simultaneous P-solubilizing and biocontrol activity of microorganisms: potential and future trends, *Appl. Environm. Microbiol.* **2006**, *71*, 137–144.

[7] C. J. Beauchamp, Mode of action of plant growth-promoting rhizobacteria and their potential use as biological control agents, *Phytoprotection* **1993**, *71*, 19–27.

[8] J. W. Kloepper, Plant-growth-promoting rhizobacteria as biological control agents, in: *Soil Microbial Ecology* (Ed: F. B. Jr. Metting Marcel Dekker Inc., N.Y. (USA) **1993**, 255–273.

[9] Z. S. Omer, R. Tombolini, A. Broberg, B. Gerhardson, Indole-3-acetic acid production by pink-pigmented facultative methylotrophic bacteria, *Plant Growth Regul.* **2004**, *43*, 93–96.

[10] B. R. Glick, D. M. Karaturovíc, P. C. Newell, A novel procedure for rapid isolation of plant growth-promoting pseudomonads, *Can. J. Microbiol.* **1995**, *41*, 533–536.

[11] S. Mayak, T. Tirosh, B. R. Glick, Plant growth promoting bacteria that confer resistance to water stress in tomato and pepper, *Plant Sci.* **2004**, *166*, 525–530.

[12] A. C. Zeven, P. M. Zhukovsky, *Dictionary of Cultivated Plants and their Centres of Diversity*, Centre for Agricultural Publishing and Documentation, Wageningen **1975**.

[13] E. Aga, T. Bryngelsson, E. Bekele, B. Salomon, Genetic diversity of forest arabica coffee (*Coffea arabica* L.) in Ethiopia as revealed by random amplified polymorphic DNA (RAPD) analysis, *Hereditas* **2003**, *138*, 36–46.

[14] D. Muleta, F. Assefa, U. Granhall, In vitro antagonism of rhizobacteria isolated from *Coffea arabica* L. against emerging fungal coffee pathogens, *Eng. Life Sci.* **2007**, *7*, 577–586.

[15] T. Jimenez-Salgado, L. E. Fuentes-Ramirez, A. Tapia-Hernandez, M. A. Mascarua-Esparza, E. Martinez-Romero, J. Caballero-Mellado, *Coffea arabica* L., a new host plant for *Acetobacter diazotrophicus* and isolation of nitrogen- fixing-acetobacteria, *Appl. Environm. Microbiol.* **1997**, *63*, 3676–3683.

[16] C. C. H. Sakiyama, E. M. Paula, P. C. Pereira, A. C. Borges, D. O. Silva, Characterization of pectin lyase produced by an endophytic strain isolated from coffee cherries, *Lett. Appl. Microbiol.* **2001**, *33*, 117–121.

[17] F. E. Vega, M. Pava-Ripoll, F. Posada, J. S. Buyer, Endophytic bacteria in *Coffea arabica* L., *J. Basic Microbiol.* **2005**, *45*, 371–380.

[18] D. Muleta, F. Assefa, S. Nemomissa, U. Granhall, Composition of coffee shade tree species and density of indigenous arbuscular mycorrhizal fungi (AMF) spores in Bonga natural coffee forest, southwestern Ethiopia, *Fore. Ecol. Manage.* **2007**, *241*, 145–154.

[19] N. Requena, I. Jimenez, M. Toto, J. M. Barea, Interactions between plant-growth-promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi and *Rhizobium* spp. in the rhizosphere of *Anthyllis cytisoides*, a model legume for revegetation in Mediterranean semi-arid ecosystem, *New Phytol.* **1997**, *136*, 667−677.

[20] A. Pandey, P. Trivedi, B. Kumar, L. M. S. Palni, Characterization of a phosphate solubilizing and antagonistic strain of *Pseudomonas putida* (B0) isolated from a sub-Alpine location in the Indian Central Himalaya, *Curr. Microbiol.* **2006**, *53*, 102–107.

[21] W. Höfner, Nährstoffstatus der Böden in den Kaffeeanbaugebieten Äthiopien, in *20 Jahre Agrarforschung des Tropeninstituts in Äthiopien* (Ed: H. E. Matter, A. Westpal), Tropeninstitut Giessen **1987**, 55–56.

[22] E. O. King, M. K. Ward, D. E. Raney, Two simple media for the demonstration of pyocyanine and fluorescein, *J. Lab. Clin. Med*. **1954**, *44*, 301–307.

[23] J. R. Cole, B. Chai, R. J. Farris, Q. Wang, A. S. Kulam-Syed-Mohideen, D. M. McGarrell *et al*., The ribosomal database project (RDP-II): Introducing myRDP space and quality controlled public data, *Nucleic Acids Res*. **2007,** *35*(*Database issue*): D169–D172; doi: 10.1093/nar/gkl889.

[24] J. Felsenstein, *PHYLIP (Phylogeny Inference Package) version 3.6. (distributed by the author)*, Department of Genome Sciences, University of Washington, Seattle (USA) **2005**.

[25] R. D. M. Page, TREEVIEW: An application to display phylogenetic trees on personal computers, *Comput. Applic. Biosci*. **1996**, *12*, 357–358.

[26] J. M. Bric, R. M. Bostock, S. E. Silverstone, Rapid in situ assay for indole acetic acid production by bacteria immobilized on nitrocellulose membrane, *Appl. Environ. Microbiol*. **1991**, *57*, 535–538.

[27] M. Dworkin, J. Foster, Experiments with some microorganisms which utilize ethane and hydrogen, *J. Bacteriol*. **1958**, *75*, 592–601.

[28] D. M. Penrose, B. R. Glick, Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria, *Physiol. Plant*. **2003**, *118*, 10–15.

[29] J. E. Loper, M. N. Schroth, Influence of bacterial sources of indole-3-acetic acid on root elongation of sugar beet, *Phytopathol*. **1986**, *76*, 386–389.

[30] C. L. Patten, B. R. Glick, Role of *Pseudomonas putida* indole acetic acid in development of the host plant root system, *Appl. Environm. Microbiol*. **2002**, *68*, 3795–3801.

[31] J. R. de Freitas, M. R. Banerjee, J. J. Germida, Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brassica napus* L.), *Biol. Fertil. Soils* **1997**, *24*, 358–364.

[32] S. Timmusk, B. Nicander, U. Granhall, E. Tillberg, Cytokinin production in *Paenibacillus polymyxa*, *Soil Biol. Biochem*. **1999**, *31*, 1847–1852.

[33] B. R. Glick, D. M. Penrose, J. Li, A model for lowering of plant ethylene concentrations by plant growth-promoting rhizobacteria, *J. Theor. Biol*. **1998**, *190***,** 63−68.

[34] J. A. Hall, D. Peirson, S. Ghosh, B. R. Glick, Root elongation in various agronomic crops by the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2, *Isr. J. Plant Sci*. **1996**, *44*, 37–42.

[35] D. Saravanakumar, R. Samiyappan, ACC deaminase from *Pseudomonas fluorescens* mediated saline resistance in groundnut (*Arachis hypogea*) plants, *J. Appl. Microbiol*. **2007**, *102*, 1283–1292.

[36] C. Wang, E. Knill, B. R. Glick, G. Défago, Effect of transferring 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase genes into *Pseudomonas fluorescens* strain CHA0 and its gacA derivative CHA96 on their growth-promoting and disease-suppressive capacities, *Can. J. Microbiol*. **2000**, *46*, 898–907.

[37] D. Gemechu, *in Aspects of Climate and Water Budget in Ethiopia*, Addis Ababa University Press, Addis Ababa **1977**.

[38] C. Ruiz-García, V. Béjar, F. Martínez-Checa, I. Llamas, E. Quesada, *Bacillus velezensis* sp. nov., a surfactant producing bacterium isolated from the river Vélez in Málaga, southern Spain, *Int. J. Syst. Evolut. Microbiol*. **2005**, *55*, 191–195.

[39] J. Debode, K. De Maeyer, M. Perneel, J. Pannecoucque, G. De Backer, M. Höfte, Biosurfactants are involved in the biological control of *Verticillium* microsclerotia by *Pseudomonas* spp., *J. Appl. Microbiol*. **2007**, *103*, 1184–1196.

[40] L. Gardan, H. Shafik, S. Belouin, R. Broch, F. Grimont, P. A. D. Grimont, DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremae* sp. nov. and *Pseudomonas cannabina* sp. nov. (*ex* Sutic and Dowson 1959), *Int. J. Sys. Bacteriol*. **1999**, *49*, 469–478.

[41] S. C. Verma, J. K. Ladha, A. K. Tripathi, Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deepwater rice, *J. Biotechnol*. **2001**, *91*, 127–141.

[42] M. Lebuhn, W. Achouak, M. Schloter, O. Berge, H. Meier, M. Barakat *et al*., Taxonomic characterization of *Ochrobactrum* sp. isolates from soil samples and wheat roots, and description of *Ochrobactrum tritici* sp. nov. and *Ochrobactrum grignonense* sp. nov., *Int. J. Syst. Evol. Microbiol*. **2000**, *50*, 2207–2223.

[43] A. Ngom, Y. Nakagawa, H. Sawada, J. Tsukahara, S. Wakabayashi, T. Uchiumi *et al*., A novel symbiotic nitrogen fixing member of *Ochrobactrum clade* isolated from root nodules of *Acacia mangium*, *J. Gen. Appl. Microbiol*. **2004**, *50*, 17–27.

[44] E. Taye, *Report on Woody Plant Inventory of Yayu National Forestry Priority Area*, IBCR/GTZ, Addis Ababa **2001**.

[45] R. M. N. Kucey, H. H. Janzen, M. E. Leggett, Microbially mediated increases in plant available phosphorus, *Adv. Agron*. **1989**, *42*, 199–221.

[46] A. Khan, Mycorrhizoremediation–an enhanced form of phytoremediation, *J. Zhejiang University Sci*. **2006**, *B7*, 503–514.

