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Identification and Characterization of Nitrogen Fixing Bacteria Associated with Kudzu Root Nodules

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IDENTIFICATION AND CHARACTERIZATION OF NITROGEN FIXING BACTERIA ASSOCIATED WITH KUDZU ROOT NODULES

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ABSTRACT

Pueraria montana, better known as kudzu, is an invasive species rapidly spreading throughout the southeastern United States. This plant can form root nodules which house nitrogen-fixing bacteria, allowing atmospheric nitrogen to be converted into biologically available forms of nitrogen for use by the plant host. Given the centrality of these bacteria to the spread of kudzu, isolates from nodules were characterized after collection from seven different locations across the metropolitan Atlanta area. Twenty-five isolates were grown on two different variants of nitrogen free media. Four different carbon sources were evaluated as well. Finally, growth under both aerobic and anaerobic conditions was investigated. Almost all isolates grew better under anaerobic conditions. Additionally, the carbon source and other components of the composition of the media affected growth. These data suggest significant metabolic diversity inside a relatively small geographic area posing questions about the relative contribution of nitrogen fixing bacteria to kudzu's invasive expansion in this region. In addition, four possible "promiscuous ineffective" isolates were identified using data evaluating relative growth, possibly reflecting reduced nitrogen fixation and corresponding benefit to the host. The kudzu can be described as "promiscuous ineffective" because it allows nodulation of bacteria that have very poor nitrogen fixing capabilities. Two sequences, 16S rRNA and the gene *nif*D, were amplified from these four isolates. The 16S rRNA sequence reveals minor evolutionary diversity amongst isolates. Analysis of *nifD* reveals variations between isolates and some correspondence with an ability to fix nitrogen. With these data, further characterization of the "promiscuous ineffective" isolates may reveal the mechanism of reduced fixation rates and provide insight into possible bioremediation of kudzu.

Keywords: phylogenetics, nitrogen fixation, bacterial symbionts, kudzu

INTRODUCTION

A prevalent theory about microbial biogeography is that "everything is everywhere, but the environment selects" (O'Malley 2007). However, recent work has begun to indicate that selection may be insufficient to explain the distinct distribution of taxa found

in different locations for certain environments (Martiny et al. 2006; Livermore and Jones 2015). For example, hot springs with near-identical environmental parameters can have very different microbial communities if the sites experienced different historical disturbances (Martiny et al. 2006; Livermore and Jones 2015). Soil appears to be one of the habitats where local factors like dispersal may play an important role in species richness and abundance (Martiny et al. 2006; Livermore and Jones 2015; O'Malley 2007). This observation is especially important for plants that are involved in horizontally transferred mutualisms, such as legumes.

Legumes recruit N_2-fixing bacterial symbionts from the surrounding soil and form specialized structures, nodules, that facilitate the exchange of photosynthate with glutamate. This symbiosis requires N_2-fixing members of specific groups of α- and β-proteobacteria. These symbiont bacteria possess a suite of genes including the essential *nod* and *nif* genes responsible for the establishment of the symbiosis and subsequent nitrogen fixation. This could suggest a strong level of specificity between plant and symbiont taxonomy. But among legumes, both promiscuous and stringent patterns of symbiont-plant interactions can be observed (Andrews and Andrews 2017).

Members of the Fabaceae family, including vetches, kudzu, and chickpeas, reflect a diversity of host-symbiont strategies. Some members of the Fabaceae family, such as rooibos (*Aspalathus linearis*), form nodules with quite divergent lineages, while others, like chickpeas (*Cicer arietinum*), can only form nodules with a single species (*Mesorhizobium*) (Livermore and Jones 2015). However, promiscuity could be an advantage for potentially invasive legumes like kudzu (genus *Pueraria*). Work on the tropical kudzu (*Pueraria phaseoloides*) suggested that this legume was capable of nodulating with a variety of different symbionts (Sylvester-Bradley et al. 1991). However, the strain of symbiont appeared unrelated to the total nitrogen yield at the time of harvest. These data indicate that this plant is "promiscuous ineffective" meaning it can nodulate with many partners but not all nodules improve nitrogen acquisition by the plant. Recent work with *P. phaseoloides* suggests that inoculation with some strains of symbionts may significantly improve plant biomass (an 81% increase in dry weight between inoculated and uninoculated controls) (Sylvester-Bradley et al. 1991).

Symbionts of invasive legumes are especially important because of the ecological disturbance the host can cause. In addition to outcompeting native species, exotics can induce serious biogeochemical changes (Liao et al. 2008). This disturbance is even more pronounced when the invasive plants are woody or associated with nitrogen fixation (Liao et al. 2008). The legume kudzu (*Pueraria montana*) is an invasive species originally from Japan and now found throughout the southeastern United States. Kudzu alters biogeochemical cycles in the Southeast profoundly, doubling the nitric oxide emissions from soil after invasion of fallow fields (Hickman et al. 2010). Nitric oxide can photoreact to form ground level ozone, thus representing a serious negative impact on regional air quality. Also, *P. montana* has been shown to alter carbon flux in pine forests upon invasion by causing a 28% decrease in soil-sequestered carbon and a 50% increase in the proportion of oxidation-resistant soil organic matter (Tamura and Tharayil 2014).

Pueraria montana poses a unique problem given predicted range expansion due to anticipated alterations in climate (Hickman and Lerdau 2013). With the expected northward spread of kudzu, both ecosystem integrity and air quality may be impacted. If *Pueraria montana* is both promiscuous and affected by the efficacy of the partnering symbiont, understanding how this legume recruits suitable symbionts for nitrogen

depleted soils can contribute to possible strategies for prevention of the worst effects of expansion.

In this study, we isolated twenty-five *P. montana* nodule symbionts from around metropolitan Atlanta, Georgia, for biochemical and phylogenetic characterization. We examined correlations between geography and growth dynamics using two different selective media, each amended with different carbon sources and aerobic versus anaerobic growth conditions. We postulated that, given geographic proximity, these strains would share growth dynamics with nodules isolated from nearby plants. We also probed the cause of exceptionally slow growth from one location via phylogenic analysis of the 16S rRNA sequence as well as *nifD* (the alpha subunit of nitrogenase) gene sequences.

MATERIALS & METHODS

Pueraria montana nodules were collected from seven different locations around metropolitan Atlanta: Lawrenceville, Winder, East Point, Duluth, Marietta, College Park, and Henderson Park (Figure 1). Briefly, nodules were washed in sterile water, soaked in 95% ethanol for 5 min while vortexing, washed twice with sterile water, and macerated in a sterile mortar and pestle with 1 ml of sterile water. The mixture was then plated onto nutrient agar (Fisher Scientific).

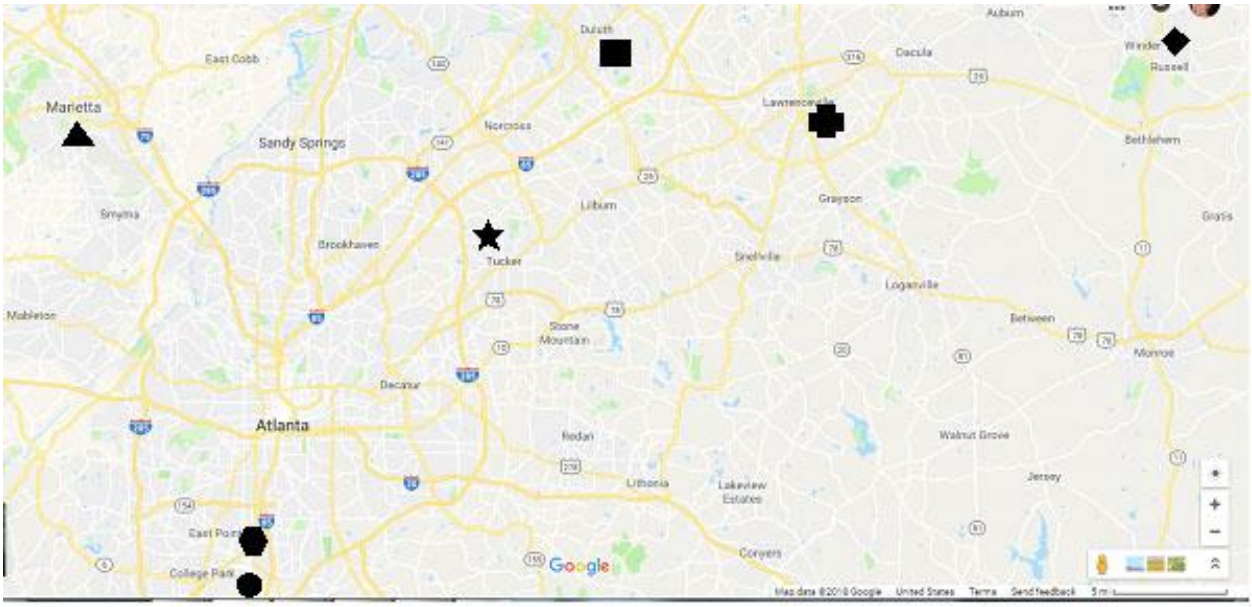

Figure 1. Locations in metropolitan Atlanta area where kudzu (*Pueraria montana*) was sampled. A total of twenty-five nodule-associated bacteria were isolated from College Park ●, East Point ⬢, Marietta ▲, Henderson Park ★, Duluth ■, Lawrenceville ✚, and Winder ◆.

Colony morphology, Gram stain, and motility were assessed from the nutrient agar plates. Isolates that were not small (< 5 μm) Gram-negative rods capable of growing on nitrogen free ($CaCl_2$, 1g; K_2HPO_4, 1g; $Mg_2SO_4 \cdot 7H_2O$, 0.2g; NaCl, 0.2g; $FeSO_4 \cdot 7H_2O$, 0.1g; $Na_2MoO_4 \cdot 2H_2O$, 5 mg) media were discarded from further study. Growth rate was

assessed using a spectrophotometer using four different media under both aerobic and anaerobic conditions (Genesys 20, Fisher Scientific). To ensure growth rates of different cultures could be compared using spectrophotometry, standard curves of CFU/ml corresponding to OD were made using exponentially growing cultures of five of the twenty-five isolates. All cultures assessed were inoculated to a standardized optical density of 0.01 +/- 0.005. The optical density of cultures was measured in the range of 1 to 0.1 OD; if turbidity exceeded 1, the sample was diluted. Time points of 24 hours and 48 hours were used for analysis because all isolates had achieved detectable levels of growth at these time points.

Nitrogen free media with 20% sterile filtered glucose or sucrose as well as Norris media (K_2HPO_4, 1 g; $Mg_2SO_4 \cdot 7H_2O$, 0.2 g; $CaCO_3$, 1 g; NaCl, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.1 g; and $Na_2MoO_4 \cdot 2H_2O$, 5 mg) with sterile filtered 20% glucose or 20% mannitol was added to the media for a final concentration of 1%. Growth of 25 isolates (4 Winder, 5 Lawrenceville, 5 Henderson Park, 4 Marietta, one East Point, 4 Duluth, and 2 College Park) was assessed by measuring optical density at 24 and 48 hours after inoculation. All cultures were grown at room temperature in triplicate under microoxic conditions using a GasPak (Becton Dickinson & Company), inside an anaerobe jar or under oxic conditions without agitation. Biochemical capabilities were assessed by growth on nitrate reduction broth (Difco Laboratories) and Triple Sugar Iron slants (Hardy Diagnostics). Analysis of growth data was done in Excel. These growth and biochemical data were analyzed in Excel using a one-way ANOVA, Tukey's honestly significant difference Scheffé multiple comparison as well as a Bonferroni and Holm multiple comparison.

Three isolates from Henderson Park were identified as the slowest growers under several conditions and were selected for further study. Cultures were grown overnight under anaerobic conditions at room temperature and 1 ml of cell suspension was pelleted through centrifugation (1 min, 13,000 rpm). The pellet was resuspended in 1 ml of lysis buffer (2 mg/ml lysozyme, 0.5% sodium dodecyl sulfate, and 0.5 mg/ml Proteinase K) and incubated at 37 °C for 20 min. Phenol chloroform extraction and ethanol precipitation were then conducted and the DNA pellet was resuspended in Tris EDTA buffer before calculating DNA yield and purity via spectrophotometry.

Both the 16S rRNA gene and the *nifD* gene were amplified from the DNA of five isolates. 16S rRNA genes were amplified using primers 8F and 1492R and PCR conditions previously reported (Brosius et al. 1991; Adane 1991). The alpha chain of nitrogenase (*nifD*) was amplified using NifDF (5' ATGACCSYCAMGKTYGAGGAA 3') and NifDR (5' AGGTCCTCGTAGGCGCCGATG 3') based on an alignment on 10 α- and β-proteobacteria (96 °C, 30 s; 54 °C, 20 s; 72 °C, 1 min; 30 cycles). Sequencing was conducted at the Georgia Genomics and Bioinformatics Core facility (Athens, Georgia). Geneious software was used for alignment and phylogenetic tree construction (Lane 1991) and Quick 2D was used for secondary structure analysis (Zimmerman et al. 2017; Kearse et al. 2012).

RESULTS

Multiple bacterial isolates from kudzu nodules were collected throughout metropolitan Atlanta. Growth was assessed by optical density using a standard curve. Analysis of a subset of the isolates using serial dilutions indicated that the CFU/ml corresponded to the optical density for this range of isolates with no deviation greater than 10-fold. Generation time was determined in nitrogen free media for twenty-five

isolates from seven different locations around Atlanta, Georgia. Isolates were also grown in nitrate reduction broth as well as triple sugar iron media. There were no significant differences between the location of the isolates and their ability to reduce nitrate or ferment glucose, sucrose, or lactose under aerobic conditions (data not shown). However, both the growth conditions and the location of isolation had significant relationship with the isolates' generation times in nitrogen free media (Figures 2 and 3) (ANOVA $P = 0.0004$ and $P = 0.00350$ respectively). Subsequent analysis to identify any specific samples that could account for these differences (Tukey's Honest Standard Deviation, Scheffé multiple comparison as well as a Bonferroni and Holm multiple comparison) suggested that pairwise comparisons of the same media and carbon source, with or without oxygen, showed significant differences (minimum $P < 0.05$). An exception to this trend was found in samples grown on mannitol. The presence or absence of oxygen had no pronounced effect on generation times when this carbon source was used.

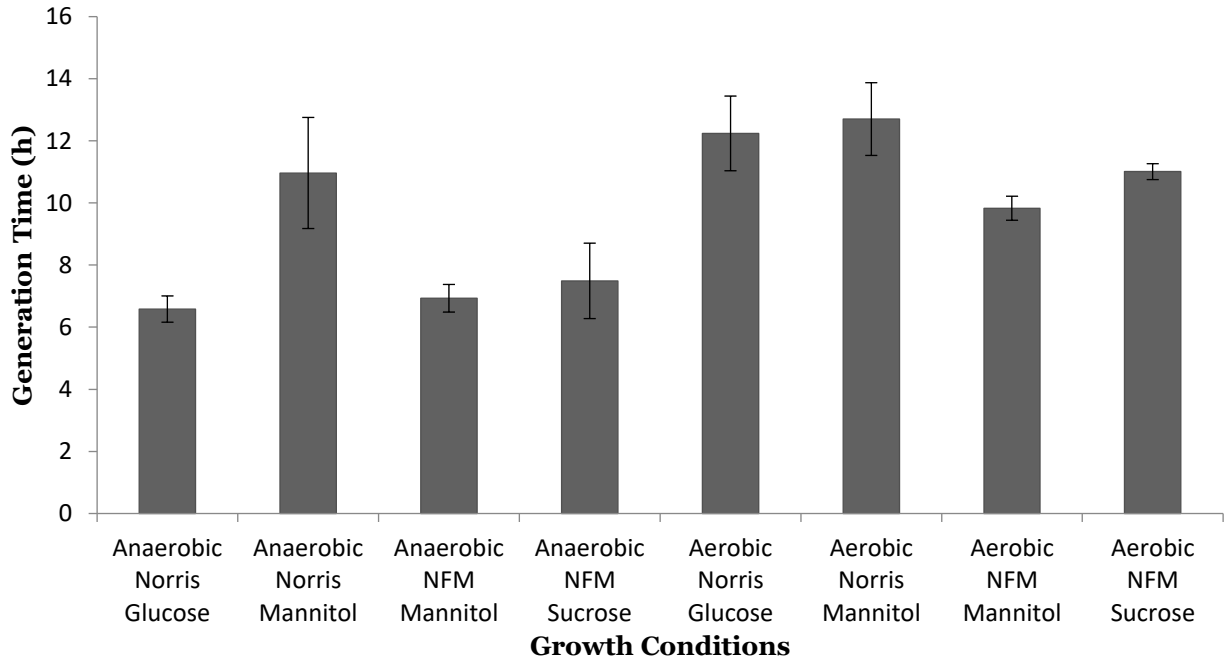

Figure 2. Generation time by growth conditions. Generation times for all isolates were determined under eight different conditions, varying the media composition, the carbon source, and the oxygen availability (ANOVA $P < 0.01$). Average anaerobic growth of isolates from all locations was faster than aerobic growth and there was a significant effect of carbon source in Norris medium (ANOVA $P < 0.05$).

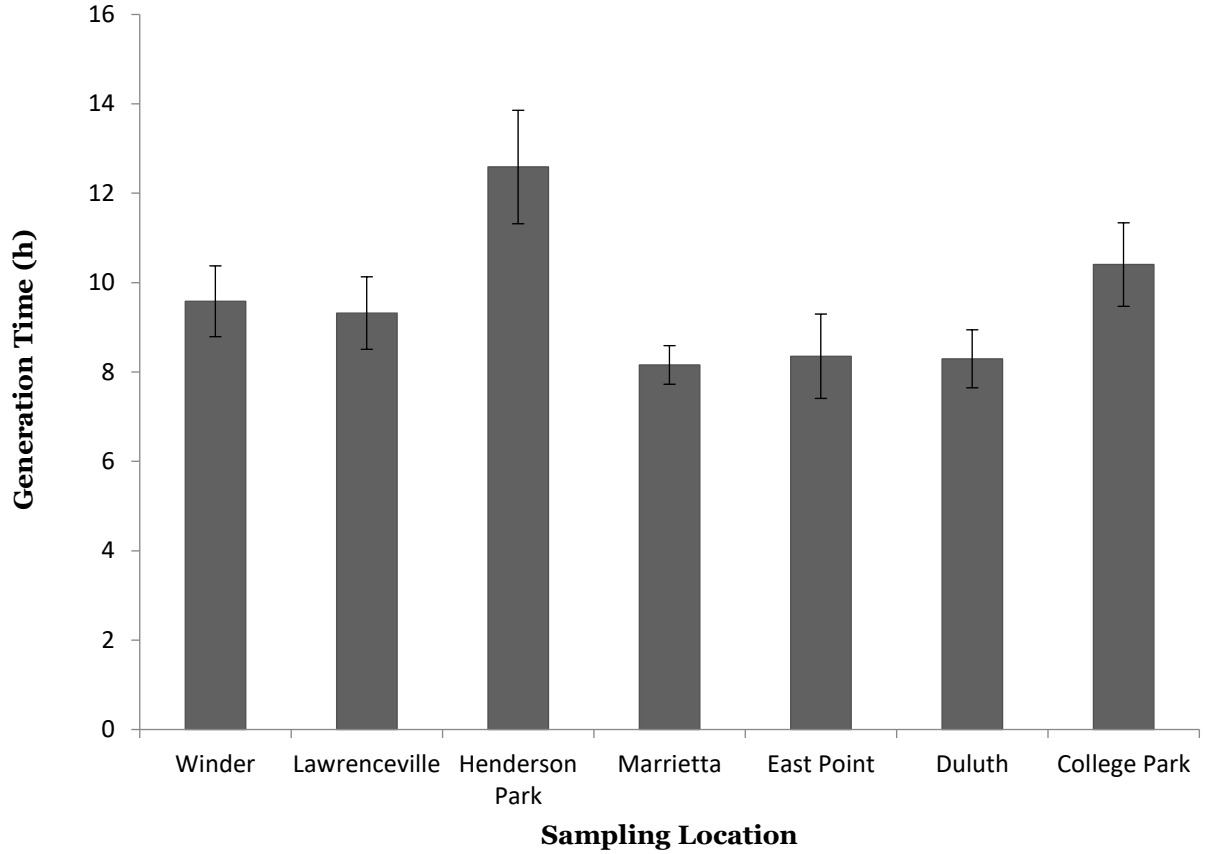

Figure 3. Generation time by location. The generation times of isolates under all conditions were significantly impacted by the location from which they were taken (ANOVA $P < 0.01$). Henderson Park had the longest average generation time of all locations and was significant in comparison to both Marrietta and Duluth (Tukey's HSD $P < 0.01$).

Trends were also observed when examining growth rate by location (ANOVA $P = 0.0035$). The average generation time of Henderson Park isolates was the longest (the slowest growth) and significantly different from both the Duluth and Marietta isolates (Tukey's HSD $P < 0.01$). Because differences were found between growth conditions, each location was analyzed separately under each condition. Generation times from samples obtained from Henderson Park differed from all other locations under all growth conditions examined (ANOVA $P < 0.05$).

Thus, the three isolates from Henderson Park were subject to molecular analyses: both the 16S rRNA sequence and the *nifD* genes were examined (Figure 4). The isolates H1 and H4 were 99.6% identical to *Rhizobium* speciesTAL182 and 99.8% identical to *Bradyrhizobium diazoefficiens*, but they clustered with NifD protein sequences alongside other *Bradyrhizobium*. Additionally, isolate H3-1 was 99.8% identical to *Rhizobium etli* but contained a *nif*D sequence more commonly associated with *Sinorhizobium* species.

We found that the 16S and *nifD* sequences of these isolates were not associated with lineages known for slow growth or poor nitrogen fixation efficiency. However, the phylogenetic affiliation of the 16S and *nifD* sequences were not always congruent with each other, suggesting horizontal gene transfer. More notable, the three isolates cultured

from the same location were each genetically distinct combinations of 16S and *nifD* phylogenies. Although we are unable to ascertain the cause of the exceptionally slow growth from this region, our data does indicate promiscuity in symbiont pairing with *P. montana*.

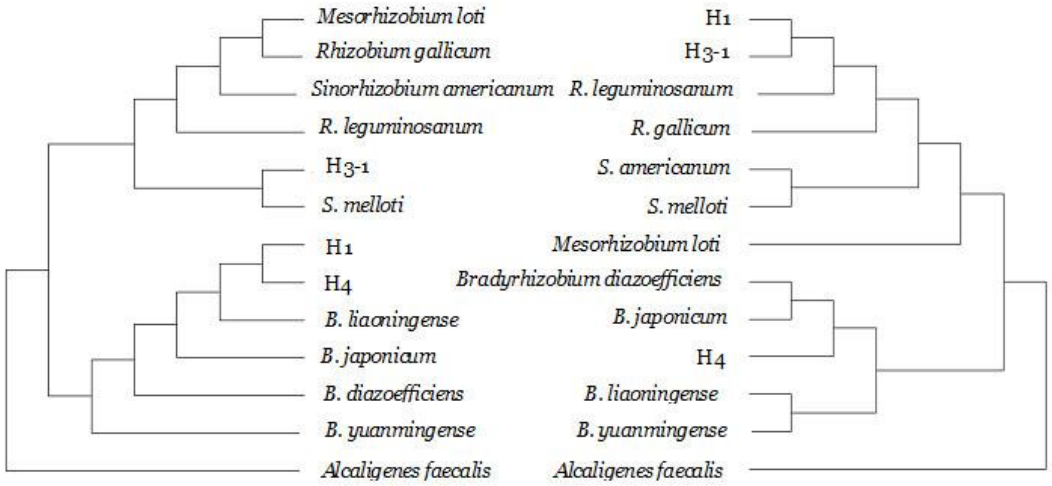

Figure 4. Phylogenetic relationships using 16S *rRNA* and *NifD* genes. There appears to be little correspondence among these isolates between the phylogenetic relationships derived from the 16S rRNA sequence (right) and the protein *Nif*D (left). Only isolate H4 has both the 16S sequence and the NifD protein-coding sequences associated with the same genus, *Bradyrhizobium*.

DISCUSSION

Given the well-known effect of growth conditions on nitrogen fixation capability, this study attempted to mitigate that effect when attempting to compare locations by examining many different media combinations. The slow growth of rhizobia on nitrogen free media in aerobic conditions is well established (Dreyfus et al. 1983). This is caused by the competitive inhibition of the nitrogenase enzyme by O_2. In symbiosis with legumes, rhizobia produce leghemoglobin that sequesters oxygen and express genes for cytochrome oxidases that have a very high affinity for oxygen in order to continue aerobic respiration (Bueno et al. 2012). Nodule conditions have been estimated to provide only 30 nM O_2 (Delgado et al. 1998).

Medium composition and carbon source have differential impacts on different groups of nodule-associated rhizobia (Fikri-Benbrahim et al. 2017). In these analyses, the only significant effect observed among all isolates was found between glucose and mannitol as a carbon source in Norris medium. There is a long-standing association with poor mannitol utilization and the slow-growing variants of rhizobia (Allen and Allen 1950; Kuykendall and Elkan 1977). Given the long generation times observed among our isolates, this may account for these differences.

Investigation of kudzu symbiont biogeography required examination of growth under a variety of conditions to increase the likelihood of observing location-specific trends. However, our data suggest that this was an unnecessary caution; separate analysis of all locations by conditions showed the same trend seen in the aggregate data. In all analyses, Henderson Park isolates were significantly different from those from one or more of the other locations, possessing longer generation times (ANOVA $P < 0.05$).

Henderson Park contains a soccer field which is fertilized occasionally, raising the possibility that kudzu growth in this location is not under nitrogen-limiting conditions. This would prevent any natural selection against nodulation with less efficient symbionts. In this environment, spontaneous mutations in the gene suites responsible for nitrogen fixation could occur with relaxed competitive sanction. These less-efficient gene suites could also spread via horizontal gene transfer. Horizontal gene transfer of components of the nitrogen fixation gene suite is well documented (Haukka et al. 1998). This sequence of events could explain a regional localization of distinct slow-growing symbionts.

In order to explore this possibility, three of the slowest growing nodule-associated bacteria were selected for 16S rRNA and *nifD* gene sequencing. Isolates H1, H3-1 and H4 had generation times of 10, 16, and 14 hours respectively when averaged across all tested conditions. Although H1 and H4 shared identical *nifD* sequences, the H3-1 sequence was quite divergent. Also, predicted secondary structures of these *nifD* were insufficient to explain the slow growth observed (data not shown). This refutes the hypothesis that slow growth was caused by a shared plasmid with a single mutation in the *nifD* gene, the alpha subunit of nitrogenase.

Incongruence was found between the 16S rRNA sequences and *nifD* sequences in these regionally localized isolates. Isolates H1 and H4 were more closely related to *Bradyrhizobium* species when analyzing the 16S rRNA gene. However, the inferred protein sequence analysis for NifD suggests that isolate H1, like H3-1, has a nitrogenase suite more closely related to *Rhizobium*. It could be implied that species H1 and H3-1 did undergo conjugation of the same plasmid. This phenomenon has been observed in previous studies as well (Qian and Parker 2002; Parker et al. 2002).

An analysis of *Bradyrhizobium* isolates from four different continents revealed divergent 16SrRNA sequences but strong similarities in *nifD* sequences for co-localized symbionts (Haukka et al. 1998). The authors suggest that the incongruence of the sequences may arise from horizontal gene transfer of the nitrogen-fixation gene suite. A logical supposition would be that the gene suite conferred an evolutionary advantage in that particular region. However, our findings suggest that any shared nitrogenase gene suites do not provide a growth rate advantage. The evolutionary pressures affecting these symbionts in areas without nitrogen limitation should continue to be explored.

These studies leave unresolved the question of “everything is everywhere” despite regional localization of growth dynamics. Precisely because there may be different mechanisms for the slow growth of the Henderson Park isolates, a distinct biogeography of symbiont strains has not been demonstrated in this work. Despite this, one important facet of this symbiosis has been elucidated: *P. montana* can be categorized as promiscuous. Three genetically distinct nodule-associated isolates were found within a one-mile radius.

This promiscuity has implications for ecosystems far beyond Atlanta, Georgia. If, as recent research indicates, there is a distinct biogeography for bacterial species, it could be inferred that invasive N_2 fixing species expansion could be limited by available symbionts under nitrogen-limited conditions. However, if the legume is capable of partnering with many distinct symbionts, the ability to expand its range increases under the selective pressure of “survival of the fittest”. Given the predictions of kudzu expansion to other parts of the United States as a response to climate change, the relative promiscuity of *P. montana* should be the subject of increased study.

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