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ORIGINAL PAPER

Aromatic compounds degradation plays a role in colonization of *Arabidopsis thaliana* and *Acacia caven* by *Cupriavidus pinatubonensis* JMP134

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Abstract Plant rhizosphere and internal tissues may constitute a relevant habitat for soil bacteria displaying high catabolic versatility towards xenobiotic aromatic compounds. Root exudates contain various molecules that are structurally related to aromatic xenobiotics and have been shown to stimulate bacterial degradation of aromatic pollutants in the rhizosphere. The ability to degrade specific aromatic components of root exudates could thus provide versatile catabolic bacteria with an advantage for rhizosphere colonization and growth. In this work, *Cupriavidus pinatubonensis* JMP134, a well-known aromatic compound degrader (including the herbicide 2,4-dichlorophenoxyacetate, 2,4-D), was shown to stably colonize *Arabidopsis thaliana* and *Acacia caven* plants both at the rhizoplane and endorhizosphere levels and to use root exudates as a sole carbon and energy source. No deleterious effects were detected on these colonized plants. When a toxic concentration of 2,4-D was applied to colonized *A. caven*, a marked resistance was induced in the plant, showing that strain JMP134 was both metabolically active and potentially beneficial to its host. The role for the β-ketoadipate aromatic degradation pathway during plant root colonization by *C. pinatubonensis* JMP134 was investigated by gene inactivation. A *C. pinatubonensis* mutant derivative strain displayed a reduced ability to catabolise root exudates isolated from either plant host. In this mutant strain, a lower competence in the rhizosphere of *A. caven* was also shown, both in gnotobiotic in vitro cultures and in plant/soil microcosms.

Keywords *Acacia caven* · *Arabidopsis thaliana* · Aromatic compounds · *Cupriavidus pinatubonensis* JMP134 · Plant growth · Rhizosphere

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Introduction

Terrestrial plants provide a habitat for a wide variety of microorganisms (Bais et al. 2006; Lugtenberg et al. 2002). Microbial colonization may cover the complete surface of the host (Lindow and Brandl 2003), and concentrates largely in the rhizosphere (Berg and Smalla 2009; Costa et al. 2006), where it can produce beneficial, presumably neutral, or even harmful effects on the plant, depending on the microbe involved, the

plant species and its nutritional status (Mercado-Blanco and Bakker 2007). Some microorganisms establish a more intimate association with plants and even colonize the internal tissues of the root (endorhizosphere) without causing harm or signs of infection (Hallmann et al. 1997; Mendes et al. 2007; Ryan et al. 2008; Sturz et al. 2000). A strong influence on root colonization and soil ecology has been ascribed to organic components of root exudates (Badri and Vivanco 2009; Bais et al. 2006), which can inhibit the growth of certain microorganisms, while stimulating the proliferation of others, thus making the rhizosphere a selective environment (Bais et al. 2006; Kiely et al. 2006). Although their identities are not well characterized, some of the major organic components of exudates have been described in a few plant species (Uren 2000; Walker et al. 2003). Readily available carbon sources secreted by plant roots include sugars or simple polysaccharides, amino acids, and phenolic compounds (Bertin et al. 2003; Nguyen 2003), some of which can also be found in root tissues (Tan et al. 2004).

Catabolically versatile bacteria have been frequently found associated with plant roots (Chen et al. 2005; Elliot et al. 2009; Pérez-Pantoja et al. 2010a, 2011), and enhanced biodegradation of organic compounds and xenobiotics has been observed in rhizosphere soil (Shaw and Burns 2003). The competence and catabolic activity of such soil bacteria is presumed to be associated with their ability to take up specific components of exudates as pathway inducers and/or carbon sources (Shaw and Burns 2004, 2005). Plant derived phenolics, produced by the phenylpropanoid pathway (Graham 1998) are highly abundant in root secretions and tissues of most plants (Bertin et al. 2003; Narasimhan et al. 2003; Tan et al. 2004), and share structural similarity with aromatic xenobiotics, including a few herbicide compounds (Shaw et al. 2006). Since catabolic versatility against aromatic pollutants has been correlated with good rhizosphere colonization ability (Kuiper et al. 2001; Narasimhan et al. 2003), it is tempting to speculate that degradation of exuded aromatic compounds favours the establishment of catabolically versatile bacteria, by stimulating their growth and competence in the vicinity of the roots.

Cupriavidus pinatubonensis (ex *C. necator*) JMP134 (pJP4) is a well-characterized β-proteobacterium that is able to degrade a wide variety of aromatic and chloroaromatic compounds (Pérez-Pantoja et al. 2008). Its genome possesses a vast array of genes encoding degradation of aromatic compounds like cinnamic, salicylic, 4-hydroxybenzoic, protocatechuic, and phenylacetic acids, to name a few (Lykidis et al. 2010; Pérez-Pantoja et al. 2008). Recent surveys have shown that *C. pinatubonensis* is a good representative of the aromatic compounds degradation potential present in a number of proteobacterial strains, especially those of the *Burkholderiales* order (Pérez-Pantoja et al. 2010a, 2011). Several relevant aromatic compounds present in plant root exudates are catabolised through aromatic ring cleavage pathways that converge in the formation of β-ketoadipate, an intermediate that can be incorporated into the central metabolism (Harwood and Parales 1996; Pérez-Pantoja et al. 2010b). In order to investigate the role of catabolic functions related to the β-ketoadipate pathway in degradation of root exudates components and plant colonization, the interaction of *C. pinatubonensis* JMP134 with the well established plant model *Arabidopsis thaliana* and the leguminous shrub *Acacia caven*, a representative plant host for β-proteobacteria (Barret and Parker 2006; Diouf et al. 2007; Hoque et al. 2011), was investigated. Rhizosphere and internal tissue colonization was assessed for catabolic mutant derivatives of strain JMP134, and their possible effect on plant growth was determined.

Materials and methods

Bacterial strains and culture

Cupriavidus pinatubonensis JMP134 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, and routinely grown in Dorn minimal saline medium (Dorn et al. 1974) containing 2 mM fructose. Mutants of *C. pinatubonensis* JMP134 in catabolic functions belonging to the β-ketoadipate pathway were obtained by recombinational inactivation of selected genes (*benA*, *pcaH*, or *pcaIJ* gene mutants) with internal gene fragments cloned in the pCR2.1-TOPO system (Invitrogen Life Technologies, Carlsbad, CA), or by successive allelic replacement (BCA triple mutant) with a gene version interrupted by a gentamycin resistance cassette in plasmid pCM351 (Marx and Lidstrom 2002). The gentamycin marker was removed

by transforming each derivative with plasmid pCM157, expressing the Cre recombinase system (Marx and Lidstrom 2002). The corresponding clones were electroporated in strain JMP134 to produce the desired mutants. Gene inactivation was confirmed by PCR with primer pairs flanking the insertion site, and sequencing of each amplified product.

Green fluorescence protein labelling and bacterial inocula preparation

Strain JMP134 was tagged with the gfp marker gene using a mini-Tn5 system, which produces stable genomic insertions (Mathysse et al. 1996). Wild-type JMP134 was conjugated with the *E. coli* strains PRK2073, as helper, and S17, containing a mobile mini-Tn5GFP construct. Transconjugants carrying the gfp marker were selected on Luria–Bertani (LB) medium containing 10 μg of tetracycline per ml. Colonies of the gfp labelled strains were examined using Optical Fluorescence microscope (model Nikon Labophot 2) equipped with a UV light source (100W HBO Fluorescence lamp housing) and a B-2A fluorescent filter. For plant colonization and growth effects experiments, strain JMP134::gfp was routinely grown on fructose for 24 h at 30°C. Cell suspensions from each inoculum were then obtained and inoculated homogenously to approximately 10^4 colony forming units (CFU) per ml on 0.8% agar plates containing 50% Murashige and Skoog (MS) basal salt mixture.

Bacterial growth with plant root exudates

Plant root exudates were obtained from 1 week old hydroponic cultures of *A. thaliana* and *A. caven*, prepared by germinating 16–20 surface sterilized seeds on a plastic support grid, and placing them over the surface of 50 ml of pure water, inside of sterile magenta flasks. After 1 week of incubation, 25 ml of liquid medium were removed, filtered through a 0.22 μm pore size filter (Millipore Inc. MA, USA) and frozen for later use. Dilutions of these exudates were carried out in Dorn medium. Bacterial growth on dilutions of plant root exudates was followed as the increase in optical density at 600 nm (OD 600 nm) after 48 h of incubation at 30°C on a rotary shaker. Equivalent volumes of pure water were used as controls.

Plant growth

Acacia caven seeds were stratified by 2 h treatment with concentrated H_2SO_4, with occasional stirring to prevent seed aggregation, and washed three times with an equivalent volume of sterile water, while *A. thaliana* seeds were stratified by incubation at 4°C for 2 days. Afterwards, seeds from either plant were surface sterilized with 5% (vol/vol) commercial chlorine bleach for 7 min and washed three times in sterile distilled water. Seeds were then sown on sterile agar plates. After 5 days, plantlets were transplanted to fresh 0.8% agar plates containing 50% MS basal salt mixture, homogenously inoculated or not with 10^4 CFU/ml of gfp labelled *C. pinatubonensis* JMP134. One seed was sown per plate. Plates without seeds and inoculated with bacteria were used as plant-free, non-rhizosphere controls. For determination of plant growth parameters, seeds were sown in agar contained in Magenta flasks (PhytoTechnology Laboratories, KA, USA), with or without bacterial inoculum. The plant growth chamber was run with a light:dark period of 12:12 h and a temperature of 22 ± 4°C.

Rhizoplane and endophytic colonization

For rhizoplane colonization tests, 3 weeks old plants were removed from the inoculated agar and samples of agar that were in contact with the roots (rhizosphere agar: at a distance lower than 1 cm) were taken. Agar samples from plates without plantlets were also obtained as controls. Non-inoculated agar samples were homogenized by vortexing and serially diluted with Dorn minimal medium on R2A plates, to rule out bacterial contamination. CFU/ml of the inoculated bacteria: *C. pinatubonensis* JMP134, and its mutant derivatives strains BenA, PcaH, PcaIJ, and BCA were determined after 24 h of incubation at 30°C. Rhizosphere ratios were calculated dividing CFU/ml in rhizosphere agar by CFU/ml found in agar from the corresponding not sown plates. For endophytic colonization tests (adapted from Compant et al. 2005), 3 weeks old plantlets were removed from agar plates, and surface sterilized with 70% ethanol for 1 min, followed by 1% commercial bleach and a 0.01% Tween 20 solution for 1 min, and then washed three times in sterile water. The sterilized plant material was macerated and the disrupted tissue was resuspended in 1 ml of 50 mM phosphate buffer with 1%

Polyvinylpyrrolidone. CFU/ml of *C. pinatubonensis* strains were determined by serial dilutions of these extracts in R2A agar plates. Extracts from non-inoculated control plants produced none or negligible CFU values compared to inoculated plants.

Rhizoplane colonization in plant-soil microcosms

In order to track bacteria inoculated in plant-soil microcosms by a culture dependent strategy, spontaneous rifampicin resistant mutant derivatives of *C. pinatubonensis* strains JMP134, PcaIJ, and BCA, were obtained by plating overnight fructose grown cultures of each strain in LB plates supplemented with 100 μg/ml of the antibiotic. Mutant derivatives were selected in each case and, as an additional marker, the kanamycin resistant plasmid pBBRMCS-2 (Kovach et al. 1995) was introduced in each strain by electroporation, and plasmid containing derivatives were selected by plating in LB plus 50 μg/ml of kanamycin. Plant-soil microcosms were prepared using 50 g of a loam soil, containing 60.5% sand, 7.5% clay and 32% silt, sown with *A. caven* seedlings pre-germinated in vitro for 1 week, as described above. Plant-soil microcosms were then incubated in a plant growth chamber at 23–25°C with a light:dark period of 12:12 h, and routinely irrigated with 5 ml of sterile water every 5 days. After 4 weeks, microcosms were irrigated with 5 ml of a bacterial suspension containing 1×10^7 CFU/ml prepared in sterile water, to obtain a final bacterial concentration of approximately 1×10^6 CFU/g of soil. At least four plant-soil microcosms were prepared for each inoculum type, i.e., *C. pinatubonensis* JMP134 and its derivative strains PcaIJ, and BCA, all tagged with the rifampicin and kanamycin resistance markers. Control, plant-free soil microcosms inoculated with bacteria but not sown with *A. caven*, as well as non-inoculated plant-soil microcosms, were prepared in each case. Two weeks after inoculation, rhizoplane colonizing bacteria were extracted by harvesting the plants, collecting 0.5 g of the soil adhering directly to the root surface, and preparing a cell suspension by mixing them with 5 ml of Dorn medium with two sterile 5 mm glass beads for 2 h in an overhead shaker at 50 rpm/min. This soil suspension was allowed to stand for 1 min, in order to decant large soil aggregates, and then a 0.1 ml sample was used to prepare serial dilutions in Dorn medium for quantification of CFU in LB plates supplemented with kanamycin (50 μg/ml) and rifampicin (100 μg/ml). Rhizoplane colonization numbers were compared to those obtained in control soil microcosms containing only bacteria (bulk soil). Background bacterial growth in plates containing both antibiotics was negligible for non-inoculated plant soil microcosms.

Microscopy analyses of rhizoplane and endophytic colonization

To assess colonization of the rhizoplane by gfp marked bacterial strains, inoculated and non-inoculated plant root surfaces were examined by Optical Fluorescence microscope and photographs were taken with a digital camera (C5060; Olympus). To determine endophytic colonization by gfp marked bacterial strains, fresh roots from inoculated and non-inoculated plantlets, were prepared for examination by a Nikon Optiphot C200 reflected light confocal microscope, with a Mar 2488 and HeNe G 543 laser. GFP fluorescence was collected through a long pass filter (BA-505-525) and root autofluorescence through a second filter (BA-56IF).

Determination of plant growth parameters

Twenty inoculated and twenty non-inoculated plantlets from each treatment were removed from the growth agar medium, and aerial part lengths were determined by measuring the total stem length from the root transition zone up to the apical bud. Root lengths were determined directly in harvested plants, and fresh weights were recorded as previously described (Compant et al. 2005; Ryu et al. 2005). Chlorophyll was extracted from leaves of *A. caven* or *A. thaliana* in *N*,*N*-9-dimethylformamide for 24 h at 4°C in the dark, and chlorophyll *a* and chlorophyll *b* concentrations were measured simultaneously by spectrophotometry, based on a described procedure (Porra et al. 1989).

Statistical analysis

Data for plant growth parameters and bacterial enumeration were statistically analyzed using one-way analysis of variance. When analysis of variance showed significant treatment effects, the Tukey's HSD (Honestly Significant Difference; $P < 0.05$) test was applied to make comparisons between treatments.

Results

Cupriavidus pinatubonensis JMP134 inoculation on *A. thaliana* and *A. caven* does not produce deleterious effects on plant growth

To explore the interaction between *C. pinatubonensis* and plants, we first measured the effect in plant growth of the inoculation of *A. thaliana* and *A. caven* plantlets with *C. pinatubonensis* JMP134, using gnotobiotic cultures. The effects on plant fresh weight, root length, and chlorophyll content were measured after 4 weeks of incubation and compared to mock inoculated controls. As can be seen on Fig. 1, *C. pinatubonensis* JMP134 inoculation did not affect the weight of *A. caven* plants, though it did produce a minor reduction in *A. thaliana* plants. However, it did not modify the length of the root system or the total chlorophyll content in plant leaves of either of the hosts to a significant extent, as compared to control plants. Accordingly, no differences were found in the general aspect of the plants to suggest any other alteration in their morphology or health. These results were confirmed for *A. caven* by experiments performed in plant soil microcosms (data not shown).

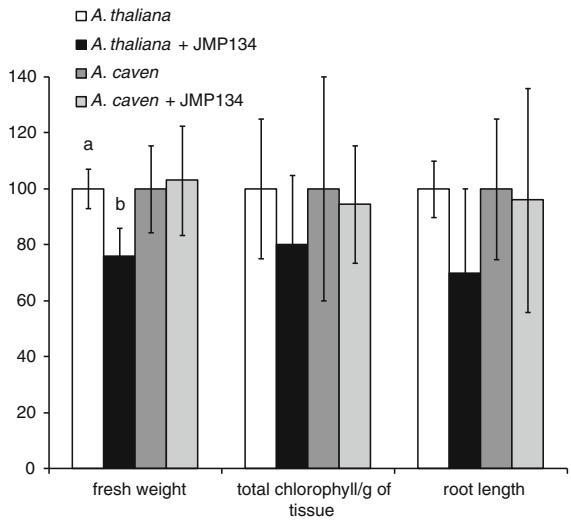

Fig. 1 Comparison of plant growth in gnotobiotic *Arabidopsis thaliana* or *Acacia caven* cultures, inoculated with *Cupriavidus pinatubonensis* JMP134. Growth parameters were measured 4 weeks after inoculation. The *bars* show mean percentage values relative to the control (0.5 g fresh weight; 3.12 μg of chlorophyll per mg of leaf tissue; 59 mm of root length), and the *error bars* indicate standard deviations from experiments with 20 plants in two independent biological replicates. *Letters* (*a* and *b*) were included to indicate the only case where a statistically significant difference between treatments was found, for either of the plants under study (One way ANOVA Tukey's HSD tests; $P < 0.05$)

Cupriavidus pinatubonensis JMP134 stably colonizes the rhizosphere and endorhizosphere of *A. thaliana* and *A. caven*

Any interaction involving *C. pinatubonensis* and either of the plants under study should start at the root level, through the establishment of a rhizospheric bacterial population and later, if possible, an endorhizospheric population. Therefore, we compared bacterial numbers in the presence or absence of plant roots, in gnotobiotic culture systems. After 3 weeks of culture, bacterial numbers in plant-free agar were in the order of 1.1×10^5 CFU/ml, whereas counts in the vicinity of each plant roots reached levels three orders of magnitude higher (2.7×10^8 CFU/ml for *A. thaliana* and 7.8×10^8 CFU/ml for *A. caven*). Epifluorescence microscopy of root surface from plants inoculated with gfp-tagged JMP134 cells revealed green fluorescence on the surface of the root system with high densities of bacterial cell aggregates at the root tip and the sites of emergence of lateral roots and root hairs (data not shown).

Endophytic colonization by *C. pinatubonensis* JMP134 was also assessed in gnotobiotic cultures of *A. thaliana* and *A. caven*: first, sterilizing the surface of plants grown in inoculated cultures and then homogenizing them separately to obtain a plant tissue extract. *Cupriavidus pinatubonensis* JMP134 was detected in the extract of inoculated plants (0.1 g of fresh tissue) with an average abundance of 1.07×10^3 CFU/ml, while mock inoculated plant controls showed no bacterial colonization. Confocal microscopy analyses showed *C. pinatubonensis* JMP134-gfp in inspected tissues reaching about 11 μm from the root surface, probably accessing epidermal and cortex tissue apoplast, but not necessarily crossing towards the vascular tissue (Fig. S1, supplementary material).

Cupriavidus pinatubonensis JMP134 grows on *A. thaliana* and *A. caven* root exudates

To assess possible benefits for *C. pinatubonensis* JMP134 interacting with *A. thaliana* or *A. caven* roots, bacterial growth was measured in liquid cultures

supplemented with different dilutions of root exudates obtained in vitro. After 2 days of incubation, *C. pinatubonensis* JMP134 grew on exudates at every dilution tested (Fig. 2). To get an idea of the role of relevant aromatic catabolism features on the ability of *C. pinatubonensis* JMP134 to grow on root exudates, selected catabolic mutants of this strain were compared with the wild type for their ability to grow on root exudates of *A. thaliana* or *A. caven*. The mutated enzyme activities catalyze key steps in the β-ketoadipate pathway (benzoate dioxygenase, BenA; protocatechuate dioxygenase, PcaH; β-ketoadipate succinyl CoA transferase, PcaIJ) which is involved in catabolism of many aromatics generally assumed to be released by plants, like 4-hydroxybenzoate, protocatechuate, benzoate, and phenylpropanoids like cinnamic, coumaric and caffeic acids (Fig. 2a), by strain JMP134 and other proteobacteria. Since gene redundancy is found for several catabolic functions in the β-ketoadipate pathway (Pérez-Pantoja et al. 2008), a triple β-ketoadipate:succinyl CoA transferase mutant (JMP134-BCA) was generated to ensure inactivation of genes contributing to β-ketoadipate catabolism (Fig. 2a). Inactivation of BenA, PcaH and PcaIJ genes alone did not produce a significant reduction in the growth of *C. pinatubonensis* on plant exudates (Fig. 2b, c). However, inactivation of the three β-ketoadipate: succinyl CoA transferase genes resulted in a significant reduction of the final biomass obtained with both root exudates.

Cupriavidus pinatubonensis also establishes a significant rhizospheric population in planted soil

As gnotobiotic culture systems do not fully represent the complexity of the natural situation, for instance the soil matrix is absent, we further studied the interaction of *C. pinatubonensis* with plants, using plant-soil microcosms. Due to its faster growth in soil, and the higher resistance of its root system to mechanical manipulation, *A. caven* (rather than *A. thaliana*) plants were selected for studying colonization by *C. pinatubonensis* in soil conditions, i.e., in the presence of other members of the soil microbiota. For this, non-sterile plant-soil microcosms were prepared. Four weeks old plantlets grown under greenhouse conditions were inoculated by irrigation with 2×10^6 CFU of *C. pinatubonensis* per g of soil. After 3 weeks, inoculated plants showed a mean distribution of 2.9×10^5 CFU/g in rhizospheric soil and 1.4×10^4 CFU/g in non-rhizospheric soil, which means an average rhizosphere/non rhizosphere soil (R/S) ratio of 20.7. When *C. pinatubonensis* JMP134 was inoculated at 2×10^2 CFU/g, its distribution at equilibrium was 2.4×10^5 CFU/g in rhizospheric soil and 1.1×10^4 CFU/g in non-rhizospheric soil (averaged R/S ratio of 21.8), quite similar to those of plant-soil microcosms inoculated with 10^6 bacterial cells per gram of soil.

To address if *C. pinatubonensis* interacting with *A. caven* roots in plant soil microcosms was metabolically active and functional, we challenged these microcosms with increasing, toxic concentrations of the herbicide 2,4-D. *C. pinatubonensis* is able to efficiently degrade, and to grow with, this chloroaromatic compound. Four weeks old *A. caven* plants were inoculated with 1×10^6 CFU/g of strain JMP134 and, at the same time, irrigated with 0 (control), 25, or 50 ppm of 2,4-D. As can be seen in Fig. 3, 4 weeks after 2,4-D exposure, 100% of the plants treated with 50 ppm of the herbicide were killed, while the totality of inoculated *A. caven* plants survived during the treatment period. This strongly indicates that colonizing *C. pinatubonensis* cells are metabolically active protecting the plant from the toxic effect of the herbicide.

Cupriavidus pinatubonensis mutants in the β-ketoadipate pathway have reduced competitivity in the *A. caven* rhizosphere

As indicated above, a *C. pinatubonensis* triple mutant in the β-ketoadipate pathway showed a significant decrease in the ability to use plant root exudates as a growth substrate. We compared the triple and one selected single *C. pinatubonensis* β-ketoadipate pathway mutants with the wild type strain regarding their ability to interact with *A. caven* in plant-soil microcosms. *A. caven* soil microcosms were homogenously inoculated with either the wild type or mutant *C. pinatubonensis* strains. Rhizosphere and non-rhizosphere soil were extracted at different times, and CFU values were determined for each strain (Fig. 4a, b). These results show that the wild type strain JMP134 and the PcaIJ and BCA mutants decay at a similar rate and to a similar abundance in non-rhizosphere soil. In contrast, the BCA mutant exhibited a reduction of one order of magnitude in the abundance in rhizosphere soil ($1.4 \times 10^6 \pm 5 \times 10^5$), compared to the wild type

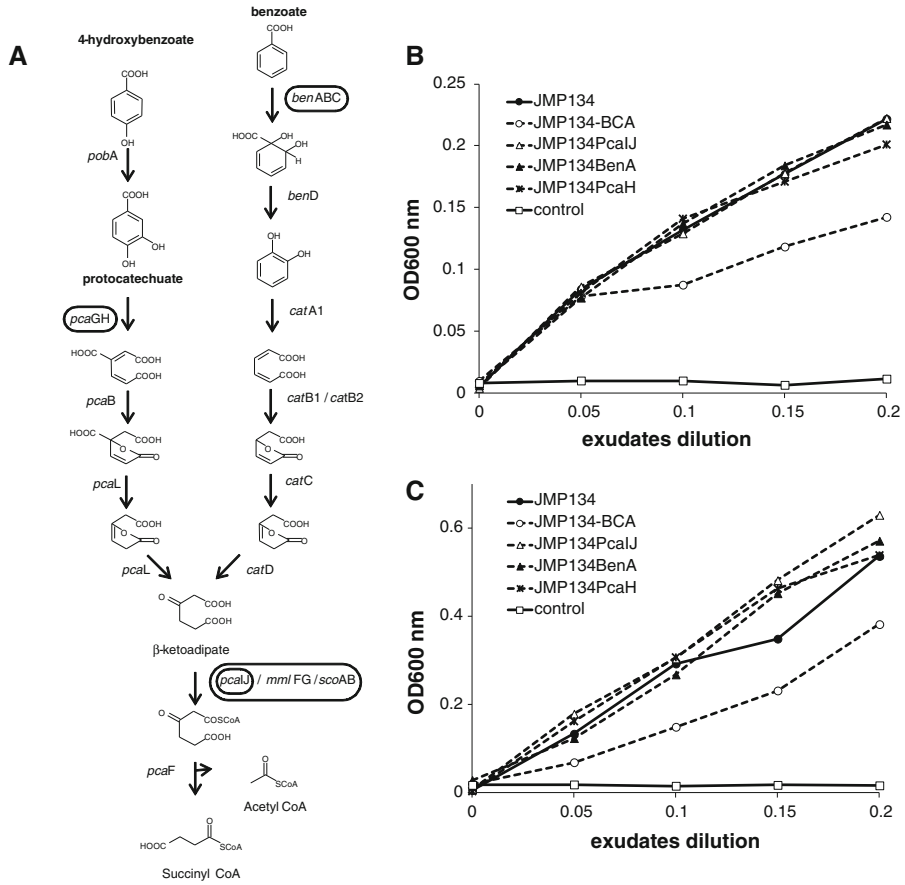

Fig. 2 The *β*-ketoadipate pathway in *C. pinatubonensis* JMP134 and its growth on plant exudates as a sole carbon source. **a** General scheme of the *β*-ketoadipate catabolic pathways in *C. pinatubonensis* JMP134. The genes encoding the enzymes responsible for each conversion are shown. These are separated with a slash whenever two or more genes (or operons) display redundant or additive functions. Genes that have undergone recombinational inactivation in the respective mutant derivative strains of *C. pinatubonensis* JMP134 are enclosed in open ovals. The triple mutant strain JMP134-BCA has lost the *pcaIJ*, *mmlFG* and *scoAB* gene functions. Growth of *C. pinatubonensis* JMP134 and its catabolic mutants in different *A. thaliana* (**b**) or *A. caven* (**c**) root exudates dilutions. Liquid minimal saline medium cultures supplemented with different dilutions of root exudates, were inoculated with JMP134 (*filled circle*), or the mutant derivative strains (dotted lines) BenA (*filled triangle*), PcaH (*), PcaIJ (*open triangle*) and BCA (triple mutant) (*open circle*). Strain JMP134 was inoculated in dilutions of medium without plants as a control (*open square*). OD600 was registered at the stationary phase after 48 h cultivation. Values are means from three replicates, and are representative for three different experiments. The standard deviations were less than 5%

JMP134 ($1.2 \times 10^7 \pm 2 \times 10^6$) (Fig. 4a). Based on the corresponding values for non-rhizosphere colonization ($1.7 \times 10^4 \pm 3 \times 10^3$ and $1.5 \times 10^4 \pm 5 \times 10^3$, respectively, Fig. 4b), a significant decrease in the rhizosphere effect is observed for this mutant strain. Essentially the same behaviour was observed for these mutants in *A. caven* gnotobiotic cultures (Figure S2, supplementary material).

Discussion

The results of this work show a stable association, even if unilateral, between *C. pinatubonensis* JMP134 and the two plant species used in this study. Although no putative functions presumed to be involved in interaction with plants are found in its genomic sequence (Lykidis et al. 2010), *C. pinatubonensis*

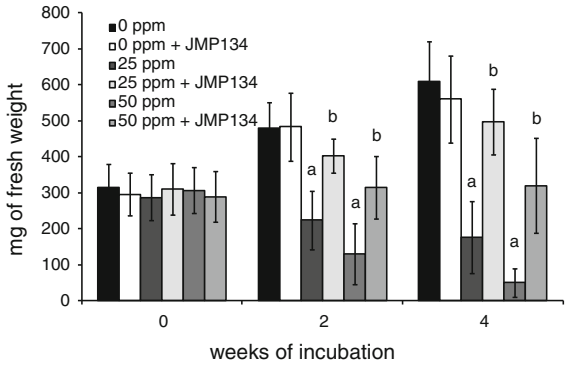

Fig. 3 Growth of inoculated *A. caven* plants in microcosms treated with toxic concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D). Fresh weight of plantlets inoculated with *C. pinatubonensis* (+JMP134), or control plantlets, was measured after treatment with 0, 25 or 50 ppm of 2,4-D. Results show mean values of six plants per treatment measured at 0, 2 and 4 weeks of incubation. *Letters* (*a* or *b*) are added to indicate statistically significant differences between groups (inoculated and non inoculated) within each 2,4-D treatment concentration (One way ANOVA Tukey's HSD tests; $P < 0.05$), and are absent in groups that show no significant differences between each other

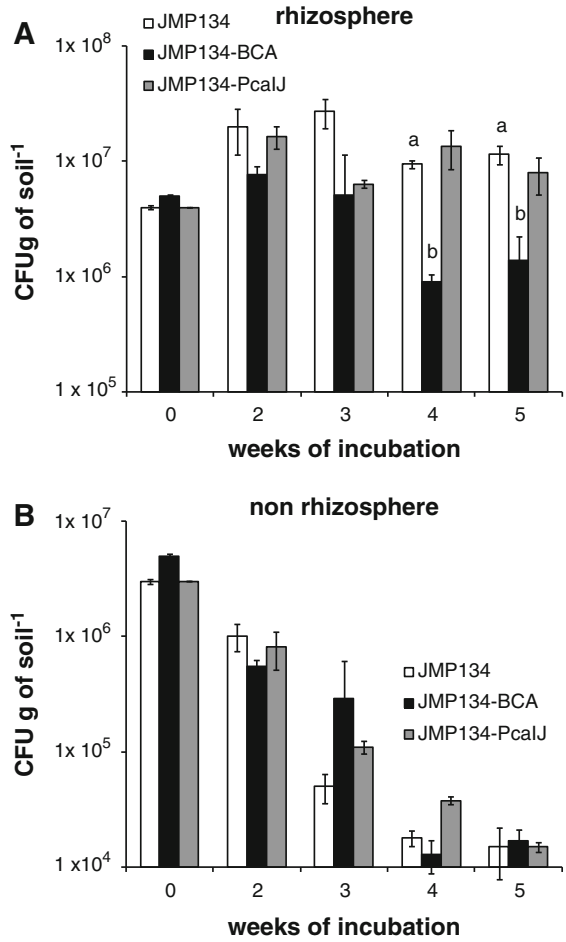

Fig. 4 Colonization of the *A. caven* rhizosphere by *C. pinatubonensis* JMP134 catabolic mutants, in plant soil microcosms. Abundance of selected *C. pinatubonensis* catabolic mutants of rhizosphere (**a**) and non-rhizosphere (**b**) soil in *A. caven*—soil microcosms. Bacteria were homogenously inoculated in soil to 1×10^6 CFU/g and soil samples were analyzed after 2, 3, 4 and 5 weeks. Results are shown for four biological replicates. *Letters* (*a* or *b*) indicate statistically significant differences between the wild type and the BCA tripe mutant (One way ANOVA Tukey's HSD tests; $P < 0.05$)

JMP134 is able to interact and colonize *A. thaliana* and *A. caven* plants to a similar extent to that of other well characterized plant colonizers, such as *B. phytofirmans* PsJN (Compant et al. 2005; Zúñiga, Donoso, Ledger, Gutiérrez and González, unpublished results). On the other hand, inoculated plants are not adversely affected by colonization of *C. pinatubonensis*, which could suggest that the activity of the strain in the plant root surface and internal tissues of the plants is not mainly saprophytic, and population numbers are maintained to a level that is not harmful to the host. However, when the herbicide 2,4-D is added to plant soil microcosms, the presence of strain JMP134 prevents toxic effects on the plant host, which suggests that the bacterium can be potentially beneficial, given the right set of circumstances, and that it is metabolically active during colonization.

Growth in the rhizosphere environment has been identified as an important factor for root colonization (Simons et al. 1996), and catabolism of exudates components has been previously proposed to play an important role in rhizosphere colonization (Lugtenberg et al. 2002; Lugtenberg and Kamilova 2009). However, major organic components of exudates such as sugars, have been proved to be of little relevance in rhizosphere colonization by beneficial *Pseudomonas* strains (Lugtenberg et al. 1999). In addition, carbon sources like citrate and malate have shown to be effectively used by plant colonizing bacteria in vitro (Kamilova et al. 2006), but growth on such substrates in soil can be restricted by competition for this readily available carbon sources with the bulk of the rhizosphere microbiota (Badri and Vivanco 2009), and by the presence of phenolic components of exudates, described as selective growth inhibitors (Shaw et al. 2006). Degradation of specific phenolic compounds has been implicated in lateral root crack colonization

of *Brassica napus*, *Triticum aestivum*, *A. thaliana* and *Oryza sativa* by certain growth promoting bacteria in vitro (Gough et al. 1997; Jain and Gupta 2003; O'Callaghan et al. 2000; Webster et al. 1998). Thus, the ability to catabolise and cope with variable amounts of an assortment of structurally related aromatic compounds (as found in plant exudates), can provide versatile degrading bacteria, such as *C. pinatubonensis*, in the vicinity of the roots with a double selective advantage over those that are restricted to more conventional carbon sources (like carboxylic acids and/or sugars), or may be more susceptible to inhibition by plant derived phenolics.

The β-ketoadipate pathway has been implicated in the degradation of several aromatic compounds that can be released by plants into the rhizosphere, like cinnamic acids, 4-hydroxybenzoate, and protocatechuate (Bertin et al. 2003; Narasimhan et al. 2003), some of which have been shown to negatively affect colonization and survival of other plant colonizing bacterial species (Chao and Yin 2009; Schnitzler et al. 1992). Our results show that *C. pinatubonensis* does rely partially on the function of β-ketoadipate pathway to degrade exudates compounds, and that this activity is relevant to achieve wild type rhizosphere colonization levels. This is consistent with previous reports showing expression of catabolic functions belonging to this pathway in response to exudates obtained from plant roots and seeds (Espinosa-Urgel et al. 2000; Matilla et al. 2007; Ramos-González et al. 2005). Taken together, these lines of evidence support a novel role for the degradation of plant derived phenolics as a relevant factor mediating proliferation and colonization of plants by catabolic bacteria like *C. pinatubonensis* JMP134, and they can help achieve a better understanding of the actual environmental factors driving catabolic versatility in rhizosphere colonizing *Burkholderiales*, and other proteobacteria.

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