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ORIGINAL PAPER

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Taxonomic diversity of bacteria associated with the roots of modern, recent and ancient wheat cultivars

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Abstract Breeding programs for crop plants are designed to improve agronomic characteristics such as yield, fertilizer use efficiency and disease resistance. These programs do not typically consider interactions between plants and soil microflora. This study assessed the bacterial communities associated with roots of various spring wheat (*Triticum* spp.) cultivars of related lineage. Bacteria (*n*=ca. 1900) were isolated from the rhizosphere and root interior of *Triticum moncoccum* PI 167549 (an ancient land race that originated in Turkey), *T. aestivum* cv. Red Fife (historical spring wheat cultivar released in Canada ca. 1845) and *T. aestivum* cv. CDC Teal (modern cultivar registered in Canada in 1991) grown at two different field sites. Bacteria were identified by gas chromatography-MIDI (microbial identification software) fatty acid methyl ester analysis. Twenty-eight bacterial genera were identified as being associated with the three wheat cultivars, but only *Aureobacter* species differed significantly between cultivars with 16 isolates identified from the root interior of PI 167549 compared to one isolate from Red Fife and two from CDC Teal. In contrast, the bacterial endophytic community of the more modern cultivars was more diverse than that seen for the ancient land race. Increases in diversity were not limited to a single genus and some species were selected against. For example, pseudomonads were more numerous and diverse in the root interior (11 species identified in 117 isolates) compared to the rhizosphere (eight species identified in 94 isolates), but *Pseudomonas fluorescens* abundance decreased in the root interior compared to the rhizosphere. The fact that the roots of newer wheat cultivars were aggressively colonized by endophytic pseudomonads suggests that these bacteria might be exploited as plant growth-promoting rhizosphere bacteria or as a means to establish specific catabolic activities in these plants.

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Introduction

Plants influence the composition and dynamics of microbial communities present in the rhizosphere, and recent studies have assessed factors that influence the composition of these root-associated communities (Germida et al. 1998; Grayston et al. 1998; Lemanceau et al. 1995; Westover et al. 1997). Such studies are important as the composition of the rhizosphere community can significantly influence the development of phytopathogens (Nehl et al. 1997), nutrient acquisition (Lynch 1990), heavy metal resistance (Bradley et al. 1981) and ecological fitness of plants (Bever et al. 1997; Parker 1995). Recently, it has become apparent that root endophytic bacteria (i.e., bacteria colonizing the root interior) influence plant health (Chanway 1996; Hallmann et al. 1997). For example, some endophytic bacteria are known to promote the growth of plants (Shishido et al. 1999) whereas others can have an indirect effect on plant disease resistance (Wei et al. 1996). However, the function of many endophytic bacteria and their potential interaction with other endophytes such as arbuscular mycorrhizal fungi is not known. Our recent studies indicate that endophytic bacteria colonizing roots of canola and wheat represent a specific subset of the normal rhizosphere community (Siciliano et al. 1998).

It is likely that conventional and biotechnological plant breeding practices, which increase the agronomic potential of wheat, will also alter the root-associated microbial community. These alterations may point to novel ways to increase the agronomic potential of wheat by highlighting beneficial associations between plants and bacteria. In this context, several investigators have examined the influence of soil type (Lemanceau et al. 1995; Sato and Jiang 1996a, 1996b), cultivation techniques (Lupwayi et al. 1998), distance from root, i.e., the "rhizosphere effect" (Mavingui et al. 1992; Semenov et

al. 1999) and plant genotype (Rengel et al. 1998) on root-associated bacteria (Micanovic et al. 1997; Miller et al. 1990; Neal et al. 1970; Neal et al. 1973). These studies indicate that even a single chromosome substitution in the plant genotype (Neal et al. 1973) may alter the composition of the microbial community. However, the soil type in which the plant grows modulates the plant effect on the microbial community and the influence of the plant on the root-associated microflora changes during the growing season.

Recently, we assessed the microbial community associated with *Triticum moncoccum* PI 167549 (an ancient land race that originated in Turkey) (Hetrick et al. 1992), *T. aestivum* cv. Red Fife (historical spring wheat cultivar released in Canada ca. 1845) and *T. aestivum* cv. CDC Teal (registered in Canada in 1991) (Siciliano et al. 1998). These cultivars represent a continuum from ancient land races to modern cultivars. We analysed the fatty acid methyl esters (FAME) extracted from roots and rhizosphere soil, as well as the community level physiological profiles (CLPP) of the root and soil communities. Those results indicated that there was no difference in the microbial FAMEs or the utilization rate of Biolog functional guilds in the roots of the three wheat cultivars. We did find, however, that the correlation between the ability of endophytic and rhizosphere microbial communities to utilize carbon substrates was lower in CDC Teal compared with earlier cultivars. Here, we extend our previous FAME and CLPP analysis to species level analysis through the isolation and identification of culturable bacteria associated with the roots of these three cultivars. Our intent was to identify specific bacterial species that might explain our previous findings, and also to assess the impact of wheat breeding programs on potential beneficial plant-bacteria associations.

Materials and methods

Experimental design

The wheat cultivars PI 167549, Red Fife and CDC Teal were assessed using a randomized complete block design replicated 4 times at two different sites, i.e., each site had four replicates of all three cultivars. An individual replicate consisted of four randomly chosen plants from one block that were bulked together such that each replicate was a composite of four plants. Plants were harvested 41 days after planting. Field sites were considered random factors, i.e., sites were not chosen for a specific reason but rather as two fields drawn at random from available Saskatchewan sites, with cultivars (n=3) as fixed treatments and replicates (n=4) as the blocks. Field sites were near Kernen (Black soil climatic zone) and Saskatoon (Dark Brown soil climatic zone), Saskatchewan, Canada; selected soil characteristics are given in Table 1.

Sample processing

Plants and their associated root material were removed from soil with a trowel and placed in a plastic bag. Samples were immediately transported to the laboratory and processed <2 h after removal from the ground. The shoot was removed with a scalpel and the roots with adhering soil were sieved (5 mm) for 5 min. Rhizosphere and endophytic microorganisms were extracted as previously described (Siciliano and Germida 1999). To extract rhizosphere microorganisms, a 5-g portion of root with adhering soil was placed in a 1-l Erlenmeyer flask containing 495 ml phosphate-buffered saline (PBS; 1.2 Na_2HPO_4; 0.18 NaH_2PO_4; 8.5 NaCl (g l^{-1}); pH 7.6] and placed on a rotary shaker (200 r.p.m.) at 22°C for 20 min (Holl and Chanway 1992). This solution was serially diluted and 0.1 ml of the 10^{-4}, 10^{-5} and 10^{-6} dilutions spread plated onto triplicate plates containing 1/10 typticase soy broth (3 g l^{-1}) solidified with 1.5% agar (1/10 TSA).

Root-endophytic organisms (i.e., isolated from the root interior) were recovered after removing rhizosphere organisms and surface disinfecting. Roots (from rhizosphere sampling) were transferred into a 500-ml Erlenmeyer flask containing 200 ml NaClO (1.05% v/v) in PBS and placed on a rotary shaker (200 r.p.m.) at 22°C for 10 min. Roots were rinsed 4 times with 200 ml sterile PBS and 0.1 ml of the final wash diluted in 9.9 ml of 1/10 TSB to check for contamination (McInroy and Kloepper 1995). The roots were chopped into 1-cm sections and then triturated with a sterile mortar and pestle containing 10 ml sterile PBS. The root/PBS mixture was serially diluted in sterile PBS and 0.1 ml of the 10^{-1}, 10^{-2} and 10^{-3} dilutions spread plated onto triplicate plates of 1/10 TSA.

Isolation and identification of bacteria

After 72 h incubation at room temperature, one enumeration plate containing 50–300 colonies (i.e., typically the 10^{-2} root-endophytic and 10^{-5} rhizosphere dilutions) was selected from each replicate and the bacterial colonies numbered. A random number table was consulted and 50 colonies isolated from one plate for each plant replicate (Germida et al. 1998). Isolates were streaked twice on 1/10 TSA and purified strains stored on 1-ml stabs containing 1/10 TSA, overlaid with sterile glycerol and maintained at 4°C.

Isolates were identified based on whole-cell fatty acids, derivatized to methyl esters (i.e., FAMEs) and analysed by gas chromatography (Germida et al. 1998). Bacterial isolates were analysed using the MIDI microbial identification software (Sherlock TSBA Library version 3.80; Microbial ID). The FAME profile of *Xanthomonas maltophilia* ATCC 13637 was used as a reference strain for the MIDI determinations. Strains with a similarity index (SIM) of 0.3 or greater were considered positively identified, whereas strains with a SIM of <0.3 were considered tentatively identified.

Table 1 Selected characteristics[a] of the A horizon soils. *EC Electrical conductivity*

Site	Texture	pH	EC (dS m^{-1})	NO_3-N (µg g^{-1})	P	K	SO_4-S
Kernen	Clay loam	7.5	0.9	21	12	>270	7
Saskatoon	Clay loam	7.5	0.9	22	24	>270	22

[a] Soil characteristics determined by Enviro-Test Laboratory Services, Saskatoon, Saskatchewan: pH, 1:2 soil water; EC, 1:2 soil:water (Janzen 1993); NO_3-N, $CaCl_2$ (Maynard and Lalra 1993); P, modified Kelowna (Qian et al. 1994); K, modified Kelowna (Qian et al. 1994); SO_4-S, $CaCl_2$ (Combs et al. 1998)

Statistical analyses

The genera composition of rhizosphere and root-endophytic communities were compared using the Shannon-Weaver diversity index (H'), Carmago's evenness index (E_{var}) and genus richness (Germida et al. 1998). The Shannon-Weaver index combines measurements of richness with those of evenness, whereas Carmago's evenness index is an estimate of the variance in genera abundance over the number of genera, with 1 being the maximum evenness and 0 the minimum (Smith and Wilson 1996).

Results and discussion

Isolation and identification of bacteria obtained from field-grown wheat

The taxonomic identities of 28 genera from approximately 1,900 root-associated bacteria isolated from the roots of the wheat cultivars PI 167549, Red Fife and CDC Teal grown at two field sites were determined. Of these 1,900 isolates, 32% (604 isolates) could not be identified by the MIDI system. A further 13% (240 isolates) were identified with a SIM <0.3 which indicates a tentative identification, and were not included in further analysis. Identifying endophytic bacteria (i.e., 60% identified) was more successful than rhizosphere isolates (i.e., 50% identified) with no difference seen between cultivars. These results, i.e., 55% of isolates identified, were considerably better than what was previously seen for isolates from canola, in which only 30% of isolates could be identified (Siciliano and Germida 1999), but were similar to that previously found for wheat (41%) (Germida et al. 1998). It is difficult to compare these results to other studies which report identification rates of 80–95%, as those studies do not report the SIM level required for identification (Lilley et al. 1996) or use a SIM level (McInroy and Kloepper 1995) considerably lower (e.g., SIM >0.1) than the one used in this study. Nevertheless, MIDI libraries allow identification of approximately 60% of culturable root-associated bacteria, and this level will improve as the database is expanded.

The endophytic community of the ancient land race, PI 167549, was less diverse than that of recent cultivars such as CDC Teal or Red Fife (Fig. 1). In contrast, the diversity of rhizosphere bacteria was higher in ancient land races compared to cultivars that are more recent, but this difference was not statistically significant. Plant root exudation significantly influences bacterial diversity in the rhizosphere (Atkinson et al. 1975; Di Cello et al. 1997; Latour et al. 1996; Miller et al. 1990; Rovira 1965), and differences in exudates between recent and ancient land races may explain why bacterial diversity in the rhizosphere differs between these cultivars. The overall diversity of endophytic bacteria did not differ from that of rhizosphere bacteria (P<0.280). This result is similar to that seen with sugar beet (*Beta vulgaris*) (Lilley et al. 1996) but differs from our previous results for canola in which the rhizosphere communities were more diverse than endophytic ones (Siciliano and Germida 1999). Both sugar beet and canola have coarse root systems, suggesting that the difference in root architecture between wheat and canola is not the sole explanation for the higher endophyte diversity observed in wheat. It is likely that bacterial diversity in the rhizosphere and root interior depends on the interaction between root exudates composition and quantity as well as root system architecture.

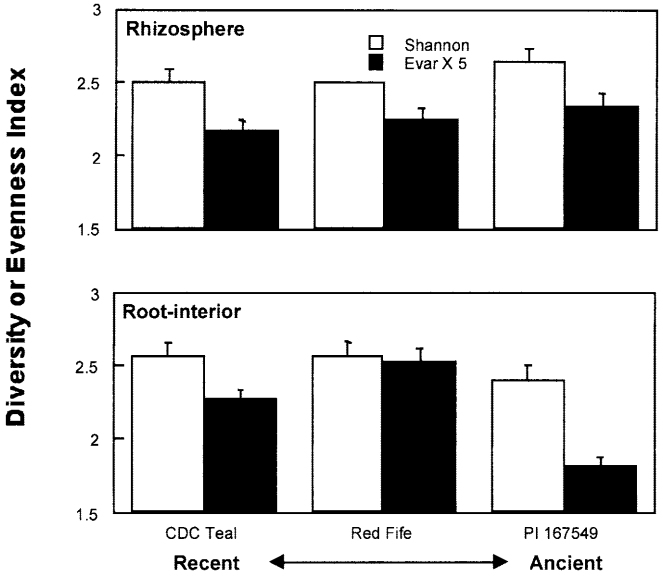


Fig. 1 Shannon diversity index and Carmago's evenness index (E_{var}) of bacterial communities associated with the roots of three wheat cultivars. *Bars* are the mean of bacterial communities (50 isolates) obtained from eight plant samples of each cultivar. *Error bars* are SEMs

The effect of plants on the diversity of root-associated bacteria cannot be generalized, but instead is dependent on the bacterial genera, and perhaps the species, being studied (Sato and Jiang 1996a, 1996b). In this study, the diversity of pseudomonads increased in the root interior compared to the rhizosphere. Eleven species were found amongst the 117 endophytes isolates compared to only eight species identified in 94 rhizosphere isolates (Fig. 2). *Pseudomonas cichorii* (one isolate), *P. mendocina* (five isolates) and *P. viridiflava* (three isolates) were all found in the root interior but not the rhizosphere of wheat. Perhaps more important is the fact that *P. putida* was an aggressive endophyte, as this organism is well recognized as a beneficial plant growth-promoting rhizosphere bacterium (Shah et al. 1998). In contrast to the effect of plants on pseudomonad diversity, the diversity of bacilli was greatest in the rhizosphere with 11 genera found among 44 isolates, whereas only eight genera were found among 25 endophytic isolates (Fig. 2). Noteworthy is the dominance of *Bacillus megaterium* in the rhizosphere as this species is well known for its plant growth-promoting activity (Chanway et al. 1988) as well as its ability to degrade starch and other plant carbohydrates. Other investigators have also found that the diversity of bacilli (e.g., *Bacillus polymyxa*) was greatest in non-rhizosphere and rhizosphere soil compared to that for isolates from the rhizoplane (Mavingui et al. 1992).

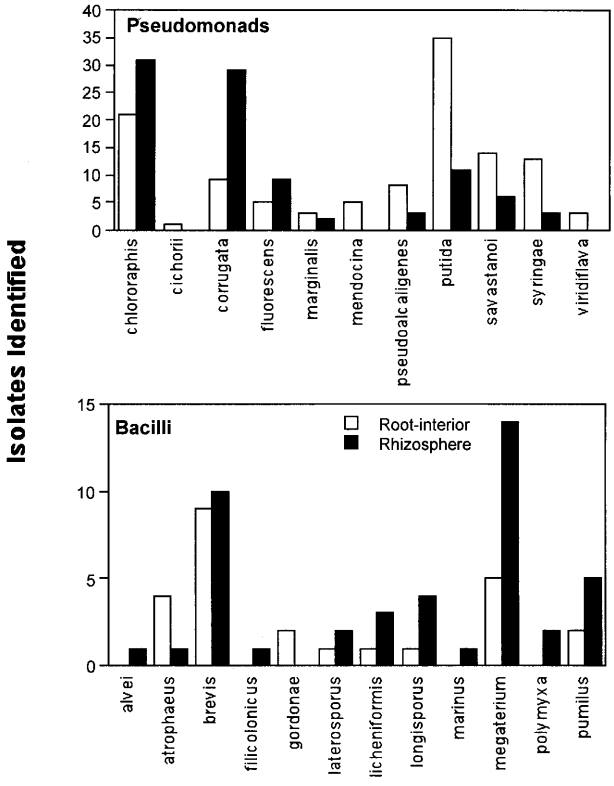


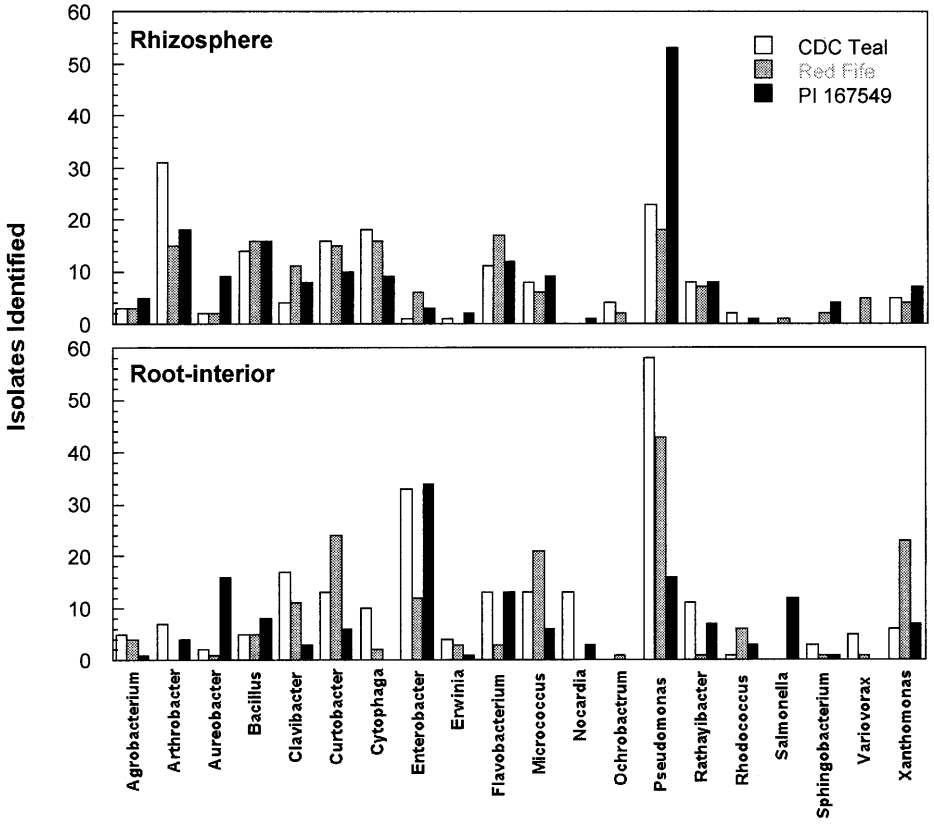
Fig. 2 Species abundance of pseudomonads and bacilli associated with the rhizosphere or root interior of wheat cultivars. Approximately 470 rhizosphere and 510 root-interior isolates were identified from 24 plant samples of three different wheat cultivars, CDC Teal, Red Fife and PI 167549

Differences in the effects of plants on bacterial abundance are also seen at the species level (Sato and Jiang 1996a, 1996b). For example, we found that despite the generalized increase in pseudomonad diversity in the root interior, only five isolates of *P. fluorescens* were found in the root interior but nine were found in the rhizosphere. Miller et al. (1990) also found very low levels of fluorescent pseudomonads in the root interior compared to the rhizosphere of spring wheat (Miller et al. 1990). Similarly, Latour et al. (1996) found that the diversity of fluorescent pseudomonads in the root interior of tomato or flax was lower than that seen in the rhizosphere (Latour et al. 1996). This decrease in diversity highlights opportunities for the new development of plant growth promotion by identifying which pseudomonad species are best capable of colonizing the root interior. For example, Wei et al. (1996) and Shishido et al. (1999) demonstrate the importance of endophytes to plant fertility and growth promotion.

Effect of cultivar on root-associated bacteria

There were two genera, *Aureobacter* and *Salmonella*, associated with roots of the ancient land race which were detected only rarely in the more modern cultivars (Fig. 3). Sixteen isolates of *Aureobacter* were identified from the root interior of PI 167549, but only one isolate in Red Fife and two in CDC Teal. This trend was also seen in the rhizosphere where more *Aureobacter* isolates were obtained from PI 167549 (nine identified) com-

Fig. 3 Cultivar dependence of genera abundance in three different wheat cultivars, CDC Teal, Red Fife and PI 167549. Fifty bacterial isolates were identified from eight plant samples of each cultivar. In total, 470 rhizosphere and 510 root-interior isolates were identified with a similarity index >0.3



pared to Red Fife (two identified) or CDC Teal (two identified). Lilley et al. (1996) found that the prevalence of *Aureobacter* was greater in the root interior of sugar beet compared to rhizosphere soil, which is similar to that seen here. Similarly, 12 isolates of *Salmonella* were identified in the root interior of PI 167549, but no similar isolates were found in Red Fife or CDC Teal. The presence of *Salmonella* in the rhizosphere was unexpected. To the best of our knowledge, *Salmonella* has not been found in association with plant roots by other investigators nor in our previous investigations. In fact, 11 of the 12 *Salmonella* isolates had SIM indices below 0.42 and one isolate was identified with a 0.535 SIM as a *Salmonella typhimurium* GC subgroup B. However, all of the *Salmonella* isolates were within 0.1 SIM or less of being keyed out and identified as *Enterobacter agglomerans* (*Erwina herbicola* group), a more typical rhizosphere bacterium. Endophytic pseudomonads were predominant in more modern cultivars like CDC Teal (58 identified) or Red Fife (43 identified) compared to PI 167549 (16 identified). In contrast to the results seen for the root interior, pseudomonads were dominant in the rhizosphere of PI 167549 (53 identified) but not in Red Fife (18 identified) or CDC Teal (23 identified) (Fig. 3). Similarly, *Salmonella/Enterobacter* isolates commonly found in the root interior of PI 167549 were not found in the rhizosphere of this cultivar and one isolate was identified in the rhizosphere of Red Fife. Atkinson et al. (1975) postulated that differences in the composition of root exudates, rather than quantity, control bacterial diversity in the rhizosphere and may explain differences commonly seen between cultivars of wheat.

In this study, the effect of plants on the diversity of root-associated microorganisms was best resolved at the genera level. Furthermore, there was significant variation between the bacterial genera associated with modern and older wheat cultivars. For example, some bacteria such as pseudomonads were more abundant in the rhizosphere of older cultivars, but were the most dominant endophytes in the newer cultivars. The reasons for these differences are not known. The CLPP analyses of our previous study (Siciliano et al. 1998) suggested that modern cultivars had root morphologies or chemical composition that affected the ability of certain rhizosphere bacteria to colonize the root interior. The abundance of pseudomonads detected in this study confirms this idea. Furthermore, the fact the roots of newer wheat cultivars are aggressively colonized by endophytic pseudomonads suggests that these bacteria could be exploited as plant growth-promoting rhizosphere bacteria or as a method of increasing the catabolic versatility of plants in phytoremediation systems.

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