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An increasing opine carbon bias in artificial exudation systems and genetically modified plant rhizospheres leads to an increasing reshaping of bacterial populations

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Abstract

To investigate how exudation shapes root-associated bacterial populations, transgenic *Arabidopsis thaliana* plants that exuded the xenotopic compound octopine at low and high rates were grown in a nonsterile soil. Enumerations of both cultivable and octopine-degrading bacteria demonstrated that the ratios of octopine degraders increased along with octopine concentration. An artificial exudation system was also set up in which octopine was brought at four ratios. The density of octopine-degrading bacteria directly correlated with the input of octopine. Bacterial diversity was analysed by *rrs* amplicon pyrosequencing. *Ensifer* and *Pseudomonas* were significantly more frequently detected in soil amended with artificial exudates. However, the density of *Pseudomonas* increased as a response to carbon supplementation while that of *Ensifer* only correlated with octopine concentrations possibly in relation to two opposed colonization strategies of rhizosphere bacteria, that is, copiotrophy and oligotrophy.

Keywords: copiotrophy, DESEQ, genetically modified plants, oligotrophy, opine, pyrosequencing, root exudates

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Introduction

Microbial populations of the rhizosphere are influenced by various parameters, of abiotic [e.g. physicochemical soil characteristics (Fierer & Jackson 2006)] and biotic [e.g. plant species or genotype (Hartmann *et al.* 2009)] origins (Philippot *et al.* 2013). Since the early work of Hiltner in 1904, several authors reported results from field or microcosm experiments that clearly demonstrated the influence of plants as selective agents of bacterial populations and communities (Bais *et al.* 2006; Jones *et al.* 2009). Root-deposited carbon in soil is most likely the major determinant of the microbial diversity. The carbon sources are diverse in nature and complexity. First, roots release exudates that consist mostly of organic and amino acids and sugars. Second, root growth involves mechanical constraints and root cell shearing that led to the release of high molecular weight compounds (e.g. polysaccharides, peptides, proteins) in soil. These two forms of carbon input constitute rhizodeposition (Berg & Smalla 2009), a major phenomenon in rhizosphere ecology, that provides 'diverse substrate to soil microbial communities and is responsible for the well recognized stimulation of microbial biomass size and activity around roots' (Millard & Singh 2010).

The role of exudates in the reshaping of soil bacterial community structure was evidenced by stable isotope probing at the rhizosphere of four plant species labelled with $^{13}CO_2$. This led to the identification of generalist colonizers (i.e. microbial groups with no 'plant preference') and to that of preferential colonizers, more fre-

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quently detected in the rhizosphere or root surface of plants (e.g. species closely related to *Azospira*, *Acidovorax* and *Variovorax* species found only in association with rape plant; Haichar *et al.* 2008). Exudate composition is, however, host- and growth condition specific. Indeed, there are multiple examples of selection of specific microbial populations by plants of different genera and species or even by plants of the same species but different ecotypes alone (Hovatter *et al.* 2011; Lundberg *et al.* 2012; Oh *et al.* 2012; Philippot *et al.* 2013) or in association (Krimi *et al.* 2002). Genetic structure and function of bacterial communities or specific clade may also be affected by the developmental stage of the plant (Mougel *et al.* 2006; Chaparro *et al.* 2014).

In most of the above-mentioned studies, the observed population or community changes have not been correlated with the presence/absence or concentration of exuded compounds (or the presence/absence/frequency of specific compounds at the plant surface) most certainly because of the complexity of the exudate composition (Uren 2007) and the impossibility to quantify the components of the exudates when plants are cultivated under 'real soil' conditions.

Molecular techniques permit the authors, however, to engineer the composition of exudates, to induce specific biases and to further investigate on the consequences of these biases. Using these techniques, the authors of this article have developed over the years a strong model that involves transgenic plants that expressed opine biosynthetic genes from *Agrobacterium tumefaciens* T-DNA region. Opines are low molecular weight carbon compounds derived from amino acid and/or organic acids. They are normally absent from plant metabolomes as they are specifically found in crown gall tumours induced by the pathogen *Agrobacterium*. These bacteria use opines as both growth substrates and inducers of the conjugal transfer of their main pathogenic element, the Ti plasmid. Their synthesis in the plant tumour proceeds from simple metabolites (e.g. arginine and pyruvate for octopine), and their breakdown in the bacteria generally yields the same constituents (Dessaux *et al.* 1998). The microflora associated with the opine-producing plant lines do not differ from parental lines in terms of cell density of root-associated, cultivable bacteria. These plants, however, clearly selected a microbial community able to degrade the opines (Oger *et al.* 1997, 2000; Savka & Farrand 1997). This selection was observed in two soils with different characteristics, irrespectively of the transgenic plant species (Mansouri *et al.* 2002).

The above correlation (i.e. the production of opine by the plants and the selection of an opine-degrading community) may be strengthened by evidencing a correlation between the magnitude of a carbon bias induced by the root exudates and that of the bacterial population reshaping. To investigate how a carbon bias may affect microbial populations in quantitative and qualitative ways, two independent but strongly complementary experiments were set up. A first experiment involved transgenic plants producing the opine octopine at two concentrations. The impact of these two lines on the bacterial microflora was analysed as above, using the parental line as a control. Because only a limited number of conditions could be generated using transgenic plants and because the amount of carbon in the exudates in soil cannot be directly estimated, a second series of experiments was set up in an artificial exudation/rhizosphere system. This artificial system involved unsterilized soil amended through a slow percolation system with a compound mixture mimicking root exudates. An increasing carbon bias was introduced in the composition of the mixture. The bias consisted in the replacement, at equivalent carbon atom concentration, of one or more of the organic acids generally found in root exudates by the opine octopine. The impact of these changes on the bacterial microflora was analysed in terms of: (i) cell density of total cultivable bacteria; (ii) cell density of octopine-degrading bacteria; and (iii) diversity of genera inhabiting the artificial rhizosphere system via an *rrs* amplicon pyrosequencing approach. Results of these investigations clearly indicate that the impact of the carbon bias on some bacterial populations and on the bacterial community degrading octopine directly correlates with the amount of carbon that characterizes these artificial or plant exudation biases. Additionally, the experiments revealed the existence of two opposed colonization strategies of rhizosphere bacteria, that is, copiotrophy and oligotrophy as suggested by Fierer *et al.* (2007), in relation to the nature of exuded carbon compounds.

Materials and methods

Bacterial growth media and bacterial enumeration

Bacteria were routinely cultivated on modified Luria-Bertani medium (LBm that contains 2.5 g/L NaCl instead of 10 g/L). Total bacterial populations were enumerated by plating serial dilutions of soil suspensions obtained in water on LBm, while octopine-degrading bacteria were enumerated on Bergensen's modified minimal medium (Bergensen 1961) that contained octopine (1.2 g/L, i.e. 5 mM) as sole C and N source. All bacteria were cultivated at 28 °C.

Chemicals

All chemicals were from commercial source except octopine that was synthesized in the laboratory according

to previously published procedure (Tempe & Goldmann 1982).

Plant cultivation

For transgenic screening and seed amplification, *Arabidopsis thaliana* plants were grown in a greenhouse under long-day conditions (16-h day/8-h night, 22 °C, 60% humidity). For the impact study, plants were grown under short-day conditions (8-h day/16-h night, 22 °C, 60% humidity) in a soil substrate that consisted in a mixture of unsterilized 'Mérantaise' soil (from a CNRS campus meadow, GPS coordinates 48.706298N 2.126495E) and sterile organic compost (1:1 v:v) that yields a sandy/loamy substrate. For composition, see Table S1 (Supporting information). Plants were watered with tap water. In vitro, plants were routinely cultivated on germination solid medium that consisted of half-strength Murashige and Skoog medium (M0221-10; Kalys, France), MES 0.5 g/L, sucrose 10 g/L, Gamborg B5 vitamin mix and agar Kalys HP696 7 g/L.

Plant transformation

The *Escherichia coli* strain XL1-Blue was used for cloning and propagation of the different vectors. *Agrobacterium tumefaciens* strain EHA105 was transformed by electroporation and used in all plant transformation experiments (Hood *et al.* 1993). The genomic region containing the octopine synthase (*ocs*) gene and its own promoter was amplified using the DNA from *A. tumefaciens* strain 15955 as template. PCR amplification was performed with the primer couple, OCSfor-HindIII, 5′-CGGCCAATACTCAAGCTTCAAGGAATCTCA-3′, and OCSrev*XBA*I, 5′-CTTACCCCATTATCTAGATTGCGATGAAGC-3′, to introduce the restriction enzyme sites *Hin*dIII and *Xba*I for subsequent cloning. The amplicon was subcloned into the pGEM-Teasy vector (Promega®) as indicated by the manufacturer. The restriction fragment *Hin*dIII/*Xba*I was subcloned into a modified MCS vector derived from pCambia1390. *Arabidopsis thaliana* ecotype Col-0 was transformed with empty vectors pCambia1390:35S or pCambia1390::*ocs* using the flower dipping method (Logemann *et al.* 2006). T1 plants were screened on germination medium (supplemented with hygromycine 30 mg/L). The presence of the T-DNA in the screened lines was monitored by PCR analysis using the primer couple, OCSfor, 5′-GCTCTTACTCTTGCAGGTGA-3′, and OCSrev, 5′-TCGTCTATTGCGGTAACACG-3′. T2 progeny analysis was performed, and lines with a single T-DNA locus were selected for further experiments.

Evaluation of octopine production by transgenic lines

The evaluation of octopine production in leaf plants was assessed by high-voltage paper electrophoresis at pH 1.9. Quantification of octopine production was achieved by phenanthrene-quinone staining of the electropherograms and comparison of spot diameters with those standards of known concentrations (Dessaux *et al.* 1992). Octopine production in exudates was assessed in 1-month-old plants grown on germination medium. The plants were transferred into 1 mL sterile water for 3 days under continuous illumination. Water containing the exudates was concentrated fivefold, and octopine was quantified as indicated earlier.

Evaluation of the consequence of the exudation bias generated by octopine-producing plants

Two homozygous transgenic lines of *A. thaliana*, one with a high level of octopine production (high-OCS) and the other with a low one (low-OCS), were obtained as indicated earlier. Opine concentrations in exudates of these two lines are given under results. Col-0 wild-type line was used as control. Seeds from Col-0 and homozygous octopine-producing lines (3rd generation) were sown in the soil mixture described above, and plants were grown in a growth chamber under short-day condition (8-h day/16-h night, 60% humidity, 22 °C). Soil mixture and plant samples were harvested 10 weeks after sowing, with plants still producing opine. Twenty gram of soil samples was resuspended in sterile water and strongly shaken (2 × 30 s, plus 1 × 2 min), and the resulting suspensions were serially diluted into sterile water. Cell density of total cultivable bacteria and that of octopine-degrading bacteria was assessed as described above. The taxonomic affiliation of the octopine-degrading bacteria was achieved by partial *rrs* gene sequencing, as described later. The experiment involved six independent biological replicates (each with two technical replicates) per condition, that is, bare soil, and soil that sustained the growth of wild-type, low-OCS and high-OCS plants.

Identification of opine-degrading bacteria by rrs gene sequencing

Single colonies of octopine-degrading bacteria were randomly picked from Bergensen-octopine plates that contained 1 to ~100 colonies. In this last case, the plates were randomly divided in quarters, and all colonies from a quarter were collected. Single colonies were diluted into 10 μL of sterile ultrapure water. One microlitre of this solution was mixed with 9 μL of a lysis buffer (10 mM Tris-HCl pH 7.6, 50 mM KCl, 0.1% Tween

20) and heated at 100 °C for 10 min. PCR amplification was performed using a Standard *Taq* polymerase (Eurobio, Les Ulis, France) and the universal eubacterial primer couple pA/pH (concentration 20 nmol/μL) that target the 16S rRNA gene (Edwards *et al.* 1989). The PCR conditions were as follows: 94 °C 3 min, followed by 35 cycles at 94 °C (30 s), 56 °C (30 s) and 72 °C (1 min 30 s). Single-strand sequence determination of the amplicon was carried out from pA or 907R primers by GATC (Mulhouse, France). The sequences were assigned at different taxonomic levels using the Ribosomal Database Project (RDP) classifier tool (http://rdp.cme.msu.edu/classifier/classifier.jsp) at a 0.95 confidence level (Cole *et al.* 2009).

Evaluation of the consequence of the carbon bias generated in an artificial exudation system

The artificial exudation system consisted of 25 g of the nonsterile Mérantaise soil mixture described above placed in a 150 × 22 mm glass tube and fed with 'synthetic exudates'. The composition of these 'synthetic' exudates was in agreement with the composition of plant exudates described in Kamilova *et al.* (2006), Kuo *et al.* (1982) and Neumann & Romheld (1999). The synthetic exudates were percolated within the soil through a sterile cotton wick fed from a reservoir (Fig. S1, Supporting information) at the rate of 330 μL every 2 days. Four compositions (oct0–oct75) of artificial exudates that were equimolar in carbon were used: they are given in Table 1. All compositions were buffered at pH 6.2 as root exudates are generally acidic. The devices were incubated at 24 °C for 8 days. The experiment consisted of three independent replicates for conditions oct0–oct75 (i.e. 3 repeats × 4 conditions). Cultivable bacteria were enumerated by determining (i) the cell density of total cultivable bacteria and (ii) the cell density of octopine-degrading bacteria, all as described earlier. The experiment was repeated completely independently another time.

The taxonomic affiliation of the octopine-degrading bacteria was achieved by partial *rrs* gene sequencing, as described earlier. The bacterial diversity was assessed by pyrosequencing of the *rrs* gene, as described later. This second experiment consisted of six independent replicates per condition (i.e. 6 repeats × 4 conditions).

Pyrosequencing analysis of rrs genes of soil bacteria recovered from the artificial exudation system

Total genomic DNA was extracted from the Merantaise's soil microcosms from bare soil and at 8 days after treatment with the different synthetic exudates (conditions oct0–oct75) using the Power Soil extraction kit (MoBio, USA). DNA quantity and quality were measured using a NanoDrop ND1000 Spectrophotometer (LabTech, France). DNA was extracted from three (randomly chosen) of the six independent repeats per condition. Pooled samples consisted of a mixture of 1 μg of DNA from each individual samples. The concentration of the extracted DNA was adjusted to 15 ng/μL in 0.1 × TE buffer using a NanoVue Spectrophotometer (GE Healthcare). PCR amplification of the *rrs* genes (variable regions V1–V3) was carried out using the universal bacterial primer 27f (5′ -AdaptorA-GAGTTTGATCMTGGCTCAG -3′) and 518r (5′- AdaptorB-X-WTTACCGCGGCTGCTGG -3′), where adaptors A and B represent the adaptor sequences for GIIX-Titanium pyrosequencing method and X the 10-mer sequence tags allowing multiplexing. The amplified region corresponds to the V1–V3 variable regions of the

Table 1 Composition of synthetic exudates

			Exudate composition‡			
Molecules	Nb. C*	Nb. N†	oct0	oct8	oct25	oct75
Amino acid						
Aspartic acid	4	1	0.18	0.18	0.18	0.18
Glutamic acid§	5	1	0.23	0.21	0.17	0.04
Homoserine	3	1	0.14	0.14	0.14	0.14
Sugar						
Fructose	6	0	2.33	2.33	2.33	2.33
Glucose	6	0	1.60	1.60	1.60	1.60
Maltose	6	0	0.11	0.11	0.11	0.11
Ribose	5	0	0.09	0.09	0.09	0.09
Xylose	5	0	0.09	0.09	0.09	0.09
Organic acid§						
Citric acid	6	0	17.94	16.32	13.08	3.38
Malic acid	4	0	28.85	26.25	21.05	5.44
Succinic acid	4	0	6.89	6.27	5.03	1.30
Fumaric acid	4	0	0.39	0.36	0.29	0.07
Oxalic acid	2	0	1.17	1.06	0.85	0.22
Opine						
Octopine	9	4	0.00	5.00	15.00	45.00
Total carbon‡			60	60	60	60
Octopine ratio (%)			0	8	25	75

*Number of carbon atoms in the molecule.
†Number of nitrogen atoms in the molecule.
‡Values expressed as μmol introduced per day into 25 g of soil. The experimental design therefore implies a constant carbon molarity of the synthetic exudates, whatever the octopine contribution to this molarity.
§Organic acids were replaced by octopine as they were the main components of exudates and as octopine consists of alpha-ketoglutarate and arginine. Arginine being degraded to glutamic acid, glutamic acid concentrations were also reduced while octopine concentrations increased.

16S rRNA gene, allowing classification of reads using the RDP Classifier (Vilo & Dong 2012). PCR amplification conditions were adapted for the Phusion high fidelity polymerase (Finnzymes, Germany), as follows: 98 °C (2 min), 25 cycles at 98 °C (20 s), 54 °C (20 s) and 72 °C (15 s), and a final elongation step at 72 °C (5 min). The PCR mixture was prepared as described by Cirou *et al.* (2012). Pyrosequencing was performed using a Roche FLX Titanium pyrosequencer (Microsynth, Switzerland). The obtained sequences for each sample were grouped according to their tag. The average read length after removal of the tag is 531 nucleotides with a total number of reads of 429 557 (Table S2, Supporting information).

Analysis of rrs gene sequences recovered from pyrosequencing

To compare the composition of the different samples, the number of analysed sequences per condition was randomly subsampled from 14 301 to 15 000. Two analyses were performed on the data set (Fig. S2, Supporting information). First, a diversity analysis was performed to determine the Shannon index, rarefaction curve and operational taxonomic unit (OTU) diversity with taxonomic assignment of representative sequence for each OTU. Second, the reads were taxonomically assigned, grouped at homogenous clade levels (class and genus). For both analyses, a trimming step was performed using CLC GENOMICS WORKBENCH 5.1 based on length and quality analysis. The first step was the removal of reads with low Phred quality as described in CLC Workbench manual. No ambiguous nucleotide was allowed in reads (sequence below ambiguous nucleotide was trimmed away). The final step filtered on length with a minimal size of 200 nucleotides. The chimera were searched using uchime algorithm (Edgar *et al.* 2011) implemented in mothur (Schloss *et al.* 2009). The Gold database (http://drive5.com/uchime/gold.fa) was used as reference for uchime analysis. The number of chimera was low and similar between each sample (Table S3, Supporting information). A preliminary analysis was performed on pooled DNA samples for all five conditions (bare soil, oct0–oct75) and also on individual samples (bare soil, oct0 and oct75) to evaluate the possible bias induced by sample pooling (Fig. S2, Supporting information). The trimmed reads were taxonomically assigned at different levels using the RDP's Classifier tool at 80% confidence level, and frequencies for each detected taxon against total number of reads for each condition were calculated. Correspondence analysis coupled with between-group analysis that involved individual and pooled samples of conditions bare soil, oct0 and oct75 was performed and revealed that the data of the pooled samples were close to the centres of mass of the areas determined by the individual samples (Fig. S3, Supporting information). Permutation test (n = 100 000) indicated that the defined groups (consisting in individual samples and pooled samples) were not randomly distributed (BGA, P-value 6.99×10^{-5}). In consequence, no bias was introduced by the sampling, and consequently, further analyses were therefore performed on pooled samples.

For the first analysis, the trimmed sequences were analysed using the RDP pyrosequencing pipeline (Cole *et al.* 2009) that gives reliable and accurate results for OTU clustering analysis (Barriuso *et al.* 2011). The following parameters were used for trimming: number of Ns: 0; minimum sequence length: 200; primers removed: true; forward primer(s): WTTACCGCGGCT GCTGG; and maximum forward primer distance: 2. The trimmed sequences were aligned, using the secondary-structure INFERNAL aligner v1.1 (Nawrocki & Eddy 2013), OTU clustered at 3% and 5% distance using complete-linkage clustering algorithm (Cole *et al.* 2009). Chimera generally clustered as singleton OTU. In consequence, singleton OTU was discarded for the analysis of the alpha diversity. The 16S rRNA gene sequences obtained from pyrosequencing were analysed using tools provided by the RDP pyrosequencing pipeline (http://pyro.cme.msu.edu/) to obtain Shannon index and rarefaction curves. A representative sequence for each OTU was selected using the representative sequence tool (http://pyro.cme.msu.edu/spring/derep.spr) and taxonomically assigned at 80% confidence level.

For the second analysis, the trimmed reads were taxonomically assigned at different levels using the RDP's Classifier tool at 80% confidence level. The data were normalized for a differential analysis using the DESEQ package (Anders & Huber 2010). This latter was initially developed for the analysis of RNAseq experiment. The goodness of fit of the statistical model with our data (the number of reads in sample i that are assigned to the clade level j) showed that our data best fit with a negative binomial distribution that is similar to the distribution of RNAseq data. The clades were defined as differentially present when $padj$ <0.05 (here $padj$ refers to the raw P-value adjusted for multiple testing using the Benjamini–Hochberg method). The conversion from RDP output to R was performed using an executable home-made Shell script. R functions allowed the automatization of the analyses and generation of graphical outputs (script and R functions are provided in Appendix S1).

Statistical analysis

All the statistical analyses were performed on R 2.14.2 (R-Development-Core-Team 2012). PVCLUST 1.2-2 package

was used for hierarchical clustering (Suzuki & Shimodaira 2006) and DESEQ package 1.9.6 for differential presence analysis (Anders & Huber 2010). Similarity in community composition between treatments was calculated using the Bray–Curtis similarity index (Bray & Curtis 1957) on normalized count of reads assigned at genus level (80% confidence level).

Results

Enumeration of cultivable bacteria isolated from the rhizosphere of Arabidopsis thaliana plants

Cultivable bacteria were enumerated at the root system of wild-type (WT) and two transgenic *A. thaliana* lines with altered exudation cultivated in a mixture of organic compost and unsterilized Mérantaise soil (CNRS campus; composition in Table S1, Supporting information). One line produced low concentration of octopine, while the second produced high concentration. The phenotype (size, life cycle, leaf colour, architecture of the plant organs, concentration of 70 organic and amino acids and sugars, data not shown) of the transgenic lines was similar to that of the parental WT lines. The exudation of octopine in the transgenic lines was quantified under axenic, hydroponic culture conditions. It ranged from ~20 μg/day/g plant dry weight for the low-OCS (OCS, octopine synthesis) line to ~440 μg/day/g plant dry weight for high-OCS line. The ratio of exuded octopine from one line to the other was therefore ~1–20 similar to the concentration of octopine detected in leaves of the same transgenic line. The cell density of the cultivable bacterial population and that of members of the octopine-degrading community was determined at the root system of transgenic *A. thaliana* (Fig. 1A–C). After 10 weeks, the density of cultivable bacteria and that of octopine utilizers as well as their ratios calculated at the root system of WT and low-OCS line did not statistically differ. However, a significant increase in both the density and ratios of opine-degrading bacteria was observed in the rhizosphere of the high-OCS line with an average of 35% of the cultivable bacteria that degraded octopine. The bias that affected the opine-degrading community therefore appears to be related to the modification of the carbon sources induced by the genetic modification of the plant.

Identification of octopine-degrading bacteria isolated from the rhizosphere of Arabidopsis thaliana plants

The consequence of the production of octopine on the bacteria diversity was evaluated with respect to the diversity of members of the octopine-degrading community. Results of the phylogenetic analysis (performed via sequence determination of the *rrs* gene) of 140 randomly picked (see Materials and Methods) members of the octopine-degrading communities selected from the rhizospheres of WT and transgenic lines are presented in Figs 1D and S4 (Supporting information). This analysis showed that the number of bacterial classes was not affected by the composition of the exudates. In contrast, the production of high amounts of octopine by the plant led to both the disappearance of the Flavobacteria and an increased density of the Gammaproteobacteria especially at the root system of the high-OCS line. At the class level, the appearance of members of the Alphaproteobacteria class amongst octopine degraders occurred only in the rhizosphere of high-OCS transgenic lines. Fisher's exact test indicated that these changes are statistically significant (Fig. 1D). At the genus level, most opine-degrading Gammaproteobacteria were pseudomonads, whatever the condition (Fig. S4, Supporting information). Therefore, high production of octopine translated into an increased density of members of this group. Amongst Betaproteobacteria, an increased number of *Castenalliena* were seen, as well as the appearance of members of the genera *Pusillomonas* and *Parapusillomonas*. The octopine-degrading Alphaproteobacteria detected in the rhizosphere of the high-OCS line belonged to the genus *Ensifer*, closely related to *Agrobacterium*. These results (octopine user and taxonomical assignment) suggest that the structure of the octopine-degrading community isolated at the rhizosphere of the high-OCS line differed more from that of the wild-type plant than did that of the low-OCS line.

Enumeration of cultivable bacteria in the artificial exudation system

To generate a better-defined change in octopine concentration without changing the total amount of carbon provided to the bacteria, an artificial exudation system was set up (Fig. S1, Supporting information). It consisted of Mérantaise soil samples percolated with the synthetic exudates described in Table 1, with enrichment in octopine set at 0%, 8%, 25% and 75% at constant carbon molarity (conditions oct0, oct8, oct25 and oct75). The supplementation of the soil with artificial exudate mixtures for 8 days increased the cell density of cultivable bacteria for condition oct8 with respect to conditions oct0, oct25 and oct75 (Fig. 2A). On the opposite, the cell density of opine-degrading bacteria was stimulated in microcosms supplemented with octopine-containing exudates (i.e. conditions oct8, oct25 and oct75 vs. condition oct0; Fig. 2B). The average ratio of opine-degrading bacteria to total cultivable bacteria

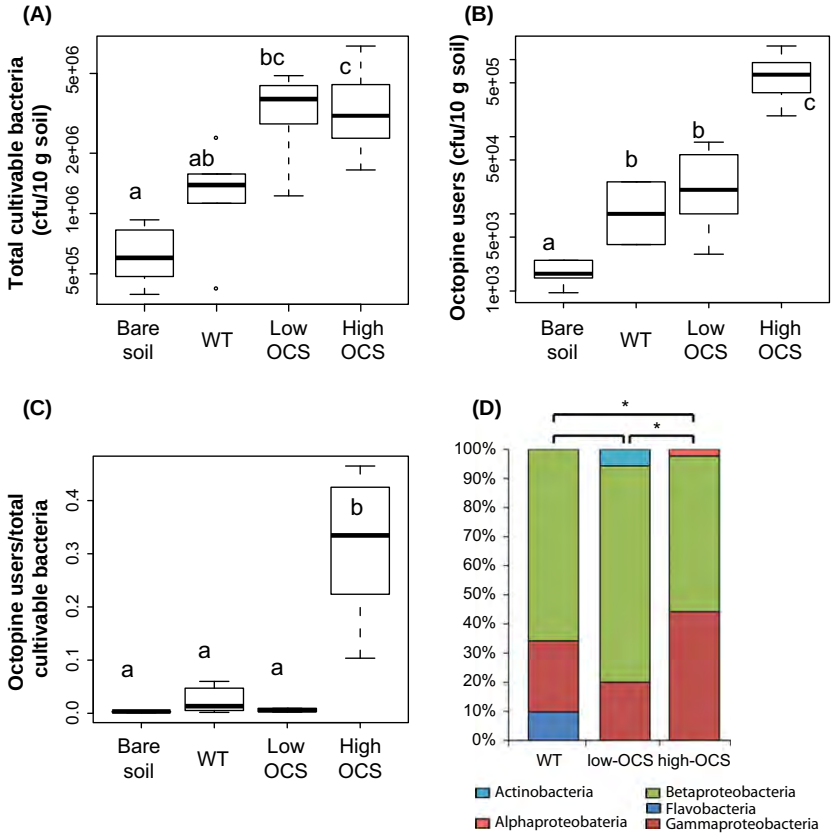


Fig. 1 Enumeration and phylogenetic analyses of opine-degrading bacteria at the rhizosphere of opine-producing *Arabidopsis thaliana* lines. Total cultivable bacteria (A), octopine-degrading bacteria (B) and ratio of octopine-degrading bacteria to total cultivable bacteria (C) were measured in bare soil and in planted soils at 10 weeks, for wild-type (WT) and transgenic low-OCS and high-OCS lines. For each panel, data were shown as box and whisker plots in which the central box represents the values from the lower to upper quartile (25–75 percentile). The middle bold line represents the median. The vertical line extends from the minimum to the maximum values. A Kruskal–Wallis one-way test (A, F = 17.4, P = 5.8e-04; B, F = 15.5, P = 1.4e-03; C, F = 14.6, P = 2.1e-03) and a post hoc Tukey's test were performed, and statistically identical values are attributed identical letters (n = 8). Phylogenetic position of the bacteria was determined by *rrs* gene sequencing. Members of the octopine-degrading community are represented per class (D). Numbers of octopine-degrading bacteria analysed were as follows: wild type (WT), 46; low OCS, 46; and high OCS, 48. Fisher's exact test was used to compare the proportions of bacterial classes between wild-type, low-OCS and high-OCS plants. The distribution was statistically different when the P-value is below 0.05 (*). Fisher's exact test values were WT vs. low-OCS (P = 0.1), WT vs. high-OCS (P = 8e-03) and low-OCS vs. high-OCS plants (P = 2e-02). OCS, octopine synthase.

increased in microcosms supplemented with octopine-containing exudates in direct proportion to the octopine concentration to reach an average value of 80% under conditions oct75 (75% of the carbon of the artificial exudate consists in octopine). The experiment was repeated once, and similar results were obtained (Fig. S5, Supporting information). A linear regression analysis was performed using the data from both experiments (Fig. 2C). The coefficient of determination was R^2 = 0.825, indicating a good fit of the data points with the linear model. Noticeably, the linear relationship between the abundance of octopine and the proportion of octopine-degrading bacteria in the microbial population was positive and statistically significant (ANOVA, F = 71.8, P-value = 6.9e-07).

Identification of cultivable octopine-degrading isolates in the artificial exudation system

Results of the phylogenetic analysis (performed via sequence determination of the *rrs* gene) for 124 members of the octopine-degrading community randomly picked from various artificial rhizospheres are presented in Figs 2D and S6 (Supporting information). Beta- (23%) and Gammaproteobacteria (53%) represented most of the octopine-degrading isolates under bare soil (untreated T0 sample) condition. Addition of synthetic exudates resulted in the appearance of octopine-degrading bacteria in additional classes, including Actinobacteria and Flavobacteria. For instance, the only octopine-degrading Gammaproteobacteria strains in

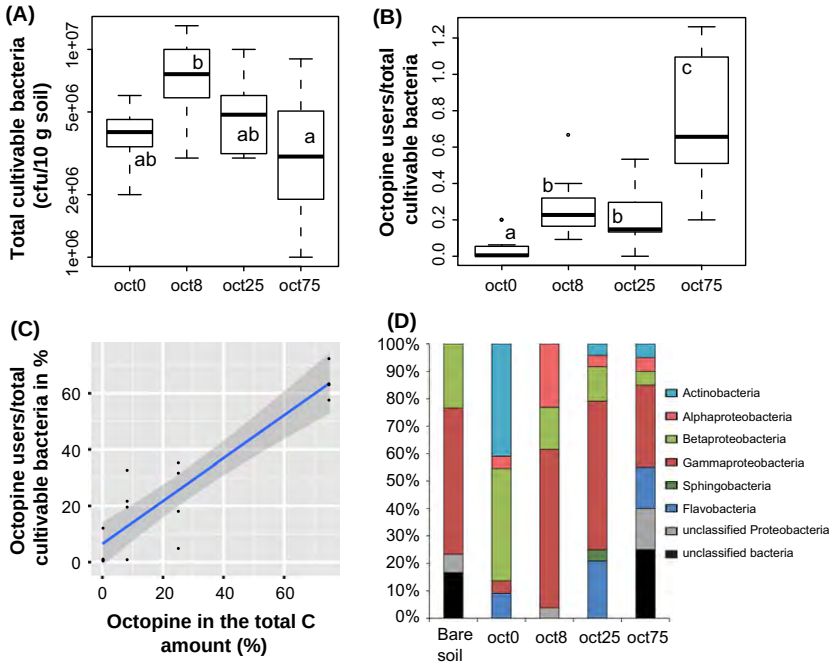


Fig. 2 Enumeration and phylogenetic analyses of opine-degrading bacteria in an artificial exudation system under four exudate compositions. Total cultivable bacteria (A), frequency of octopine user on total cultivable bacteria (B) and relation between the octopine content and the frequency of octopine user (C). Data were obtained at 8 days (T8) for the four compositions of synthetic exudates. A Kruskal–Wallis test (A, F = 13.1, P = 4.4e-03; B, F = 30.3, P = 1.1e-06) and a post hoc Tukey's test were performed; statistically identical values are attributed identical letters (n = 6). Linear regression (y = 6.51 + 0.76x, 95%) of ratio of octopine user to octopine in the total amount of carbon showed a positive linear correlation (R^2 = 0.825, ANOVA, F = 71.8, P-value = 6.9e-07). Confidence intervals are drawn in grey. Members of the octopine-degrading community are represented per class (D) and per genus amongst the three major classes (E). Numbers of octopine-degrading bacteria analysed were as follows: bare soil, 30; conditions oct0, 22; oct8, 26; oct25, 24; and oct75, 22.

bare soil belonged to the family Pseudomonaceae. In contrast, in the presence of synthetic exudates, octopine-degrading isolates amongst the genera *Acinetobacter*, *Lysobacter* and *Stenotrophomonas* were found. In a similar way, the octopine-degrading Betaproteobacteria showed strong changes as a function of octopine concentrations. In other bacterial classes, *Chitinophaga* (Sphingobacteria), *Flavobacterium* (Flavobacteria), *Paraoerskovia* (Actinobacteria) and *Streptomyces* (Actinobacteria) appeared when synthetic exudates were added. The appearance of Alphaproteobacteria only occurred in the presence of artificial exudates, with *Ensifer* and *Sinorhizobium* as opine-degrading genera (conditions oct8 to oct75; Fig. S6, Supporting information).

Diversity of soil bacterial populations in the artificial exudation system and consequence of the carbon bias on that diversity

The diversity of soil bacterial populations in the artificial exudation system was assessed by high-throughput 454 sequencing of *rrs* amplicon (variable regions V1–V3) obtained from various soil samples. Overall, after trimming, 429 557 reads were generated with an average length of 477 nucleotides (Table S2, Supporting information). To compare the composition of the different samples, the number of analysed sequences per condition eventually varied from 14 301 to 15 000 (the last value corresponding to a random subset of the sequences obtained for the conditions bare soil, oct0, oct8 and oct25) that represent a total of 175 535 analysed reads. The OTU richness and diversity indexes were calculated to determine the diversity, in the different soils (Table S4; Fig. S7, Supporting information). The numbers of OTUs (at 3% and 5%) as well as the rarefaction curves, Shannon and Bray index values were comparable in the five experimental conditions. Aside, representative sequences for each OTU were selected and taxonomically assigned at the class level (Tables 2 and S5, Supporting information). All these data indicated that the bacterial diversity of the samples did not drastically differ from one condition to the other. However, the most common OTUs varied as a function of octopine concentration, from a Bradyrhizobiaceae for bare soil, oct0 and oct25; an Alphaproteobacteria for the condition oct8; to an *Ensifer* for the condition oct75. A

Table 2 Representative sequences for each operational taxonomic unit (OTU) (3% distance) assigned to the class level (% compare to the total number of OTU)

	Pool				
Number of OTUs (%)	Bare soil	oct0	oct8	oct25	oct75
Actinobacteria	14.2	17.3	15.3	13.2	19.5
Alphaproteobacteria	9.0	10.3	9.3	9.5	12.1
Deltaproteobacteria	6.6	7.1	6.4	6.0	6.3
Acidobacteria_Gp6	5.0	6.0	4.3	4.1	4.5
Betaproteobacteria	4.7	6.4	4.5	4.6	4.7
Gammaproteobacteria	3.8	4.8	4.2	3.9	3.8
Sphingobacteria	3.5	3.9	3.5	3.0	2.0
Planctomycetacia	3.0	2.5	4.5	3.9	4.3

deeper analysis revealed that the conditions bare soil and oct0 shared the same most common OTU, which differed from the most common OTU under the condition oct25, both belonging to the Acidobacteria GP6 group. The *Ensifer*-assigned most common OTU found under the condition oct75 (194 reads/14 301 total reads) was rarely detected under bare soil (2/15 000) and oct0 (6/15 000), and more frequently under the conditions oct8 (43/15 000) and oct25 (71/15 000).

The second analysis focused on the presence of genus or classes differentially present in soil treated with artificial exudate. Of the 74 301 reads (pooled samples), 2862 (3.8 %) were listed as 'unclassified root' (i.e. unidentified sequences), 11 381 (15.3%) were assigned to unclassified bacteria, and 60 058 (80.9%) were assigned to different taxonomic clades (from genus to phylum), of which 57 618 (77.6%) were assigned at the class level and 29 282 (39.4%) at the genus level. Over 370 genera were identified. The 20 more frequent genera explain on average 62% (~2/3) of the diversity for each condition (Table 3), while the 35 more frequent explain ~75% of the diversity and the 100 most frequent genera over 93% of the diversity on average for the five analysed samples (bare soil to oct75; see Table S6, Supporting information). The relative abundance of various clades of bacteria in the five analysed samples is shown in Fig. 3A,B in the two heat maps representing the distribution of the 20 most abundant classes and 20 most abundant genera for each condition. At the class level (Fig. 3A), no major differences were seen between conditions and none appeared to be significantly influenced by the increasing concentration of octopine. Interestingly, whatever the condition (bare soil, oct0–oct75), six classes (Acidobacteria, Actinobacteria, Alpha-, Beta-, Gamma- and Deltaproteobacteria) gathered the most represented bacterial genera. Furthermore, amongst these 20 most represented bacterial classes, the class member distribution was bimodal, suggesting that overall, the bacterial population was mainly composed of members of a few highly represented bacterial classes (Fig. 3A). At the genus level (Fig. 3B), several differences were observed. The most striking one corresponds to the genus *Ensifer* (Alphaproteobacteria), the presence of which was drastically stimulated by increasing the concentrations of octopine. A hierarchical clustering analysis of the bacterial diversity was performed, taking into account all reads identified at the genus level (i.e. 29 282). The increasing amount of octopine modified the overall diversity of bacteria at the genus level (Fig. 3C). Addition of synthetic exudate without octopine did not, however, modify the overall structure as bare soil and oct0 conditions grouped together. A differential analysis was performed using the DESEQ package initially developed for RNASeq analysis. This analysis revealed that only *Ensifer* and *Pseudomonas* were significantly more frequent in oct8, oct25 and oct75 samples compared to the bare soil sample (Figs 3D and S8; Table S7, Supporting information). Moreover, the only genus differentially present between oct0 and oct75 was *Ensifer* (Fig. S8, Supporting information).

Occurrence of Ensifer OTUs is positively related to the octopine concentration

The increased number of reads assigned to *Ensifer* and *Pseudomonas* was confirmed by the analysis of individual samples (Fig. 3E,F), but the behaviour of *Ensifer* and *Pseudomonas* differed in response to exudate addition. The occurrence of reads assigned to the *Pseudomonas* genus increased upon addition of exudates to bare soil, whatever the octopine content (Fig. 3E). In contrast, the addition of artificial exudates without octopine did not lead to an increased number of reads assigned to *Ensifer* (Fig. 3F). A significant positive linear relation was evidenced, however, between the number of reads assigned to *Ensifer* and the ratio of octopine in the artificial exudates (ANOVA, $F = 122.8$, P-value = 3.92e-06).

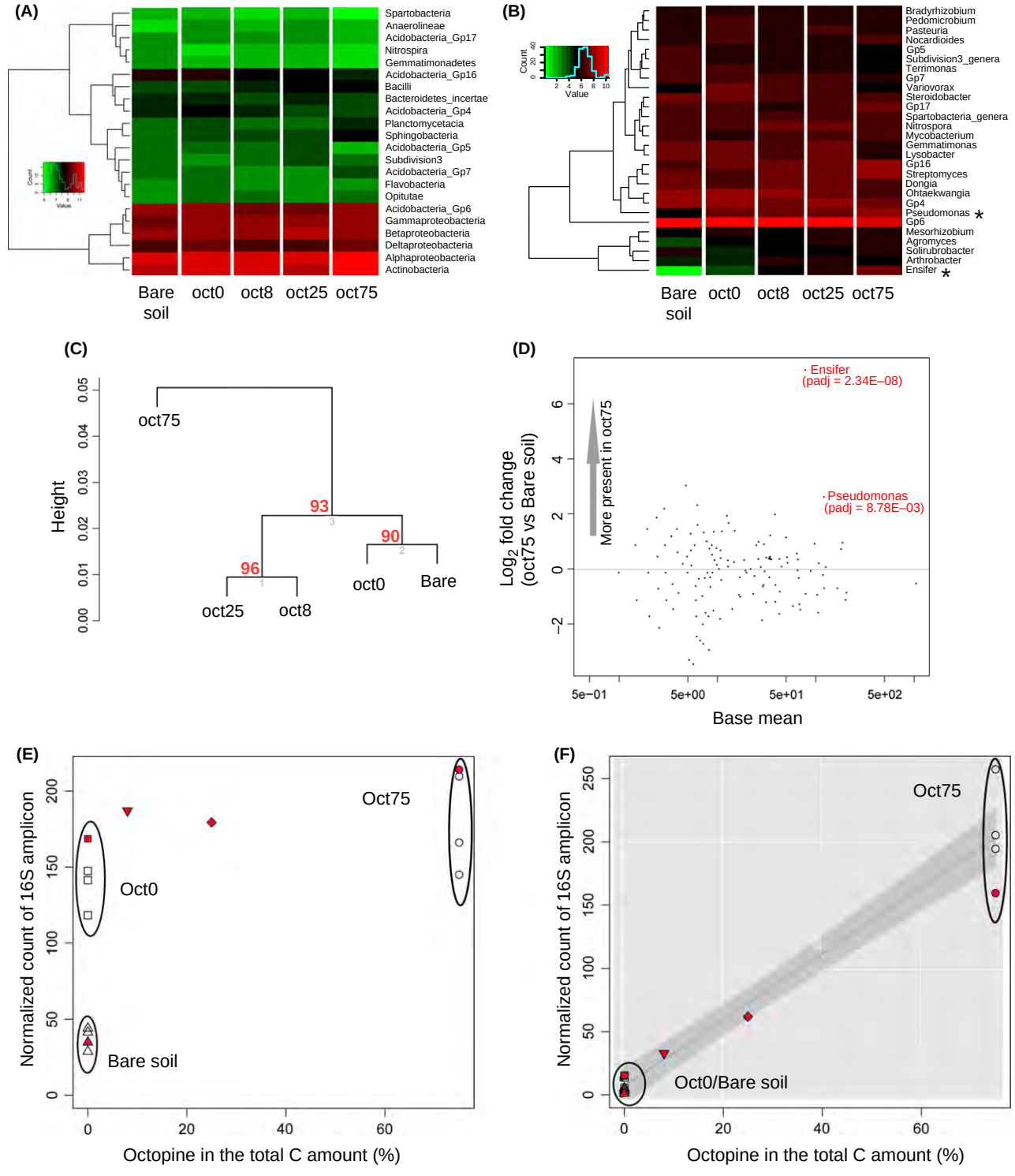
Discussion

This work evaluates whether the consequence of a modification of the exudation on root-associated bacteria depends upon the amount of carbon that typifies the exudation change. To the best of our knowledge, no study had addressed this question quantitatively and at the bacterial genus level.

The first experiment, set up with plants producing the opine octopine at two rates (in a 1:20 ratio), revealed that both the density and ratio of cultivable members of the community of octopine degraders at the rhizosphere of plants were significantly different

Table 3 Frequency of reads (%) assigned at genus level against total number of reads for each condition. The orange line corresponds to the sum of the frequency of the 20 more frequent genera (80% confidence). The ranking position of *Ensifer* and *Pseudomonas* genera is highlighted in blue and yellow, respectively

Rank	Pool_Bare soil		Pool_oct0		Pool_oct8		Pool_oct25		Pool_oct75	
1	Gp6	24.012021	Gp6	25.195682	Gp6	19.765583	Gp6	19.252014	Gp6	16.885965
2	Gp4	4.8835462	Gp4	4.2052489	Gp4	3.902504	Gp4	4.6835443	Gp16	4.8611111
3	*Gemmatimonas*	3.0353118	*Nitrospira*	2.7139715	*Pseudomonas*	3.6228023	*Pseudomonas*	3.3831991	*Streptomyces*	4.2763158
4	*Steroidobacter*	2.9902329	*Gemmatimonas*	2.6679286	*Nitrospira*	2.7703783	Gp16	3.0955121	*Pseudomonas*	4.0752924
5	*Nitrospira*	2.5093914	*Pseudomonas*	2.4684095	Gp16	2.7171018	*Nitrospira*	3.0034522	*Ensifer*	3.3625731
6	Gp16	2.4943651	Steroidobacter	2.447946	*Streptomyces*	2.4773575	*Gemmatimonas*	2.87687	Gp4	2.869152
7	*Streptomyces*	2.3741548	Gp17	2.2484269	*Mycobacterium*	2.1576985	*Streptomyces*	2.3705409	Gp17	2.503655
8	Gp17	2.3290759	*Variovorax*	2.2381951	*Variovorax*	2.1443793	Steroidobacter	2.1749137	*Nocardioides*	2.3940058
9	*Terrimonas*	2.1637866	Gp16	2.2202896	*Gemmatimonas*	2.1443793	Spartobacteria_ genera_incertae_ sedis	2.0598389	*Mycobacterium*	2.3026316
10	Gp7	2.0736289	Spartobacteria_ genera_incertae_ sedis	2.1409935	Steroidobacter	2.0644646	Gp17	1.9562716	*Nitrospira*	2.119883
11	Subdivision3_ genera_incertae_ sedis	1.9534185	*Streptomyces*	1.8161355	Spartobacteria_ genera_incertae_ sedis	1.9978689	*Mycobacterium*	1.9217491	Steroidobacter	1.754386
12	*Methylibium*	1.7881292	*Lysobacter*	1.7649767	Gp17	1.7847629	Terrimonas	1.7951669	Spartobacteria_ genera_incertae_ sedis	1.6812865
13	*Mycobacterium*	1.7580766	Terrimonas	1.672891	Terrimonas	1.7448055	*Methylibium*	1.7491369	Solirubrobacter	1.5716374
14	*Lysobacter*	1.7580766	*Methylibium*	1.5731314	Gp7	1.5982952	*Variovorax*	1.703107	*Bradyrhizobium*	1.5350877
15	Gp5	1.728024	Gp7	1.5347624	*Methylibium*	1.5583378	*Lysobacter*	1.6915995	*Mesorhizobium*	1.3888889
16	Spartobacteria_ genera_incertae_ sedis	1.5026296	*Sphingomonas*	1.4810457	Subdivision3_ genera_incertae_ sedis	1.5183804	Gp7	1.6455696	*Agromyces*	1.2244152
17	*Pirellula*	1.2021037	*Mycobacterium*	1.4605822	*Arthrobacter*	1.4651039	*Pirellula*	1.6110472	*Microlunatus*	1.2244152
18	*Opitutus*	1.1720511	Subdivision3_ genera_incertae_ sedis	1.4426766	*Pirellula*	1.3985083	*Sphingomonas*	1.5880322	*Pirellula*	1.2244152
19	*Bradyrhizobium*	1.1570248	Gp5	1.3966338	*Nocardioides*	1.3851891	*Nocardioides*	1.3233602	*Methylibium*	1.1878655
	20 more frequent	62.885049		62.689927		58.217901		59.884925		58.442982
25							*Ensifer*	1.0011507		
39	*Pseudomonas*	0.5860255			*Ensifer*	0.5993607				
84			*Ensifer*	0.1560342						
>100	*Ensifer*	0.1560342								



after 10 weeks of cultivation from one genetically modified line to the other. That production of the xenotopic compound octopine favours the development of an octopine-degrading bacterial community has indeed been reported earlier (Oger *et al.* 1997; Savka & Farrand 1997) with lotus plants and is therefore confirmed here. This result comes as a support to the opine concept that points out the trophic role played by these molecules in the *Agrobacterium*-induced tumour (review Dessaux *et al.* 1998). Although not verified in this study, the

Fig. 3 Distribution of classes and genera identified by 454 pyrosequencing in the artificial exudation system under four exudate compositions. (A) Heat map of the 20 most represented bacterial classes in each sample. The top 20 abundant classes in each sample were selected (a total of 22 classes for all five samples) and compared with their $\log_2$-transformed and normalized values. (B) Heat map of the 20 most represented bacterial genera in each sample. The top 20 abundant genera in each sample were selected (a total of 29 genera for all five samples) and compared with their $\log_2$-transformed and normalized values. The genera significantly more present in the condition oct75 compared to bare soil are indicated by asterisks (DESeq analysis). (C): dendrogram obtained after hierarchical cluster analysis of the $\log_2$-normalized values of reads assigned at the genus level. For each cluster, quantities called *P*-values were calculated via multiscale bootstrap resampling (approximately unbiased *P*-values). (D) MA plot of the differential analysis between bare soil and oct75 at the genus level. Bacterial genera significantly more or less present are highlighted in red. Relation between the octopine content and the normalized count of reads assigned to *Pseudomonas* (E) and *Ensifer* (F). Triangle = bare soil, square = oct0, cross = oct8, diamond = oct25, circle = oct75. Empty symbols are individual samples and red-filled symbols pooled sample. Linear regression ($y = 10.75 + 2.56x$) of ratio of reads assigned to *Ensifer* genus to octopine in the total amount of carbon showed a positive linear correlation ($R^2 = 0.93$, ANOVA, $F = 122.8$, *P*-value = 3.92e-06). Confidence intervals (95%) are drawn in grey.

above data also indicate that *Agrobacterium*-galled plants might have an increased number of octopine-degrading bacteria at their root system, considering that opine can be translocated from a tumour to distal plant parts including roots, and released as root exudates (Savka & Farrand 1992). Because pathogenic agrobacteria are opine-degrading bacteria, these observations may explain the increased density of these pathogens in nursery soils with frequent occurrence of crown gall disease (Krimi *et al.* 2002); this argument is, however, circular.

The novelty of the work presented here lies in the fact that the phenomenon depended upon octopine concentration. Furthermore, the present study also addressed the composition of the octopine-degrading bacterial community. The data indicated that the composition of this community appears to be more affected at the root system of plants producing high octopine concentration than at that of plants producing low concentration when compared to the composition of this community at the root system of the near-isogenic parental line that does not produce octopine (Fig. 1D–F). These series of experiments therefore demonstrate that the consequences of an altered exudation on root-associated bacteria depend upon the amount of carbon that typifies the induced exudation change. Though expected, this phenomenon had apparently not been reported earlier.

Numerous studies have reported the role of exudates in the selection of microbial populations (Bais *et al.* 2006; Hartmann *et al.* 2009) and particularly the role of secondary of specific metabolites in this selection. For instance, the plant species *Calystegium sepium* (hedge bindweed), *Morus alba* (white mulberry) or *Convolvulus arvensis* (morning glory) produces the alkaloids calystegines, and numerous calystegine-degrading bacteria can be found in the rhizosphere of this plant (Tepfer *et al.* 1988; Asano *et al.* 1994, 1996). A similar observation was made for the *Leucanea* sp. and *Mimiosa* sp. legume plants that produce mimosine, a compound toxic for several bacterial and animal species (Soedarjo *et al.* 1994; Hammond 1995). Nitrogen-fixing bacteria that induce nodule formation on plants of these two genera have, however, evolved the ability to degrade mimosine, a feature not found in nitrogen-fixing Rhizobiaceae that induce nodulation on other plant species. The data presented here performed at equivalent carbon molarity using the artificial exudation system go one step further than the description of these relations between exudates and colonizing bacteria, as they confirm the existence of a direct correlation (Fig. 2C) between the amounts of octopine released in the artificial rhizosphere system and the density of the members of the opine-degrading community, as seen with the transgenic plants The ecological consequences in terms of population and community structures at the microbial level of the carbon bias that may arise for instance from a plant mutation, a genetic modification of a plant, or a change of cultivars directly depend upon the amount of exuded carbon that characterizes this change. As seen with the high-OCS line, or earlier with *Lotus* or tobacco lines (Oger *et al.* 1997; Savka & Farrand 1997), an increased carbon bias indeed induced changes in the bacterial community structure. Conversely, if very little carbon exudates are affected by the genetic modification, the bias may remain low, close or below the detection limit as reported here for the low-OCS line, or for plants producing the bacterial quorum sensing signal (d'Angelo-Picard *et al.* 2004, 2005) or a novel nonexuded protein such as the T4 lysozyme (Lottmann *et al.* 1999; Lottmann & Berg 2001). However, a very limited carbon exudation change may still induce population changes, but these may rather result from indirect effects such as (i) a modified root architecture for transgenic alfalfa plants expressing an alpha amylase (Di Giovanni *et al.* 1999) or (ii) for transgenic poplars with altered flowering patterns (Hur *et al.* 2011) or (iii) a different soil pH, as reported for the same transgenic alfalfa plants expressing a lignin peroxidase (Donegan *et al.* 1999). In this respect, considering the key role of pH in

the possible reshaping of bacterial populations (review Hinsinger *et al.* 2009), it was important in this study to use synthetic exudates buffered at the same pH.

Bacterial clades with opine-degrading members differed in the two experimental conditions (plants producing opine and percolation with synthetic exudates) although the same soil was used in both approaches. This is not surprising because the composition of synthetic exudates probably differs from that of *Arabidopsis thaliana* exudates. For instance, the synthetic exudates did not contain any proteins (such as defence proteins) or phenolics (including bacteriostatic ones), the presence of which has been reported in *A. thaliana* exudates (Badri *et al.* 2009; De-la-Pena *et al.* 2010). Furthermore, the composition of the exudates was based on that of various plants such as pea, tomato or sweet pepper that are unrelated to *A. thaliana* (see Materials and Methods). However, our results may explain why the composition of components of the root microbiome may change during the plant lifetime (Krimi *et al.* 2002) as the quantitative composition of some exudate molecules also changes timewise.

Although valuable data have been reported (Becker *et al.* 2012), it remains difficult to link the diversity of root-associated bacteria with the functioning of the rhizosphere. In the reported study, no major changes of ratios of various bacterial classes (Fig. 3A) or diversity indexes (see Shannon and Bray–Curtiss indexes) have been detected, and therefore, limited consequences on plant could be expected. Nevertheless, this study also pointed out the selective effect of octopine on members of the *Ensifer* genus, some of them being involved in the nitrogen-fixing symbiosis with legumes (Oldroyd *et al.* 2011). In the legume root environment, *Ensifer* could be therefore regarded as a keystone species (Paine 1995; Dale & Beyeler 2001) as members of this genus can strongly favour the survival, growth and reproduction of members of this plant clade in nitrogen-deprived soils. Similarly, members of the *Pseudomonas* genus may play critical roles in plant growth promotion or plant health, including on the species *A. thaliana* (Persello-Cartieaux *et al.* 2001). The data reported here therefore confirmed that a change of exudation can readily affect important members of the plant root microbiome although the global structure of the root-associated population remains unaffected.

This study shed some light on the diversity of the bacterial populations and communities of the rhizosphere, independently of the culturability of the bacteria. The existence of a common bacterial microbiome is verified as the 100 most frequent genera (i.e. 27% of the genera) explain over 93% of the diversity. On the opposite, a rare microbiome also exists as the remaining 7% of the diversity consisted of over 270 genera (73% of the diversity). The number of reads per conditions and the ratio of unclassified bacteria (15.3%) observed in this study are comparable to those reported in similar analyses such as the investigation of the bacterial microbiome of potato (Inceoglu *et al.* 2012) or tropical tree rhizospheres or rhizoplanes (Oh *et al.* 2012). It remains impossible, however, to finely compare the results obtained in the present study with others, as the composition of the root-associated microflora strongly depends upon the soil type (Fierer & Jackson 2006) but also the plant genus, species and even genotype (Hartmann *et al.* 2009). Recent reports involving metagenomics and pyrosequencing of *rrs* genes indeed supported this assertion (Hur *et al.* 2011; Lundberg *et al.* 2012). However, the present study confirmed the relatively high abundance of Proteobacteria and Actinobacteria in the soil supplemented with the artificial exudates (see Fig. 2A), a feature that has been reported by others under different conditions (Teixeira *et al.* 2010; Lundberg *et al.* 2012). Similarly, and in agreement with other published data (e.g. Oh *et al.* 2012), some Acidobacteria groups (i.e. Acidobacteria GP6, GP4, GP16 and GP17) were highly represented amongst the five assayed conditions.

The consequences of the replacement of organic acids by octopine did not translate in visible changes of ratios of various bacterial classes (Fig. 3A) or in changes in the diversity within these classes (Tables 2 and S5, Supporting information). However, changes affecting bacterial clades were visible at the genus rank, that is, for members of the genera *Pseudomonas* and *Ensifer* (Table 3). Interestingly, while pseudomonads appeared to be stimulated by carbon compounds independently of octopine concentration, *Ensifer* was specifically stimulated by octopine (Table 3 and Fig. 3). A possibility exists that pseudomonads were favoured by their highly diverse catabolic properties (review: Silby *et al.* 2011) outcompeting the slow-growing *Ensifer* in the presence of multiple carbon sources but not when octopine was the major C source. *Pseudomonas* densities, however, remained high in the presence of increased octopine concentrations. These facts suggested that the increased density of members of these genera may indeed result from their ability to degrade the introduced octopine. In agreement, members of the two genera were also found amongst opine utilizers isolated from the artificial exudation system. These two colonization strategies, one based on a versatile metabolism and a high growth rate when resources are diverse and abundant, and the other on the use of more private (or niche) substrates, are reminiscent of r/K strategies (Pianka 1970). In microbial ecology, the r- and K-strategists are rather termed copiotrophs and oligotrophs, respectively. While oligotrophs can still maintain a significant

population in rich environments, they generally exhibit 'slower growth rates' and remain 'likely to outcompete copiotrophs in conditions of low nutrient availability due to their higher substrate affinities' (Fierer *et al.* 2007). This is precisely what was observed here with the colonization patterns of *Ensifer* and *Pseudomonas*. Additionally, members of the *Ensifer* genus possess several traits proposed as characteristics for oligotrophs/K-strategists by Fierer *et al.* (2007), that is, they exhibit a long generation time, generate slow-to-appear visible colonies, have a high glutamate reserve pool and grow poorly on rich media and best on elaborated minimal media (Y. Dessaux, unpublished). Last, this study also demonstrated that by using a progressively increasing carbon bias, the r/K-selection process was continuous and, under our experimental conditions, independent of the total carbon amount available to the bacteria (as this last parameter remained constant). The oligotroph/K-strategist *Ensifer* was indeed progressively favoured as the quality of the synthetic exudates is affected by the replacement of 'generic' substrates (that organic acids are) by the specific substrate octopine. Overall, the results reported in this study support the above-mentioned copiotroph vs. oligotroph classification of soil bacteria.

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S.M., A.L. and A.B.-C. performed the experiments. S.M. performed the bioinformatic analysis with the support of C.L. S.M. and P.R. generated the transgenic lines, D.F. and Y.D. conceived the experimental design and obtained the relevant fundings. Y.D. and S.M. wrote the manuscript.

Data accessibility

The 454 data were submitted to the Sequence Read Archive (SRA study identifier SRP036851/Bioproject PRJNA183983). The correspondence between SRA accession no. and the nature of sample (pool and individual) is listed in Table S8 (Supporting information). The Rscript described in this article is available as supplementary information.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 The artificial exudation device.

Fig. S2 Experimental design for the estimation of bacterial diversity by 454 pyrosequencing of the 16S rRNA gene.

Fig. S3 Comparison of individual and pooled samples.

Fig. S4 Phylogenetic analyses of opine-degrading bacteria at the rhizosphere of opine-producing *Arabidopsis thaliana* lines.

Fig. S5 Relation between the octopine content and the frequency of octopine user in second model experiment.

Fig. S6 Phylogenetic analyses of opine-degrading bacteria in an artificial exudation system under four exudate compositions.

Fig. S7 Rarefaction curves and Bray–Curtis similarity index comparison.

Fig. S8 MA plot.

Table S1 Soil characteristics.

Table S2 Trim summary.

Table S3 Chimera detection.

Table S4 Shannon indexes.

Table S5 OTU assignment at the class level.

Table S6 Frequency of reads.

Table S7 Pairwise comparison of the reads assigned at the genus level.

Table S8 Correspondence between SRA accession no. and the nature of sample (pool and individual).

Appendix S1 Shell script for the conversion of RDP classifier output.