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Characterization and Identification of Productivity-Associated Rhizobacteria in Wheat

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The rhizosphere is populated by a numerous and diverse array of rhizobacteria, and many impact productivity in largely unknown ways. Here we characterize the rhizobacterial community in a wheat variety categorized according to shoot biomass using 16S rRNA pyrosequencing abundance data. Plants were grown in homogenized field soil under greenhouse conditions, and DNA was extracted and pyrosequenced, resulting in 29,007 quality sequences. Operational taxonomic units (OTUs) that were significantly associated with biomass productivity were identified using an exact test adjusted for the false-discovery rate. The productivity deviation expressed as a percentage of the total mean square for regression (PMSR) was determined for each OTU. Out of 719 OTUs, 42 showed significant positive associations and 39 showed significant negative associations (*q* value, ≤0.05). OTUs with the greatest net positive associations, by genus, were as follows: *Duganella*, OTU 43 and OTU 3; *Janthinobacterium*, OTU 278; *Pseudomonas*, OTU 588; and *Cellvibrio*, OTU 1847. Those with negative associations were as follows: *Bacteria*, OTU 273; *Chryseobacterium*, OTU 508; *Proteobacteria*, OTU 249; and *Enterobacter*, OTU 357. Shoot biomass productivity was strongly correlated with the balance between the overall abundances of positive- and negative-productivity-associated OTUs. High-productivity rhizospheres contained 9.2 significant positives for every negatively associated rhizobacterium, while low-productivity rhizospheres showed 2.3 significant negatives for every positively associated rhizobacterium. Overall rhizobacterial community diversity as measured by the Chao1, Shannon, and Simpson indexes was nonlinearly related to productivity, closely fitting a wavelike cubic equation. We conclude that shoot biomass productivity is strongly related to the ratio of positive- to negative-productivity-associated rhizobacteria in the rhizosphere. This study identifies significant OTUs composing the productive and unproductive rhizobacterial communities.

The rhizosphere was defined by Hiltner as the region between the root and the soil under the influence of the plant (16). Plant productivity is greatly impacted by the plant-microbe interactions within the rhizosphere (47) with both beneficial and detrimental aspects. Despite over a hundred years of research, we still have difficulty in positively identifying rhizobacteria and rhizobacterial community parameters associated with plant productivity. The plant provides an estimated 21% of its net photosynthetic products (5) to sustain the microbial community in what is clearly a mutually beneficial relationship. The microbial community provides multiple functions, including increased nutrient availability (8), protection against pathogens (34), improvement of soil physical structure (2), and provision of plant growth-promoting substances (9). Detrimental relationships within the rhizosphere, including pathogens and deleterious rhizobacteria (DRB) (43) that reduce plant growth without showing obvious disease symptoms, also exist. Today, this interface is known as one of the most ecologically complex and important interfaces in nature, consisting of tens of thousands to a million genomes within a single gram of rhizosphere soil (33). The inherent complexity of the rhizosphere has been an obstacle for its characterization and in understanding its role in providing productivity functions for the plant. Here we seek to develop a better approach to identify specific rhizobacterial operational taxonomic units (OTUs) within the community and systemwide parameters that are associated with wheat productivity.

Past investigations to connect rhizobacteria to productivity have involved three generalized approaches, the first two being dependent on cultural technique. The problems with the use of cultural techniques are well understood when assessing whole rhizobacterial community response: cultural techniques sample only a small portion of the rhizobacterial community and ignore the substantial interaction effect associated with community function. The first generalized approach involves the isolation of hundreds to thousands of rhizobacteria through cultural means, screening them for plant growth promotion potential using a variety of plant growth assays. This approach is commonly used today and has resulted in the isolation of many plant growth-promoting organisms and biocontrol agents (40). The second functional approach involves the identification of functional characteristics that are thought to be connected to plant growth promotion using various functional assays. Characteristics include production of antibiotics, evaluation for rhizosphere competence for general community combining ability, production of siderophores to chelate nutrients such as iron, provision of nitrogen by nonsymbiotic rhizobacteria, production of hormones such as auxin or cytokinin that regulate growth and cell division, and release of compounds that improve soil properties, to name a few (40, 49). This approach often is dependent on cultural technique to functionally screen bacterial isolates and, at times, has difficulty correlating the functional assay results to plant productivity response.

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The final approach, the metagenomic approach, seeks to examine and characterize part of or the entire rhizosphere combined genome in order to understand its contribution to productivity. Other molecular approaches examine phylogenetic community structure using the 16S rRNA gene, a gene that is most often used for prokaryotic identification. The metagenomic/molecular approach is very attractive in that such approaches do not rely on cultural technique and use a wide range of powerful high-throughput advanced technologies and bioinformatic analytical tools to characterize the community in great depth. However, the information gleaned from these approaches must also be functionally connected to plant productivity response in a meaningful way, a requirement which in the past has been only partially fulfilled, if at all. Here we use high-throughput 454 pyrosequencing to characterize the abundance of specific rhizobacterial OTUs and correlate rhizobacterial abundance with wheat productivity.

In our experience, growing large numbers of wheat plants in homogenized soil, under similar care and environment, resulted in a large differential in wheat biomass. We chose to take advantage of this differential to characterize the rhizobacterial community using 16S rRNA pyrosequencing sequence data reflecting rhizobacterial abundance in plants categorized according to shoot biomass productivity. We then correlated the abundance of each rhizobacterial OTU (operational transcriptional unit) with shoot biomass productivity using a novel statistical approach. We hypothesized that the reason for this differential in wheat plant biomass was associated with the development of the rhizobacterial community during vegetative growth. This paper is meant to demonstrate this approach, in considerable depth. All in all, we identified 42 positive- and 39 negative-productivity-associated rhizobacteria significantly associated with shoot biomass productivity. Furthermore, the significant positive- and negative-productivity-associated rhizobacteria, the overall rhizobacterial community diversity, and the balance between positive- and negative-productivity-associated rhizobacteria were shown to be highly associated with shoot biomass productivity.

MATERIALS AND METHODS

Plant growth and bacterial extraction. A total of 96 wheat (*Triticum aestivum* cv. Grandin) plants were grown singly in a controlled greenhouse in large 2.8-liter Treepots (Stuewe & Sons) filled with homogenized Easpur loam soil with a prehistory of wheat production. No fertilizer was added, to force the plants to depend on the native rhizosphere microflora for productivity functions. At planting, the soil contained 30, 87, and 352 kg/ha of N, P, and K, respectively, and 2.12% organic matter. After 8 weeks of growth, shoots were cut, the roots were gently removed, and the shoots were weighed. Shoot biomass productivity was chosen because the shoot is the source of much of the organic nutrition that feeds the rhizosphere microbial food web and should be correlated with rhizosphere productivity functions. Loose soil was removed from the root by three consistent shakes, the root and shoot were weighed, and the root with clinging soil was blended three times at high speed (24,000 rpm) in eight volumes (wt/vol) of 0.1% sodium pyrophosphate for 1 min, with a 1-min icing between grindings. To minimize temporal artifacts, the rhizosphere sample was processed and placed on ice within 10 min of removal of root from the soil. From each rhizosphere a 1-ml aliquot of soil extract (250 mg of rhizosphere soil/root) was frozen at −80°C. Wheat plants were classified into five evenly spaced categories from low to high according to their corresponding shoot fresh weights, with seven plants per category. Seven 1-ml aliquots from each of the seven plants in each biomass category were combined into a single bulk extract prior to DNA extraction. Five replicate DNA extracts were extracted from each bulk extract by bead beating using the Mo Bio Power Soil extraction kit (Mo Bio, Carlsbad, CA) according to the manufacturer's directions. Replicate DNA extracts were combined to form the final bulk DNA extract. Prior to pyrosequencing, DNA quality (260 nm/280 nm absorbance ratio > 1.80) and quantity (>30 ng/μl) were determined for each DNA extract by nanodrop spectrophotometry (Thermo Scientific, Rockford, IL). Pyrosequencing was performed by the Research and Testing Laboratories (Lubbock, TX) using the (bTEFAP) FLX 454 titanium pyrosequencing procedure, 100 ng of DNA, and the 27F and 533R 16S rRNA gene universal PCR primers (11). Pyrosequencing was chosen due to its ability to return massive amounts of community sequence data in a cost-effective manner with significant phylogenetic resolution.

Sequence processing. Quality sequences were evaluated and retained using both the in-house procedure of the Research and Testing Laboratories and the RDP II pyrosequencing pipeline. Alignment and clustering were performed using the RDP II pyrosequencing pipeline, defining each OTU at a level of 1% dissimilarity (6). Here we correlate the abundance of specific OTUs with biomass productivity based on the numbers of 16S rRNA gene sequences in each category. A basic assumption of this analysis, and of all 16S rRNA sequencing work, is that the numbers of sequences are proportional to the numbers of organisms. The validity of the assumption is complicated by the multigenic copy number typical of many bacteria, from 1 to 15 (1). However, comparisons among bacteria that are defined at the subspecies level do not show significant copy number variance (22). Thus, in this study, all sequences were aligned and clustered at 1% dissimilarity. The numbers of sequences for each OTU in each biomass category were determined in a Microsoft Excel spreadsheet. A representative sequence from each OTU was selected using the dereplication function resident in the RDP II pipeline and was phylogenetically classified by the RDP II Bayesian Classifier (45). Clustering of the OTUs based on their response to productivity was performed using the SYSTAT version 10.2 (Systat Software, Inc., Chicago, IL) hierarchal classification function using Ward's distance and chi-square linkage methods. Clustering was meant to group those OTUs according to their association with productivity. No statistically based inference is suggested by the groupings of OTUs.

Correlation analysis. Correlation analysis within each OTU was based on the numbers of sequences across productivity categories for those OTUs containing six or more sequences. An exact test based on a log-linear model was utilized to compute a *P* value for each OTU (24). Exact analysis is more common in the physical or social sciences than in biology, although an increasing number of researchers are using this statistical approach, especially when confronted with a small sample size, because modeling assumptions of standard approaches are difficult to verify in this setting. An exact test is valid without making any distribution assumptions concerning the base population. Though any number of models could be considered, such as a linear or polynomial-type model, a log-linear model is common in categorical data analysis of count data because it ensures positive predictive values. False-discovery-rate-adjusted *P* values, also called *q* values, were computed using the QVALUE software in R (41) and used to determine which OTUs were significantly associated with productivity (defined by *q* values of ≤0.05).

To determine the amount of copy number variability attributable to productivity, the root mean squared regression statistic (RMSR) was computed via

$$\mathrm{RMSR} = \left(1/4\sum_{i=1}^{5}(\hat{y}_i - \bar{y})^2\right)^{1/2}$$

where $\hat{y}_i = \exp(\hat{b}_o + \hat{b}_1 X_i)$ is the expected number of genes in the *i*th biomass category assuming a log-linear relationship between productivity and abundance exists, x_i is the shoot biomass (g) of the *i*th productivity group, and $\bar{y} = \hat{b}_o$ is the expected number of genes in the *i*th biomass category assuming no relationship between biomass and abundance exists ($b_1 = 0$). Hence, we would expect $\hat{y}_i$ to be near $\bar{y}$ thereby yielding a small RMSR when the abundance of an OTU is not related to productivity, and

we expect a large RMSR value otherwise. Least-squares estimates of coefficients were obtained using the glm function in R. It should be noted that, since least-squares estimators were used, the RMSR value does not depend on any distributional assumptions for the data but does depend on the assumption that the relationship between productivity and OTUs, if it exists, is log-linear. The percent RMSR contribution for the *j*th OTU among the *n* OTUs that were declared to be positively related to productivity (q value, ≤ 0.05, $B_1 \geq 0$) was computed by

$$\mathrm{PMSR}_j = 100\left(\frac{\mathrm{RMSR}_j}{\sum_{k=1}^{n} \mathrm{RMSR}_k}\right)$$

The RMSR_j is the RMSR (productivity-associated deviation) for the *j*th OTU, and hence the PMSR_j is the proportion of productivity-associated deviation for the *j*th OTU among the total positive-productivity-associated deviation. An analogous statistic was computed for OTUs that were negatively related to productivity.

This work developed an approach to partition the productivity deviation via the RMSR statistic based upon well-established techniques for describing the viability of data with respect to some model. Specifically, the total variability for an OTU can be broken down into variability attributable to productivity (SSR) and variability not attributable to productivity (SSE) via

$$\mathrm{SST} = \sum_{i=1}^{5} (y_i - \bar{y})^2 = \sum_{i=1}^{5} (y_i - \hat{y}_i)^2 + \sum_{i=1}^{5} (\hat{y}_i - \bar{y})^2 = \mathrm{SSE} + \mathrm{SSR}$$

Dividing the left- and right-hand sides of the equation by 4 yields the mean squared error and mean squared regression, which can be thought of as the average variabilities not attributable and attributable to productivity, respectively. Taking the square root of the MSR yields the RMSR and can be thought of as the amount of average deviation attributable to productivity. The PMSR_j is then the proportion of (positive- or negative-) productivity-associated deviation among those OTUs that were determined to be (positively or negatively) associated with productivity.

Aggregate positive and negative OTUs and shoot biomass. The total number of sequences for OTUs with PMSRs of >0.1% was determined for each biomass category and correlated with biomass productivity based on either an exponential ($y = ae^{bx}$), a linear ($y = ax + b$), a logarithmic [$y = a\ln(x) + b$], or a power series ($y = ax^b$) model associated with the Excel spreadsheet trendline analysis function, where a and b represent least-squares coefficients. The sigmoidal modeling was obtained from the Graphpad PRIZM v5 software (GraphPad Software Inc., La Jolla CA) using a three-parameter logistic equation:

$$y = a + \frac{(b - a)}{1 + 10^{logEC50 - x}}$$

where a equals the lower-bound plateau, b equals the upper-bound plateau, and $EC50$ equals the x value at 50% of the y value. Once the aggregate numbers of sequences for positive- and negative-productivity-associated OTUs were determined, then the balance between positive and negative rhizobacteria was determined as the ratio between positive and negative OTUs for each biomass category. The ratio was then plotted against values for biomass categories, and linear and nonlinear correlation analysis was performed as indicated above.

Community diversity analysis. Community diversity was measured with 95% confidence intervals by the Chao1, Shannon (eH), and Simpson (1/D) indexes using EstimateS (7a) (Chao1) and PAST (Shannon and Simpson) software diversity functions (15) based on a total of 4,725 randomly chosen sequences from each biomass category. The results were expressed as a percentage of the maximum in order to fit the data in a single figure (see Fig. 6) for comparison purposes, with the actual values listed in the accompanying table insert.

RESULTS

Wheat plants were grown under defined greenhouse conditions in homogenized field soil with a prehistory of wheat production. Plants were harvested, and shoot fresh weight measurements were determined. Plants exhibited a wide range of shoot biomasses, from 0.45 to 4.06 g fresh weight/plant. Rhizospheres were combined and evenly classified into five separate categories according to their shoot biomasses. Under the above-described conditions, substantial single-plant variation was observed, with a 9-fold difference in shoot fresh weight between smallest and largest plants and a 3.4-fold difference between the averages of the lowest and highest biomass categories. Rhizobacterial DNA was extracted from each biomass category, and the 16S rRNA gene was sequenced using 454 Titanium pyrosequencing, yielding a total of 29,007 quality sequences. Each OTU with six or more sequences was analyzed by the exact test to determine the significance of the relationship adjusted for the false-discovery rate. Specific OTUs were identified after dereplication with the RDP II Bayesian Classifier using a conservative confidence threshold of 50% to delineate the taxonomic classification (45), with the result that most OTUs were matched to the genus level but some were matched only at much higher taxonomic levels, such as domain (*Bacteria*) or phylum. The degree of percent productivity-associated deviation (PMSR) was determined for each OTU as a measure of its approximate contribution to productivity in both positive and negative associations.

The relationship between biomass productivity and sequence abundance can be graphically visualized for eight representative OTUs (Fig. 1). Close examination revealed a large upward trend with increasing biomass and downward trend with decreasing biomass for positive and negative associations, respectively. Furthermore, many of the significant relationships for both positive and negative associations appeared to exhibit pronounced nonlinear responses with shoot biomass productivity, including *Cellvibrio* OTU 1847, *Rhizobium* OTU 1697, and *Lysobacter* OTU 597 for positive associations and *Enterobacter* OTU 357 and *Chryseobacterium* OTU 508 for negative associations. Some OTUs, including both *Enterobacter* 357 and *Chryseobacterium* 508, showed exclusive association in one category, in this case the low-biomass category.

Of the 719 OTUs statistically analyzed, 42 showed significant positive associations with biomass productivity. The significant OTUs were hierarchically clustered based on productivity response so that close proximity in the diagram reflects similar shoot productivity responses with the positive associations represented in Fig. 2 and the negative associations in Fig. 3. The five most significant OTUs, based on highest percent contribution as determined by the PMSR statistic in parentheses, were two *Duganella* OTUs, 3 and 43 (7.01% and 1.97%), *Janthinobacterium* 278 (2.44%), *Pseudomonas* 588 (1.51%), and *Cellvibrio* 1847 (1.44%), all accounting for 14.4% of the positive productivity deviation. Furthermore, the top five sequences comprised 73% of the total number of sequences presented in Fig. 2 and 4.3% of all 29,007 sequences represented in this study. Overall, the significant positive-productivity-associated rhizobacteria contributed 37% to the overall positive productivity deviation. Examining higher taxonomic relationships, 31 out of 42 significant positive OTUs were classified as *Proteobacteria*, with 10 as *Alphaproteobacteria* and 9 as *Gammaproteobacteria*. *Proteobacteria* represented 31% of the total productivity deviations, with *Betaproteobacteria* providing 12.6%, *Gammaproteobacteria* 8.2%, and *Alphaproteobacteria* 6.1%.

For positive associations, a total of 15 clusters were identified by hierarchal clustering with distances less than 1.0, and the total

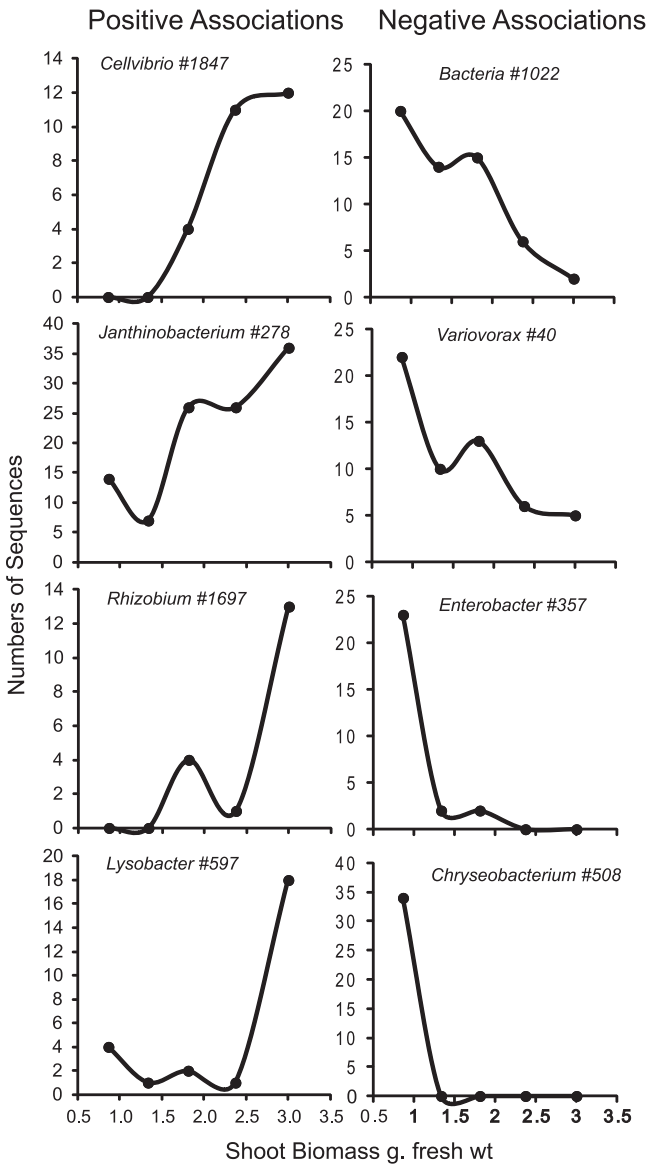


FIG 1 Composite graphs of four representative positive (left panels) and negative (right panel) OTU associations with shoot biomass productivity. The OTU taxonomic identification with sequence number is indicated at the top of each chart.

percent contribution of each cluster was determined. Two clusters showed the greatest productivity percent contribution. Cluster O, comprised of *Duganella* 43 and *Duganella* 3, contributed 8.98% toward productivity deviation, while Cluster N, comprised of *Janthinobacterium* 278, *Alphaproteobacteria* 2220, and *Polygoniaceae* 1123, provided 3.73% toward the productivity deviation. Other contributing clusters included H and I, with H including *Pseudomonas* 588 and I including *Lysobacter* 597 as their most prominent members.

Out of the 719 OTUs, 39 significant negative-productivity-associated rhizobacteria were identified in Fig. 3, ranging from 4.4% for *Bacteria* OTU 273 to 0.42% for *Aquimonas* OTU 2136. The five most significant negative associations based on the largest productivity deviation were outlined in bold, including *Bacteria* 273 (4.4%), *Chryseobacterium* 508 (4.1%), *Proteobacteria* 249 (3.9%), *Enterobacter* 357 (2.6%), and *Bacteria* 1022 (2.0%). Overall, 17% of the total negative-productivity-associated deviation came from these five OTUs. Thirteen clusters defined by a distance of less than 1.0 were evident. The clusters with the greatest sum total negative productivity deviation were cluster B at 6.8%, which included *Chryseobacterium* 508 (4.1%), and cluster I at 6.1%, which included *Bacteria* 273 (4.4%). Other clusters of negatively impacting productivity-associated rhizobacteria included F (5.4%) and K (5.2%).

Since root tissue was extracted along with rhizosphere soil, the presence of root plastid sequences was expected. A total of 9.6% of all sequences were represented by *Streptophyta*, or root plastids, comprising 59 OTUs defined at 1% dissimilarity. Out of the 59 OTUs, 30 showed significant increases with decreasing shoot biomass (Fig. 4). The shape of the productivity response curve was closely modeled by a nonlinear power series model with a dramatic increase in *Streptophyta* sequences in the lowest biomass category. *Streptophyta* sequences significantly correlated to productivity comprised 87% of the total *Streptophyta* sequences. *Streptophyta* OTUs with the greatest numbers of sequences included *Streptophyta* 42, 84, and 48, with 883, 457, and 333 sequences, respectively.

The relationship between productivity-associated taxonomic groups and shoot biomass productivity was examined, and the results are presented in Table 1. Many OTUs defined at 1% dissimilarity and classified by the RDP II Bayesian Classifier were represented multiple times; for example, *Duganella* was represented by 25 positive OTUs and 14 negative associations. A total of 127 taxonomic groups were represented and ranked according to their net percent contribution base on their aggregate PMSR statistic of >0.1%, with 68 showing a net positive association, 56 showing a net negative association and 3 evenly balanced. The net contribution for a given taxonomic group was calculated as the difference of the sum total of all positive and negative OTUs, with the top 10 positive and negative groups indicated in Table 1. *Duganella*, *Rhizobium*, *Acidobacteria* Gp6, *Janthinobacterium*, and *Cellvibrio* showed the greatest cumulative positive contribution, with 18% of the total. *Bacillus*, *Actinomycetales*, *Cellvibrio*, and *Acidobacteria* Gp6 were balanced heavily toward numbers of positive associations, with *Bacillus* showing all positive associations. Those with the greatest number of positive-productivity-associated OTUs included *Duganella* and *Acidobacteria* Gp6. The greatest cumulative negative contributions for taxonomic groups were from those classified as *Proteobacteria* and *Bacteria* followed by *Chryseobacterium*, *Enterobacter*, and *Cyanobacteria,* all totaling −27.2% productivity deviation. *Proteobacteria* and *Xanthomonadaceae* showed the strongest negative tendencies. Overall, the positive-productivity-associated rhizobacteria accounted for 93.8% of the positive deviation and the negative-productivity-associated rhizobacteria accounted for 95.9% of the negative deviation, with the net deviation tilted to the negative by 2.1%.

The productivity balance between aggregate positive and negative OTUs summed across shoot biomass categories was examined and presented in Fig. 5. The total number of sequences in each category was summed up for all positive OTUs, with a PMSR greater than 0.1% termed total OTUs. The same was done separately for negative OTUs. In addition, the total number of sequences in each category was summed up for OTUs with a significant positive and negative relationship as determined by the exact

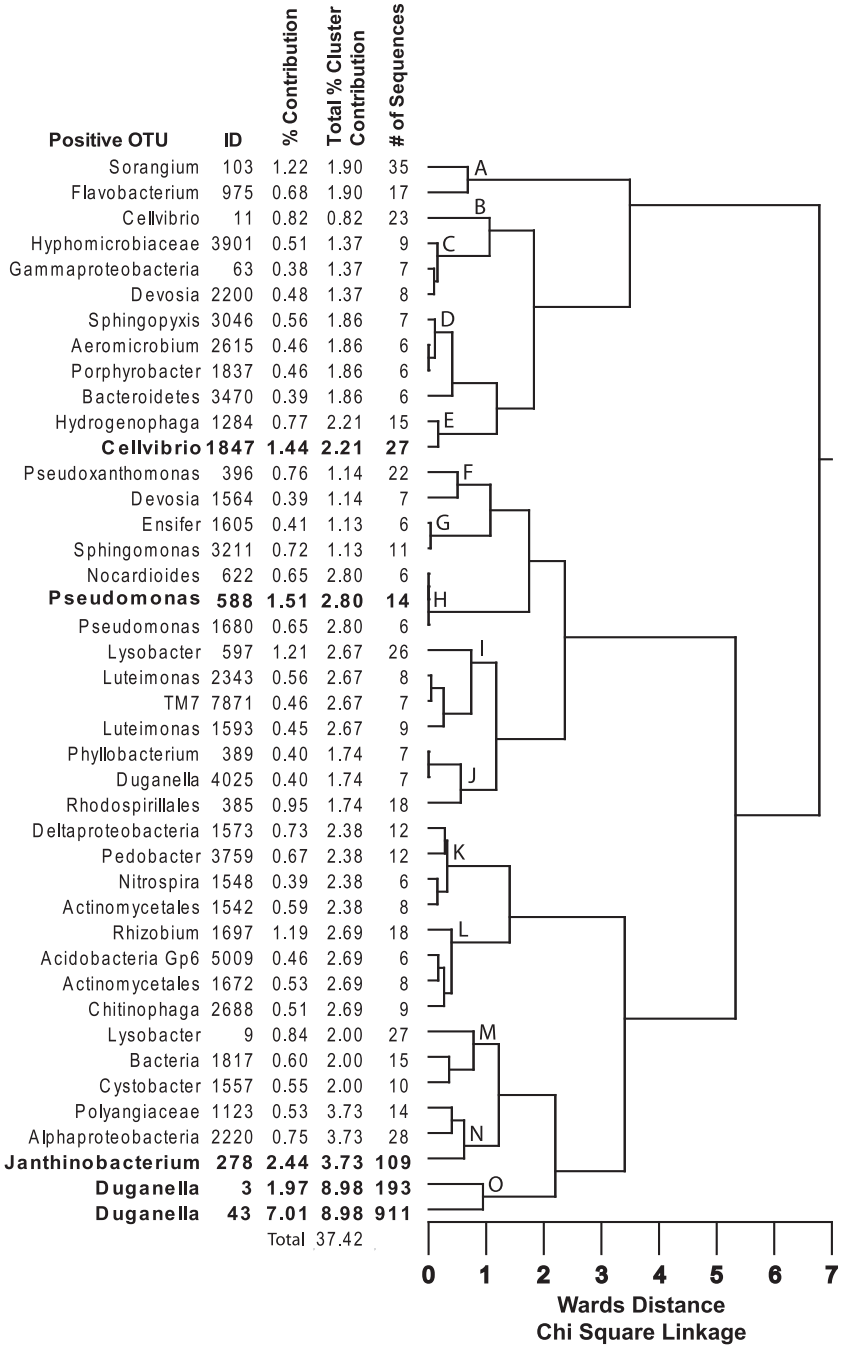


FIG 2 Clustering of 42 significant (q value, ≤0.05) positively associated OTUs, with the top five in bold type. Individual percent OTU contributions as measured by the PMSR statistic, cluster membership, percent contribution for each cluster, and number of sequences per OTU for each cluster are indicated. Cluster membership is also indicated by the letter symbol at the cluster branch. Total overall contributions of significant positive associations are indicated at the bottom of the figure.

test (q value, ≤0.05), termed total significant OTUs. From these data, the balance between the aggregate positive and negative organisms was determined as represented as the P/N ratio (positive-/negative-productivity-associated rhizobacterial ratio) for total overall and significant OTUs in Fig. 5 as defined above. Correlation analysis was performed using both linear and nonlinear models to find the best fit as determined by the R^2 values, and these are presented in the table in Fig. 5.

Of the 719 total OTUs in this study, 268 (37% of the total) showed positive PMSR while 238 (33% of total) showed negative PMSR of 0.1% or greater, and the remaining 213 OTUs (30%) had a neutral effect. In all cases, both positive and negative, and for total and significant OTUs as well as for the P/N ratio, the data fit nonlinear curves. In particular, the sigmoidal model showed the best fit in four out of six categories according to the R^2 values, ranging from 0.607 to 1.000. The other two categories showed a

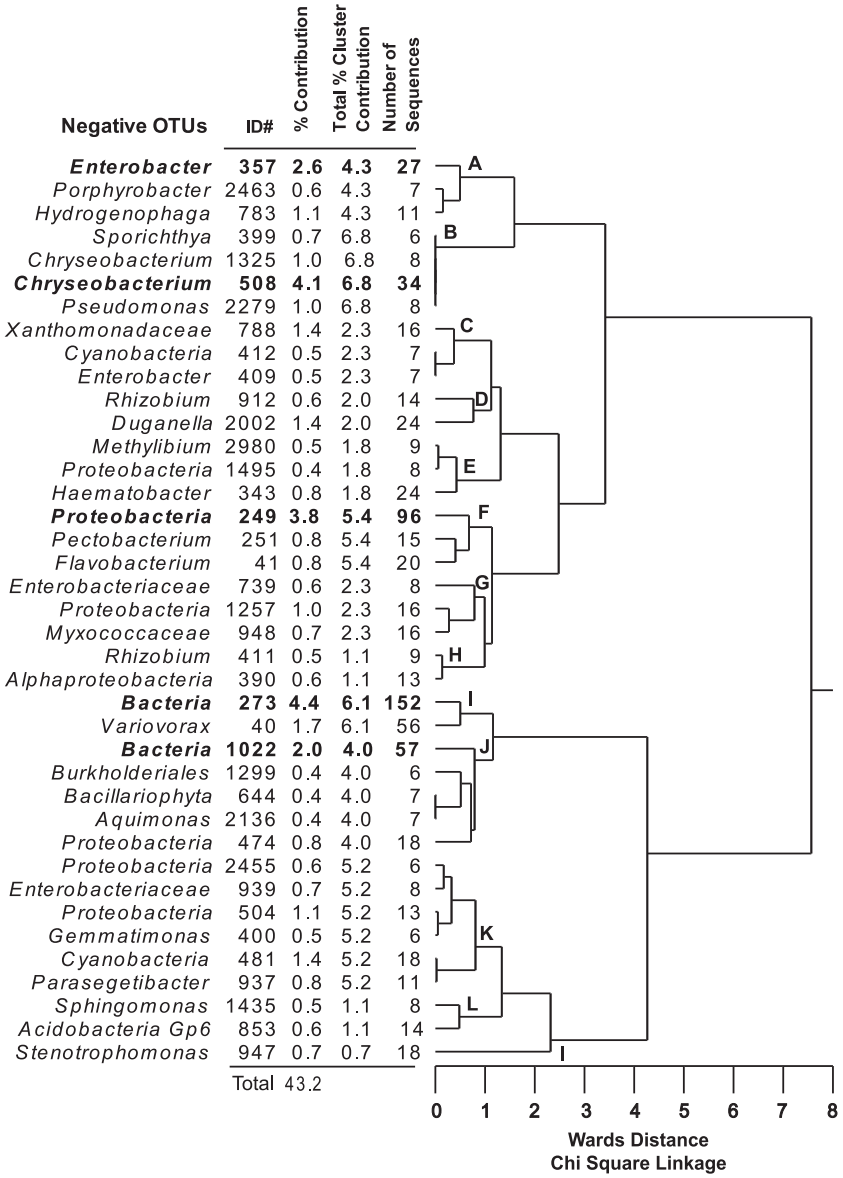


FIG 3 Clustering of 39 significant (q value, ≤0.05) negatively associated OTUs, with the top five in bold type. Individual percent OTU contributions as measured by the PMSR statistic, cluster membership, percent contribution for each cluster, and number of sequences per OTU for each cluster are indicated. Cluster membership is also indicated by the letter symbol at the cluster branch. Total overall contributions of significant positive associations are indicated at the bottom of the figure.

slightly better fit to the power series model than to the sigmoidal model. The linear slope was significantly different (P value, ≤0.05) from zero in all cases with the exception of the significant-negative-association aggregate OTUs (data not shown). The closest fit was from the total P/N ratio, which very closely matched sigmoidal, power series, and linear relationships with the R^2 ranging from 0.986 to 1.000. Based on total OTUs, there were 3.5 positive to 1 negative total OTUs in high-biomass plants and 2.3 negative OTUs for every positive in low-biomass plants. Based on significant OTUs, there were 9.2 significant OTUs for every 1 negative OTU in high-biomass plants and 2.3 negative OTUs for every positive OTU in low-biomass plants.

Rhizobacterial community diversity as determined by three diversity indicators is presented in Fig. 6 based on overall community diversity analysis in each category using Chao1, Shannon, and Simpson indexes. All three indicators showed a nonlinear wavelike response to increasing productivity from low to high. The lowest diversity was found in the lowest biomass categories, and the highest tended to be in the highest biomass category, with the exception of Chao1, which had a significantly higher diversity in the medium category. The Simpson index showed the greatest nonlinear wavelike oscillation, followed by Shannon and Chao1.

DISCUSSION

More than a hundred years of research suggests that the rhizomicrobial community plays a very significant role in plant productivity processes. Despite its importance, so much remains un-

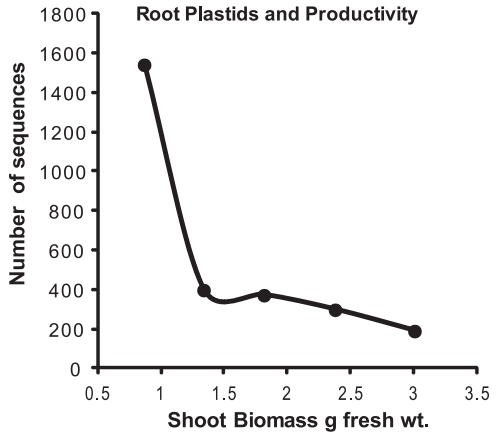


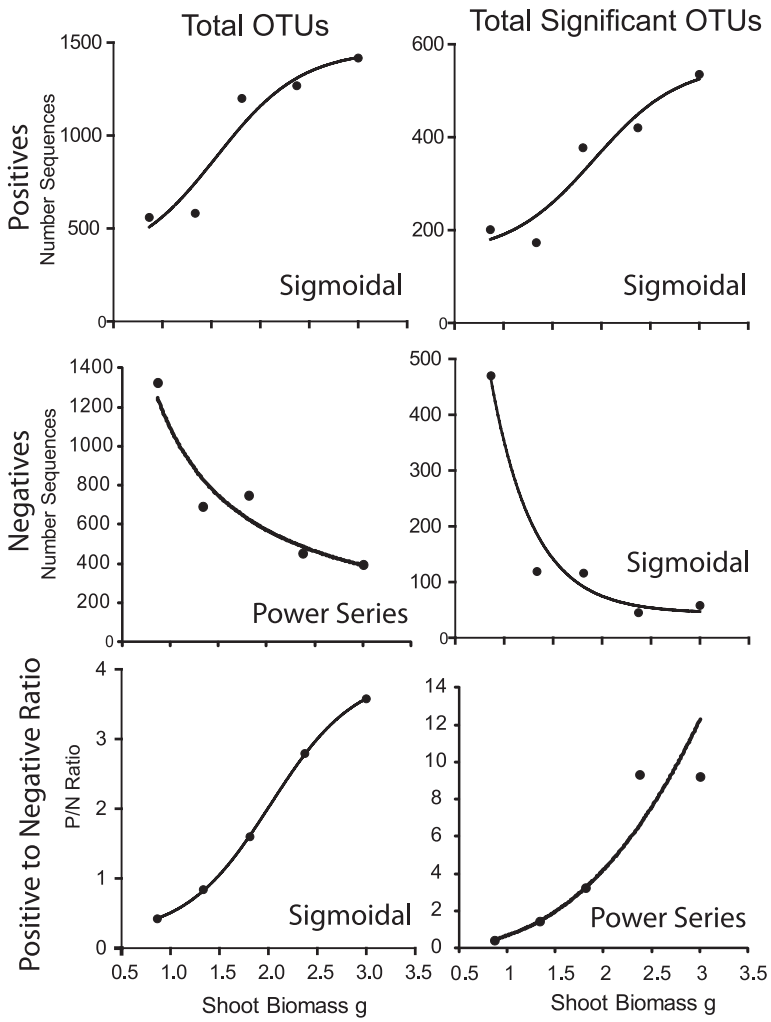
FIG 4 Relationship between root plastids (*Streptophyta*) and shoot biomass productivity.

known due to (i) the biotic and abiotic complexities and heterogeneity associated with the rhizosphere environment, (ii) the inability to culture the vast majority of rhizobacterial residents, (iii) the difficulty in evaluating the interorganismal interaction effect associated within this complex community, and (iv) the difficulty in extracting and characterizing the rhizosphere community in a unperturbed state or studying the rhizosphere community interactions *in situ*. Many previous attempts at characterizing the rhizosphere have relied heavily on reductionism-based approaches that sought to isolate and characterize specific segments of the rhizobacterial community or associated functional processes in order to understand in part the rhizosphere contribution to plant productivity. While these research projects have provided valuable information on productivity processes, they are clearly not sufficient to understand the system-wide contribution as a whole. In contrast, the research reported here represents a significant system-level advance in rhizobacterial community research in that the research (i) used a molecularly based pyrosequencing technique to identify a large number of individual OTUs from relatively unperturbed rhizosphere communities associated with productivity without resort to cultural means, (ii) identified both positive- and negative-productivity-associated rhizobacteria, (iii) used a novel nonparametric statistical approach to identify specific OTUs associated with productivity, and (iv) identified fundamental system-level parameters associated with the rhizobacterial community productivity functions. Finally, it is acknowledged that the results here represent a demonstration of the potential of this approach using one soil system and under specific environmental conditions and that under other conditions the results are likely to differ. However, we are confident that continued comparative investigations using this same approach, under a wide variety of conditions, will result in the unfolding of underlying general principles concerning rhizobacterial contribution to plant productivity. We will first discuss the specific OTUs associated with productivity followed by the community-wide aspects.

TABLE 1 Taxonomic groups with the highest positive or negative aggregate percent productivity deviation (PMSR)

Taxonomic group	Result for group					
	Positive		Negative		Total	
	No. of OTUs[a]	%PD[b]	No. of OTUs	%PD	No. of OTUs	%PD
Positive-productivity associated						
Duganella	25	14.8	14	7.0	39	7.7
Rhizobium	12	5.0	5	2.4	17	2.6
Janthinobacterium	2	2.9	1	0.3	3	2.6
Acidobacteria Gp6	20	4.5	8	1.9	28	2.6
Cellvibrio	4	2.6	1	0.4	5	2.2
Pseudoxanthomonas	3	1.6	0	0.0	3	1.6
Lysobacter	4	2.3	2	0.8	6	1.5
Sorangium	2	1.5	0	0.0	2	1.5
Actinomycetales	5	1.6	1	0.3	6	1.3
Bacillus	5	1.2	0	0.0	5	1.2
Negative-productivity associated						
Acidobacteria Gp4	12	2.4	16	3.5	28	−1.1
Flavobacterium	10	4.1	13	5.3	23	−1.1
Enterobacteriaceae	0	0.0	2	1.4	2	−1.4
Variovorax	0	0.0	1	1.7	1	−1.7
Xanthomonadaceae	2	0.7	5	2.4	7	−1.7
Cyanobacteria	0	0.0	2	1.8	2	−1.8
Enterobacter	0	0.0	2	3.0	2	−3.0
Chryseobacterium	0	0.0	2	5.1	2	−5.1
Bacteria	11	2.8	13	9.3	24	−6.5
Proteobacteria	3	0.6	18	11.3	21	−10.7
Total	268	93.8	238	95.9	506	−2.1

[a] Number of OTUs for a given taxonomic group.
[b] Percent productivity deviation associated for each taxonomic group as determined by the aggregate PMSR statistic.



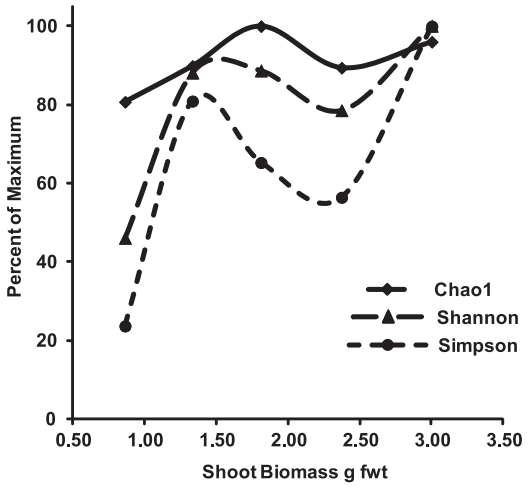
R^2 values

	Exponential	Linear	Logrithmic	Power Series	Sigmoidal
Total Positives	0.822	0.858	**0.873**	0.855	**0.920**
Total Negatives	0.877	0.780	0.876	**0.917**	**0.915**
Total P/N Ratio	0.952	**0.986**	0.930	**0.993**	**1.000**
Signficant Positives	0.838	**0.903**	0.848	0.807	**0.921**
Significant Negatives	0.762	0.607	0.759	0.869	**0.958**
Signficant P/N Ratio	0.902	0.892	0.853	**0.970**	**0.944**

FIG 5 Relationships between shoot biomass productivity and aggregate OTU sequence numbers totaled for each category for the total (left panels) and significant (right panels) OTUs and for overall positive OTUs (upper panels), negative OTUs (middle panels), and positive-to-negative OTU ratios (P/N, lower panels). The table below the composite chart provides R^2 values for linear and nonlinear models for each of the respective panels. The best-fit model is indicated in the lower right-hand corner for each panel.

Overall, the approach identified to the strain level 42 positive- and 39 negative-productivity-associated rhizobacteria identified for the most part to the genus level or higher. Cluster analysis was used to group the specific OTUs according to their response to increasing plant productivity. Many of these genera, including *Pseudomonas*, *Rhizobium*, *Flavobacterium*, and *Lysobacter*, etc., are known to include plant growth promoters or biocontrol organisms, while others, such as *Duganella*, *Cellvibrio*, *Cystobacter*, and *Luteimonas*, have rarely or never been observed with these characteristics. Many of these productivity-associated rhizobacteria could be identified only at the highest phylogenetic level such as domain or class. These poorly classified OTUs most likely represent phylogenetically novel organisms that are hard to culture and have yet to be assigned to the current taxonomic structure.

The PMSR statistic identified the OTUs that contribute most to the productivity deviation. The most significant positive associations were from the genera *Duganella* (OTUs 3 and 43) and *Janthinobacterium* (OTU 278). These two are genera that have rarely been examined for biocontrol or plant growth promotion. Furthermore, *Duganella* is taxonomically closely related to *Janthinobacterium*, both being members of the *Oxalobacteraceae* family. The large percent contribution of the two *Duganella* rhizobacterial OTUs and one *Janthinobacterium* rhizobacterial OTU most likely reflect their large numerical abundance. Both *Duganella* and



Biomass Category	Chao1	Shannon	Simpson
Low	6487 a	880 a	130 a
Med Low	7219 b	1684 b	444 b
Medium	8033 c	1694 b	358 c
Med High	7183 b	1501 c	309 c
High	7714 b	1912 d	549 d

FIG 6 Relationship between microbial diversity and wheat shoot productivity expressed as a percentage of maximum values for each diversity index. Absolute values for the Chao1, Shannon, and Simpson indexes are provided in the table below the figure. Letters to the right of the data in the table represents statistical significance. Differing letters indicate that the value lies outside the 95% confidence interval.

Janthinobacterium are producers of the antibiotic violacein, a known inhibitor of viruses, fungi, and nanoflagellates (19). Their numerical abundance may reflect the production and activity of these antipredatory or antiparasitic compounds, since predation and parasitism are major known factors shaping the rhizobacterial community (26). Furthermore, these two genera are rich in catalytic enzymes, are known degraders of a wide range of organics, and are likely participants in nutrient cycling of complex organic compounds (30, 44). It should be noted that among the significant OTUs *Duganella* OTU 2002 was associated with negative productivity and another *Duganella* OTU, 4025, was associated with less positive-productivity deviation than those mentioned above. Thus, productivity deviation can vary substantially within a given genus as indicated with *Duganella* and for other genera listed in Table 1.

Other significant positive-productivity-associated rhizobacteria include *Pseudomonas* OTU 588, a member of one of the most widely distributed genera colonizing a broad range of biological niches under a wide range of environments. In particular, pseudomonads have been widely associated with both plant growth promotion and pathogenesis, possess a wide diversity of metabolic capability, produce antibiotics such as, 2,4-diacetylphloroglucinol (DAPG), and have been used as biocontrol agents. The DAPG antibiotic has been shown to reduce root fungal infection in wheat (35). In this study, five *Pseudomonas* OTUs showed positive associations and three showed negative associations, for a net positive productivity response of 1.2% (data not shown). Another positive-productivity-associated rhizobacterium is *Cellvibrio* OTU 1847, which is a member of a genus known for its cellulose and complex carbohydrate degradation potential (23) and for this purpose has been investigated for biofuel production (13). This genus occurs in wheat fields (31) and may be associated with mycorrhizal networks facilitating AMF spore germination (36).

In 1982 Suslow identified a class of bacteria termed deleterious rhizobacteria (DRB) that reduced growth without showing obvious disease symptoms (28). DRBs, also called minor pathogens, impact plant productivity significantly by reducing growth in some way not related to obvious disease symptoms. Research progress on identifying and functionally characterizing DRBs has been slow. As with research on growth promoters, identification of DRBs depended primarily on the use of cultural technique. Results from this study indicated that 39 OTUs were negatively associated with productivity. Those most negatively associated with productivity were *Bacteria* OTU 273, *Proteobacteria* OTU 249, *Chryseobacterium* OTU 508, *Enterobacter* OTU 357, and *Bacteria* OTU 1022. The fact that three out of five of the greatest negative associations were taxonomically undetermined at the highest level of taxonomic classification indicates that the taxonomy of DRBs is not well developed. Little can be said concerning the functional attributes of these taxonomically undetermined members. However, members of the *Chryseobacterium* and *Pseudomonas* genera are associated with the wheat disease take-all. Other members of the *Chryseobacterium* genus are known saprophytes (4), plant growth promoters, and biocontrol agents (10). Members of the genera *Enterobacter* and *Pseudomonas* have previously been identified as DRBs (39, 43).

A previous work (25) using both cultural and molecularly based techniques demonstrated that both chryseobacteria and pseudomonads increase in abundance under the influence of the wheat disease take-all. Our work is consistent with theirs in that *Chryseobacterium* (OTUs 508 and 1325) and *Pseudomonas* (OTU 2279) are found to be more abundant under low-productivity conditions. The low-productivity conditions in this study may be analogous to the fungus-infected conditions in the McSpadden Gardener and Weller study (25). Furthermore, in our work the *Chryseobacterium* OTUs and the single *Pseudomonas* OTU were tightly clustered together, indicating that they had similar responses to negative productivity, which may indicate that there were some inherent interactions between the two OTUs. This would be consistent with the suggestion of McSpadden Gardener and Weller that the rise in *Chryseobacterium* may actually support the suppression of take-all-causing fungi by DAPG-producing pseudomonads. Nonetheless, sequence analyses of *Chryseobacterium* spp. and *Pseudomonas* spp. in this study and those from the McSpadden Gardener and Weller study show sizeable divergence, indicating that they are likely to be different organisms (data not shown).

In our analysis, specific OTUs were represented multiple times, indicating that some taxonomic groups may be more highly associated with productivity than others. Of these multiple associations, most showed both positive and negative contributions to productivity, while some showed exclusive association with either positive or negative contributions. By summing up the productivity deviations for both positive and negative associations and sub-

tracting the positive from the negative, it was possible to identify those taxonomic groups that showed the greatest net contribution to the productivity deviation. Our analysis identified *Duganella*, *Rhizobium*, *Janthinobacterium*, *Acidobacteria* Gp6, and *Cellvibrio* as the five taxonomic groups that showed the most net positive association in our plant growth system (Table 1). These groups may represent phylogenetic hotspots which over time have coevolved in wheat to provide productivity functions. *Duganella*, *Janthinobacterium*, and *Cellvibrio* have already been discussed above. However, *Acidobacteria* originates from a phylum that has been newly identified, mostly characterized through molecular means, with few cultural representatives. Research supports the idea that acidobacteria are very abundant in soils, comprising 10 to 50% of the organisms (46). They are very versatile heterotrophs feeding on simple and complex organic compounds in a way that makes them important contributors to the carbon cycle. These appear to be long-lived, slow-growing types that are well adapted to low nutrient environments such as in the bulk soil (20, 46) and in this case where nitrogen fertility was not provided. Furthermore, acidobacteria have been shown to be part of disease-suppressive soils (38). In this study, *Acidobacteria* subdivision 6 represented a very dominant positive OTU, with 29 showing positive associations and 12 showing negative associations, resulting in a net negative contribution of 2.8% of the total productivity deviation, the second highest next to *Duganella*.

Another genus prominent in positive productivity relationship is *Rhizobium*. *Rhizobium* spp. are primarily known for their symbiotic association with legumes. However, recent work has tied this genus to functions that affect plant growth promotion, including disease-suppressive effects through production of antibiotics, iron-chelating compounds, and stimulation of induced resistance in plants. In addition to disease suppression, *Rhizobium* is known for P solubilization from organic sources and provision of plant growth-promoting compounds (3). On the negative side, taxonomic groups including *Proteobacteria*, *Bacteria*, *Chryseobacterium*, and *Enterobacter* represented 29.2% of the negative-productivity-associated productivity deviation. Little can be said for those classified to the level of *Proteobacteria* or *Bacteria* except that these may represent novel organisms that are in need of further classification. Both *Chryseobacterium* and *Enterobacter* have been discussed earlier.

This work may facilitate the isolation of a number of novel plant growth-promoting rhizobacteria not examined before. Many PGPR or biocontrol agents have been isolated and evaluated using screening systems that rely on the ability to culturally isolate and evaluate the plant growth promotion potential of targeted bacteria (14, 32). Many of these use plant growth promotion assays in which organisms are reintroduced into the rhizosphere under highly artificial conditions, followed by a growth promotion evaluation. In contrast, here we first used noncultural techniques to identify rhizobacteria associated with biomass productivity which could then be followed up with isolation and evaluation of targeted isolates. Furthermore, identification of growth promoters through this noncultural approach may be more effective in identifying rhizosphere-competent rhizobacteria without the bias associated with cultural technique. The significant OTUs identified in this study could be isolated using specific cultural conditions guided by sequence information for a given OTU, using sequence-based hybridization probes for screening the DNA from a rhizobacterial library, or the sequences themselves could be compared with sequences generated from an isolated genus-specific library *in silico*. Genus-specific culture conditions could aid in overcoming cultural constraints with many of the identified OTUs. OTUs such as those of *Duganella* or *Janthinobacterium* were found in high abundance, and these may serve as targeted genera for PGPR isolation. Other positive-productivity-associated taxonomic groups could also serve as targets for further analysis, such as those of *Pseudoxanthomonas* or selected *Rhizobium* strains. Isolation of strains for use as PGPR is not the only way to further characterize the functional contribution of these OTUs to plant productivity, given that many taxonomic groups contain both positive- and negative-productivity-associated rhizobacteria. Comparative whole-genome analysis among OTUs that are closely related genetically but differ substantially in their contributions to plant productivity could serve to uncover genes and pathways important to plant productivity functions (42).

Probably the most significant finding of this paper is that productivity appears to be highly associated with the balance between positive- and negative-productivity-associated rhizobacteria in the rhizobacterial community. The balance between total positive- and negative-productivity-associated rhizobacteria was quantitatively represented by the ratio of the sum total of positive to negative sequences for each shoot biomass category. The correlation of this total ratio with shoot biomass productivity closely fit nonlinear and linear models, as indicated by the large R^2 values ranging from 0.930 to 1.000 (Fig. 5). Similar relationships were found when the positive-to-negative ratio was determined using statistically significant OTUs (defined by q values of ≤ 0.05) representing 42 positive and 39 negative OTUs. This ratio was also highly correlated to productivity, with large R^2 values ranging from 0.853 to 0.970. Thus, the rhizobacterial community in highly productive wheat plants appears to be dominated by as many as 9.2 significant positives for every significant negative, while low-productivity plants show a ratio of 2.3 significant negatives for every significant positive. The very close fit with total and significant OTUs indicated that the total positive-to-negative ratio explains a very large part of the productivity deviation. Furthermore, the relationship between total and significant OTUs and productivity appears to have some nonlinear nature. In fact, the best model of all those tested appeared to exhibit a slight sigmoidal relationship. The nonlinear sigmoidal response may reflect a dose-response mechanism associated with the positive-to-negative ratio and plant productivity. In this case it is conceivable that the dose represents exudates derived from shoot photosynthate and the response is the change in balance between positive- and negative-productivity-associated organisms. However, caution must be employed, because the current analysis does not differentiate between linear and nonlinear models, leaving this question to future investigations which would have to include many more data points (biomass categories) to allow for a more statistically robust analysis.

The tight balance between total and significant positive- and negative-productivity-associated rhizobacteria and plant productivity strongly suggests that the rhizobacterial community composition is tightly coupled to plant physiological processes. This strong coupling further suggests that the significant and total positively and negatively associated rhizobacteria could be considered part of the overall plant system. In an analogous system that has been much researched, gastrointestinal microflora are also tightly

linked to their host metabolism in many ways. Theorists have suggested that since symbiotic associations act in concert with their host, the sum of all the parts could be considered a single organism, or "holobiont," and that the combined genome could be considered the "hologenome" (37). Thus, plant productivity appears to be not only associated with the plant genome but also influenced strongly by the combined rhizomicrobial genomes. The fact that rhizobacteria provide numerous productivity functions argues for an important role in plant growth and development, but it does not by itself convincingly argue for a controlling cause-and-effect involvement. However, recent work on other hologenome systems suggests that the microbial symbiont is far from passive and that the microflora may have more of a controlling impact than first realized (21) and very likely have a very significant impact on the evolutionary development of the host (37). This is also very likely true for the plant system.

The ratio of positive- to negative-productivity-associated rhizobacteria mentioned above strongly supports our hypothesis that productivity in plants is strongly connected to the development of rhizobacterial community structure. How and when this balance is developed needs much more study. What factors encourage a more positive or negative balance are critical to understanding the relationship between plant and microbe. The positive-to-negative ratio might actually reflect the overall system-level productive efficiency of the rhizobacterial community. The strong coupling inherent in this balance between positive and negative OTUs, indicated in this work, may provide a metric that could be exploited, through breeding, biotechnological engineering, or improved agronomic practices, to potentially enhance rhizobacterial productive efficiency. Research examining this balance under a wide range of conditions and with a number of genotypes is necessary to determine if this metric is open to manipulations. We believe that development of this approach is just a beginning and that further work is necessary involving different soils, plant varieties, and plant cultural practices before general principles affecting rhizobacterium-plant interactions can be better understood with respect to both specific OTUs, positive and negative, and overall general community response. This approach may help better define at a very fine level what constitutes healthy or productive versus unhealthy or unproductive soil-microbe-plant systems.

Rhizobacterial community diversity is a characteristic feature of the root rhizosphere community. Ecologists have speculated that microbial diversity provides significant redundancy essential for environmental adaptation (48). The role of microbial diversity in soils as it relates to productivity and ecological sustainability is still a matter of intense discussion and research (48). It is logical to assume that greater biomass productivity will be associated with increased rhizobacterial diversity in the rhizosphere. In this study, we directly examined this hypothetical assertion with our experimental approach utilizing pyrosequencing data and three common diversity indexes (Chao1, Shannon, and Simpson). The three indexes differ in their coverage of the rhizobacterial community, with the Chao1 and Shannon indexes generally covering the rarer OTUs and the Simpson index the more dominant OTUs (7, 18). This three-tiered system was selected to provide a diverse range of analysis of the rhizobacterial community rather than relying on a single index.

Our results indicated that the relationship between diversity and productivity was nonlinear, closely resembling a cubic wave-like model with increasing and decreasing trends, most likely reflecting positive and negative impacting factors on rhizobacterial diversity. The shape of the diversity productivity curve can be rationalized as an interaction between positive and negative impacting factors. The identities of the positive factors are unknown at this time, but they may reflect aspects of both microbial and plant growth processes. Factors that increase rhizobacterial diversity with increasing productivity may be the result of increased carbon exudation and increased volumetric root growth. Increased exudation, correlated with greater shoot biomass, could support increased rhizobacterial diversity through the provision of generally metabolizable carbon compounds. Increased root growth would in effect explore a larger soil volume, incorporating a more diverse array of rhizobacteria.

The negative impacting factor acts to reduce rhizobacterial diversity which may be associated with a number of selectivity mechanisms initiated by the plant or microbial food web communities. Plants and the microbial food web have ways of selecting for specific members of the rhizobacterial community through regulation of pH, redox relationships, antibiotics, volatile chemicals, quorum sensing inhibitors, chemotropic agents, and predations or parasitism (17). Modeling using a third-degree polynomial results in an expression with both positive and negative coefficients. These coefficients may actually reflect the relative importance of both positive and negative factors on diversity and may be useful in estimating their association with productivity. Accordingly, the fact that the Simpson index showed the greatest modulation would imply that dominant OTUs are more actively selected by the plant than are rarer OTUs. Further research is necessary to confirm whither this approach quantitatively reveals aspects of the inner rhizosphere dynamics associated with rhizobacterial community diversity as affected by plant carbon exudation and rhizobacterial selectivity by the plant. However, this diversity analysis may provide another community-wide system parameter that may be useful in assessing soil-microbe-plant associations with overall soil health and productive capacity.

The pyrosequencing data from this study were based on DNA extracted from the rhizosphere soil, including roots, and were expected to contain plant DNA. Amplification of DNA using the so-called universal 16S rRNA primers would be expected to amplify plastid DNA sequences. Approximately 9.3% of all sequences recovered in this study but not included in the above analysis were identified by the RDP II Bayesian Classifier as *Streptophyta*, or root plastids. These were separated into 59 distinct OTUs, 30 of which were negatively associated with productivity. Surprisingly, numbers of root plastid 16S rRNA sequences were dramatically greater in the low-productivity group (Fig. 4). The reason for this is unknown, but it may be associated with infection or colonization by parasitic microorganisms (DRBs, fungi), especially in low-biomass plants. Plastids in roots are essential for the metabolisms of starch, amino acids, and fatty acids (29). Colonization by mycorrhizae has been shown to dramatically increase plastid production and encourage plastid reorganization (12). In low-biomass plants, increased plastid numbers may be actively stimulated and/or regulated by parasites in order to divert primary resources toward their own growth and development.

This study focuses on the rhizobacterial community exclusively and does not distinguish between endophytic bacteria and rhizosphere bacteria, since DNA extraction was performed on the whole root system. However, with minor adjustments, it is possible to identify both endophytic or rhizosphere bacteria associated

with productivity. The study is based on 29,007 sequences, which by today's standards is somewhat small. Larger studies with 10 to 20 times the numbers of sequences are easily possible, increasing the depth of sampling and the statistical reliability of the results. One study suggested that as many as 29 million reads are necessary to provide 90% coverage of the entire soil bacterial community for the 16S rRNA gene alone (33). Nevertheless, this small study identifies the most abundant of the productivity-associated rhizobacteria, which most likely represent the prime rhizobacterial drivers. Moreover, there are still recognized limitations associated with the DNA extraction and PCR amplification processes upon which this approach relies (27). Careful examination of rhizosphere and DNA extraction procedures, PCR amplification parameters, and the statistical and bioinformatic processes is necessary to further refine and enhance this procedure. Finally, this same approach can be used for the other microbial food web residents, including viruses, fungi, nematodes, protozoa, and micro- and mesofauna, in order to obtain a multitrophic view of the association with productivity. However, compared to their bacterial counterparts, the available PCR primers and databases for identifying these other organisms need much more development to be of comparable value. Nevertheless, even without identification the approach would still be informative given that individual OTUs are determined by the 16S rRNA gene sequence alone. Taxonomically unidentified OTUs would still represent unknown organisms and could still provide sequence tags to isolate the targeted organism or to obtain larger DNA fragments to supplement current DNA-based information for a more refined analysis.

Lastly, and most importantly, it must be understood that the results presented in this paper reflect the type of soil, the nutrient conditions, and the environment under which the experiment was conducted. Results may differ under different conditions. Only after extensive evaluation using an array of conditions will it be possible to identify bacteria generally associated with productivity for a given plant species, environmental condition, and soil type. Further research using this pyrosequencing-based approach will greatly assist in characterizing and utilizing the productive and the unproductive holobiont communities.

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