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Comments

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Casuarina Root Exudates Alter the Physiology, Surface Properties, and Plant Infectivity of *Frankia* sp. Strain CcI3

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The actinomycete genus *Frankia* forms nitrogen-fixing symbioses with 8 different families of actinorhizal plants, representing more than 200 different species. Very little is known about the initial molecular interactions between *Frankia* and host plants in the rhizosphere. Root exudates are important in *Rhizobium*-legume symbiosis, especially for initiating Nod factor synthesis. We measured differences in *Frankia* physiology after exposure to host aqueous root exudates to assess their effects on actinorhizal symbioses. *Casuarina cunninghamiana* root exudates were collected from plants under nitrogen-sufficient and -deficient conditions and tested on *Frankia* sp. strain CcI3. Root exudates increased the growth yield of *Frankia* in the presence of a carbon source, but *Frankia* was unable to use the root exudates as a sole carbon or energy source. Exposure to root exudates caused hyphal "curling" in *Frankia* cells, suggesting a chemotrophic response or surface property change. Exposure to root exudates altered Congo red dye binding, which indicated changes in the bacterial surface properties at the fatty acid level. Fourier transform infrared spectroscopy (FTIR) confirmed fatty acid changes and revealed further carbohydrate changes. *Frankia* cells preexposed to *C. cunninghamiana* root exudates for 6 days formed nodules on the host plant significantly earlier than control cells. These data support the hypothesis of early chemical signaling between actinorhizal host plants and *Frankia* in the rhizosphere.

Actinorhizal symbioses are mutualistic interactions that occur between actinorhizal plants and the actinomycete genus *Frankia* (27, 38). *Frankia* exists either in a free-living state in the soil or in symbiosis with actinorhizal plants (2, 4, 31). Actinorhizal plants consist of 8 different plant families, including over 200 different species of woody dicotyledonous trees and shrubs (31, 38, 42). The globally distributed actinorhizal plants are found on every continent except Antarctica and are able to grow in a diverse set of natural habitats, including arid lands, plains, tundra, and temperate forests. Actinorhizal plants are pioneer plant species that are able to grow in extremely nutrient-poor soil conditions and, with the aid of *Frankia*, are able to reclaim surrounding soil in disrupted environments. Actinorhizal plants are important in agroforestry, in soil reclamation, and as a fuel source.

While actinorhizal symbiosis has been well studied at a morphological level since the 1970s, very little is known about the molecular interactions that occur between the plant hosts and *Frankia* during the establishment of the association. The establishment of the symbiosis encompasses the infection and nodulation processes (23, 27, 38). In general, the bacterium needs to recognize a host plant and the host needs to identify the bacterium as a friend, not a foe. The bacteria enter the plant and establish the association after several steps. The infection ultimately leads to the formation of the mature nodule.

There is a paucity of information on the initial interactions occurring between actinorhizal host plants and their symbiont, *Frankia*, in the rhizosphere. In this study, we investigate whether actinorhizal host plants excrete a chemical signal in the rhizosphere that is perceived by *Frankia* and that changes its physiology prior to infection. For this study, *Casuarina cunninghamiana* and *Frankia* sp. strain CcI3 were chosen for several reasons. The *Frankia* CcI3 genome is completely sequenced (22), and it represents a narrow-host-range symbiont. The relationship between the plant host and symbiont, including the nodulation process, is well studied (17, 18, 27, 35, 41). The purpose of this study was to develop a system to identify chemical signaling between the microbe and its host plant.

MATERIALS AND METHODS

Growth media and culture conditions. *Frankia* strain CcI3 was grown and maintained in basal MP growth medium with 5.0 mM NH_4Cl as a nitrogen source and 5.0 mM propionate as a carbon source at 28°C, as described previously (33, 34). For some experiments, cells were grown in BAP medium as described previously (26). For growth under nitrogen-sufficient conditions, 5 mM NH_4CL was added to either MP or BAP medium. For growth under nitrogen-deficient conditions, N_2 was the sole nitrogen source.

Plant growth conditions. *Casuarina cunninghamiana* seeds were incubated overnight in sterile tap water and surface-sterilized with 30% hydrogen peroxide with 2 drops of Tween 20. Seeds were extensively washed with sterilized deionized H_2O (sdH_2O). Surface-sterilized seeds were germinated at 28°C with a 16-h light period and 8-h dark period in Magenta GA-7 boxes with autoclaved perlite and one-quarter-strength Hoagland's modified basal salt solution (1/4 HS). *Elaeagnus angustifolia* seeds were sterilized in 30% bleach. The treated seeds were extensively washed with sdH_2O and treated again with 30% H_2O_2. Seeds were germinated in sterilized pebbles and 25 ml of 1/4 HS.

Conditions for production and collection of root exudates. At 7 to 12 days after seed germination, *C. cunninghamiana* and *E. angustifolia* seedlings were aseptically transplanted to a Brite-Kote aluminum screen in a

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Magenta GA-7 box suspended over 50 ml of 1/4 HS medium. For each Magenta box, spent growth medium was replaced weekly with fresh, sterile 1/4 HS medium. Root exudates were collected at 3 weeks, 1 month, and 2 months of plant growth and filter sterilized. At each point, root exudates were collected over a 1-week period from these aged plants. The root exudates were stored at −20°C until use. The same procedure was repeated with seedlings incubated in 50 ml without a source of nitrogen in one-quarter-strength Hoagland's solution [1/4 HS(N_2); 22.5 μM Na_2EDTA, 22.5 μM $FeSO_4 \cdot 7H_2O$, 0.5 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM K_2SO_4, 0.625 mM $CaSO_4 \cdot 2H_2O$, and Hoagland's micronutrients, at a final pH of 5.5]. Hoagland's micronutrients contained 0.115 mM boric acid, 0.08 μM $CuSO_4 \cdot 5H_2O$, 2.3 μM $MnCl_2 \cdot 4H_2O$, 0.19 μM $ZnSO_4$, and 0.026 μM $Na_2MoO_4 \cdot 2H_2O$.

For root exudates collected from soil-grown plants, *C. cunninghamiana* seeds were sown into soil and incubated at 25°C on a 16-h light cycle for 2 months. After the seedlings were washed in tap water and sdH_2O, they were aseptically transferred to 15-ml culture tubes containing 14 ml of 1/4 HS medium. After a 1-week acclimation period, root exudates were collected and filter sterilized. For plants under nitrogen-deficient conditions, the process was repeated with 1/4 HS(N_2) medium.

Plant nodulation studies. After *C. cunninghamiana* seeds were germinated and seedlings were placed in Magenta boxes containing 5 seedlings, as described above, plants were incubated at 28°C with a 16-h light period for 2 weeks in 1/4 HS(N_2) medium. In order to be used as an inoculum, 14-day-old *Frankia* sp. CcI3 cultures grown in MP medium with 5.0 mM NH_4Cl and 5.0 mM propionate were harvested and washed 2 times with 10 ml of 1/4 HS(N_2) medium to remove all traces of nitrogen. The washed cells were resuspended in a 25-ml solution of aqueous root exudates or 1/4 HS(N_2) medium (the control) and incubated at 28°C for 6 days. The treated *Frankia* suspensions were adjusted to equivalent final protein concentrations (60 μg/ml) in 1/4 HS(N_2) medium. Spent plant growth medium was decanted from each Magenta box and replaced with 50 ml of the *Frankia* suspension. Control boxes contained 50 ml fresh 1/4 HS(N_2) medium. After inoculation, the plants were incubated at 25°C with a 16-h light period and plant growth medium was replaced weekly for the duration of the experiment. The plant roots were monitored daily for nodule formation. Both the number of plants with nodules and the total number of nodules formed were recorded.

Experiments on the effect of root exudate exposure on *Frankia* physiology. A 24-well growth assay was used to determine the effects of root exudates on *Frankia* growth and physiology as described previously (10). Briefly, cells were grown in MP medium for 14 days and growth was measured by total cellular protein content or total cellular dry weight as described below. Growth yield was determined by subtracting the protein or dry weight content of the inoculum.

Total cellular protein and dry weight determination. Protein content was measured by the bicinchoninic acid (BCA) method (32). Total cellular dry weight was determined using tarred polycarbonate membranes (34).

Measurement of total phenolic and flavonoid content. The total phenolic content of root exudates was measured by a modification to the Waterhouse method (40). Root exudates (25 μl) were added to 125 μl Folin-Ciocalteu reagent, and samples were mixed by agitation with a Vortex-Genie 2. After the mixture was incubated for 5 min, 375 μl of 20% sodium carbonate and 475 μl of H_2O were added to each sample. The samples were mixed and incubated at room temperature (RT) for 2 h. Phenolic content was determined by measuring the A_{760} on a UV-2401 PC spectrophotometer, with Gallic acid as a standard (10 to 200 μg/ml).

Flavonoid content of root exudates was determined by a modification of the method used by Chang et al. (5). Root exudate samples (300 μl) were added to 900 μl of 95% ethanol, 60 μl of 10% aluminum trichloride, and 60 μl of 1 M potassium acetate. The reaction mixtures were mixed and incubated at RT for 30 min. Flavonoid content was determined by measuring the A_{415} on a UV-2401 PC spectrophotometer, with quercetin as a standard (10 to 200 μg/ml).

GC. The volatile organic acid (C2 to C5 acids) content of root exudates was measured by gas chromatography (GC) and the use of an 80/120 carbopack column (14). Root exudate samples (1 μl) were injected into the HP6890 gas chromatograph fitted with an 80/120 carbopack B-DA Carbowax 20 M packed column and flame ionization detector. The following GC parameters were used: injector port temperature of 200°C, column temperature of 175°C, column N_2 carrier flow rate of 24 ml/min, and flame ionization detector temperature of 200°C. The retention time and area under the peak of the samples were analyzed and compared to those of the volatile acids standards (retention time [min]/area [pA · s], mM): formic acid (0.654/106.5, 10 mM), acetic acid (1.722/1,562, 10 mM), propionic acid (3.377/2,490, 10 mM), isobutyric acid (5.706/2,414, 10 mM), butyric acid (7.4/2,738, 10 mM), isovaleric acid (13.9/2,410, 10 mM), and valeric acid (17.3/2,481, 10 mM).

Ammonium and nitrate measurements. Ammonium content in root exudates was determined by the indophenol blue assay (6), with NH_4Cl (0.01 to 0.5 μg/ml) as a standard. Nitrate content in root exudates was determined spectrophotometrically as previously described (15), with KNO_3 (0.01 to 0.2 mM) as a standard.

Microscopy of *Frankia* cultures. Photomicroscopy of *Frankia* cultures was performed by the agar method of Pfenning and Wagener (28). Cultures were observed under phase-contrast microscopy at ×400 magnification.

Congo red dye binding assay. Bacterial surface property changes were measured by a modification of the Congo red dye binding assay (8). *Frankia* cultures were incubated in MP growth medium at 28°C for 11 days. At day 11, 10 μl of Congo red (10 mg/ml) was added to the 1-ml culture. After 3 more days of incubation at 28°C, *Frankia* mycelia were collected by centrifugation at 13,000 × *g* for 10 min and washed 3 times with sdH_2O. The washed cells were resuspended in 200 μl acetone and incubated with shaking for 2 h at RT. Acetone was separated from the debris by centrifugation at 13,000 × *g* for 10 min and transferred to a flat, transparent 96-well plate. The Congo red concentration was determined by measuring the A_{488}.

Fourier transform infrared spectroscopy (FTIR). For these experiments, 5-day-old cultures were harvested by centrifugation at 13,000 × *g* for 10 min and washed 3 times in sdH_2O. The washed cells were frozen at −80°C and lyophilized for 48 h. FTIR was performed as described previously (10). Briefly, FTIR spectra were determined from three biological replicates on a diamond attenuated total reflectance (ATR) Nicolet iS10.

Statistical analysis and reproducibility. Statistical tests were performed using JMP software utilizing analysis of variance (ANOVA), Dunnett's test, or Student's *t* test. The significance threshold was set at a *P* value of 0.05. Experiments were performed in triplicate with the use of three biological replicates.

RESULTS AND DISCUSSION

Production of aqueous root exudates and general properties. Although previous studies on root exudates have used solvent-extracted samples, aqueous root exudates were chosen for use in this study (1, 13, 29). Root exudates from plants grown under nitrogen-deficient and nitrogen-sufficient conditions were collected from both 2-month-old soil-grown plants and 3 week-, 1 month-, and 2-month-old axenically grown plants. The soil- and axenically grown *C. cunninghamiana* root exudates contained similar levels of phenolic (2.0 to 4.9 μg/ml) and flavonoid (9.9 to 18.6 μg/ml) compounds (data not shown). Flavonoid compounds have been identified from extracts of *Alnus glutinosa*, *Myrica gale*, and *Casuarina glauca* (1, 29). Although flavonoid compounds were detected in the *C. cunninghamiana* root exudates, we did not want to exclude other possible plant-signaling molecules and used aqueous extracts throughout this study. Besides phenolic and flavonoid content, the root exudates were analyzed for ammonium and nitrate content. All of the root exudates collected under

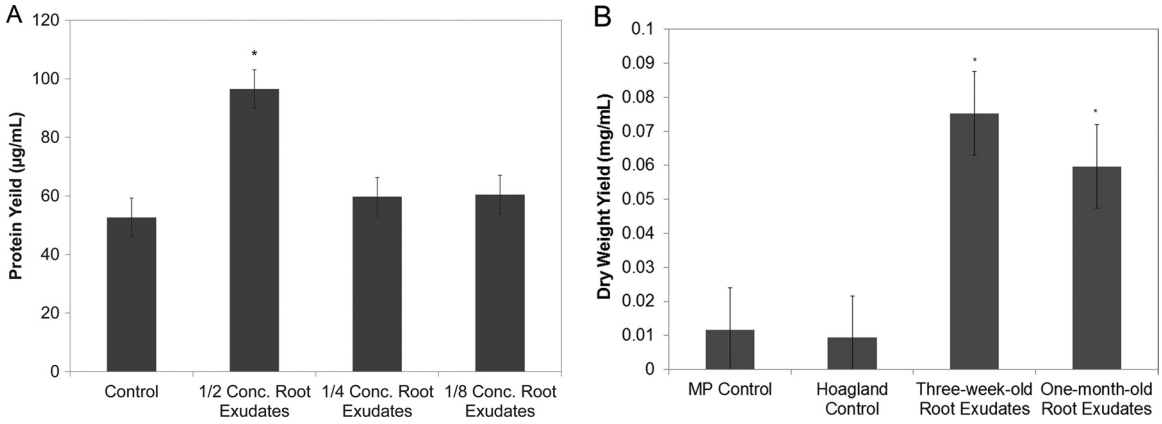

FIG 1 The effect of *C. cunninghamiana* root exudates on *Frankia* CcI3 growth yield. (A) Effect of root exudates from 2-month-old soil-grown *C. cunninghamiana* plants on growth yield, as measured by protein content. (B) Effect of one-half-strength root exudates from axenically grown *C. cunninghamiana* plants on *Frankia* CcI3 growth yield, as determined by total cellular dry weight. Root exudates were collected over 1-week time periods for the different ages of plants, as described in Materials and Methods. *Frankia* was grown with propionate-MP medium supplemented with root exudates. Control conditions contained 1/4 HS(N_2) medium. After 14 days, cellular protein content or cellular dry weight was determined as described in Materials and Methods and corrected for the inoculum value. A control Dunnett's test was used to determine statistical significance. *, *P* value of less than 0.05.

nitrogen-deficient conditions contained ammonium concentrations below detectable levels and 0.04 to 0.07 mM nitrate concentrations, while root exudates collected under nitrogen-sufficient conditions had ammonium concentrations ranging from 0.14 to 0.45 μg/ml and nitrate content concentrations below detectable levels. The root exudates were also analyzed for C2 to C8 organic acids by gas chromatography (GC). For 3-week-old axenic plants, acetic and propionic acid were found in the root exudates at very low concentrations (0.25 to 0.9 mM and 0.03 to 0.17 mM for acetic and propionic acids, respectively). For the older plants (axenic and soil-grown), the acetic and propionic acid contents of the root exudates were below the detection limits of the instrument. These levels may be insufficient to support growth of *Frankia*. A small amount of formic acid was identified in 3-week-old axenic root exudates collected under nitrogen-deficient conditions. Isobutyric, butyric, isovaleric, and valeric acids were not found in the axenic root exudates.

Effect of root exudates on *Frankia* CcI3 growth. The effect of *C. cunninghamiana* root exudates on *Frankia* CcI3 cell growth was tested, and growth yield was determined by total cellular protein after 14 days (Fig. 1A). In growth medium devoid of a carbon source, root exudates were unable to support the growth of *Frankia* CcI3. Under these conditions, the protein content was lower and significantly different than that of the inoculum (data not shown). This protein reduction was proportional to the concentration of root exudates, suggesting an inhibitory effect under these conditions. In the presence of an additional carbon source (propionate), the addition of root exudates caused an increase in growth yield. Two-month-old soil-grown *C. cunninghamiana* root exudates caused a 1.8-fold increase in protein yield (Fig. 1A). Similar growth yield results were obtained for all of the root exudates. Supplementing the growth medium with nitrogen did not reduce *Frankia*'s growth response to root exudates (data not shown). This suggests that the nitrogen content of the root exudates was not a significant factor in the enhanced growth. These results show that *Frankia* CcI3 was unable to utilize *C. cunninghamiana* root exudates as a sole carbon and energy source for growth, but growth in the presence of an exogenous carbon source was enhanced when supplemented with root exudates.

Growth yield was also determined by measuring cellular dry

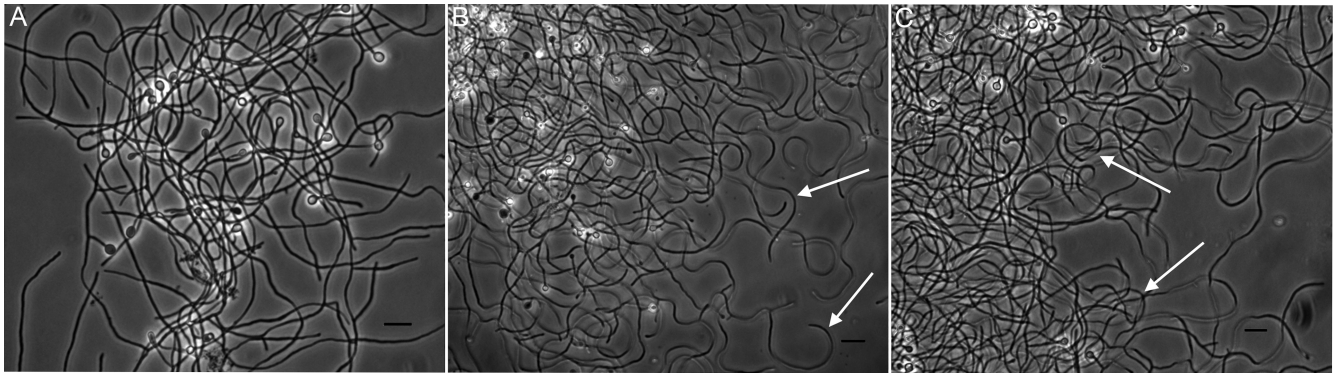

FIG 2 Hyphal curling response of *Frankia* CcI3 upon exposure to host root exudates. *Frankia* CcI3 cells incubated for 14 days in propionate-MP medium containing 1/4 HS(N_2) medium (control) (A), soil-grown 2-month-old (nitrogen-sufficient) *C. cunninghamiana* root exudates (B), or axenic 1-month-old (nitrogen-deficient) *C. cunninghamiana* root exudates (C). Cultures were observed under phase-contrast microscopy at a magnification of ×400. Bar, 10 μm. Arrows point to regions showing curling effects.

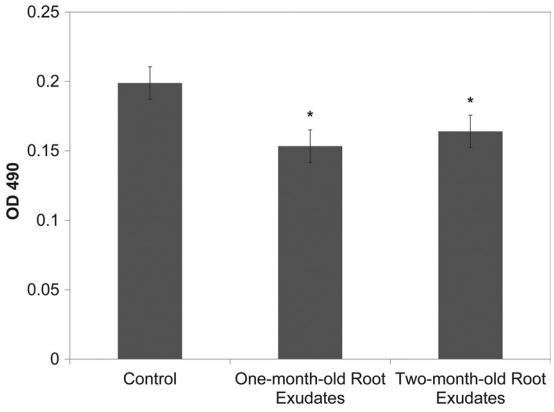

FIG 3 Effect of exposure to host root exudates on *Frankia* CcI3 Congo red binding. Cultures were incubated for 14 days in propionate-MP medium supplemented with axenic (nitrogen-deficient) *C. cunninghamiana* root exudates or 1/4 HS(N_2) medium (control). Congo red dye was added during the last 3 days of incubation and treated as described in Materials and Methods. A control Dunnett's test was used to determine statistical significance. *, *P* value of less than 0.05.

weight (Fig. 1B). In the presence of a carbon source, 3-week-old axenic *C. cunninghamiana* root exudates caused an 8-fold increase in the total dry weight yield. One-month-old axenic root exudates produced a 6-fold increase in growth yield. The overall patterns of the dry weight results were similar to those of the total protein results: *Frankia* growth was enhanced by the addition of *C. cunninghamiana* root exudates. However, the growth yields for total mass (dry weight yield) showed a larger fold increase than those measured for protein yield. These results would suggest that the root exudates influence *Frankia* physiology by affecting cellular components beyond total protein levels. Our results with aqueous root exudates confirm previous studies on flavonoid effects on the growth of *Frankia* with methanol extracts or purchased phenolics (25, 29, 37). Although aqueous root exudates alone did not support *Frankia* growth, plant compounds in the root exudates enhanced the growth of *Frankia*. This effect suggests that *C. cunninghamiana* aqueous root exudates provide a growth nutrient or plant-signaling compound(s) influencing *Frankia* physiology.

We tested whether this effect could be mimicked by flavonoids known to be involved in *Rhizobium*-legume symbiosis and to affect *Rhizobium* growth *in vitro* (7). The addition of 1 μM luteolin, chrysin, or naringenin to BAP medium did not cause an increase in *Frankia* growth yield compared to that of the control culture without flavonoids (data not shown). Hence, the stimulating effect of *C. cunninghamiana* root exudates on *Frankia* growth was not due to any of these flavonoid molecules that are known to be active on rhizobia.

Root exudates cause hyphal curling. Since *C. cunninghamiana* root exudates affected growth of *Frankia* CcI3, their effects on cell morphology were investigated. *Frankia* cultures exposed to root exudates of 2-month-old soil-grown plants (Fig. 2B) or root exudates of 1-month-old axenic plants (Fig. 2C) exhibited a hyphal curling response. The tips of the hyphae were bent or curled. Control cells did not exhibit hyphal curling (Fig. 2A). Hyphal curling was induced by root exudates collected under nitrogen-deficient and -sufficient conditions. Root exudates collected from the same plants at different ages also induced the response but did not show an age-dependent response (data not shown). The curling response by *Frankia* CcI3 was specific for root exudates from its host plant. *Frankia* CcI3 did not respond upon exposure to *E. angustifolia* (a nonhost actinorhizal plant) root exudates (data not shown). The hyphal curling could be an *in vitro* observation of a chemotrophic response and/or changes to *Frankia* surface properties. Root exudates from legumes are a chemoattractant for motile *Rhizobium* bacterium, but *Frankia* is a nonmotile bacterium (12, 31). *Frankia* CcI3 grows in the rhizosphere of *C. cunninghamiana*, which suggests that a chemotrophic response is possible, but it has not yet been studied (20, 36, 37).

Changes in *Frankia* surface properties. During the analysis of *Frankia* growth, we noticed that cells exposed to root exudates were more difficult to pellet than control cells, suggesting a change in their surface properties. Dye absorption changes have been used extensively to investigate bacterial surface property changes (8, 19, 24). Cell surface changes in mycobacteria are detected by

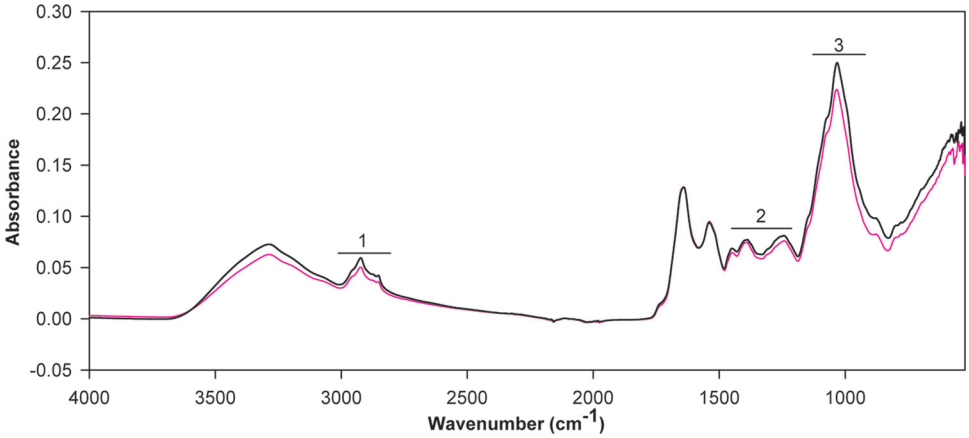

FIG 4 Effect of host root exudates on the FTIR spectra of *Frankia* CcI3. Cultures were incubated for 5 days in propionate-MP medium containing 2-month-old soil-grown *C. cunninghamiana* root exudates (pink) or 1/4 HS(N_2) medium (control) (black). After incubation, the cultures were collected and treated as described in Materials and Methods. FTIR scans of lyophilized cells were taken, and the averaged scans are presented ($n = 3$). Numbers in the figure represent areas of change and correspond to specific chemical signatures of fatty acids (1), fatty acids and proteins (2), and cell wall carbohydrates (3). Areas of change were determined by the variance of individual conditions.

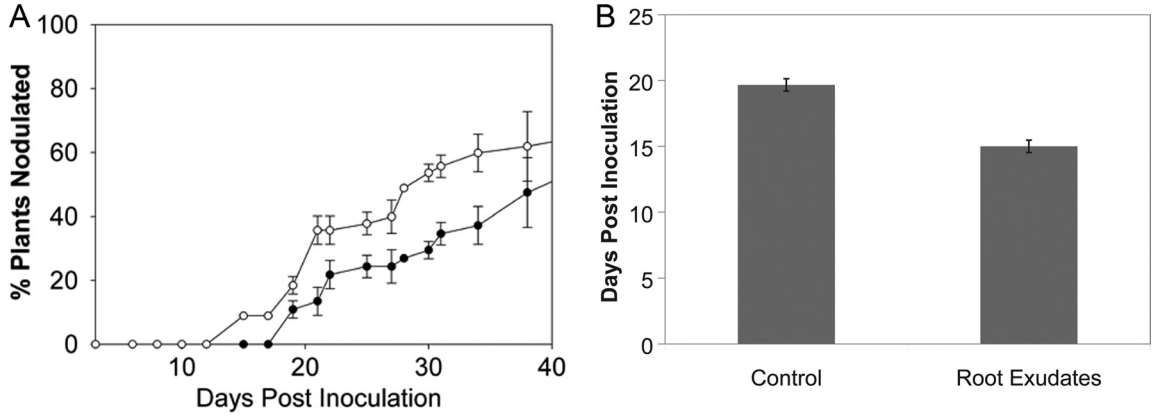

FIG 5 The effect of host root exudate pretreatment on plant nodulation frequency. (A) Time course of plant nodulation. Prior to plant inoculation, *Frankia* CcI3 cells were pretreated with *C. cunninghamiana* root exudates from nitrogen-deficient conditions (white circles) or 1/4 HS(N_2) medium (black circles) for 6 days. The percentages of plants with nodules were determined as described in Materials and Methods. Data presented are the averaged percentages of three independent experiments. The average number of plants per condition in the three experiments was 10. (B) Average number of days required to observe the first appearance of nodules on a plant in each treatment ($n = 3$). *Frankia* CcI3 cells were pretreated with *C. cunninghamiana* root exudates from nitrogen-deficient conditions or 1/4 HS(N_2) medium (control) for 6 days. ANOVA showed this to be a significant difference (P value of <0.05).

Congo red dye, which binds to lipids and lipoproteins (3). Congo red binding was used to identify any broad changes to *Frankia* surface properties in response to host root exudates. Exposure of *Frankia* CcI3 to axenic *C. cunninghamiana* root exudates reduced Congo red binding compared to that of the control cells (Fig. 3). These results imply that the lipid or lipoprotein content of the *Frankia* CcI3 cell surface changed in response to host root exudates.

To further examine surface property changes, FTIR analysis was used to characterize the general types of molecules present in microbes based on specific wavenumber areas (21, 39). FTIR spectra were collected for *Frankia* CcI3 cells exposed to host root exudates and control cells (Fig. 4). Exposure to root exudates caused several changes in the spectral pattern. Specific changes in the wavenumber regions of 2,850 to 2,960 cm^{-1}, 1,370 to 1,400 cm^{-1}, and 906 to 1,170 cm^{-1} indicate alterations in fatty acids, fatty acids and proteins, and cell wall carbohydrates, respectively. The bacterial fingerprint region (600 to 900 cm^{-1}) was also altered by root exudate exposure. However, root exudate exposure did not alter the spectra in the regions of the protein peaks (1,600 and 1,500 cm^{-1}) and the H_2O peak (3,200 cm^{-1}). These spectral changes were observed for cells grown with root exudates from nitrogen-deficient plants cultivated in soil or under axenic conditions. The average spectral changes were conserved in cells grown with root exudates collected under nitrogen-sufficient conditions, but the variance was too large to determine if they were statistically significant.

Altogether, the Congo red and FTIR assay results, as well as the observed change in the ability of cells to be pelleted, provide several lines of evidence to indicate that surface property changes occurred in response to plant host nutrients and/or signaling compounds in addition to the observed change in the ability of cells to be pelleted. Because of the intimate nature of the intracellular infection pathway between *Frankia* CcI3 and *C. cunninghamiana*, surface property changes in *Frankia* were expected in response to infection and nodulation events. The lipid and carbohydrate surface property changes seen in this study are similar to those of other host-microbe recognition systems that were deemed necessary for many pathogenic and symbiotic infection pathways (9, 16, 30). Although the specific type of molecules being produced remains unknown, these changes could be used as tools to identify the corresponding compound(s) and its structure.

Root exudate exposure influences plant nodulation. Since surface property changes suggest major modifications in the bacterial exterior, we asked whether the changes in *Frankia* surface properties in response to root exudates correlated with effects on plant-microbe interactions. *Frankia* cultures were preexposed to axenic *C. cunninghamiana* root exudates (nitrogen-deficient) or 1/4 HS(N_2) medium for 6 days without an additional carbon source. Figure 5A shows the time course for nodulation. The data presented are the averaged percentages of plants with nodules from three independent experiments. Plants inoculated with *Frankia* CcI3 cells pretreated with host root exudates produced their first root nodules 15 days after inoculation, while those inoculated with control cells initiated nodule formation at day 19.6 (Fig. 5B). The frequency of nodulation increased over time, reaching 60% and 55% for treated and control cells, respectively. There was no difference in the number of nodules produced per plant between the pretreated cultures and the control (data not shown). Here, we report that preexposure to root exudates allowed *Frankia* to nodulate host plants earlier than untreated cells, suggesting that physiological changes to *Frankia* from host root exudate exposure were beneficial to the infection and nodulation process. This result is in agreement with the observed reduction in nodulation time by *Frankia* sp. strain BCU110501 under plant crowding conditions, perhaps due to an increase of the signaling molecule(s) in root exudates (11). We still do not know what changes in *Frankia* physiology allow for symbiosis with the plant, but it is clear that the observed physiological changes benefit the infection and nodulation process. Future studies will establish what changes are the most beneficial.

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