Bacillus megaterium* Rhizobacteria Promote Growth and Alter Root-System Architecture Through an Auxin- and Ethylene-Independent Signaling Mechanism in *Arabidopsis thaliana

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Soil microorganisms are critical players in plant-soil interactions at the rhizosphere. We have identified a *Bacillus megaterium* strain that promoted growth and development of bean (*Phaseolus vulgaris*) and *Arabidopsis thaliana* plants. We used *Arabidopsis thaliana* as a model to characterize the effects of inoculation with *B. megaterium* on plant-growth promotion and postembryonic root development. *B. megaterium* inoculation caused an inhibition in primary-root growth followed by an increase in lateral-root number, lateral-root growth, and root-hair length. Detailed cellular analyses revealed that primary root-growth inhibition was caused both by a reduction in cell elongation and by reduction of cell proliferation in the root meristem. To study the contribution of auxin and ethylene signaling pathways in the alterations in root-system architecture elicited by *B. megaterium*, a suite of plant hormone mutants of *Arabidopsis*, including *aux1-7*, *axr4-1*, *eir1*, *etr1*, *ein2*, and *rhg6*, defective in either auxin or ethylene signaling, were evaluated for their responses to inoculation with this bacteria. When inoculated, all mutant lines tested showed increased biomass production. Moreover, *aux1-7* and *eir1*, which sustain limited root-hair and lateral-root formation when grown in uninoculated medium, were found to increase the number of lateral roots and to develop long root hairs when inoculated with *B. megaterium*. The ethylene-signaling mutants *etr1* and *ein2* showed an induction in lateral-root formation and root-hair growth in response to bacterial inoculation. Taken together, our results suggest that plant-growth promotion and root-architectural alterations by *B. megaterium* may involve auxin- and-ethylene independent mechanisms.

Additional keywords: cell cycle, root architecture, shoot development.

Roots provide the plant with physical support as well as nutrients and water, which they take up from the soil. Root morpho-

genesis and growth are under the control of both environmental stimuli and endogenous developmental programs. In both cases, plant hormones coordinate adaptive changes in cell division and differentiation that lead to changes in root-system architecture (López-Bucio et al. 2003, 2005).

Arabidopsis thaliana is a dicotyledonous plant that harbors a typical taproot system. Initially, growth is restricted to the primary root, which is formed during embryogenesis. Later in development, the root system expands by forming lateral roots. Under optimal environmental conditions, the primary root of *Arabidopsis* grows steadily downward, due to the ability of the root apical meristem cells to divide continuously. After division, a population of cells leaves the meristem and increases in size, thus contributing to root growth. Both cell division and elongation processes contribute to the length and final configuration of the root system (Baluska et al. 1996; Dolan and Davies 2004).

Several environmental stimuli, such as low nutrient or water availability, can reduce or halt cell division or elongation, leading to an arrest of primary-root growth accompanied by a stimulation of lateral-root emergence (López Bucio et al. 2002; Sánchez Calderón et al. 2005). In addition, plant roots live in close association with a myriad of bacterial and fungal species at the rhizosphere. Some of these microbial species can alter root development and plant growth by producing plant growth-regulating substances including auxin, ethylene, and cytokinins (Bowen and Rovira 1999), by producing volatile compounds such as acetoin (Ping and Boland 2004; Ryu et al. 2003), or by modulation of plant ethylene levels by the bacterial enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Glick 2005). The impact of bacterial hormones on root morphogenesis includes overproduction of root hairs and lateral roots (Persello-Cartiaux et al. 2003), which are commonly associated with the increased performance of plants to respond to the challenging environment (Lynch and Ho 2005).

Auxin and ethylene are known to regulate several of the same processes that modify root-system architecture, including primary-root elongation (Alonso et al. 2003; Swarup et al. 2002) and root-hair formation and elongation (Pitts et al. 1998; Rahman et al. 2002). Plant growth-promoting activity of certain rhizosphere microorganisms has been related to the production of substances that modify root morphogenesis, also termed phyto-stimulators (Bloemberg and Lugtenberg 2001). For instance, Loper and Schroth (1986) found that 12 of 14 plant growth-promoting rhizobacteria (PGPR) isolates produced indoleacetic acid (IAA) in culture filtrates and there was a significant rela-

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tionship between IAA production and decreased root elongation and increased shoot-to-root ratios of sugar beet seedlings. A positive correlation between auxin production and growth-promoting activities of PGPR has been reported (Asghar et al. 2002; Khalid et al. 2004). Auxins are quantitatively the most abundant phytohormones secreted by *Azospirillum* species, and it is generally agreed that auxin production is the major factor responsible for the stimulation of rooting and enhanced plant growth by this bacterium (Bloembergen and Lugtenberg 2001; Steenhoudt and Vanderleyden 2000).

Arabidopsis thaliana has been chosen as a model system to study plant-microbe interactions that occur at the rhizosphere because of its short life cycle, its suitability to be grown in vitro, and the availability of many mutants impaired in hormone-response pathways, including auxin, ethylene, and cytokinin (Persello-Cartieux et al. 2001; Ryu et al. 2003, 2005). From these studies, a picture is beginning to emerge in which a complex array of bacterial metabolites participates in plant-growth promotion. For instance, elicitation of *Arabidopsis* growth promotion by PGPR strains in vitro involved signaling of cytokinins, brassinosteroids, auxin, salicylic acid, and gibberellins (Ryu et al. 2003, 2005). Besides this important information, very little is known of the cellular, developmental and physiological responses of plants to rhizobacteria.

To elucidate the signaling mechanisms by which PGPR modify plant development, we initiated a survey to identify new PGPR strains by using *Arabidopsis thaliana* as a model plant. Our search identified a new strain of *Bacillus megaterium* (UMCV1), which is able to promote growth of bean and *Arabidopsis* plants. We show here that plant-growth promotion elicited by *B. megaterium* in *Arabidopsis* correlates with dramatic changes in root-system architecture that include increased growth of lateral roots and increased root-hair elongation. Detailed cellular measurements and *CycB1,1:uidA* expression analyses in the root meristem suggest that bacterial inoculation inhibits both meristematic activity and cell elongation in the primary root and promotes epidermal-cell differentiation. The involvement of auxin and ethylene in mediating the observed developmental alterations was tested using the auxin-responsive marker *DR5:uidA* and auxin- and ethylene-related mutants.

RESULTS

Effect of *B. megaterium* inoculation on plant growth.

The effect of *B. megaterium* inoculation on plant growth was investigated in bean (*Phaseolus vulgaris*) and in *Arabidopsis thaliana* (Col-0 ecotype). Bean plants were grown both

in vitro and in sterilized soil. In these growth conditions, *B. megaterium* inoculation significantly increased the fresh and dry weights of the plants. Interestingly, plant-growth promotion was related to modifications in root architecture, including an increase in lateral-root number and in lateral-root length (Table 1). To further study the plant growth-promoting activity of *B. megaterium* on plant morphogenesis, we used *Arabidopsis* as a model. A transplanting method was devised in which *Arabidopsis* seedlings were germinated and grown for a 4-day period in petri plates containing solidified Murashige-Skoog (MS) 0.2× nutrient medium. At day 4 after germination, the seedlings were transferred to solidified MS 0.2× medium (control medium) or to the same medium inoculated with *B. megaterium* UMCV1 strain. The seedlings were transferred 5 and 2 cm from the site of bacterial inoculation to test the possibility that diffusible bacterial compounds could be affecting plant growth in a concentration-dependent way. After 6 days of growth in the presence of *B. megaterium*, in plants transferred at a distance of 5 cm from the bacterial culture, a significant increase in shoot and root biomass production was observed (Fig. 1A to D). Interestingly, bacterial inoculation had an effect on the *Arabidopsis* root-system architecture by altering primary-root growth and promoting lateral-root formation (Fig. 1A to D). A significant deviation in root gravitropism was also recorded in plants transferred 5 cm from *B. megaterium* when compared to uninoculated plants (Fig. 1C). For control plants, the average angle and standard deviation of growth towards the gravity vector was of 107.5 ± 6 degrees, whereas inoculated plants showed an angle of 139.7 ± 9 degrees (Fig. 1A and C and data not shown).

The effects of inoculation on growth of *Arabidopsis* plants varied among their distances from the bacterial inoculation site. For instance, *B. megaterium* inoculation inhibited shoot growth when plants were placed at a distance of 2 cm from the inoculum (Fig. 1E). At this distance, inoculation dramatically stimulated lateral-root formation and root-hair growth but repressed both primary and lateral-root growth (Fig. 1E), an effect reminiscent of altered auxin or ethylene responses (Alonso et al. 2003; Pitts et al. 1998).

Effect of inoculation distance on *B. megaterium* growth.

To define whether plant exudates could have an effect on *B. megaterium* growth, we performed experiments to measure bacterial growth at 5 or 2 cm from the root, using the *Arabidopsis* lines Col-0, *ein2*, and *aux1-7*. We measured total protein content from different streak sections at different times after inoculation, as indicative of bacterial biomass. Our data clearly show that the total protein content of the bacterial inoculum from Col-0, *ein2*, and *aux1-7* cultures increases with time, suggesting that the bacteria continue to grow after streaking (Table 2). In these experiments, however, we did not observe a clear effect of the inoculation distance from the root on bacterial biomass as revealed by the protein assay. In addition, we did not observe significant differences between wild-type (WT) and mutant plants on bacterial growth at the same time-points. These results suggest that the bacterial inoculum, independently of their distance to the roots remains viable after several days of growth on the petri plates and that the differential effects of the inoculation distance on plant growth are likely due to one or more diffusible substances produced by *B. megaterium*.

B. megaterium inoculation inhibits meristematic activity and reduces cell elongation in primary roots of *Arabidopsis*.

To understand further the effect of *B. megaterium* inoculation on *Arabidopsis* primary-root growth, we carried out kinetic

Table 1. Effect of inoculation of bean plants with *Bacillus megaterium* UMCV1

Parameter	Axenic bean plants	Inoculated bean plants
Bean plants grown in vitro		
Fresh weight (g)	1.46 ± 0.35	1.97 ± 0.22 ^a
Dry weight (g)	0.076 ± 0.024	0.111 ± 0.020 [*]
Shoot/root biomass ratio ^b	1.74 ± 0.43	1.96 ± 0.41
Stem length (cm)	8.0 ± 2.2	10.3 ± 1.2 [*]
Root length (cm)	6.7 ± 1.3	11.6 ± 2.6 [*]
Lateral root number per plant	11.0 ± 2.7	17.6 ± 4.4 [*]
Lateral root length (cm)	1.2 ± 0.4	2.3 ± 0.4 [*]
Bean plants grown in sterilized soil		
Fresh weight (g)	1.42 ± 0.35	1.92 ± 0.32 [*]
Dry weight (g)	0.13 ± 0.02	0.17 ± 0.04 [*]

^a Values shown represent the average of ten replicates ± standard deviation. Asterisks are used to indicate statistically significant differences by *t* test (*P* < 0.05).

^b The shoot/root biomass ratio was calculated on a fresh weight basis.

assays to compare the early temporal effects of inoculation on root growth. During the first two days after transfer, the primary roots of control and inoculated plants had similar root-growth rates (Fig. 2). However, 3 days after transfer, a gradual reduction in primary-root growth in inoculated plants was observed as compared with control plants, which sustained a relatively constant growth rate (Fig. 2).

We next investigated whether the primary-root growth inhibition observed after inoculation with *B. megaterium* could be caused by altered cell growth or cell division. The root tip contains the root-growth zone, which consists of the meristem, a population of small dividing cells, followed by a cell-growth region composed by gradually enlarged elongated cells. More

distal to the tip, a cell differentiation region can be identified by the differentiation of epidermal cells into root hairs (Baluska et al. 1996). Using a dissection microscope, we observed that root tips of inoculated plants had a similar structure to that of uninoculated root tips. In inoculated plants, however, the cell-differentiation zone was located closer to the root meristem region and formed longer root hairs (Fig. 3A and B).

To test whether *B. megaterium* inoculation could alter cell division or elongation or both, we analyzed the expression of the cell-cycle marker *CycB1:uidA* (Colón-Carmona et al. 1999) in *Arabidopsis* transgenic plants. In uninoculated plants, *CycB1:uidA* showed a patchy expression in the root meristem (Fig. 3C). This expression was clearly reduced in plants inocu-

Fig. 1. Effect of *Bacillus megaterium* at two different distances from the point of bacterial inoculation on growth of *Arabidopsis* seedlings. **A** and **B**, *Arabidopsis* (Col-0) plants grown on the surface of agar plates with 0.2× Murashige and Skoog (MS) medium. **C** and **D**, *Arabidopsis* plants that were inoculated with *B. megaterium* at a distance of 5 cm from the root tip 4 days after germination and grown for a further 6-day period. Arrow indicates the point of inoculation. **E**, *Arabidopsis* plants inoculated with *B. megaterium* at a distance of 2 cm from the root tip 4 days after germination and grown for a further 6-day period. Note the elicitation of growth by bacterial inoculation 5 cm from the root and the formation of branched root systems when plants were inoculated at a closer distance. Photographs are representative individuals from four plates per treatment. The experiments were repeated three times with similar results.

lated with *B. megaterium* (Fig. 3D). We also counted the cells present in the root meristematic zone that expressed the *CycB1:uidA* marker. It was observed that in uninoculated plants, around 37 cells in the root meristem expressed the marker. Interestingly, bacterial inoculation decreased by 80% the number of cells expressing the *CycB1:uidA* marker (Fig. 4A).

We analyzed the effect of *B. megaterium* inoculation on cell elongation by measuring the epidermal-cell size in the differentiation region of primary roots in WT (Col-0) plants. These measurements showed that epidermal cells in plants inoculated are, on average, 70% shorter than those of uninoculated plants (Fig. 4B). Therefore, the observed inhibitory effect of *B. megaterium* inoculation on primary-root growth is caused both by a reduction in cell proliferation in the meristem and by limited elongation of the cells that exit the meristem.

Table 2. Growth of *Bacillus megaterium* on agar plates at different distances from *Arabidopsis thaliana* roots

Culture	Protein from inoculum section (measured in μg) ^a		
	1	2	3
Col-0			
2 cm	0.20 $\pm$ 0.21	0.52 $\pm$ 0.40	0.63 $\pm$ 0.54
5 cm	0.44 $\pm$ 0.44	0.62 $\pm$ 0.40	0.90 $\pm$ 0.90
ein2			
2 cm	0.09 $\pm$ 0.08*	0.32 $\pm$ 0.08	0.92 $\pm$ 0.57
5 cm	0.49 $\pm$ 0.21	0.58 $\pm$ 0.31	0.67 $\pm$ 0.11
aux1			
2 cm	0.14 $\pm$ 0.03	0.50 $\pm$ 0.27	0.79 $\pm$ 0.63
5 cm	0.15 $\pm$ 0.06	0.35 $\pm$ 0.28	0.64 $\pm$ 0.24

^a At the indicated number of days after inoculation. Inoculum samples (1.5 cm) were assayed for total protein content using the Bradford protein determination assay. Values shown represent the mean $\pm$ standard deviation of four replicate experiments. Asterisks are used to denote means that are statistically different by a least significant difference test ($P \geq 0.5$).

Fig. 2. Effect of *Bacillus megaterium* on *Arabidopsis* primary-root growth. *Arabidopsis* Col-0 seedlings were germinated and grown 4 days on the surface of agar plates containing 0.2 $\times$ Murashige-Skoog (MS) medium. At day 4, seedlings were transferred to control (uninoculated) fresh 0.2 $\times$ MS medium. Half of the plates were inoculated with *B. megaterium* at a distance of 5 cm from the tip of the primary root and grown for the indicated number of days. Day 0 indicates the length reached by the primary at the moment of inoculation. Mean $\pm$ standard deviation values were plotted at the indicated days in the kinetic experiment ($n = 30$).

B. megaterium inoculation reduces *DR5:GUS* expression in primary-root tips.

Many bacterial species produce auxin or auxin-like compounds and ethylene (Bowen and Rovira 1999). Exogenous auxin and ethylene have been found to repress primary-root growth in *Arabidopsis* (López-Bucio et al. 2002). To determine whether the diffusible factor from *B. megaterium* responsible for primary-root inhibition in *Arabidopsis* is likely to be auxin or ethylene, seedlings of *DR5:uidA*, a reporter line for auxin- and ethylene-inducible gene expression (Sabatini et al. 1999; Stepanova et al. 2005; Ulmasov et al. 1997) were included in the inoculation assays. *DR5:uidA* seedlings were grown for 4 days in MS 0.2 $\times$ medium and then transferred 5 or 2 cm from the *B. megaterium* inoculum for an additional 6-day period of growth. At the end of this period, transgenic seedlings were stained for β -glucuronidase (GUS) activity and were cleared to determine changes in expression. Although a clear reduction in root growth and a decrease in the number of meristematic cells were observed for inoculated *DR5:uidA* plants (data not shown), no increase in GUS expression in the root tip was observed in plants transferred 5 or 2 cm from the inoculation site, suggesting that *B. megaterium* did not increase auxin or ethylene responsiveness in primary-root tips (Fig. 5A and B). On the contrary, a very clear reduction in *DR5:uidA* expression was observed in inoculated plants (Fig. 5B and D), thus suggesting that bacterial inoculation negatively affects auxin- or ethylene-induced gene expression in primary-root tips.

Fig. 3. Effect of *Bacillus megaterium* on cell differentiation and division in *Arabidopsis* primary roots. **A**, Primary root tip of a representative *Arabidopsis* plant grown in uninoculated 0.2 $\times$ Murashige-Skoog medium. **B**, Primary root tip of an inoculated plant. Note the increased growth of root hairs and their closer location to the root tip in plants inoculated with *B. megaterium*. **C**, Expression of cell division marker *CycB1:uidA* in the primary-root meristem of an uninoculated plant. **D**, *CycB1:uidA* expression in the meristem of a plant inoculated with *B. megaterium*. Note the decrease in the number of cells expressing the marker in inoculated roots, indicative of reduced proliferative activity of meristem cells. Photographs were taken at day 6 in a primary-root growth kinetic experiment.

Fig. 4. Cellular parameters in *Bacillus megaterium*-inoculated plants. **A**, Number of cells expressing the *CycB1:uidA* marker in the primary-root meristem. Mean $\pm$ standard deviation (SD) is shown for 10 plants. **B**, Epidermal cell length $\pm$ SD. At least 20 epidermal cells were measured from 30 independent primary roots. Mean values were plotted at day 6 in the kinetic primary-root growth experiment.

Fig. 5. Effect of *Bacillus megaterium* inoculation on auxin-regulated gene expression. Twelve-hour β -glucuronidase (GUS) staining of *DR5:uidA* primary roots of *Arabidopsis* seedlings grown for 6 days in **A** and **C**, 0.2 $\times$ Murashige-Skoog medium or **B** and **D**, in medium inoculated with *B. megaterium*. Lateral-root primordia (LRP) at two developmental stages in **E** and **G**, control or **F** and **H**, inoculated plants. Note the decrease in GUS expression in primary-root tips and the increase in expression in LRP of inoculated plants. Photographs are representative individuals of at least 20 plants stained. Scale bar = 100 μm .

We further examined GUS expression in lateral-root primordia (LRP) at early developmental stages in *DR5:uidA* seedlings inoculated with *B. megaterium*. Interestingly, inoculated plants showed increased GUS activity in LRP when compared with uninoculated plants (Fig. 5E to H). These data suggest that *B. megaterium* inoculation may enhance auxin or ethylene responsiveness in LRP.

Effect of bacterial inoculation on growth of ethylene and auxin *Arabidopsis* mutants.

We used the transfer system to study the effects of *B. megaterium* inoculation on the growth of *Arabidopsis* WT plants

and mutants defective on ethylene (*etr1-1*, *ein2-1*) or auxin (*axr4-2*, *aux1-7*) response. At day 4 after germination and growth on 0.2× MS medium, WT and mutant plants were placed 5 cm from the bacterial inoculation site, and the shoot and root fresh weights were quantified at a further 8-day growth period. In this assay, *B. megaterium* significantly increased shoot and root fresh weights in WT plants and in the four mutant lines tested (Fig. 6A and B), suggesting that plant-growth promotion by this rhizobacteria may not directly involve the ethylene and auxin pathways.

Effect of *B. megaterium* inoculation on the root-system architecture of ethylene and auxin *Arabidopsis* mutants.

To further define the particular role of ethylene and auxin in the *Arabidopsis* developmental responses to *B. megaterium* inoculation, we tested the root-architectural responses to inoculation of *etr1-1*, *ein2-1*, *aux1-7*, *eir1-1*, and *axr4-2* *Arabidopsis* mutants. WT and mutant plants were placed 5 cm from the bacterial inoculation site, and the primary-root length, the primary-root diameter, and the number of lateral roots were determined at day 8 after inoculation. It was found that inoculation with *B. megaterium* similarly inhibited the growth of primary roots in WT and mutant plants (Fig. 7A). No significant differences were found among control and inoculated plants in terms of primary-root diameter for all genotypes tested (Fig. 7B), suggesting that inoculation did not cause root swelling. It was found that the *etr1* and *ein2* mutants, which are defective in ethylene sensing, produce normal numbers of lateral roots in uninoculated medium and respond to bacterial inoculation by increasing the production of lateral roots (Fig. 7C). The mutants affected in the auxin influx and efflux carriers *aux1-7* and *eir1-1*, respectively, and the auxin response mutant *axr4-2* produced a reduced number of lateral roots in uninoculated medium when compared with WT but showed increased numbers of lateral roots when inoculated with *B. megaterium* (Fig. 7C). Taken together, these data indicate that the ethylene and auxin mutant lines tested retain normal root developmental responses to bacterial inoculation.

Effect of *B. megaterium* inoculation on root-hair development of ethylene and auxin *Arabidopsis* mutants.

Root hairs are root epidermal cells that increase the total absorptive surface of the root system and participate in nutrient and water uptake. Root-hair development is particularly sensitive to both biotic and abiotic stimuli and is a useful marker of differentiation processes that take place in the root (López-Bucio et al. 2005). To analyze whether *B. megaterium* inoculation could alter root-hair development, we performed experiments in which *Arabidopsis* WT and *etr1-1*, *ein2-1*, *aux1-7*, *eir1-1*, *axr4-1*, and *rhd6* mutants were grown on the surface of agar plates with or without bacterial inoculation. Root-hair parameters were analyzed 6 days after inoculation on primary roots of control and inoculated plants. Bacterial inoculation increased by 2.5-fold the length of root hairs in WT plants (Fig. 8A). Uninoculated *etr1-1*, *ein2-1*, *aux1-7*, and *eir1-1* mutants showed a substantial reduction in root-hair length when compared with WT plants, whereas *axr4-2* seedlings sustained normal growth of root hairs under this condition (Fig. 8A). It was found that all mutant lines analyzed showed increased length of root hairs when inoculated with *B. megaterium* (Fig. 8A). Further analysis of root-hair development in plants grown in uninoculated medium showed that *aux1-7* and *eir1* mutants had a reduced number of root hairs when compared with WT (Fig. 8B). In these experiments, inoculation significantly increased the number of root hairs in WT plants and restored

Fig. 6. Effect of *Bacillus megaterium* on growth of wild-type *Arabidopsis* (Col-0), ethylene perception, and auxin-resistant mutants. Plant material was harvested 8 days after bacterial inoculation at 5 cm from the root tip. Roots and shoots were excised at the root-shoot junction and the fresh weight was determined on an analytical balance. **A**, Shoot fresh weight. **B**, Root fresh weight. Values shown represent the mean of four groups of 10 seedlings $\pm$ standard deviation. Different letters are used to indicate means that differ significantly ($P < 0.05$).

Fig. 7. Effect of *Bacillus megaterium* on root architecture in wild-type *Arabidopsis* (Col-0), ethylene perception, and auxin-resistant mutants. Plants were grown for 6 days in uninoculated or inoculated medium, on vertically oriented agar dishes. Data are given for the **A**, length of the primary root, **B**, primary-root diameter, and **C**, lateral-root number. Values shown represent the mean of 15 seedlings $\pm$ standard deviation. Different letters are used to indicate means that differ significantly ($P < 0.05$).

normal root-hair responses in *aux1-7* and *eir1*. Interestingly, the *rhd6* mutant, which is defective on root-hair initiation and has been previously shown to be rescued by both auxin and ethylene (Masucci and Schiefelbein 1994), was completely devoid of root hairs both in control and inoculated medium (Fig. 8A and B), thus suggesting that the increase in root-hair number by bacterial inoculation was not due to increased initiation of root hairs but rather to a reduction in epidermal-cell length.

Fig. 8. Effect of *Bacillus megaterium* on root-hair development in wild-type *Arabidopsis* (Col-0), ethylene perception, and auxin-resistant mutants. Plants were grown for 6 days in uninoculated or inoculated medium on vertically oriented agar dishes. Data are given for the **A**, length of root hairs formed in the primary root, and **B**, root-hair number. Values shown represent the mean of 60 root hairs $\pm$ standard deviation. ($n = 15$). Different letters are used to indicate means that differ significantly ($P < 0.05$).

***B. megaterium* UMCV1 produces acetoin and other volatile compounds.**

We conducted experiments aimed at identifying IAA in the bacterial culture supernatant. With this aim, samples of *B. megaterium* culture supernatant medium were prepared for gas chromatographic-mass spectrometry (GC-MS) analysis. Our GC-MS analysis, however, failed to detect this auxin in the culture supernatant from *B. megaterium* (data not shown).

Bacterial volatiles have been recently considered important signals involved in plant-growth promotion by rhizobacteria (Ryu et al. 2003; Ping and Boland 2004). Therefore, we carried out volatile determinations from *B. megaterium* cultures. Our search revealed a suite of volatile compounds produced by this bacterium (Table 3). Interestingly, *B. megaterium* was found to produce acetoin, a substance previously reported to stimulate growth of *Arabidopsis* in vitro (Ryu et al. 2003).

DISCUSSION

Rhizosphere microorganisms can have marked effects on plant performance in agricultural and marginal soils by influencing growth and development of roots and improving nutrient availability in the rhizosphere (Tinker 1984). For instance, phosphate solubilizing bacteria have been found to play an important role in plant-growth promotion (Rodríguez and Fraga 1999). PGPR, which belong to diverse genera such as genera *Pseudomonas* and *Bacillus*, are rhizosphere-inhabiting bacteria that have a positive influence on plant growth (Persello-Cartieux et al. 2003). These organisms have been recognized from a wide range of plant species, such as barley, rice, canola, and bean. Their contribution can be exerted through direct and indirect methods. Direct methods include those by which bacteria directly influence root-system architecture and shoot development by secretion of plant growth-promoting substances such as auxins and cytokinins (Arkhipova et al. 2005; Persello-Cartieux et al. 2001). Indirect methods include the effects of products such as antibiotics and hydrogen cyanide, which promote plant growth by inhibiting the growth of deleterious microorganisms in the rhizosphere (Bowen and Rovira 1999). Most studies on mechanisms for plant-growth promotion by PGPR have focused on bacterial traits without examining the detailed phenotypic, cellular, and physiological responses of the host plant. This is due, at least in part, because root architecture is difficult to observe, quantify, and interpret. Roots grow in soil, an opaque medium from which they cannot be extricated or readily observed without introducing artifacts, destroying the native root architecture, or precluding subsequent analysis of the same individuals. In this work, we used *Arabidopsis thaliana* as a model system to further characterize the

phenotypic and physiological basis by which *B. megaterium* regulates plant growth and root architecture.

Mechanisms for plant-growth promotion elicited by the UMCV1 strain of *B. megaterium*.

B. megaterium UMCV1 was found to stimulate the growth of bean plants in vitro and in soil and of *Arabidopsis* in vitro, suggesting that this bacterial strain likely acts as a novel plant growth-promoting bacterium. It was previously reported by Ryu and associates (2005) that *Arabidopsis* growth promotion by several PGPR involves complex hormonal signaling. We used the *Bacillus megaterium* UMCV1 strain to gain a better understanding of the mechanisms of plant-growth promotion by PGPR. Our results show that the growth promotion is dependent on the *B. megaterium* inoculation distance from the plant. Positive effects of inoculation on plant growth were observed when *Arabidopsis* seedlings are placed at a distance of 5 cm from the inoculation site, whereas repressing effects were found at a distance of 2 cm. These results suggest that a diffusible bacterial metabolite, possibly a plant growth-regulating substance caused the described alterations on growth and development.

Inoculation with *B. megaterium* affected the root system in *Arabidopsis* WT plants in a way that suggests the effects mediated by auxin (Fig. 1). It has been reported that IAA-producing bacteria can stimulate or inhibit root growth depending on bacterial concentrations (Persello-Cartieux et al. 2001). Similarly, the primary roots of WT plants reduced their growth after inoculation with *B. megaterium*. Unlike inoculated roots, the roots of uninoculated WT plants sustained normal indeterminate growth (Fig. 2). It was also found that, in parallel or as a consequence of primary root-growth inhibition, many lateral roots emerge in inoculated plants. In previous experiments, root caps have been eliminated by surgical removal or by expression of a diphtheria toxin gene (Tsugeki and Fedoroff 1999). Roots with damaged meristems grew much more slowly, differentiated prematurely, and showed extensive root branching (Tsugeki and Fedoroff 1999). Our microscopical survey showed that the meristems of inoculated roots appear normal, as revealed by the presence of small dividing cells that express the *CycB1:uidA* marker and because primary roots did not increase in diameter (Fig. 3A to D; Fig. 7B). The count of meristem cells that express the *CycB1:uidA* marker showed, however, that bacterial inoculation causes a reduction of cell proliferation (Fig. 3C and D). These results suggest that attenuation of meristematic activity in the primary root may be sufficient to elicit major developmental alterations in the whole root system, including the premature differentiation of root hairs and maturation of lateral roots.

The role of auxin and ethylene on *Arabidopsis* growth and developmental responses to *B. megaterium* inoculation.

The described developmental alterations in *Arabidopsis* plants inoculated with *B. megaterium* are very similar to those reported for auxin accumulating in root tips or by ethylene as well (López-Bucio et al. 2002; Stephanova et al. 2005). Both primary-root elongation and lateral-root formation require auxin transport, and there is evidence that an auxin gradient is present at the tip of primary roots (Sabatini et al. 1999). A recent study indicates that ethylene plays a key role regulating auxin production and opens the possibility that several root architectural responses, including primary-root elongation and root-hair development, which are regulated by auxin, could be also controlled by ethylene (Stepanova et al. 2005). The analysis of *DR5:uidA* marker in transgenic *Arabidopsis* plants revealed the presence of an auxin gradient in uninoculated roots

Table 3. SPME gas chromatographic-mass spectrometry profile of volatile compounds produced by *Bacillus megaterium* UMCV1^a

Compound	Retention time (min)	Area (%)
Acetoin	5.34	0.29
Phenylethyne	6.80	0.11
Acetic acid	8.61	4.81
Propanoic acid, 2-methyl	10.63	4.37
Butanoic acid, 2-methyl	12.28	8.23
Thymol	19.19	0.13
Tetradecanoic acid	24.11	12.00
Pentadecanoic acid	25.69	5.36
Hexadecanoic acid	27.75	40.13
9-Hexadecanoic acid	28.57	21.82
Octadecanoic acid	34.99	2.74

^a Reported compounds include those positively identified in the growth medium of *Bacillus megaterium* UMCV1 but not present in uninoculated medium.

(Fig. 5A and C). Inoculation with *B. megaterium* did not increase *DR5:uidA* expression in primary-root tips indicating that the primary-root growth inhibition observed after inoculation was not caused by increased auxin accumulation at this region. Interestingly, reduced *DR5:uidA* expression in inoculated primary roots correlated with increased expression of this marker in lateral-root primordia (Fig. 5A to H), suggesting that increased growth of lateral roots after inoculation may be mediated by auxin redistribution. Consistent with this hypothesis, we found that auxin (*aux1-7*, *eir1*, *axr4*) and ethylene (*etr1*, *ein2*) mutants retain normal growth and developmental responses to bacterial inoculation. The *aux1-7*, *eir1*, *axr4*, *etr1*, and *ein2* mutants showed primary root-growth inhibition and lateral root-growth stimulation when subjected to *B. megaterium* inoculation, suggesting that the products of these genes are not directly involved in root architectural responses to this bacterium.

Regulation of root-hair development by *B. megaterium* requires a functional *RHD6* gene.

WT plants inoculated with *B. megaterium* showed both an increased number and an increased length of root hairs, an effect reminiscent of altered ethylene or auxin responses (Fig. 8). The analysis of mutants revealed that the *aux1-7*, *eir1*, *etr1*, and *ein2* plants have reduced length of root hairs when grown in uninoculated medium, indicating that auxin and ethylene are important regulators of hair growth under these conditions. Inoculation of all these mutants with *B. megaterium*, however, increased both root-hair number and length, indicating that auxin or ethylene are not directly involved in root-hair developmental regulation by this bacterium. This conclusion is also supported by our finding that the *rhb6* mutant failed to respond to inoculation by forming root hairs. The *rhb6* mutant was isolated in screens aimed at identifying *Arabidopsis* mutants defective in root-hair development (Masucci and Schiefelbein 1994). *rhb6* is defective in root-hair initiation, and the lack of root hairs in this mutant can be reversed by treatment with auxin or by the ethylene precursor ACC (Masucci and Schiefelbein 1994). Under our growth conditions, inoculation with *B. megaterium* 5 or 2 cm from *rhb6* plants did not rescue the root-hair phenotype of this mutant, suggesting that the increased numbers of hairs observed after inoculation in WT, ethylene, and auxin mutants were not caused by ectopic formation of root hairs. This result also suggests that one or more of the metabolites produced by *B. megaterium* likely acts upstream of *rhb6* in the signaling cascade that leads to alterations in root-hair development.

Arabidopsis responses to *B. megaterium* inoculation reveal a novel mechanism for plant growth regulation by rhizobacteria.

B. megaterium is not often studied as PGPR. Kishore and coworkers, however, have recently isolated a *B. megaterium* strain that significantly increased the growth of *Arachis hypogaea* plants (Kishore et al. 2005). Interestingly, the authors did not find a correlation between auxin production by this bacterium and its plant growth-promotion ability. Our search for IAA in the culture medium of *B. megaterium* failed to detect this auxin (data not shown). This information together with our data obtained from the analysis of auxin and ethylene mutants and the *DR5:uidA* reporter line reveal a novel mechanism for plant growth regulation by *B. megaterium*.

The analysis of bacterial volatiles showed the production of acetoin by *B. megaterium* UMCV1. A recently discovered mode of growth promotion and activation of induced systemic resistance is based on airborne volatiles (i.e., butanediol, acetoin), which are released from certain microorganisms. In particular, certain *Bacillus* strains that produce acetoin were found

to stimulate *Arabidopsis* growth (Ryu et al. 2003). Whether acetoin production by *B. megaterium* accounts for growth and developmental responses elicited in *Arabidopsis* remains to be determined.

Many PGPR display a combination of different modes of actions. We cannot exclude the possibility that a combination of different mechanisms may account for plant growth promotion by *B. megaterium*. For instance, molecules of bacterial and fungal origin have been found to alter root-system architecture in several plant species. For example, Souleimanov and associates (2002) showed that the Nod factors of *Bradyrhizobium japonicum* can stimulate development of soybean root systems by increasing their total lengths, surface areas, and dry weights, suggesting that these substances may have a general plant growth-regulating activity in addition to their symbiotic roles in nodulation. Nod factors were also found to increase mycorrhizal colonization in *Medicago truncatula* and to stimulate lateral-root formation in this plant species (Olah et al. 2005). Interestingly, a diffusible factor from arbuscular mycorrhizal fungi was also found to stimulate lateral-root formation in *M. truncatula*, suggesting that substances of bacterial and fungal origin regulate lateral-root proliferation (Olah et al. 2005). Whether Nod factors and mycorrhizal factors regulate root-system architecture interacting with auxin or ethylene remains to be determined.

The data presented in this work and other recent evidence from the literature suggest the existence of new plant growth-regulating substances whose molecular mechanisms of action remain unknown. We are currently working on the identification of one or more of the *B. megaterium* factors to test their biological activity in *Arabidopsis* and other plant species. The incorporation of *Arabidopsis* as a model system to study plant-bacterial symbiosis shows great promise in investigating the molecular mechanisms of plant-growth promotion by PGPR.

MATERIALS AND METHODS

Plant material and growth conditions.

Arabidopsis ecotype Col-0, the *Arabidopsis* transgenic lines *CycB1;1:uidA* (Colón-Carmona et al. 1999) and *DR5:uidA* (Ulmasov et al. 1997), and the mutant lines *etr1-3* (Hua and Meyerowitz 1998), *ein2-1* (Guzmán and Ecker 1990), *eir1-1* (Roman et al. 1995), *axr4-2* (Hobbie and Estelle 1995), *aux1-7* (Pickett et al. 1990), and *rhb6* (Masucci and Schiefelbein 1994) were used for the different experiments. Seeds were surface-sterilized with 95% (vol/vol) ethanol for 5 min and 20% (vol/vol) bleach for 7 min. After five washes in distilled water, seeds were germinated and grown on agar plates containing 0.2× MS medium (Murashige and Skoog basal salts mixture, Cat. M5524; Sigma, St. Louis). Plates were placed vertically at an angle of 65 degrees to allow root growth along the agar surface and to allow unimpeded aerial growth of the hypocotyls. Plants were placed in a plant-growth chamber with a photoperiod of 16 h of light, 8 h of darkness, light intensity of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and temperature of 24°C.

Isolation of the *B. megaterium* UMCV1 strain.

The UMCV1 strain was initially isolated from the rhizosphere of maize plants grown under greenhouse conditions, from an andosol (pH 6.2) obtained in the municipality of Uruapan, Michoacan, México (19°23'56.69" N, 102°02'41.17" W). Soil characteristics are sand/silt/clay 64.7:24.7:7.8%, and total organic matter content of 8.9%. Soil samples were taken from the upper soil layer (0 to 20 cm), were air dried. Each sample was then meshed (≤ 4 mm) and maintained at 4°C until their use as substrate for greenhouse experiments. Rhizosphere soil suspensions were obtained from bean roots according to a modi-

fication of the method of Picard and associates (2000). Plants were carefully taken from the soil, and bulk soil was separated from the roots by vigorously shaking in 100 ml of sterile water for 20 s, using a Vortex; rhizosphere soil was separated from the roots by vigorously shaking (200 rpm) in 20 ml of sterile phosphate-buffer for 2 h, using an orbital shaker. Bacterial UMCV1 strain was isolated from tubes containing nutritive broth supplemented with 20 mM HEPES, 0.1 mM ferrozine, and 400 $\times$ μ M ferric citrate, and was inoculated with serial dilutions of rhizosphere soil suspensions. Tubes were incubated 3 days at 30°C in the dark.

Molecular characterization of *B. megaterium* UMCV1.

Fragments of an average of 374 bp from the 5' and 3' ends of 16S rDNA sequences from bacterial isolates were amplified and sequenced using the primers fd1 and rd1 (Weisburg et al. 1991). 16S rDNA amplification and sequencing was repeated twice as previously described (Valencia-Cantero et al. 2003). Amplified sequences were deposited in the NCBI GenBank (accession numbers AY553859 and AY553860) and were compared with sequences from the NCBI database, using the blast algorithm (Altschul et al. 1990). Homology of 100% was found between sequences from UMCV1 strain and sequences from different strains of *B. megaterium*.

Inoculation experiments.

Seeds of *Phaseolus vulgaris* cv. Flor de Mayo Bajio were superficially sterilized as reported by Masalha and associates (2000). Plants were grown in glass culture tubes (25 mm diameter, 250 mm height) containing 10 g of autoclaved (1 h/day $\times$ 7 days) alkaline soil (pH 8.0), or 20 ml of mineral culture media (Schmidt 1994) solidified with Phytagar (commercial grade; Gibco-BRL, Gaithersburg, MD, U.S.A.). Seedlings were inoculated with approximately 1×10^8 CFU of UMCV1 bacteria and were grown for a 12-day period at a temperature of 25°C under continuous illumination. At the end of this period, plants were sectioned at the root and shoot interface to quantify root and shoot weight. The fresh weight was measured on an analytical scale immediately after plant harvest, stem and root lengths were measured with a ruler, and lateral roots were counted and measured under a dissection microscope.

B. megaterium UMCV1 was evaluated in vitro for their plant growth-promotion ability, using the *Arabidopsis* Col-0 ecotype. Bacterial densities of 2.5×10^8 CFU were inoculated by streaking on agar plates containing 0.2 $\times$ MS medium. Four-day-old germinated *A. thaliana* seedlings (8 seedlings per plate) were transferred to one side of the plate, opposite to the inoculation site. Plates were sealed with parafilm and were arranged in a completely randomized design.

Bacterial growth measurement.

Bacterial growth on petri dishes with plant-bacterial cultures was determined as bacterial protein production. *B. megaterium* UMCV1 was inoculated at a distance of 5 or 2 cm from *A. thaliana* Col-0, *ein2*, and *aux-1* seedlings. Samples covering 1.5-cm sections from the bacterial inoculum were carefully recovered from the growth medium, were resuspended in 1 ml of distilled water, and were frozen at -30°C until protein determination. Total protein was determined by the Bradford method using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, U.S.A.), according to the instructions of the manufacturer. In order to ensure cellular lysis, bacterial suspensions were boiled for 10 min prior to protein determination.

Histochemical analysis.

For histochemical analysis of GUS activity, *Arabidopsis* seedlings were incubated overnight at 37°C in a GUS reaction

buffer (0.5 mg/ml of 5-bromo-4-chloro-3-indolyl- β -D-glucuronide in 100 mM sodium phosphate, pH 7). The stained seedlings were cleared using the method of Malamy and Benfey (1997). For each marker line and for each treatment, at least 10 transgenic plants were analyzed. A representative plant was chosen and photographed, using Nomarsky optics on a Leica DMR microscope.

Analysis of bacterial volatile compounds.

Bacterial growth medium (2 ml) was placed in a 4-ml vial for a 60-min time period. At the end of this period, the volatiles were collected using a blue SPME fiber (PDMS/DVB) (Supelco, Inc., Bellefonte, PA, U.S.A.) and desorbed for 60 s in the injector port of a gas chromatograph (Agilent 6850 Series II; Agilent, Foster City, CA, U.S.A.), equipped with a MS detector 5973 from Agilent and a free fatty acid-phase capillary column (HP-FFAP) 25 m $\times$ 0.52 mm I.D., film thickness of 0.32 μ m. Operating conditions used helium as the carrier gas (1 ml/min), detector temperature of 250°C, and injector temperature of 180°C. The column was held for 5 min at 40°C, and the temperature was programmed to rise at a rate of 3°C per minute to a final temperature of 220°C, which was maintained for 5 min. Three independent determinations were made for each sample.

Auxin determination.

Bacterial growth medium (500 μ l) was evaporated to dryness under a stream of nitrogen and then was diluted in 1 ml of dichloromethane and redried; this procedure was repeated three times. The sample was first treated with acetyl chloride in methanol (2 ml/500 μ l), was sonicated for 15 min and was then heated for 1 h at 75°C. After cooling, the derivatized sample was evaporated and redissolved in 100 μ l of dichloromethane.

For GC-MS analysis, derivative samples were analyzed in a 6850 Series II gas chromatograph equipped with an Agilent MS detector model 5973 and a 30 mm $\times$ 0.25 mm $\times$ 0.25 μ m, 5% phenyl methyl silicone capillary column (HP-5 MS). Operating conditions used helium as carrier gas (1 ml/min), detector temperature of 250°C, and injector temperature of 180°C. The volume of injected sample was of 1 μ l. The column was held for 3 min at 80°C and was programmed to rise at 6°C/min to a final temperature of 230°C for 5 min.

Data analysis.

Arabidopsis root systems were viewed with an AFX-II-A stereomicroscope (Nikon, Tokyo). All lateral roots emerging from the primary root and observed under the 3 $\times$ objective were taken into account for lateral root-number data. Primary root length was determined for each root using a ruler. The lengths of 20 epidermal cells (only those cells that give rise to root hairs) were measured on images of cleared roots taken with a digital camera connected to a microscope using the Scion Image Free Software (Scion Corp., Frederick, MD, U.S.A.).

For all experiments, the overall data were statistically analyzed in the SPSS 10 program (SPSS, Chicago). Univariate and multivariate analyses with a Tukey's post hoc test were used for testing differences in growth and root developmental responses in WT and mutant plants. Different letters are used to indicate means that differ significantly ($P < 0.05$).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

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Scion Image Free Software: www.scioncorp.com