Microbiome of the Cool Season Forage Grass Timothy (*Phleum pratense* L.) and Its Potential Role in Stress Tolerance

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Key words: Timothy; endophytes; biofilm; chemotaxis; growth promoting bacteria; *Brachypodium*.

Abstract

The functional attributes of the microbiome associated with timothy for growth promotion properties, antimicrobial and biosurfactant capacities were characterized. A total of 254 culturable bacteria were identified by 16S rRNA sequencing and grouped into 16 taxa that shared high homology of 98–99% with other known sequences. The majority of bacterial isolates exhibited multifunctional growth promotion attributes and plant stress improvement. The selection of competent and compatible strains for application in forage production is dependent on the recognition of root exudates and motility towards the roots, attachment to the root surface, formation of biofilm, penetration, and colonization of internal tissues. We selected competent rhizospheric bacteria to generate a multispecies consortium made of three strains that displayed growth-promoting abilities in timothy through the production of IAA, volatile organic compounds that increased root biomass, the production of siderophores and antibiotic resistance, as well as the ability to colonize plants. This study demonstrated that the multispecies consortium displayed biofilm formation and chemotactic behaviour towards several organic acids and towards root exudates released from the model grass *Brachypodium distachyon*. Organic acids were successful in stimulating the formation of biofilm of the multispecies consortium. In particular, fumaric and malic acids enhanced selective recruitment of the multispecies consortium in a dose-dependent manner thereby, promoting biofilm formation on root surface as demonstrated in SEM micrographs. The multispecies consortium exhibited biofilm-related traits including the production of exopolysaccharides (EPS) and alginate. EPS amounts were comparable in single strains and consortium forms and alginate production increased by 160% when the consortium was subjected to drought stress. These findings demonstrated that plant-microbe interaction is the hub of various factors directly affecting this balanced dual relation and that root exudates could be very selective in recruiting highly qualified multispecies consortium.

Introduction

Plant-growth-promoting rhizobacteria (PGPR) are associated with plant roots and augment plant productivity and immunity.. The significant beneficial effect of rhizobacteria on plant growth are achieved by phosphorus solubilization, more nitrogen fixation, production of siderophores and biosurfactants along with their capacity to synthesize an array of metabolites like hormones and organic acids capable of assisting the plants and increasing their productivity under biotic and abiotic stress (Numan et al. 2018).

Successful and effective colonization of plant roots by rhizospheric PGPR depends on the exudates excreted by roots in the rhizosphere. Root exudates consisting of organic acids, free sugars and amino acids are essential components of rhizodeposits (Jones et al. 2009). It is established that low molecular weight carbon compounds such organic acids from the roots represent one of the essential drivers of bacterial activity and diversity in the rhizosphere (Eilers et al. 2010) that influence the microbial community surrounding the root system in the rhizosphere Components of root exudates are reported to mediate both positive and negative interactions in the rhizosphere chemotactic response and biofilm formation of rhizospheric bacteria (Rudrappa et al. 2008).

Soil bacteria belonging to different genera, exist in their natural environment in close proximity where fitness of a single cell depends on the interaction and cooperation with other cells in the population (Cavaliere et al. 2017). This cooperation, referred to as syntropy, among different bacterial genera stimulates key processes that benefit plant growth and health and governs metabolism and growth among diverse microbes in natural settings (Kouzuma et al. 2015). Thus, a combination of microorganisms in the form of mixed inoculants that interact synergistically is a feasible strategy for increased activity and better viability of plant-growth-promoting rhizobacteria (PGPR). Additionally, studies reported that multispecies consortia are more effective in their biological function than monocultures of single species (Seneviratne et al. 2008). Therefore, it is essential to use selective PGPR as consortia, which are compatible and able to produce certain phytohormones and exhibit assorted biochemical functions. The understanding of application multispecies consortium capable of producing phytohormone will serve as the basis for future research to elucidate the role of bacterial communities in crop productivity and sustainable agriculture.

The overarching goal of this study was to (i) characterize at the molecular and biochemical levels the bacterial isolates associated with tissues of timothy cultivars grown under field conditions, (ii) select the top performing isolates, in relation to phytohormone production and other growth promoting attributes, in the form of multispecies consortium, (iii) determine whether organic acids released from root exudates of the model grass *Brachypodium* act as an attractant to the multispecies consortium by inducing chemotactic response and biofilm formation of the consortium compared to single species inoculum.

Methods and Study Site

Three field sites located in Sainte-Anne-de-Bellevue, Québec, Canada were selected for sampling. Sampling was conducted in October 2016. A total of three replicate samples (four plants per sample), their associated rhizosphere soil and surrounding bulk soil were randomly collected from every field and processed within 48 h. Leaves and crowns from each sample were cut into sections of 0.5 cm in width and surface sterilized by stepwise washing procedure with ethanol and sodium hypochlorite (Schulz et al. 1993) and processed separately. Samples of the rhizosphere and bulk soils of each field site were pooled and three subsamples (10 g each) were processed separately. Bacterial colonies were grown on specific media and the emerging bacterial colonies from plant tissues and from both types of soils were preserved in glycerol and stored at -80°C.

A range of functional biochemical tests were performed in triplicate to characterize the endophytes' traits for growth promotion These tests include the estimation of indole 3-acetic acid (IAA), 1-aminocyclopropanecarboxylic acid (ACC), the production of volatile organic compounds (VOC), and a series of enzymatic tests to select bacterial isolates exhibiting plant's colonization and antimicrobial traits and stress tolerance tests as detailed in Saleh et al. (2019).

Bacillus species strains 28 and 144, and *Microbacterium* sp. strain 50 isolated from the rhizosphere of timothy were selected because of their growth-promoting abilities in timothy. The compatibility and interaction among the three bacteria of the consortium was tested using the Burkholder agar diffusion assay (Burkholder et al. 1944) and through the co-culture plating method. The response of the multispecies consortium to organic acids was quantified using a modified capillary chemotaxis assay (Mazumder et al. 1999) and qualitatively evaluated using the drop assay in carbon-free medium (Kadouri et al. 2003). To study the effect of root exudates of plants on the chemotaxis and biofilm formation of the selected strains, *Brachypodium distachyon* Bd21 was grown as previously described (Saleh et al. 2020). The effect of root exudates and organic acids on the formation of bacterial biofilm was determined in 96 microtiter plates. Additioanlly, biofilm traits, including exopolysaccharide and alginate production, hydrophobicity and swimming and swarming motility were studied .

Results

Diversity, distribution, and multi-functional attributes

A total of 476 culturable bacterial isolates were recovered from three replicate samples of bulk and rhizosphere soils and crown and leaf tissues (endosphere) of timothy grown in the different fields. Percent distribution of bacterial isolates in the three fields was comparable, with field 3 having the greater proportion of the total number of isolates (36%). The genus *Arthrobacter* was cultivar-specific to Champ, while the genus *Pedobacter* was predominantly associated with the cultivar AC Alliance. More than 60% of the culturable isolates were recovered from different fractions of the soil with the majority (41%) of them recovered from the rhizosphere soil. Leaf and crown tissues harbored 106 and 65 isolates of culturable endophytes respectively. A total of 254 isolates were associated with five different phyla (Actinobacteria, α- and β-Proteobacteria, Bacteroidetes and Firmicutes) and 16 discrete genera that shared high homology (91 to > 99%) with known bacterial sequences in GenBank. The most frequently isolated bacteria belonged to the Gram-positive bacterium, *Bacillus* (28%), the Actinobacteria *Streptomyces* (6%) and the Gram-negative genera, *Pseudomonas* (7%), *Pedobacter* (5%) and *Pantoea* (5%). Our results show that a greater portion of the relative abundance of the bacterial microbiome (64%) is associated with the bulk and rhizospheric soil fractions than the plant tissue and that the proportion and diversity of the genera inhabiting the rhizosphere soil (41%) was greater when compared with the surrounding bulk soil (24%). Of particular interest, is the enrichment and sole presence of rhizosphere competent isolates such as *Curtobacterium*, *Pedobacter*, *Streptomyces* and *Variovorax*, indicating that selectivity of timothy root to certain rhizosphere bacterial taxa could be influenced by root exudation and hostderived metabolites leading to differences in the bacterial community structure between rhizosphere and bulk soil.

OPLS-DA analysis revealed a variable grouping among the bacterial isolates based on the recorded biochemical features. Bacteria sharing the same biochemical attributes were clustered close together (95% confidence interval). In total, 60 isolates belonging to 16 different genera were selected for additional assays related to volatile compounds production, antifungal activity, and biosurfactant-producing properties. Calcite solubilization, the production of siderophores showing varying intensities of CAS substrate hydrolysis and biosynthesis of the plant growth promoting hormone IAA, are the most commonly occurring functional traits of isolates across the 60 isolates. The release of volatile organic compounds by isolates (28, 50, 63, 120, 144, 295, 464) significantly ($P \leq 0.05$) triggered growth promotion in grass seedlings for a period of 12 days compared to the control treatments. The best performing isolate was *Bacillus* (28) which caused a 32-fold increase in seedling's dry weight when compared to the control treatment. The same isolates were excellent IAA producers. Out of 60 isolates, many bacterial isolates exhibited varying degrees of antifungal activity against five phytopathogens of different lifestyles. Surprisingly, the same isolates (isolates 33, 70, 12, 17 and 48) that displayed strong antifungal activities consistently displayed a strong biosurfactant activity and an antimicrobial activity. The highest resistance was detected with hygromycin which means that these microbes are capable of competing with the surrounding bacteria in the microbiome without being suppressed by their presence. Furthermore, these beneficial microbes had clear phenotypic effects on their hosts. Timothy seedlings inoculated with live bacterial isolate (120) had more root mass compared to the control treatments. SEM micrographs revealed that more hair roots are formed in bacterized roots compared to non-bacterized roots.

Organic acids and root exudates of Brachypodium distachyon: their effects

Assessment of the compatibility of strains (i.e., 28, 50 and 144) showed that they all grew similarly whether inoculated alone or in combination with one or two strains. Other traits were also evaluated like EPS production, swarming and swimming characteristics. The amount of EPS produced by individual strains in monoculture and when co-cultured in a multispecies consortium was similar and the increase in the hydric stress has led to substantial increases in alginate ranging from 160% increase in multispecies consortium to 80 % in *Microbacterium* strain 50 compared to media without PEG. On the other hand, the swimming and swarming motility of each of the bacteria was quantitatively measured at 24 and 48 h of incubation. After 24 h of incubation, strains 28, 50 and 144 displayed similar swimming and swarming motility ($p \leq 0.05$) while after 48 hours of incubation, swimming and swarming motility of bacteria increased. The hydrophobicity of the bacterial strains as monoculture and as multispecies consortium was quantified as the fraction of bacteria adhered to the hydrocarbon phase. All strains were similar in their hydrophobic capacity. The capillary chemotaxis assay revealed that the multispecies consortium was attracted to a variety of organic acids. Generally, the consortium cells migration into the syringe were positively correlated to certain organic acid at specific concentrations. The capillary chemotaxis assay revealed that the multispecies consortium was attracted to a variety of organic acids. Significantly high numbers of cells compared to their relative control were observed in both concentrations of fumaric acid at 25 and 50 µM/L. Chemotaxis was also evaluated qualitatively. All tested organic acids (succinic acid, fumaric acid, citric acid, oxalic acid and malic acid) and concentrated root exudates of *Brachypodium* initiated a chemotaxis response on cells from the multispecies consortium compared to bacteria and chemotaxis buffer. Compared to the buffer solution that served as control, consortium cells formed an unusual large ring of turbidity near the center of each organic acid within the first 30 min indicating that a chemotactic response of the consortium was triggered. The *Brachypodium* root exudates (concentrated 50 times) had a similar but intense pattern. The ring of turbidity formed indicating that exudates were actively attracting bacteria. Also, the biofilm formation by single species and by the multispecies consortium was evaluated in microtiter plates at 24 and 48 h. The biofilm formation by single species depended on the organic acid type and concentration, and time of incubation. In parallel, the effect of root exudates on biofilm development in the multispecies consortium was also studied on microtiter plate. Biofilm development increased at 10µL of root exudates (50× concentrated) originating from *Brachypodium* seedlings inoculated with the consortium by 32% compared to the control at 48 h. Bacterial strains were visualized for their single and mixed biofilm formation on glass coverslips and on the root surfaces of *Brachypodium*. Strains 28, 50 and 144 formed strong but different biofilm phenotypes as well as when mixed in the consortium.

Discussion

This study reported for the first time the abundance, diversity and distribution of bacteria associated with different tissues of two cultivars of timothy grass (*Phleum pratense* L.) grown under field conditions, along with their biochemical and molecular characterization. Despite several studies related to bacteria associated with grasses, an in-depth study on the use of bacteria on cool season grasses, such as timothy is lacking. Hence, studies such as ours using strains isolated from timothy on the model grass *Brachypodium* help to bridge the gaps leading to a possible overview for the kind of relation between bacterial isolates and forages.

Following a series of cultural and biochemical tests, bacterial strains were studied for their growth promoting attributes and the capacity to sustain biotic and abiotic stress conditions through the production of indole-acetic acid, siderophores, HCN and a large array of enzymes that are capable of making nutrients more available to the grass, as well as enzymes capable of degrading cell walls of fungi. These attributes have accelerated the selection of efficient strains successfully colonizing the internal parts of plants tissues.

This study allowed us to gain insight on the effect of individual organic acids and root exudates released from *Brachypodium distachyon* on the multispecies consortium. Features, like the competency and compatibility of single free cells behaving like a multicellular entity were documented. In addition, the study provided information on bacterial biofilm traits, including motility, alginate and exopolysaccharide production and their chemotactic behavior towards pure organic acids and root exudates. All these traits enhanced the chances of rhizospheric bacteria to successfully colonize the host. And further, presented knowledge on specific organic acid(s) released from *Brachypodium* roots which may have modulated the chemotactic response and biofilm formation of multispecies consortium,

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