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Rhizosphere Microbial Community Structure in Relation to Root Location and Plant Iron Nutritional Status

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Root exudate composition and quantity vary in relation to plant nutritional status, but the impact of the differences on rhizosphere microbial communities is not known. To examine this question, we performed an experiment with barley (*Hordeum vulgare*) plants under iron-limiting and iron-sufficient growth conditions. Plants were grown in an iron-limiting soil in root box microcosms. One-half of the plants were treated with foliar iron every day to inhibit phytosiderophore production and to alter root exudate composition. After 30 days, the bacterial communities associated with different root zones, including the primary root tips, nonelongating secondary root tips, sites of lateral root emergence, and older roots distal from the tip, were characterized by using 16S ribosomal DNA (rDNA) fingerprints generated by PCR-denaturing gradient gel electrophoresis (DGGE). Our results showed that the microbial communities associated with the different root locations produced many common 16S rDNA bands but that the communities could be distinguished by using correspondence analysis. Approximately 40% of the variation between communities could be attributed to plant iron nutritional status. A sequence analysis of clones generated from a single 16S rDNA band obtained at all of the root locations revealed that there were taxonomically different species in the same band, suggesting that the resolving power of DGGE for characterization of community structure at the species level is limited. Our results suggest that the bacterial communities in the rhizosphere are substantially different in different root zones and that a rhizosphere community may be altered by changes in root exudate composition caused by changes in plant iron nutritional status.

Root exudates selectively influence the growth of bacteria and fungi that colonize the rhizosphere by altering the chemistry of soil in the vicinity of the plant roots and by serving as selective growth substrates for soil microorganisms. Microorganisms in turn influence the composition and quantity of various root exudate components through their effects on root cell leakage, cell metabolism, and plant nutrition. Based on differences in root exudation and rhizodeposition in different root zones, rhizosphere microbial communities can vary in structure and species composition in different root locations or in relation to soil type, plant species, nutritional status, age, stress, disease, and other environmental factors (8, 16, 22, 23). During the growth of new roots, exudates secreted in the zone of elongation behind the root tips support the growth of primary root colonizers that utilize easily degradable sugars and organic acids. In the older root zones, carbon is deposited primarily as sloughed cells and consists of more recalcitrant materials, including lignified cellulose and hemicellulose, so that fungi and bacteria in these zones are presumably adapted to crowded, oligotrophic conditions. Other nutritionally distinct sites include the sites of lateral root emergence and the secondary, nongrowing root tips, which are relatively nutrient-rich environments colonized by mature communities.

Current models based on nutritional competition for iron in the rhizosphere suggest that plant iron nutritional status may influence rhizosphere community structure, particularly at the root tips, where plants and microorganisms secrete siderophores to solubilize and transport iron (7, 24, 25). In monocot grasses, iron stress results in the release of phytosiderophores that are secreted behind the root tips to mobilize inorganic iron that can be taken up by the plant roots (24). Certain bacteria in the rhizosphere can utilize these phytosiderophores as a source of iron or may alter iron availability to plants and other competing microorganisms (7, 35). Chemical analyses of root exudate components produced by barley (*Hordeum vulgare*) have shown that the quantities of exudate increase threefold during mild iron stress, which involves increased exudation of organic acids and phytosiderophores (10). As iron stress becomes more severe, the proportion of phytosiderophores in the root exudate increases so that the plant iron chelators comprise 50% of the total root exudate. These changes in root exudate composition should have a considerable impact on microbial community structure in the rhizosphere. In several studies workers have suggested that rhizosphere competence may be influenced by siderophore production (6, 25, 27, 31). However, no studies have been conducted to examine the effect of iron competition and plant iron nutritional status on microbial community structure in the rhizosphere.

Although not yet routinely used for rhizosphere studies, culture-independent analysis of microbial communities is readily accomplished by analyzing 16S ribosomal DNA (rDNA) profiles generated by denaturing gradient gel electrophoresis (DGGE) (28, 29). The resulting DNA band pattern provides a fingerprint of the microbial community structure, in which each band represents a group of bacteria having 16S rDNA sequences with a similar melting temperature (18, 30). Individual bands can be excised from the gel and used to generate clones, which may then be sequenced and used to identify the predominant bacterial species present in individual DNA bands. The methods that are currently used still have some pitfalls, as there are potential problems with PCR bias of selected sequences and the formation of PCR artifacts (36). However, the ability to obtain a snapshot of rhizosphere community structure at selected sites in the rhizosphere that re-

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veals both culturable and nonculturable microorganisms has great potential for studies of rhizosphere ecology.

In this study, we examined differences in microbial communities associated with specific root locations and the impact of plant iron nutritional status on the communities by using barley plants grown in an iron-limiting soil. Plant iron nutritional status was altered by treating Fe-limited plants with foliar iron to ameliorate iron deficiency and suppress phytosiderophore production. Bacterial communities associated with different root locations were characterized by using PCR-DGGE. Similarities in the microbial communities associated with different root locations and plant iron nutritional status were compared by performing an image analysis of the DNA fingerprints and a correspondence analysis of the 16S rDNA band data. Finally, some of the predominant bacteria that were revealed by the PCR-DGGE analysis of 16S rDNA were identified by cloning and sequence analysis.

MATERIALS AND METHODS

Plant culture. Barley seeds (*Hordeum vulgare* cv. CM72) were placed on moist germination paper. After 4 days, the seedlings were transferred to root box microcosms. Each microcosm (295 by 175 by 15 mm) had a removable transparent acrylic front plate that was covered with a sheet of black acrylic plastic to keep the root system dark (25). The root boxes were packed with sieved (mesh size, 2 mm) Dello loamy sand (15% silt, 0.5% clay; pH 7.7; total organic C content, 0.9%). To water the plants and avoid preferential water percolation along the soil-plate interface during watering, a polyester cloth was placed in the back of each compartment. A small piece of this cloth emerged from the top of the box and was inserted into a 20-ml plastic vial. The cloth was moistened by filling the vial with modified 1× Hoagland's solution (24). After planting, the microcosms were maintained at a constant moisture content of 15% by adding water daily. To alter the plant iron nutritional status during the iron-sufficient and iron stress treatments, the foliage of the plants was sprayed with iron citrate and distilled water, respectively, daily. An iron citrate solution was prepared by dissolving equimolar $FeCl_3$ and $(NH_4)_2$ citrate in double-distilled water to a concentration of 0.14% Fe at pH 4.8. Two drops of detergent were added to the solution. The plants were grown in a controlled environment, a plant growth chamber, with a relative humidity of 65% at 22°C. The daily light schedule consisted of 15 h of light and 9 h of darkness, and the light intensity was 370 microeinsteins m^{-2} s^{-1}. Three replicates were prepared for each treatment.

Community sampling. Root samples were obtained from four locations, including actively growing primary root tips, nonelongating secondary root tips, sites of lateral root emergence, and mature root sections 5 to 10 cm distal from the root tips. All of the root samples were obtained at one time, 30 days after transplanting. Each root portion was 0.5 cm long and was collected with the adhering rhizosphere soil. The samples were each placed in a bead beater tube (Bio 101, Vista, Calif.). To lyse the bacterial cells in the rhizosphere soil, soil samples were disrupted with a Fastprep model FP120 bead beater (Bio 101) set at 5.5 m/s for 30 s. Total DNA was isolated from the soil by using a Fast DNA kit as described in the protocols of the manufacturer (Bio 101). Samples from three locations in the same root area were obtained from each plant, and a total of 72 rhizosphere samples were analyzed in the experiment. For the subsequent statistical analyses in which correspondence analysis was used, we selected a subset consisting of one sample from each root location for each of the replicate plants, and the samples were then electrophoresed on two DGGE gels that were prepared and electrophoresed simultaneously with the DGGE apparatus. This smaller sample set, which consisted of only 24 samples, allowed us to compare root locations in replicate plants and eliminated problems associated with analyzing images of different DGGE gels that differ slightly in denaturant gradient, running time, and staining intensity.

PCR primers and DGGE analysis. Primers PRBA338f and PRUN518r, located in the V3 region of 16S rRNA genes of bacterioplankton, were used in this study (30). PRBA338f consists of a region that is conserved in the domain *Bacteria*, and PRUN518r is located in a universally conserved region. Ready-To-Go PCR beads obtained from Amersham-Pharmacia Biotech (Piscataway, N.J.) were used for PCR amplification. The PCR mixtures used for bacterial 16S rDNA sequence amplification contained 5 pmol of each primer, 4 μg of bovine serum albumin, and sterile distilled water in a final volume of 25 μl. The PCR program used for amplification was as follows: 92°C for 2 min, followed by 30 cycles consisting of 92°C for 1 min, 55°C for 30 s, and 72°C for 1 min and a single final extension step consisting of 72°C for 6 min.

DGGE was performed with 8% (wt/vol) acrylamide gels containing a linear chemical gradient ranging from 30 to 70% denaturant (7 M urea plus 40% [vol/vol] formamide). The gels were prepared by using 8% (wt/vol) acrylamide stock solutions (acrylamide/bisacrylamide ratio, 37.5:1) containing 0 and 100% denaturant (7 M urea plus 40% [vol/vol] formamide). The gels were electrophoresed for 3 h at 200 V with a DCode universal mutation detection system (Bio-Rad Laboratories, Hercules, Calif.).

Bacterial identification. rDNA bands were excised from DGGE gels and placed into sterilized vials. Twenty microliters of sterilized distilled water was added to each of the vials, which were then kept at 4°C overnight to allow the DNA to passively diffuse out from the gel strips. Ten microliters of eluted rDNA was used as a DNA template along with the primers and PCR conditions described above. The sizes of the PCR products were checked by using an agarose gel, and the DNAs were then cloned into the pGEM-T Easy vector (Promega, Madison, Wis.) and transformed into *Escherichia coli* JM109. Plasmids were isolated from *E. coli* by using standard protocols and a QIAprep Miniprep kit (QIAGEN, Inc., Santa Clarita, Calif.). The purified plasmids were sequenced with a model 4000 L automatic sequencing system (Li-COR, Lincoln, Nebr.). The sequencing reaction was carried out by performing cycle sequencing with a SequiTherm Excel II Long-Read DNA sequencing (type LC; kit Epicentre, Madison, Wis.). Sequence analyses were performed by using the BLAST database (29a). The overall levels of similarity of 16S rDNA sequences to sequences of previously described bacteria were determined by using the programs FRIENDLY and GCG (Genetics Computer Group, Oxford Molecular Company, Madison, Wis.).

Statistical analyses. We prepared DGGE gels containing samples obtained from the same root locations in iron-stressed and nonstressed plants. The DNA fingerprints obtained from the 16S rDNA band patterns on the DGGE gels were photographed and digitized by using a computer scanner. The gel images were straightened and aligned by using iPhoto (Ulead Systems Inc., Taipei, Taiwan), and then they were imported into an image analysis program (Scion Image; Scion Corp., Frederick, Md.), with which they were converted into *x/y* plots, and transferred to EXCEL files (Microsoft Inc., Seattle, Wash.). The line plots were analyzed and integrated by using peak analysis software (PeakFit version 4; SPSS, Inc., Chicago, Ill.). Baselines were subtracted from each image prior to peak detection by using the AutoFit 2nd Deriv Zero program with the Best fit option. This baseline correction used assumes that baseline points tend to exist where the second derivative of the data is both constant and zero and selects from eight different parametric models. After baseline correction, the peaks were resolved with a deconvolution curve fit, which defined a visible peak as a peak that produced a local maximum in the input data. In the deconvolution option, hidden peaks were detected by the sharpening achieved by Gaussian deconvolution of the raw data. A standard peak width was assigned to all peaks by using the default parameter "full width at half maximum" that is utilized for fitting Gaussian curves to peaks.

Community similarities based on peak areas and R_f values for the different bacterial groups (16S rDNA bands) were analyzed by performing a correspondence analysis (CANOCO 4.0; Microcomputer Power, Ithaca, N.Y.); each peak represented a different bacterial group or species, and the area under each peak represented the relative abundance of the group. Community similarities were graphed by using ordination biplots with scaling focused on intersample differences (17). This type of diagram allows interpretation of distances between centroid points for individual samples. The effects of plant iron status at each root location were analyzed statistically by performing a canonical correspondence analysis with Monte Carlo permutation tests. In this procedure, the first ordination axis was constrained to include plant iron status as a covariable. The Monte Carlo tests were based on 199 random permutations of the data in which we tested the null hypothesis (that iron did not have a significant effect on the distribution of the species data).

RESULTS

Community structure at specific root locations. A visual comparison of DNA band profiles obtained from denaturing gradient gels containing 16S rDNA showed that there were distinct communities associated with the different root locations (Fig. 1A). Inspection of the band profiles revealed that the communities consisted of several predominant bacterial groups that were present at all root locations and that the remainders of the communities were composed of groups or species that were represented by less predominant 16S rDNA bands. One of the predominant bands was subsequently determined by sequence analysis to be a band containing plastid DNA, which was amplified from the root tissue DNA extracted along with the bulk microbial community DNA from the adhering rhizosphere soil. When analyzed by BLAST sequence analysis, this band was found to have a high match value with the *Zea mays* chloroplast genome. The plastid DNA band was most intense at the new root tips and was less predominant at the sites of lateral root emergence and older root zones. This band was subsequently removed from the data set before we performed correspondence analyses of the bacterial commu-

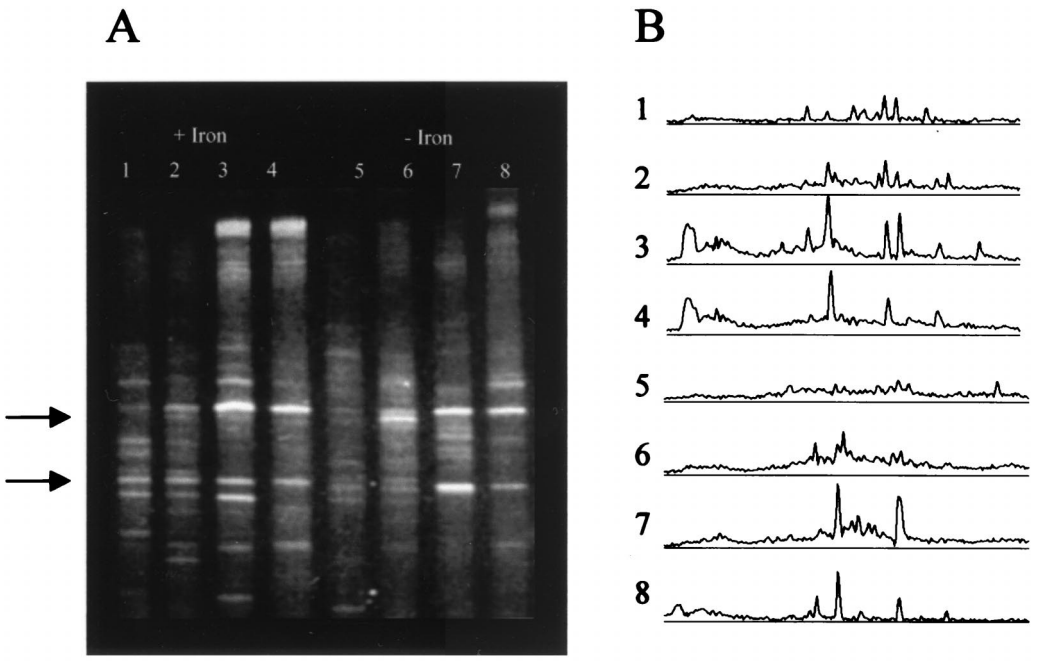

FIG. 1. (A) Microbial community 16S rDNA fingerprints of bacteria from different locations on iron-stressed (+ Iron) and iron-sufficient (− Iron) barley roots as determined by PCR-DGGE. (B) Line image profiles generated by image analysis. The arrows indicate a common band determined to be plastid DNA (top arrow) and a second predominant band (bottom arrow) that was cloned and sequenced for all root locations. Lanes: 1 and 5, old roots; 2 and 6, sites of lateral root emergence; 3 and 7, nongrowing root tips; 4 and 8, elongating new root tip.

nities in the different root zones, but it was a useful marker for aligning the DNA lanes prior to image analysis.

To quantitatively examine the relative similarities of the communities, the 16S rDNA band profiles were analyzed by peak fitting. Different sets of data were compared, and each set consisted of samples from a root location electrophoresed on separate gels. The gels revealed that the band patterns for the same root location in plants were very similar (data not shown). Because of the inherent problems associated with analyzing the images of completely different gels in which there are subtle differences in the gel gradients, running times, and DNA staining procedures, another analysis was performed with a reduced sample set. The gels used in the latter analysis were gels that were prepared with one sample per root location and plant rather than three, and two gels were prepared and electrophoresed simultaneously with the gel apparatus.

In a gel image in which different root locations were compared (Fig. 1), we detected bands at 49 R_f locations, with each sample containing 21 to 29 bands. Four DNA bands were obtained only with iron-stressed plants, and six bands were obtained only with iron-sufficient plants. Eight bands appeared only once as minor peaks, and 14 bands appeared to be randomly associated with the rhizosphere and not affected by the root location. The remaining 17 DNA bands were observed with both iron-stressed and nonstressed plants, and the image intensity changed depending on the root location and plant iron stress status.

In the ordination diagram in Fig. 2, individual points on the two-dimensional biplot represent the microbial communities associated with specific root locations used to prepare the gel shown in Fig. 1. Seventy-four percent of the variation in community structure was explained by four eigenvectors. The first and second eigenvectors, plotted on the *x* and *y* axes, explained 23 and 19% of the variation, respectively; thus, 42% of the cumulative variation was explained. When iron was included as a factor in the canonical analysis, it explained 100% of the variation associated with eigenvector 1. Thus, 23% of the variation for all root locations could be explained by plant iron nutritional status. When the centroid rule was used, the difference between the communities with respect to the first and second eigenvectors was the distance between the points representing the communities on the ordination diagram. A comparison of the distances paired with respect to root location and differences in plant iron nutritional status showed that the microbial communities associated with the old roots were the most similar and were separated by less than 0.5 standard deviation. The communities associated with the sites of lateral root emergence from iron-stressed and iron-sufficient plants were separated by approximately 1 standard deviation. The most dissimilar communities were the communities from the root tips of iron-stressed and nonstressed plants, which were separated by 1.5 and 2 standard deviations from the communities at the nongrowing secondary tips and new tips, respectively.

Community structure in relation to plant iron nutritional status. At each of the root locations examined, the microbial community structures were very similar, and for most bands plant iron nutritional status did not have a significant influence. As shown by the line profiles for the 16S rDNA bands obtained with the new roots tips in Fig. 3, the communities associated with this root zone were almost identical for the two iron treatments. There were 33 distinct 16S rDNA bands, and 25 of these were produced at least once by roots of iron-stressed and iron-sufficient plants. Five major bands other than the plastid band were present in all replicate samples. In some instances, individual bands were not present in one or two replicate samples for a treatment, suggesting that colonization by certain bacterial groups was stochastic. An analysis of the overall differences between iron-treated and nontreated plants in which canonical correspondence analyses were performed revealed

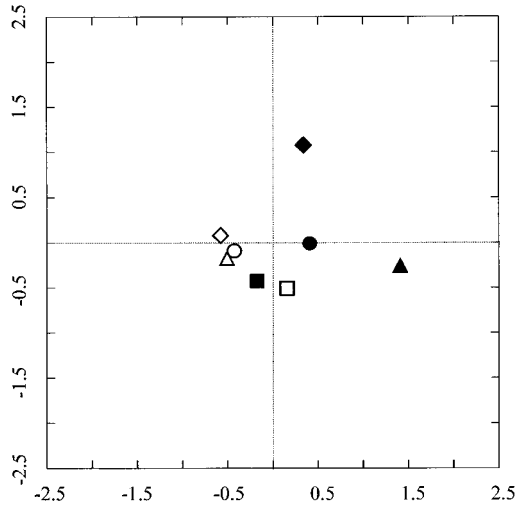

FIG. 2. Ordination diagram of microbial communities associated with different root locations on iron-stressed and nonstressed barley plants generated by correspondence analysis of 16S rDNA profiles for individual root segments and adhering rhizosphere soil. Symbols: ▲ and △, new root tips; ◆ and ◇, old root tips; ● and ○, lateral emergence sites; ■ and □, old roots; open symbols, iron-sufficient plants; solid symbols, iron-deficient plants.

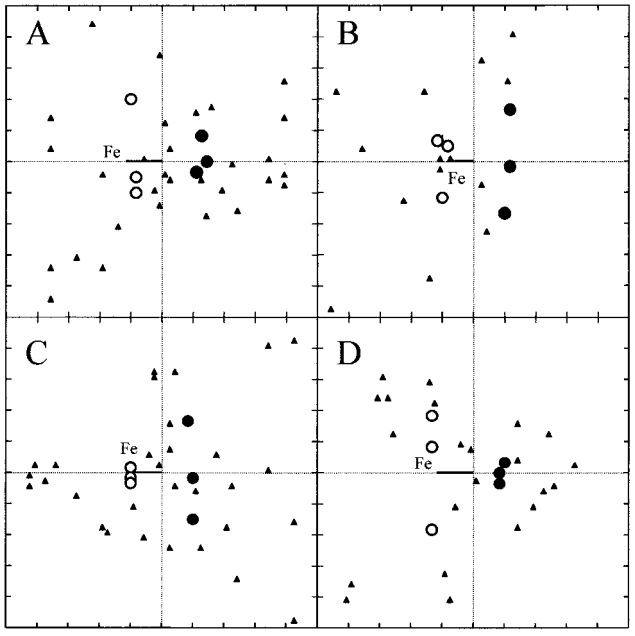

FIG. 4. Canonical correspondence analysis, showing the relative similarities of microbial communities associated with specific barley root locations as affected by plant iron nutritional status. Iron is included as a covariable on the *x* axis. (A) New root tips. (B) Secondary, nongrowing root tips. (C) Sites of lateral root emergence. (D) Older roots axes distal from root tips. Symbols: ▲, individual 16S rDNA bands ordinated with respect to plant iron nutritional status; ○, centroids representing 16S rDNA from communities associated with iron-sufficient plants; ●, centroids representing 16S rDNA samples from iron-deficient plants.

that approximately 40% of the variation in the communities could be attributed to plant iron nutritional status.

In ordination diagrams in which the effects of iron at all root locations were compared (Fig. 4), iron was included as a variable, so that the first ordination axis (*x* axis) was constrained as a linear combination of the effect of iron and the variation described by the first eigenvector. Bacterial communities associated with the actively growing root tips of stressed and nonstressed plants were the communities affected most strongly by plant iron nutritional status. This was revealed visually in line plots of the gel image in which we statistically compared the eigenvector values for the axis with iron as the covariable. In the data set obtained for actively elongating tips, 99% of the variation for all of the rhizosphere communities was explained by four eigenvectors. Seventy percent of the variation was explained by the first and second eigenvectors, which described 38 and 32% of the variation, respectively, and which are plotted on the *x* and *y* axes in Fig. 4A. When iron was included as a covariable, it explained 100% of the variation described by eigenvector 1 on the *x* axis. The results of a Monte Carlo permutation test for iron showed that plant iron nutritional status was statistically significant for explaining variation along this axis ($P = 0.005$).

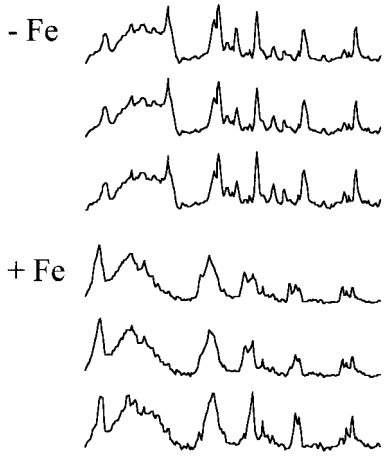

FIG. 3. Line graph profiles of 16S rDNA band patterns resulting from DGGE of microbial communities associated with iron-stressed (− Fe) and nonstressed (+ Fe) barley root tips. Each profile shown was generated from root tips of replicate plants in different containers. The profiles reveal the very consistent community structure associated with this root zone and the impact of iron stress on community species composition.

The effect of iron on the microbial species composition of the rhizosphere is also shown by the distribution of the DNA band data points around the centroid points for the new root tips (Fig. 4A). Data points representing DNA bands to the far left of each plot were strongly influenced by the rhizosphere of iron-sufficient plants, whereas data points located to the far right represented DNA bands obtained only with iron-stressed plants. Points closer to the origin represented DNA bands that were obtained with the rhizosphere communities of both iron-stressed and nonstressed plants. Several of the DNA band points occurred along the vertical line passing through the origin and represented bacterial species that were not influenced by plant iron nutritional status.

Communities associated with the secondary nongrowing root tips produced 22 bands (Fig. 4B). Sixty-five percent of the variation in this data set was explained by the first and second eigenvectors, and iron had a significant effect, as shown on the *x* axis. Eubacterial communities associated with the sites of lateral root emergence produced 34 different bands, and the band intensities for iron-stressed and nonstressed plants were different (data not shown). The first and second eigenvectors explained 67% of the variation between the iron-stressed and

TABLE 1. Bacteria in randomly selected clones generated from a predominant 16S rDNA band found at all root sample locations in the barley rhizosphere

Location	Bacteria obtained with:	
	Iron-stressed roots	Nonstressed roots
Old roots	Uncultured soil bacterium C032 (accession no. AF128720) (97)[a]	Uncultured member of the gamma subclass of the *proteobacteria* (accession no. AF141542) (92)
Lateral emergence sites	*Microbacterium* sp. strain B19 (accession no. AB027702) (97)	*Microthrix parvicella* (accession no. XA2546) (92)
Old tips	*Microbaterium barkeri* (accession no. X77446) (97)	*Microbacterium* sp. strain B19 (accession no. AB027702) (97)
New tips	*Spirosoma linguale* (accession no. M62789) (85)	*Hyphomicrobium vulgare* (accession no. Y14302) (97), unidentified eubacterium (accession no. UEU232845) from *Lolium perenne* and *Trifolium repens* roots (91)

[a] The values in parentheses are overall levels of 16S rDNA similarity to previously described bacteria, expressed as percentages.

nonstressed plants (Fig. 4C). Twenty of the bands occurred in one-half or more of the samples, whereas 12 bands occurred in fewer than one-half of the samples.

Similar analyses of communities associated with the old roots showed that 67% of the total variation was explained by the first and second eigenvectors; iron explained 100% of eigenvector 1 or 37% of the variation in the communities obtained from iron-stressed and iron-sufficient plants (Fig. 4D). Twenty-four different DNA bands were detected in a peak-fitting analysis of DNA bands from the old roots, and 19 of these were produced by both iron-stressed and nonstressed plants (data not shown). In contrast to the root tip communities, in the communities associated with the older root parts the distribution of the different species was relatively even, and there were relatively few predominant bands. Twelve of the 25 bands or bacterial groups were found in at least two-thirds of the root samples. In three cases, unique bands appeared in one of the three root samples tested after the same treatment and thus appeared to represent randomly appearing community members that were present at relatively low levels.

Bacterial identities. The identities of microbial species associated with a single predominant DNA band present in all of the root samples (Fig. 1) were determined by isolating this band from the gel and then cloning and sequencing it. We sequenced two separate clones from each root location. Taxonomically distinct bacteria were identified in this band when we examined samples from each of the root locations (Table 1). Clones obtained from old roots of iron-stressed and nonstressed plants had sequences similar to the sequences of members of unidentified eubacterial genera. Four of the five clones obtained from the sites of lateral emergence and nongrowing root tips were *Microbacterium* sp. In the same band, *Microthrix* sp. was identified at the sites of lateral root emergence in nonstressed roots. Finally, clones obtained from the new root tips belonged to four different genera, and these organisms included an unidentified eubacterium, *Hyphomicrobium vulgare*, and *Spirosoma linguale*.

DISCUSSION

The plant rhizosphere is a dynamic environment in which many factors may affect the structure and species composition of the microbial communities that colonize the roots. It has been shown previously that rhizosphere communities vary spatially in a radial direction from the root surface, including the endorhizosphere, rhizoplane, and rhizosphere zones (1, 3, 14, 19), as well as in specific root locations along the root axis (8, 14, 19). Microbial communities associated with the rhizosphere also vary depending on the plant species (15), the soil type (5), and cultural practices, such as crop rotation or tillage (22). Understanding the structure and species composition of these communities is fundamental to understanding how soil biological processes are influenced by environmental factors and cultural practices.

Until recently, in most studies of the plant rhizosphere workers have used a culture-based approach that provides a snapshot of overall community structure at a single moment. In general, this method is biased towards culturable microorganisms, and the procedures are labor-intensive (33, 34). Typically, rhizosphere soil is collected by shaking soil that is loosely adhering to the roots, and then the soil is used to prepare serial dilutions in water that are plated onto selective agar media. Individual isolates are then identified by using metabolic tests (32) or procedures such as metabolic fingerprinting (5, 13), fatty acid methyl ester analysis (20), and genetic analysis of individual isolates by 16S rDNA gene sequencing (26). Often several hundred to more than 1,000 isolates are analyzed in an analysis of one sample. Given these limitations, new non-culture-based methods for fingerprinting microbial communities have provided rhizosphere ecologists with powerful new tools for rapidly analyzing microbial community structure with plants grown under different conditions or samples collected in different root locations.

For some time, competition for iron has been considered a major factor which affects microbial community structure and disease interactions in the plant rhizosphere (4, 7). Microorganisms compete for iron based on differential utilization of the predominant siderophore types, and the effects of siderophores on survival, fitness, and disease-suppressing activity of rhizosphere pseudomonads have been thoroughly investigated (12, 21, 31). In grasses, production of phytosiderophores, which can comprise a large fraction of the root exudate under iron-deficient conditions, can also influence the availability of iron to microorganisms (24). These compounds are secreted primarily behind the root tips, along with sugars and organic acids that can be readily utilized for microbial growth. In previous attempts to determine the complex microbial interactions involving siderophore production and iron, workers have studied microbial cultures in vitro or have used other model systems that have been criticized as being simplistic (7). Thus, it was interesting in this study to determine the effect of plant iron nutritional status on microbial community structure and species composition in the rhizosphere by using culture-independent methods for community analysis.

Based on microsite sampling of roots at different locations in the rhizosphere, we found that distinct and very consistent microbial community structures occurred in different root

zones of barley plants grown in an iron-limiting soil. Iron also affected the community composition at each root location. Analysis of the distribution of 16S rDNA bands showed that many bands were present in all gels, suggesting that certain dominant bacterial species were ubiquitous. In one case, this included a dominant band which represented plastid DNA amplified from the root tissue. Another band which might be expected to occur in all profiles but which was not identified here is a band representing the small-subunit rDNA of mitochondria. In addition to several common DNA bands, there were also bands which were obtained only at specific root locations. However, when unique bands appeared, they were not always produced by replicate samples obtained from the same location, which suggests that certain components of the rhizosphere community may appear randomly. This variability may reflect stochastic events that occur during root colonization as the sterile, elongating root tips grow past soil and organic matter particles, which are colonized by various bacteria and fungi that are transferred to the root tissue. Another explanation may be method-inherent inconsistencies in extraction and amplification of DNAs represented by the unique bands.

The data obtained in this study are in contrast to the data of Duineveld and coworkers, who showed that there was very little change in the microbial communities associated with the roots of chrysanthemum over time and very few differences between root parts (9). The differences in the data may be due to differences in plant species and root exudation patterns or to sampling methods. In the study of Duineveld et al., differences in the microbial communities were compared by performing cluster analysis, and each species was represented by the absolute presence or absence of a band. In our study we performed correspondence analyses, which took into account both the presence of a band and its relative staining intensity.

Although many factors may affect the growth of specific bacteria at different root locations, plant iron nutritional status explained approximately 20 to 40% of the total variation in community structure at all of the root locations that were sampled. The fact that several predominant bands were not influenced by iron suggests that siderophore and phytosiderophore production may have relatively minor effects on certain ubiquitous rhizosphere colonizers but result in consistent and reproducible shifts in community structure. The degree to which plant iron nutritional status directly influenced the microbial community structures at the sites of lateral root emergence or on older roots was somewhat surprising as phytosiderophores are produced primarily behind root tips. One possible reason for the similar effects of iron at all of the locations is that the primary colonizers at the root tips did not disappear from the communities through death or grazing by the soil microfauna but instead were retained as part of the community fingerprint as the communities matured and underwent succession. Alternatively, the primary rhizosphere communities that develop on root tips may somehow influence the succession and species composition of communities that develop on the older root parts as the roots mature. As indicated by the appearance of multiple, taxonomically different bacteria at the same band location, answering these questions will require techniques that provide greater resolution than the resolution provided by PCR-DGGE.

Using 16S rDNA fingerprints proved to be a powerful method for exploring structural variation in microbial communities in the rhizosphere, but this technique still provides relatively low resolution and is based on assumptions that may lead to misinterpretation (11). As pointed out by Muyzer and Smalla, bacteria detected by PCR-DGGE of 16S rDNA must comprise at least 1% of the total population (29). Certain bacteria may be represented by more than one band, and as shown here, some bands may contain DNA contributed by several different bacterial species. In this respect, 16S rDNA profiles are similar to fatty acid profiles in which any one peak may contain fatty acids extracted from various different bacteria. Nonetheless, the advantage of DGGE is that it can be used to clone DNA in discrete bands and thereby determine which species contribute to selected bands of interest that change in relation to specific experimental parameters or environmental conditions.

One predominant DNA band obtained at all root locations was cloned and sequenced, and this band provided intriguing insight into the extent of microbial diversity in the rhizosphere. On the older root parts, unidentified eubacteria were identified twice on the mature root axes. On the old root tips and sites of lateral root emergence, *Microbacterium* sp. (previously *Aureobacterium* sp.) was identified in three of the four clones obtained from these locations. It has been reported previously that members of this genus comprise 9% of the culturable bacteria in the rhizosphere of sugar beet (20). Among the other clones that were obtained from the new root tips and sites of lateral root emergence and identified were several clones that have not been found previously in the rhizosphere, including *Hyphomicrobium* and *Spirosoma* clones. *Hyphomicrobium* strains are dimorphic prosthecate bacteria that are ubiquitous in brackish water and soil (2). They are denitrifiers and generally are enriched on methanol nitrate medium. The genus *Spirosoma* is classified as a member of the *Chlorobiaceae* branch of the gamma subclass of the class *Proteobacteria*. As a group, these bacteria are generally associated with anaerobic muds. Almost all of these bacteria have unique cultural requirements and may not be readily cultured on tryptic soy agar under aerobic conditions. The function of these species and still-to-be-discovered species in the rhizosphere remains an open question. In future analyses, PCR-DGGE of 16S rDNA will probably provide many new insights into microbial community structure in the rhizosphere.

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