Associative Nitrogen Fixation Ability Does Not Predict Symbiotic Nodulation Ability in *Burkholderia tropica*

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Abstract

Symbiotic nodulation of legumes by diazotrophic bacteria was long believed to be the exclusive realm of a few alphaproteobacteria closely related to *Rhizobium*. Recent work on tropical legumes has revealed nodulation ability in many strains of *Burkholderia*, a ubiquitously distributed genus of betaproteobacteria that was previously noted mostly for its opportunistic pathogens of plants and humans. Previous work at Reed College has showed that *Burkholderia tropica* is capable of endophytic nitrogen fixation in dune grasses (*Ammophila arenaria* and *Elymus mollis*) from Oregon. To determine whether Oregon *Burkholderia* might also have the ability to induce nodulation, a greenhouse inoculation trial was performed. Red Alder (*Alnus rubra*), Scotch Broom (*Cytisus scoparius*), Yellow Bush Lupine (*Lupinus arboreus*), Miniature Lupine (*Lupinus bicolor*), and Siratro (*Macroptilium atropurpureum*) were inoculated with *B. tropica* strain Aa1 (isolated from dune grasses) and grown under N-limited hypoxenic greenhouse conditions. Inoculated *Macroptilium* and *Cytisus* showed small increases in size, but no nodulation was observed. Diverse bacteria were recovered from surface-sterilized *Cytisus* roots, but none of the isolates fixed nitrogen in liquid culture and no strain clearly corresponding to *Burkholderia* was recovered. In a concurrent study, PCR was used to probe *Burkholderia tropica* for the presence of the core nodulation genes *nodABCD*. Products apparently corresponding to *nodA* and *nodC* were recovered, but *nodB* and *nodD* were not. *Burkholderia tropica* strain Aa1 seems to be incapable of nodulating plants and may lack a functional *nodB*.

Dedicated to the memory of Julia Orth, who convinced me to throw in my lot with the biologists. She died a better scientist than I will ever be.

Introduction

0.1 Plants have a nitrogen problem

Plants need nitrogen in order to make most of their essential constituent parts. Nitrogen is a key component of proteins, nucleic acids, chlorophyll, and many secondary metabolites. Only carbon, oxygen, and hydrogen are needed in greater quantities than N, but normal growing conditions provide an effectively unlimited supply of these elements from air and water, whereas biologically available N is often insufficient to meet the plant's needs. Therefore, limited N availability is the most common factor limiting plant growth.

The presence of widespread N limitation may seem surprising, given that plants grow in an atmosphere which contains about 78% dinitrogen gas and that the reduction of N_2 to ammonia is exothermic (-33.39 kJ mol^{-1}), but the extraordinarily stable triple bond (dissociation energy 945.33 kJ mol^{-1}) between the atoms of N_2 means atmospheric nitrogen is kinetically inert (Sprent and Sprent, 1990). Although this is a problem for N-hungry plants, human readers can be reassured that it also means the explosive tendencies of our atmosphere are happily minimal.

To make N biologically available, the N_2 triple bond must be reduced to a more reactive form such as ammonia (NH_3), a process known as nitrogen fixation. Eukaryotes are not themselves capable of performing this reduction, and are therefore entirely dependent on N fixed abiotically during lighting strikes, industrially through the Haber-Bosch process (section 0.1.1) and as a byproduct of fossil fuel combustion, or biologically by certain prokaryotes such as *Rhizobium* and *Frankia*, which produce a remarkable enzyme called nitrogenase (section 0.1.2). Biological nitrogen fixation (BNF) is the largest, most complex, and arguably most important of these pathways. This thesis will focus on the highly effective symbiotic nutrient exchanges that lie at the heart of BNF, in which some carbon-fixing plants pair with certain nitrogen-fixing microbes to drive the nutrient flow of entire ecosystems. Before exploring these associations in depth, it may be helpful to consider a few more details about nitrogen

flux at the global scale.

There are three main reservoirs of nitrogen on Earth: the atmosphere, soil and water, and biomass. Atmospheric N_2 forms the largest and least reactive pool and can be seen as the starting point of a complex process of reduction, nitrification, biological use and reuse, mineralization, transport, and eventual denitrification to N_2 that controls and balances N availability in the biosphere. Human activities in the last century have caused major changes in the magnitude and dynamics of this cycle, doubling the rate of N fixation and increasing overall N mobility (Vitousek et al., 1997). It is important to note that even in places where low N availability limits growth, the ecological effects of anthropogenic N are not always positive. In addition to fertilizing plant growth, human changes in N cycling can increase levels of smog-forming NO and of the greenhouse gas N_2O, reduce availability of other nutrients, acidify soils and waterways, reduce diversity of organisms such as mycorrhizal fungi adapted to low-nitrogen environments, and increase the amount of organic carbon stored in terrestrial ecosystems (Vitousek et al., 1997; Schlesinger, 1997; Galloway et al., 2004). Furthermore, these effects can cascade, with a single atom of N affecting all these factors in series (Galloway et al., 2003).

Reliable estimates of N cycling, as of most global cycles, are hard to make. Biological fixation is highly heterogeneous according to location and conditions, and in many cases the available data come from extremely small-scale studies (Cleveland et al., 1999). Denitrification rates are even harder to quantify; Galloway et al. (2004) give up and merely assume no long-term N storage (fixation equal to export) in pre-anthropogenic systems. Human influences have probably led to wide-scale net N storage, but due to excessive application and fast leaching from croplands, much of this N is quickly exported to riverine and marine environments, where it may increase net primary productivity and increase oceanic CO_2 uptake (Schlesinger, 1997).

Natural and anthropogenic processes fixed approximately 389 Tg N yr^{-1} to biomass, soil and water during the mid-1990s (Galloway et al., 2004). By far the largest share of this can be attributed to BNF, which fixed a total of 260 Tg N yr^{-1} in marine (121 Tg N yr^{-1}), uncultivated terrestrial (107 Tg N yr^{-1} including both symbiotic and asymbiotic fixation), and agricultural terrestrial (32 Tg N yr^{-1}, mostly symbiotic) environments. Industrial N fixation accounted for another 100 Tg N yr^{-1}, byproducts of fossil fuel combustion 25 Tg N yr^{-1}, and abiotic fixation through oxidation of N_2 during lightning storms contributed about 6 Tg N yr^{-1} (Galloway et al., 2004). Anthropogenic sources are expected to continue increasing, with industrial fixation overtaking BNF by 2050. By mid-century, Haber-Bosch, agricultural BNF, and com-

bustion byproduct fixation are expected to account for 165, 50, and 53 Tg N yr^{-1} respectively. Agricultural gains will come at the expense of BNF in uncultivated ecosystems, which will drop to 98 Tg N yr^{-1} for a predicted grand total of 492 Tg N yr^{-1} by 2050 (all from Galloway et al., 2004).

0.1.1 The Haber-Bosch process: Brute-force chemistry

Plants in crop systems are subject to the same nitrogen limitations as those in natural ecosystems, with the significant additional constraint that they are actively managed for continuous removal of N in the form of food proteins (Vitousek et al., 2002). Therefore it is no surprise that agriculturists have long focused on increasing N availability in order to raise crop yields. Fritz Haber's 1913 discovery of a practical method to synthesize ammonia from N_2, quickly commercialized by Carl Bosch and known as the Haber-Bosch process, enabled large-scale production of nitrogen fertilizer and was one of the major factors responsible for the "green revolution," a massive increase in worldwide agricultural productivity that helped allow the world's human population to expand from under 2 billion in 1900 to over 6 billion today. For this reason the Haber-Bosch process can make a credible claim on the title "most important invention of the 20th century" (Smil, 2004).

Industrial N fixation has grown rapidly throughout the 20th century and is now the primary nitrogen input to agricultural systems, adding 100 Tg N yr^{-1} to the biosphere in the mid-1990s. That is over three times more than the 31.5 Tg N yr^{-1} fixed by human-induced biological activity, and a quarter of the 389 Tg N yr^{-1} fixed by all sources worldwide (Galloway et al., 2004). Global population, and therefore food consumption, is projected to continue rising for the foreseeable future; by 2050, industrial ammonia production is expected to reach 165 Tg N yr^{-1}.

The Haber-Bosch process is conceptually simple, but energy-intensive (c. 28 GJ per ton (Appl, 1992, cited in Kirova-Yordanova (2004))) and relatively inefficient. Natural gas is first steam-reformed to CO_2 and H_2. The hydrogen then combines with N_2 over an iron catalyst to form ammonia:

$$3CH_4 + 6H_2O \rightarrow 3CO_2 + 12H_2$$

$$4N_2 + 12H_2 \underset{600°C,\ 200\ atm}{\overset{Fe^{3+}}{\rightleftharpoons}} 8NH_3$$

The reaction is reasonably fast at high temperatures, but is complicated by an unfavorable equilibrium; even when pressurized to 200 atm, the single-pass yield is only

about 25%, necessitating multiple passes to process all the reactants. Every step of the process requires significant energy inputs in addition to the natural gas used as a feedstock, resulting in the production of a total of 0.7 tons carbon (as CO_2) for every ton of ammonia produced (Jenkinson, 2001). This total does not include the energetic cost of shipping and application: 1.5 kg of fuel oil are burned for every 1 kg of N applied to fields (Sprent and Sprent, 1990). Ammonia production used about 5% of all the natural gas produced in the mid-1990s (Jenkinson, 2001).

0.1.2 Nitrogenase

As detailed above, the industrial synthesis of ammonia is performed at about 600 °C and 200 atmospheres of pressure. Considering that a standard autoclave sterilizer runs at a mere 121 °C and about 2 atmospheres, and easily kills everything alive inside it, it is all the more remarkable that most of the ammonia entering the biosphere each year comes not from the Haber-Bosch process but from prokaryotic organisms living at room temperature. These plucky organisms, known as diazotrophs, are armed not with pressure vessels but with a very powerful enzyme complex called nitrogenase, which reduces N_2 and protons to NH_3 with an obligate side-product of H_2:

$$N_2 + 8e^- + 8H^+ + 16\text{ATP} \longrightarrow 2NH_3 + H_2 + 16\text{ADP} + 16P_i$$

This equation assumes ideal conditions. Realistically, each electron transfer often costs more than two ATP and extra electrons may also "leak through" to form more than one H_2 for each N_2 reduced (Sprent and Sprent, 1990). After reduction, NH_3 is quickly converted to ammonium (NH_4^+) and then passed to the cell's glutamate synthesis pathway for assimilation.

Nitrogenase is a large complex built up of two proteins: the 220-240 kDa MoFe protein (also known as dinitrogenase) that binds to an iron-molybdenum cofactor (the sonorously named 'FeMoco') to form the active site for nitrogen reduction, and the 60-70 kDa Fe protein (known as dinitrogenase reductase) that passes electrons to the MoFe protein (Burris, 1991). The Fe protein is composed of two identical subunits encoded by the *nifH* gene, while the MoFe protein is an $\alpha_2\beta_2$ heterotetramer encoded by the *nifD* (α subunit; 50 kDa) and *nifK* (β subunit; 60 kDa) genes (Burris, 1991; Sprent and Sprent, 1990).

Nitrogenase is expensive for the cell, both to produce and to operate. The fully assembled 500-600 kDa nitrogenase complex can form up to 30% by weight of the protein in a cell, and typically requires 20-30 ATP for each reduction cycle performed

(Haaker and Klugkist, 1987; Sprent and Sprent, 1990). For this reason, most diazotrophs do not reduce N_2 when other forms of N are available. To make matters worse, nitrogenase is relatively non-specific and will happily reduce many substrates other than N_2, including oxygen. Nitrogenase is permanently inactivated when it binds to O_2, meaning that it must operate in a cellular environment that is anaerobic or microaerobic ($pO_2 < 1$ kPa) (Burris, 1991). This requirement is strongly at odds with the cell's need for oxidative phosphorylation to produce the large amounts of ATP used in N_2 reduction (Dalton and Kramer, 2006).

The same promiscuous reduction tendencies that make nitrogenase oxygen-sensitive can also be exploited to provide a very useful assay for nitrogenase activity: Nitrogenase will reduce acetylene ($HC{\equiv}CH$) to ethylene ($H_2C{=}CH_2$). By exposing suspected diazotrophs to a known concentration of acetylene and monitoring (by gas chromatography) its conversion to ethylene over time, a relatively good quantitative estimate of nitrogenase activity can be obtained.

0.2 Diazotrophs have a carbon problem

Fixing nitrogen is, as described in section 0.1.2, energetically expensive. To maintain fixation, diazotrophs require a good energy source, usually in the form of large amounts of carbon. N-fixers have adopted a variety of strategies to maintain this carbon supply; for the purpose of analysis we can divide this continuum into three subjective modes of fixation: free-living, associative, and symbiotic.

0.2.1 Free-living N fixation

Autotrophic N fixers can fix both carbon and nitrogen for themselves and are thus able to live off little more than N_2, CO_2, and a few metal ions. Free-living chemoheterotrophs are not quite so spartan; they require some externally supplied organic carbon and tend to be found in rotting logs, leaf litter, compost, or other environments where high carbon and low nitrogen availability give diazotrophs a free lunch and a competitive advantage over non-fixers (Balandreau, 1986). Both these groups of free-living N fixers are scientifically interesting and ecologically important—a large portion of marine N fixation is probably attributable to free-living aerobic cyanobacteria (Stewart, 1973)—but diazotrophs that do not interact directly with plants will not be considered further here.

0.2.2 Associative N fixation

Many diazotrophs are found loosely allied with plants, living in the rhizosphere or on the surface of the plant or endophytically within plant tissue. This associative fixation is different from free-living fixation only as a matter of degree; the key characteristic of associative fixation is that the cooperation is real but informal and not obligatory. Both the plant and the diazotroph gain from the association, but neither one has invested in specialized signaling or differentiated structures (Klucas, 1991). Associative fixation is probably quite common worldwide, but the characteristically loose host-microbe affiliations involved in associative fixation tend to make claims about its diversity, scale and effect on N budgets quite difficult to prove (Dalton and Kramer, 2006). Associative fixation has probably been best studied in tropical grasses such as sugar cane, rice, and Kallar grass, which often contain large endophytic communities of *Azoarcus*, *Herbaspirillum*, *Acetobacter*, and *Burkholderia* (Baldani and Baldani, 2005; Weber et al., 1999; Reis et al., 2000; Junior et al., 2000).

0.2.3 Symbiotic N fixation

Although the difference between symbiotic and associative fixation is, like that between free-living and associative fixation, mostly a matter of degree, symbiotic fixation has generally been defined somewhat more strictly. Symbiotic fixation is an intimate, mutually beneficial association of plant and microbe, generally characterized by concerted signaling between the symbionts and the presence of differentiated structures to house the association (Klucas, 1991). A successful symbiosis requires that the microsymbiont be able to infect and colonize the host plant, that the host plant be able to supply the microsymbiont with energy and nutrients, and that plant and microbe working together be able to both regulate O_2 flux and transfer fixed N from microsymbiont to host (Vessey et al., 2005). Legumes nodulated by rhizobia are the classic and most heavily studied example of a highly specialized, tightly coevolved symbiosis, but plants and diazotrophs form a wide variety of other symbiotic associations, notably including non-rhizobial root nodules formed by the actinomycete *Frankia* and cyanobacterial fixation in lichens, cycads, and one species of fern (Denison, 1979; Lindblad and Costa, 2002; Moore, 1969).

0.3 Prokaryotic taxonomy is hard

Although microbes vary widely between taxa, their simple physiology means that most of their observable phenotypic features are not reliable taxonomic indicators (Woese et al., 1984). Metabolic profiles and cell morphology, long the standard methods for identifying bacterial species, do not cluster reliably in related species. Molecular genotyping has been touted as a straightforward way to reveal the true phylogenetic relationships of the prokaryotes, but this approach has its own set of drawbacks. Many bacterial genomes are highly fluid and engage in extensive lateral gene transfer, bringing in new genetic material that can range in size from a few hundred base pairs to whole suites of genes, and in origin from close relatives to wholly unrelated organisms. This genetic mishmash is what underlies the uninformative phenotypes mentioned above. Even ribosomal genes, once touted as the "ultimate molecular chronometers," can become mosaic through lateral tranfer and recombination events (Woese, 1987; Wang and Zhang, 2000). Despite these difficulties, molecular genotyping has proved to be generally more tractable than other taxonomic approaches, and many of the classically recognized bacterial groupings have been rearranged extensively in the last several decades as taxonomists begin to rely more extensively on genotypic data.

This shift has been especially visible in two groups of interest to the current project. The rhizobia are a formerly monophyletic group whose definition has been diversified greatly by closer molecular study, while the pseudomonads are a famously catch-all group that was obviously heterogeneous from its inception but required genetic data before it could be teased apart. This teasing-apart is of course ongoing; eventually all N-fixing groups were removed from *Pseudomonas* into their own genera, but further developments have since identified new *Pseudomonas* species which are unambiguously N-fixing. A recent proposal based on 16S sequencing by Young and Park (2007) serves to further muddy the waters by claiming synonomy of *Azotobacter*, long considered morphologically distinct, with *Pseudomonas*.

0.4 Rhizobia

Bacteria were first isolated from legume root nodules in 1888 by M.W. Beijerinck, who called his strain "*Bacillus radicola*," but it was not until Frank's 1889 publication of the name *Rhizobium leguminosarum* that rhizobia gained their permanent title. After some early taxonomic confusion, in 1932 all then-known nodule bacteria

were assigned to six species of *Rhizobium* grouped largely according to their host specificity, and this arrangement remained unchanged until 1982, when some slow-growing, metabolically distinct strains were separated into *Bradyrhizobium* (Young and Haukka, 1996). Further re-groupings have followed rapidly since then, with the result that the α-proteobacterial family Rhizobiaceae now contains 70 species scattered across eight genera, most of which were once considered one of the six classic *Rhizobium* pecies (Willems, 2006; Euzéby, 2008a).

Even aside from this bewildering diversification, the term "rhizobia" has often been used imprecisely and hence can have a confusing range of meanings. Loosely speaking, rhizobia are bacteria that nodulate and perform symbiotic nitrogen fixation in legumes. This becomes messy because it includes most, but not all, of the Rhizobiaceae (*Agrobacterium*, a non-symbiotic bacterium that causes crown galls in many crop plants, is a sister genus or possibly a subclade to *Rhizobium* roper (Young et al., 2001)), and also a number of species from other families and even from outside the α-proteobacteria. Current usage seems to favor considering the rhizobia to include any diazotroph that can produce functional N-fixing nodules on some member of the legume family, and additionally any non-nodulating species that is or was once a member of the genus *Rhizobium* (Willems, 2006; Young and Haukka, 1996). With the advent of molecular phylogenetics and particularly with the widespread use of 16S rDNA sequences to assign taxonomic status, it has become increasingly clear that the rhizobia are a grouping with strictly functional, rather than taxonomic, value. The emergence of the "beta-rhizobia," discussed in section 0.6, may provide the final proof of this distinction.

The key defining feature of the rhizobia, then, is that with one known exception (*Parasponia*, a member of the Ulmaceae, forms rhizobial nodules (Trinick, 1973)), they form tightly mutualistic symbioses with legumes exclusively. Since the legumes (family Fabaceae) are an extremely diverse clade with about 650 genera that comprise about 18,000 species (Sprent, 1995), there is considerable potential for diversity among the rhizobia that nodulate them. However, very little of this potential has been examined in detail. Until 1982 so little rhizobial diversity was recognized that all known rhizobia were lumped into a single genus of Gram-negative α-proteobacteria known as *Rhizobium* (Young and Haukka, 1996). Even today most knowledge about symbiotic N fixation still comes from the nodulation of *Rhizobium* and its former congeners *Bradyrhizobium*, *Allorhizobium*, *Mesorhizobium*, and *Sinorhizobium* in a few crop legumes such as alfalfa, soybeans and peas. The development of molecular phylogenetic methods such as sequencing of 16S rDNA genes has resulted in the

recognition of many new, poorly studied taxa of rhizobia. Additionally, it is important to note that although rhizobia only nodulate legumes (and *Parasponia*), the same species that nodulate legumes are also capable of living independently or in associative interactions with non-legumes. For example, Doty et al. (2005) report isolating the well-known legume symbiont *Rhizobium tropici* from stems of *Populus*.

0.4.1 Legume nodulation: the classical mechanism

Plants initiate the nodulation process by releasing inducers, usually flavonoids such as daizein, luteolin or chalcone derivatives, into the surrounding soil. These induce expression of nodulation genes in rhizobia by a mechanism not yet described, and may also serve as chemoattractants to induce migration towards the root. The rhizobia then respond by assembling Nod factors, which are lipo-chitooligosaccharides that in turn signal the plant to initiate the infection process and activate nodule formation (Denarie et al., 1996). Infection begins with curling of the root hair, followed by the development of an "infection thread" down which the rhizobia migrate between cells until they reach the base of the root hair and are released into plant cells that have differentiated into nodule primordia, where the rhizobial cells differentiate into nitrogen-fixing bacteroids (Oke and Long, 1999; Prell and Poole, 2006). The entire infection process is under the control of the host plant.

There are four "core" *nod* genes (*nodABCD*) that are present in at least one copy in every nodulating rhizobial strain. They seem to provide the basic skeleton of the Nod factor, which is further modified by a suite of species-specific *nod* gene products to generate a wide range of molecules that accounts for a great deal of the variation in species specificity of nodulation (Spaink et al., 1991). NodD is a transcriptional activator which binds to a conserved "nod box" ($ATCN_9GAT$) and induces transcription of downstream *nod* genes through DNA bending (Goethals et al., 1992; Fisher and Long, 1992). NodC controls the oligomer length (generally 4-6 glucosamines) of the Nod factor. NodB (a specific deacetylase) and NodA (an *N*-acyl transferase) perform intermediate transformations in species-specific methylation and fatty acid addition reactions (Denarie et al., 1996). Nod factors may also have other functions; Fujishige et al. (2008) have recently reported an intriguing finding that the core *nod* genes are involved in rhizobial biofilm formation but species-specific *nod* genes are not, suggesting that biofilm formation on the root surface may be an important early step in the infection process.

0.5 *Burkholderia*

Although the genus *Burkholderia* was not formally recognized until 1992, many of its constituent species have been known for much longer. The type species, *B. cepacia*, was first named '*Pseudomonas cepacia*' in 1950 when Walter Burkholder showed it to cause soft rot in onions (Burkholder, 1950). In subsequent years, the pseudomonads were shown to contain several distinct hybridization groups; further interest in the taxon came with the realization in the 1960s that certain pseudomonads were distinguished by their ability to utilize a very wide array of carbon compounds, and when '*Pseudomonas cepacia*' was found to be an opportunistic respiratory pathogen in patients with cystic fibrosis (Stanier et al., 1966; Lessie et al., 1996). By this time is was clear that *Pseudomonas* was becoming something of a catchall taxon, but resolution of this untidy situation had to wait until Yabuuchi et al. (1992) moved 7 species of *Pseudomonas* entirely out of the gammaproteobacteria to establish the new beta-proteobacterial genus *Burkholderia*.

Burkholderia as currently defined contains over 50 species of gram-negative, rod-shaped, obligately aerobic, non-sporulating, usually motile β-proteobacteria with near-ubiquitous distribution in soils, plant rhizospheres, water, and even iron ore (Yabuuchi et al., 1992; Gillis et al., 1995; Euzéby, 2008b; Valverde et al., 2006). The *B. cepacia* genome is unusually large (variable from 5 to 9 Mb) and complex; it consists of three chromosomes and at least one major plasmid, and shows evidence of frequent large-scale rearrangements (Rodley et al., 1995; Lessie et al., 1996).

The genus contains several human pathogens such as *B. mallei* and *B. pseudomallei* as well as a number of notable plant pathogens including *B. caryophylli*, *B. plantarii*, *B. glumae* and *B. andropogonis*, but most species seem to be either harmless or beneficial soil bacteria (reviewed in Coenye and Vandamme, 2003). *Burkholderia* is also commonly found as a plant endophyte in diverse species such as grapevine (Compant et al., 2008), *Cyclopia* (Elliott et al., 2007), maize (Perin et al., 2006), banana and pineapple (Cruz et al., 2001; Weber et al., 1999), coffee (Estrada-De los Santos et al., 2001), rice (Gillis et al., 1995), and dune grasses (Dalton et al., 2004). Diazotrophy seems to be nearly ubiquitous in *Burkholderia*; the endophyte was reported to fix nitrogen in all of these examples except the grapevine.

The example of dune grass is particularly intriguing; *Burkholderia* growing endophytically in European beach grass (*Ammophila arenaria*) on the Oregon coast (Dalton et al., 2004; Kramer, 1999) provides both the first well-established evidence of endophytic diazotrophy outside the tropics, and may explain a significant portion of

Ammophila's success in an extremely harsh and nutrient-poor environment. *Burkholderia* produce an extremely wide array of extracellular products, many of which have the potential to strongly affect their host environment, including enzymes, hemolytic compounds, siderophores, toxins, antifungals, and phytohormones (reviewed in Vial et al., 2007).

0.6 The β-rhizobia emerge

Widespread interest in rhizobial diversity has led to an extensive effort at characterizing the rhizobia found in the nodules of various legumes. In 2001, Moulin et al. examined the 16S rRNA sequence of an isolate from the South African legume *Aspalathus carnosa*, which was widely thought to be nodulated by *Bradyrhizobium*, and found that, unexpectedly, their isolate was a β-proteobacterium from the genus *Burkholderia*. Their isolate contained *nod* genes which were probably obtained by horizontal transfer from more conventional α-rhizobia, and was capable of inducing ineffective nodules when inoculated onto *Macroptilium atropurpureum*. Soon after that, *Burkholderia* was found to induce effective nodules in *Mimosa* (Vandamme et al., 2002), and it is now clear that the β-rhizobia *Burkholderia* and *Ralstonia* are the specific and routine symbionts of many tropical *Mimosa* (Chen et al., 2003, 2005; Barrett and Parker, 2006; Pandey et al., 2005). Effective nodulation has also been reported in *Dalbergia* (Rasolomampianina et al., 2005) and *Cyclopia* (Elliott et al., 2007). There is thus far no clear evidence for β-rhizobia nodulating any temperate legume taxa, but the ubitious distribution of *Burkholderia* in temperate zones and the strong evolutionary rewards for successful N-fixing symbioses make it reasonable to ask why tropical legumes should get to have all the fun.

0.7 Experimental design

The emerging view of β-rhizobia as important plant symbionts capable of both symbiotic and associative nitrogen fixation suggests that their influence must be considered in any complete description of nitrogen cycling within an ecosystem. This study attempts to extend knowledge about nitrogen fixation in Oregon by testing whether *Burkholderia tropica* strain Aa1, an endophyte isolated from dune grasses on the Oregon coast, is also capable of inducing nodulation in five species of N-fixing plants.

Sterilized seeds from three Oregon legumes (*Cytisus scoparius*, *Lupinus arboreus*, and *Lupinus bicolor*), one actinorhizal tree (*Alnus rubra*), and one promiscuously

nodulating tropical forage legume (*Macroptilium atropurpureum*) were grown in the Reed College greenhouse under very low-nitrogen conditions. Half the plants of each species were inoculated with a culture of *Burkholderia* Aa1. At harvest, plant size was measured as a proxy for plant nitrogen status, and roots were examined for nodulation. Root and nodule tissue were checked for nitrogenase activity using the acetylene reduction assay. Bacteria were re-isolated from surface-sterilized roots, grown in N-free liquid culture, and tested again for nitrogenase activity.

Concurrently with the growth trials, a molecular analysis of *Burkholderia tropica* Aa1 nodulation genes was undertaken. PCR primers were designed to amplify and sequence the four core *nod* genes in order to determine whether all four are present and intact in the *Burkholderia* Aa1 genome.

0.7.1 Plants used in this study

Red Alder, *Alnus rubra* Bong. (Betulaceae), is a medium-sized hardwood tree native to the Pacific Northwest. It is a relatively fast-growing, early-successional tree that fixes nitrogen in wet floodplains and disturbed areas through nodular symbioses with various actinomycetes of the group *Frankia*. This early-successional nitrogen can have important, lasting effects on species which come later in the succession (Klain, 2003). *Burkholderia cepacia* has previously been shown to act as a nonessential aid to the *Frankia* infection process on *Alnus* root hairs (Berry and Torrey, 1983; Knowlton and Dawson, 1983), and *Burkholderia* spp. are capable of using citrulline, the main amino acid found in the in root nodules of *Alnus glutinosa*, as their sole nitogen source (Blom et al., 1981; Estrada-De los Santos et al., 2001). Thus it is interesting to ask whether *Burkholderia* might be able to associate with *Alnus rubra* when *Frankia* is not present.

Scotch Broom, *Cytisus scoparius* (L.) Link. (Fabaceae), is an aggressively invasive leguminous shrub that has been been introduced from the Mediterranean region to North America, Australia, and New Zealand. In Oregon, *Cytisus scoparius* forms thick chaparrals especially in coastal and hill areas, where it is fire-tolerant and crowds out native plant assemblies. Although it fixes nitrogen, it regenerates quickly and may have chemical inhibitory effects such that few other plants can compete to gain any benefit from the nutrients (Haubensak and Parker, 2004). The root diazotrophs seem to be mostly *Bradyrhizobium* (Parker et al., 2006), although *Rhizobium*, *Mesorhizobium*, and *Ochrobactrum* have also been reported (Lafay and Burdon, 2006; Zurdo-Pineiro et al., 2007). The spread of *Cytisus scoparius* may be partly mediated by

symbiont availability (Parker et al., 2006). If *Burkholderia*, which is already known to be present in coastal environments (Kramer, 1999), were found to be a potential *Cytisus scoparius* symbiont, this information could be of interest when deciding on control strategies for invasive *Cytisus scoparius*.

Lupine, *Lupinus* L. (Fabaceae), is an extremely large and diverse genus that has been widely used for previous nodulation research. There are close to 60 species of *Lupinus* in Oregon and Washington alone, and they range in size from small annual herbs to midsized perennial shrubs. *Lupinus* is the only known legume that is not mycorrhizal. *Lupinus* seems to be mostly nodulated by *Bradyrhizobium* (Barrera et al., 1997), although a variety of other α-proteobacteria have also been reported (Trujillo et al., 2005; Andam and Parker, 2007; Valverde et al., 2005). Two species were selected for this study: *Lupinus arboreus*, a small perennial shrub that grows in coastal areas of California and into southern Oregon, and *Lupinus bicolor*, a small annual herbaceous lupine that is widespread in Oregon. The diversity and wide ecosytem distribution of *Lupinus* in Oregon make it an obvious choice when testing potential Northwest *Burkholderia* hosts.

Siratro, *Macroptilium atropurpureum* (DC.) Urb. (Fabaceae), is an annual tropical legume native to Central America that is used for agricultural forage throughout the tropics. It will nodulate in response to an exceptionally wide range of microsymbionts, although the nodules are commonly ineffective, and has been used in several previous studies to demonstrate the nodulation ability of putative rhizobia (Moulin et al., 2001; Bottomley et al., 1994; Barrett and Parker, 2005; Elliott et al., 2007).

Chapter 1

Methods and Materials

1.1 Seed sources

Initial *Alnus rubra* sterilization tests were performed using seeds collected on October 8, 2007, from Powell Butte Nature Park, Portland, Oregon (45.4881 N, 122.4999 W). Cones were dried in paper bags for two weeks at 20 °C, then crushed, sieved, and separated from cone fragments by shaking in deionized water; scales sank while seeds floated. Seeds were spread in a single layer on a paper towel, allowed to dry overnight, and stored in tightly closed 50 mL conical tubes at 20 °C. These seeds exhibited extremely poor germination and were not used in the inoculation trials.

Pre-cleaned *Alnus rubra* (collected in 2007 from wild plants near Sixes, Oregon) and *Lupinus arboreus* (collected in 2007 from wild plants near Ophir, Oregon) were purchased from Jonny Native Seed, Albany OR.

Cytisus scoparius seed was collected from wild plants in several locations near Port Townsend, Washington (c. 48.1 N, 122.8 W) on October 13 & 15, 2007. Whole seed pods were picked into grocery bags and either shelled immediately, followed by air-drying for one week (Oct. 13), or air-dried in the pod for one week before shelling (Oct. 15, pods collected during rainy weather). After shelling, seeds from both collection days were pooled, screened through a 2.83 mm sieve, and stored in tightly closed 50 mL conical tubes at 20 °C. The collection dates were well after the normal *Cytisus scoparius* seed-dispersal season, and most pods had already shattered. Of the remaining pods, most were poorly filled. Thus the seed sample may have contained a high proportion of immature seeds.

Lupinus bicolor seed was purchased from Elkhorn Native Plant Nursery, Moss Landing, California. These seeds were shipped with a clay coating for commercial-scale broadcast seeding; this clay was removed immediately before sterilization by

rinsing seeds in deionized water with vigorous shaking until the supernatant ran clear (c. 10 changes of water for 2 g seeds in a 50 mL conical tube).

Macroptilium atropurpureum was purchased from Educational Concerns for Hunger Organization (ECHO), an agriculture and technology charity in North Ft. Myers, FL.

1.2 Seed sterilization

1.2.1 *Alnus rubra*

This procedure was adapted from Brunner and Brunner (1990). Seeds were surface-sterilized for 30 minutes in 30% H_2O_2, rinsed 10 times with sterile water, allowed to soak in sterile water overnight at 4 °C, and plated in a monolayer on 1.5% water agar in petri dishes sealed with Parafilm. After germination in petri dishes under greenhouse light for 10–20 days, seedlings were transplanted directly to nonsterile vermiculite in either 8″ plastic pots (10–15 seedlings per pot) or 444 mL "Deepot" growth tubes (3 seedlings per tube). The *Burkholderia*-inoculated group was drizzled with approximately 1 mL per seedling of a 1–5 day old culture of *B. tropica* Aa1 in LB medium 1–2 days after planting.

1.2.2 *Cytisus scoparius*

For the initial planting in November, seeds were soaked for 30 minutes in 12 M H_2SO_4 to surface-sterilize them and soften the seed coat (Meyer, 1974), rinsed eight times in deionized water, surface-sterilized again for 30 minutes in 30% H_2O_2, rinsed 10 times, and allowed to soak at 4 °C overnight. Seeds were then planted directly into nonsterile vermiculite in 164 mL "Cone-tainer" growth tubes at a rate of 2–3 seeds per tube. The *Burkholderia*-inoculated group was drizzled with approximately 1/2 mL per tube of a 3 day old culture of *B. tropica* Aa1 in LB medium at the time of planting.

For the second planting in February, 400 seeds were soaked for 45 minutes in 12 M H_2SO_4 to surface-sterilize them and soften the seed coat, and rinsed 20 times in sterile water. The water from the final rinse was found to have a pH of 5. All seeds were then transferred to a laminar flow hood, where each seed was nicked at the cotyledon end using a flame-sterilized razor blade and handling with bare hands rinsed in 70% isopropyl alcohol. Seeds were then soaked overnight at 4 °C and planted as described above, then the *Burkholderia*-inoculated group was inoculated with approximately 1 mL per tube of an overnight culture of *B. tropica* Aa1 in LB medium 4 days

after planting. In addition, the uninoculated control group was also drizzled with approximately 1 mL per tube of sterile LB medium.

1.2.3 *Lupinus arboreus*

Seeds were soaked for 45 minutes in 12 M H_2SO_4 to surface-sterilize them and soften the seed coat (Riemenschneider et al., 1974), rinsed five times in deionized water, surface-sterilized again for 45 minutes in 30% H_2O_2, rinsed 10 times, and allowed to soak at 4 °C overnight. Seeds were then planted directly into nonsterile vermiculite in 444 mL "Deepot" growth tubes at a rate of 1–2 seeds per tube. Twelve seeds were plated on 3% maltose with 1.5% agar and monitored for several days to track germination rate and sterilization efficiency. The *Burkholderia*-inoculated group was drizzled with approximately 1 mL per tube of an overnight culture of *B. tropica* Aa1 in LB medium at the time of planting.

1.2.4 *Lupinus bicolor*

These seeds were purchased already embedded in a clay pelleting material. They were rinsed 10–12 times in deionized water to remove the clay, then surface-sterilized for 20 minutes in 12 M H_2SO_4 with the tube placed in a beaker of cold water to absorb excess heat (adapted from Riemenschneider et al., 1974). The seeds were then rinsed five times in sterile water, surface-sterilized for 10 minutes in 5% sodium hypochlorite, rinsed 10 times, allowed to soak in sterile water at 4 °C overnight, and planted directly in nonsterile vermiculite in 164 mL "Cone-tainer" growth tubes at a rate of six seeds per tube. Betweeen 10 and 20 seeds from each batch sterilized were plated on 3% maltose with 1.5% agar and monitored for several days to track germination rate and sterilization efficiency. The *Burkholderia*-inoculated group was drizzled with approximately 1 mL per seed of a 3 day old culture of *B. tropica* Aa1 in LB medium at the time of planting.

1.2.5 *Macroptilium atropurpureum*

Seeds were soaked for 5 minutes in 12 M H_2SO_4 to surface-sterilize them and soften the seed coat, rinsed 15 times in deionized water, and planted immediately into nonsterile vermiculite in 8″ plastic pots (Moulin et al., 2001). The *Burkholderia*-inoculated group was drizzled with approximately 3 mL of an overnight culture of *B. tropica* Aa1 in LB medium 5–7 days after planting. The uninoculated controls were drizzled

with a similar quantity of sterile LB medium.

1.3 Plant growth

4 × 21 cm UV-stabilized polyethylene growth tubes (164 mL Ray Leach "Conetainers") and 5 × 18 cm polypropylene growth tubes (444 mL "Deepots") were obtained from Stuewe and Sons, Corvallis, Oregon. All containers were rinsed with a 10% bleach solution before filling. Bleach-rinsed fiberglass window screen was used in both growth tubes and plastic pots to prevent the vermiculite from escaping through drainage holes.

All plants were raised in the Reed College greenhouse under combined natural and sodium vapor lighting with a 15-hour day length. Temperature was maintained at 24 °C during the day and 21 °C at night. Plants were either top-watered five days per week with an N-free nutrient solution optimized for soybeans (Appendix A) and with tap water on weekends (*Macroptilium atropurpureum*, February *Cytisus scoparius*), or bottom-watered by keeping the bottom 3 cm of the container in a tray of standing nutrient solution, supplemented with tap water applied from above on weekends. Plants of *Alnus*, *Cytisus*, and *Lupinus* planted in November and grown in 8″ plastic pots were bottom-watered until early February (c. week 12–14), then switched to daily top-watering as described above. Nutrient feeding was discontinued in late March; *Alnus* (week 20), *Macroptilium* (week 7), and February-planted *Cystisus* (week 7) were given tap water only for the remainder of the time to harvest.

1.4 Bacterial cultures

Burkholderia tropica strain Aa1 was originally isolated from dune grasses (*Ammophila arenaria* and *Elymus mollis*) on the Oregon coast and identified by its equivalent name *Burkholderia tropicalis* (Kramer, 1999).

For genotyping and strain maintenance, *Burkholderia tropica* strain Aa1 and *Pseudomonas fluorescens* were plated on LB medium with 1.5% agar and grown at 30 °C for 2–3 days until colonies were well-developed, then stored at 4 °C until needed. *Rhizobium* strain NGR234 and *Bradyrhizobium japonicum* strain DES122 were plated on yeast-mannitol medium (Appendix A) with 1.5% agar and grown at 30 °C for 3–5 days until colonies were well-developed, then stored at 4 °C until needed. For plant inoculation, *B. tropica* strain Aa1 was grown overnight in liquid LB medium at 30 °C with shaking.

For extraction of *Cytisus scoparius* root endophytes, root samples ranging from 0.1 to 2 g were taken from three *Cytisus scoparius* plants in each treatment group. The roots were surface-sterilized in 10% chlorine bleach for 30 seconds, rinsed with a large quantity (> 200 mL) of sterile water, and macerated in a microcentrifuge tube with c. 1 mL of sterile water using a sterile plastic pestle for approximately five minutes. The resulting suspension was vortexed briefly, buzz-spun to precipitate the very largest root pieces, and 100 µL of both the straight extract and a 100-fold dilution was plated on LB with 1.5% agar. Samples were allowed to grow at 30 °C for two days, at which time colonies which differed visibly from each other were re-streaked onto separate plates. These plates were allowed to grow at 30 °C for four days in order to detect slow-growing contaminants, then re-streaked again as putatively pure cultures.

To check the *Cytisus scoparius* root endophytes for nitrogenase activity, the liquid culture method described by Cahill (2003) was adapted. One colony of each strain was inoculated into 50 mL of nitrogen-free broth supplemented with ammonium nitrate (NfbN; Appendix A) and grown with shaking at 30 °C for one day. The whole volume of overnight culture was then spun down at 6500 RPM for 10 minutes, and the pellet was then resuspended in 25 mL of Nfb without N. Note that Cahill (2003) recommends resuspending in 50 mL of Nfb; due to limited reagent supplies, 25 mL was used in order to conserve media. The resuspended cells were then returned to the shaker at 30 °C overnight, pelleted again as before, resuspended in 2 mL Nfb, and 40 µL of the resulting nitrogen-starved cells were then used to aseptically inoculate 10 mL Nfb in sterile 24.3 mL serum bottles.

The ullage above the inoculated medium was then flushed with pure nitrogen gas for one minute and the bottle was stoppered with a sterile rubber septum. The airspace inside each bottle was then adjusted to 2% oxygen and 10% acetylene by using a 1-mL syringe to remove 1.7 mL of the nitrogen gas and replace it with 0.3 mL oxygen and 1.4 mL acetylene. The bottles were shaken upright at 30 °C for 90 minutes, then triplicate 0.5 mL samples were removed for acetylene reduction analysis as described in section 1.5. A bottle prepared as above but not inoculated was used to provide no-reduction control samples.

1.5 Plant harvest and nitrogenase analysis

The plant harvest procedure was substantially similar for all species. Every set of plants was photographed and its general condition noted immediately before beginning harvest. Each pot or growth tube was then inverted over a waste tub and all

plants were gently separated from the vermiculite growth medium, rinsed gently in DI water, and blotted dry with paper towels, then divided into root and shoot portions by cutting at the root crown with a razor. The weight and overall length of each segment was recorded. Many of the bottom-watered plants were found to have extensive root growth out the bottom of their pots. These in-water roots were both extremely long relative to plant size and highly susceptible to damage during the harvest process. For this reason, root lengths obtained by the longest-root measurement method were highly variable and probably not useful as a metric of overall plant health.

Samples of root and nodule tissue were saved for nitrogenase activity assays. The weight of each sample was recorded and it was then folded inside a damp paper towel to keep it moist while the other plants in the batch were harvested. Samples were then placed in glass serum bottles with a total volume of 24.3 mL, sealed with rubber septa, and a syringe was used to replace 2.4 mL of the air in the bottle with acetylene (generated by adding calcium carbide to water) to obtain a final concentration of 10% acetylene.

After incubating at room temperature for 15 minutes, a syringe was used to remove three 0.6 mL gas samples from each bottle. Syringes were stabbed into a large rubber stopper for transport between the harvest room and the gas analysis room, where 0.5 mL of each sample were injected into a Varian model 3300 gas chromatograph fitted with a $2.5' \times 1/8'' \times 0.085''$ stainless steel column containing 100/120 mesh Porapak (Alltech C-5000). The column temperature was maintained at 50 °C. Because acetylene generated from carbide contains trace amounts of ethylene, nitrogenase-free control samples were taken every time a fresh batch of acetylene was generated. The resulting background ethylene measurement (generally on the order of 20 pmol ethylene per mL sample) was subtracted from subsequent sample readings.

For *Lupinus bicolor*, *Lupinus arboreus*, and November *Cytisus scoparius*, all plant tissue not used for ARA was flash-frozen in liquid nitrogen and saved at −20 °C for planned analyses of total plant N, crude protein content, and chlorophyll concentrations. After reviewing the ARA results and the lack of any visible differences in plant size, it became apparent that these analyses were unlikely to add any new information about the nitrogen status of the plants. The nitrogen, chlorophyll, and protein tests were therefore not pursued further.

1.6 Molecular analysis

Four sets of oligonucleotide primers for the four "core" nodulation genes were retrieved from the literature. The four sets selected were judged to be likely candidates on the grounds that three of them had been already been used to amplify *nod* genes in other β-rhizobia, and the fourth (pair D) was designed against an intragenic region that shows little variation between rhizobial species.

Pair A (5′-TGGARVBTNYSYTGGGAAA-3′ and 5′-TCAYARYTCNGRNCCR-TTYC-3′) was used by Chen et al. (2003) to amplify a ∼500-bp fragment containing *nodA* from *Ralstonia taiwanensis* LMG19424 and LMG19425, *Rhizobium* J171, and *Burkholderia* TK182. Pair B (5′-CAGATCNAGDCCBTTGAARCGCA-3′ and 5′-CTNCGNGCCCARCGNAGTTG-3′) was used by Chen et al. (2003) to amplify a 2.1 kb fragment containing all of *nodB* and some of *nodC* from *Ralstonia taiwanensis* LMG19424. Pair C (5′-TAYRTGGTYGAYGACGGWTC-3′ and 5′-CCATAC-GCACCGTGGTGCTCTTGC-3′) was used by Moulin et al. (2001) to amplify a 1 kb fragment containing *nodC* and part of *nodI* from *Burkholderia* strain STM678. Pair D (5′-CTCGTCGCGCTCGACGCATTGA-3′ and 5′-TGCCCCATGGACATGTA- 3′) was used by Rivas et al. (2002) to amplify a 484-bp intragenic fragment of *nodD1* from *Devosia neptuniae*.

Crude cell extracts were prepared by picking one colony of the appropriate bacterial strain off a plate, suspending it in 200 µL sterile water in a microcentrifuge tube, boiling for 4 minutes, vortexing for one minute, and spinning down the cell lysate for 3 minutes at 13,000 xg. The supernatant was then aliquoted into clean tubes and stored at −20 °C until used directly as a PCR template at the rate of 0.5 µL per 25 µL reaction.

PCR cocktails were prepared as follows: 12.5 µL RedTaq® ReadyMix™ (Sigma R2523), 0.5 µM each primer, 0.5 µL cell extract, nanopure water to make 25 µL. Samples were amplified in either a PTC-100 thermocycler (MJ Research) or a Eppendorf Mastercycler Gradient thermocycler (Eppendorf 5331-10377) using the following program settings: 94 °C initial denature for 5 minutes; 30 cycles of 94 °C for 1 minute, 45 (or 40) °C for 2 (or 3) minutes, 72 °C for 3 minutes; hold at 15 °C. Products were visualized on 1% agarose/TAE gels. SYBR Green was added directly to the sample before loading.

PCR products were ligated into the pCR® 2.1-TOPO™vector and cloned using One Shot®TOP10 chemically competent *E. coli* (Invitrogen K4500) according to the kit directions. Plasmid recovery in order to obtain sequence data was attempted

using Quiagen's CompactPrep plasmid midi kit; failure to follow the manufacturer's directions resulted in an unsuccessful plasmid extraction. Due to time constraints, only a single attempt at plasmid extraction was made and no sequences were obtained.

Chapter 2

Results

2.1 Plant growth

2.1.1 *Alnus rubra*

Of six 8″ plastic pots per treatment transplanted with c. 20 seedlings each, five contained 4–9 living seedlings when harvested at 23 weeks (total of 26 control and 34 inoculated plants). Five plants from the control group and one from the inoculated group were conspicuously taller and greener than the other seedlings, with healthy, fully-expanded leaves and no chlorosis. These plants were found to have active root nodules of the classic *Frankia* form. Nodules were always found at the very bottom of the pot, and no morphological differences were evident between the single inoculated nodule and nodules from control plants (Fig. 2.4). Control nodules produced an average of 7.2 μmol ethylene · g nodule^{-1} · hr^{-1} when placed under 10% acetylene. The single inoculated nodule produced somewhat more (12.1 μmol ethylene · g nodule^{-1} · hr^{-1}), but this number is only from a single sample and furthermore is the mean of triplicate readings that ranged from 4.5 to 15.7 μmol ethylene · g nodule^{-1} · hr^{-1} on the same sample. Triplicate readings on nodules from control plants showed much higher internal consistency. Uninfected root material from both nodulated and non-nodulated plants showed no acetylene reduction activity.

Of 100 growth tubes transplanted with healthy *Alnus rubra* seedlings, 41 control and 36 inoculated were still alive when harvested after 22 weeks. All plants were extremely stunted and chlorotic, and in most cases the roots proved to be badly entangled with the screens at the bottom of the growth tubes, preventing reliable retrieval of the whole root system. Given the dramatic differences observed between nodulated and non-nodulated *Alnus rubra* in the potted samples, total plant weight

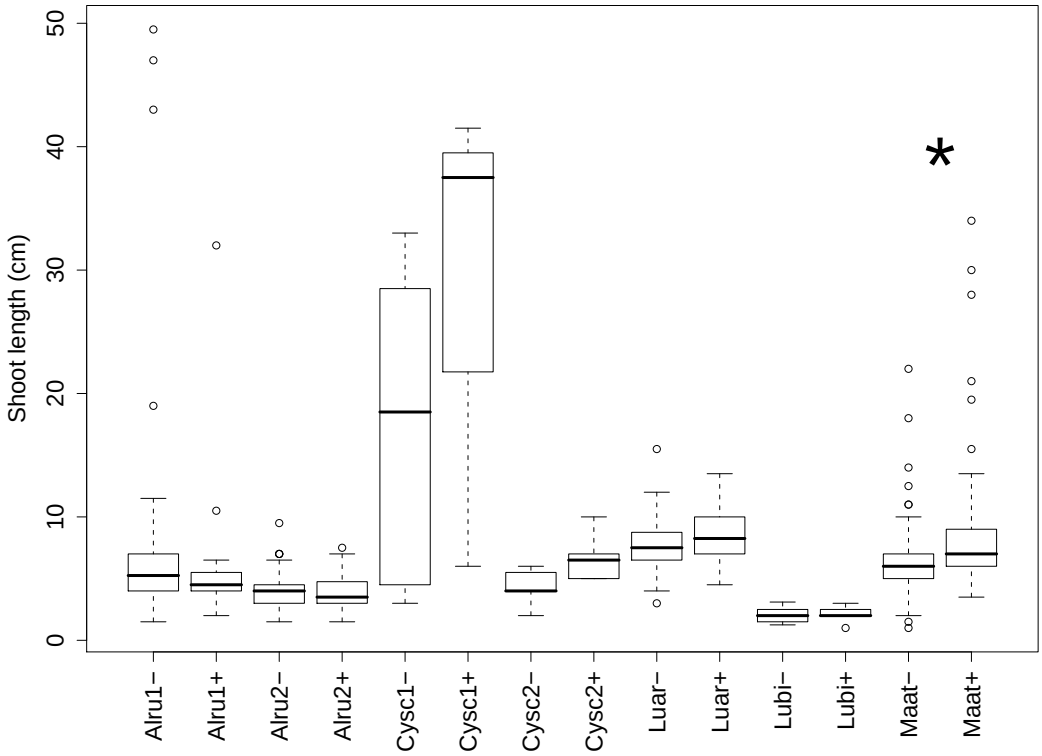

Figure 2.1: Shoot length at harvest time for five species of N-fixing plants grown under hypoxenic conditions (−) or inoculated with *Burkholderia tropica* strain Aa1 (+). Alru = *Alnus rubra*, Cysc = *Cytisus scoparius*, Luar = *Lupinus arboreus*, Lubi = *Lupinus bicolor*, Maat = *Macroptilium atropurpureum*. Mean lengths were significantly different between treatments only for *Macroptilium atropurpureum* (t=2.9, $p < 0.001$).

and root:shoot ratio were not measured. There was no significant difference in shoot length between inoculated and control tubes (Fig. 2.1). No nodulation was observed, and three randomly selected samples from each group showed no acetylene reduction activity.

2.1.2 *Cytisus scoparius*

For the initial planting in November, germination was extremely poor (< 1%) in both 8″ plastic pots and individual growth tubes. Only 8 control and 3 inoculated plants survived until harvest at 20 weeks (plastic pots) or 19 weeks (growth tubes). Surviving plants had few leaves and some chlorosis, but exhibited strong stem growth and extensive branching (Fig. 2.7). Control and *Burkholderia*-treated plants showed no significant difference in plant fresh weight (Fig. 2.2), shoot length (Fig. 2.1), or root:shoot ratio (Fig. 2.3). One inoculated and two control plants exhibited very small pesudo-nodules too small to pick off the roots (Fig. 2.5), but neither these nor

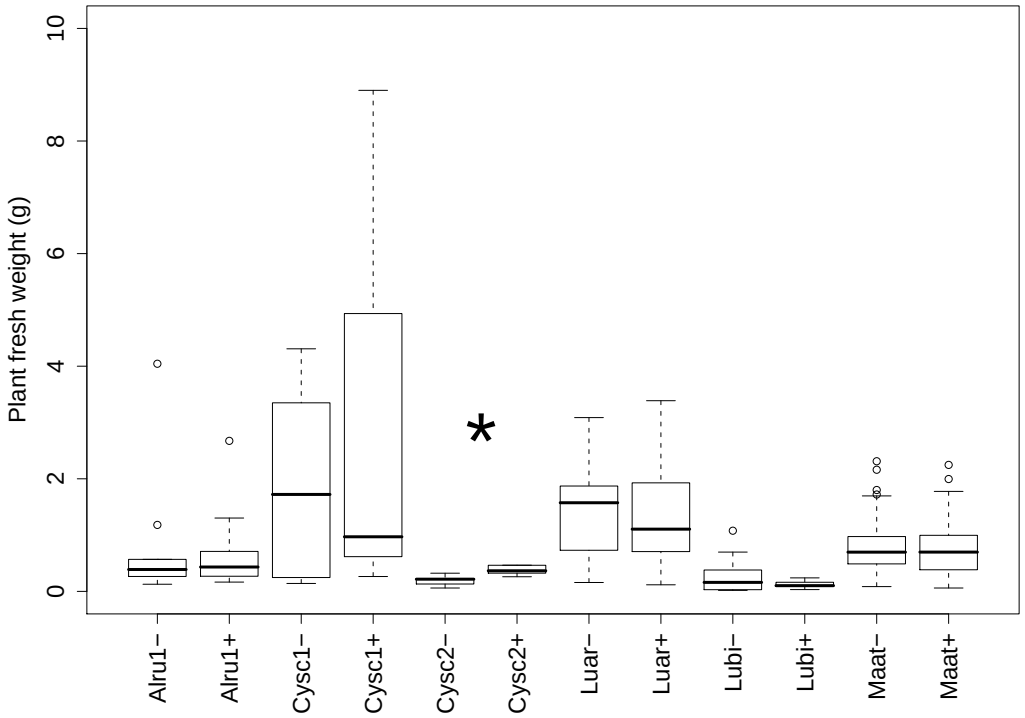

Figure 2.2: Plant fresh weight at harvest time for five species of N-fixing plants grown under hypoxenic conditions (−) or inoculated with *Burkholderia tropica* strain Aa1 (+). Alru = *Alnus rubra*, Cysc = *Cytisus scoparius*, Luar = *Lupinus arboreus*, Lubi = *Lupinus bicolor*, Maat = *Macroptilium atropurpureum*. Mean weights were significantly different between treatments only for the second planting of *Cytisus scoparius* ($t = 3.09$, $p < 0.02$). To preserve scale, *Alnus rubra* with active root nodules (4 plants (13–35 g) in Alru-, 1 plant (30 g) in Alru+) are not shown.

non-nodulated roots reduced acetylene.

For a second planting in February, the seed coats were nicked with a razor before soaking the seeds in water overnight. These plants germinated markedly faster and more completely, and five plants from each treatment group survived until harvest at 11 weeks. Inoculated plants had a significantly higher root:shoot ratio ($t = 3.91$, $p < 0.02$; Fig. 2.3) and significantly higher fresh weight ($t = 3.09$, $p < 0.02$; Fig. 2.2) than control plants. Mean shoot length was higher in inoculated plants (6.7 cm compared to 4.3 cm for controls) but the difference was not significant ($t = 2.08$, $p = 0.07$; Fig. 2.1). No nodulation was observed, and none of the roots reduced acetylene.

2.1.3 *Lupinus arboreus*

Although *Lupinus arboreus* germinated more successfully than *L. bicolor*, only 36% (inoculated) and 52% (control) of 50 tubes contained recoverable plants at harvest

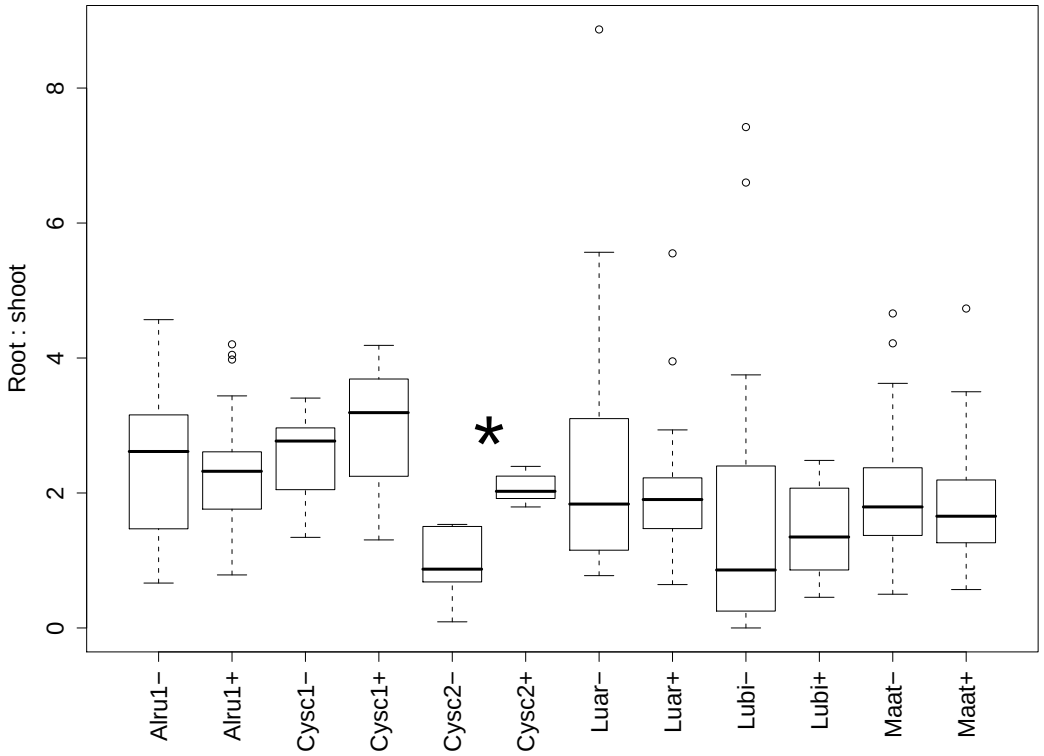

Figure 2.3: Root:shoot ratio for five species of N-fixing plants grown under hypoxenic conditions (−) or inoculated with *Burkholderia tropica* strain Aa1 (+). Alru = *Alnus rubra*, Cysc = *Cytisus scoparius*, Luar = *Lupinus arboreus*, Lubi = *Lupinus bicolor*, Maat = *Macroptilium atropurpureum*. Mean weights were significantly different between treatments only for the second planting of *Cytisus scoparius* ($t = 3.91$, $p < 0.02$).

time. Of these, 6 and 7 plants, respectively, were already dead when the plants were harvested at 14 weeks. All *Lupinus arboreus* plants exhibited vigorous root growth, with mean root:shoot ratios near 2 (ranging as high as 8.8), but poor leaf retention; plants had grown and dropped several whorls of leaves, and most living plants had only a few leaves attached to the stem. Control and *Burkholderia*-treated plants showed no significant difference in shoot length (Fig. 2.1), plant fresh weight (Fig. 2.2), or root:shoot ratio (Fig. 2.3). Roughly half the plants in each group (15 out of 27 control plants, 8 of 18 inoculated plants) displayed nodule-like root structures up to 1 mm in diameter (Fig 2.1.3), although about on about half of these (7 and 4 plants respectively) the pseudonodules were too small to pick off the roots. For the nodules that were picked, mean nodule mass per plant was higher on inoculated plants (10 mg compared to 4.9 mg for control plants), but was highly variable (standard deviation = 8.9 and 5.5 respectively) and the difference was not significant ($t = 1.0$, $p = 0.3$) The pseudonodules appeared to be determinate in structure and grew anywhere along

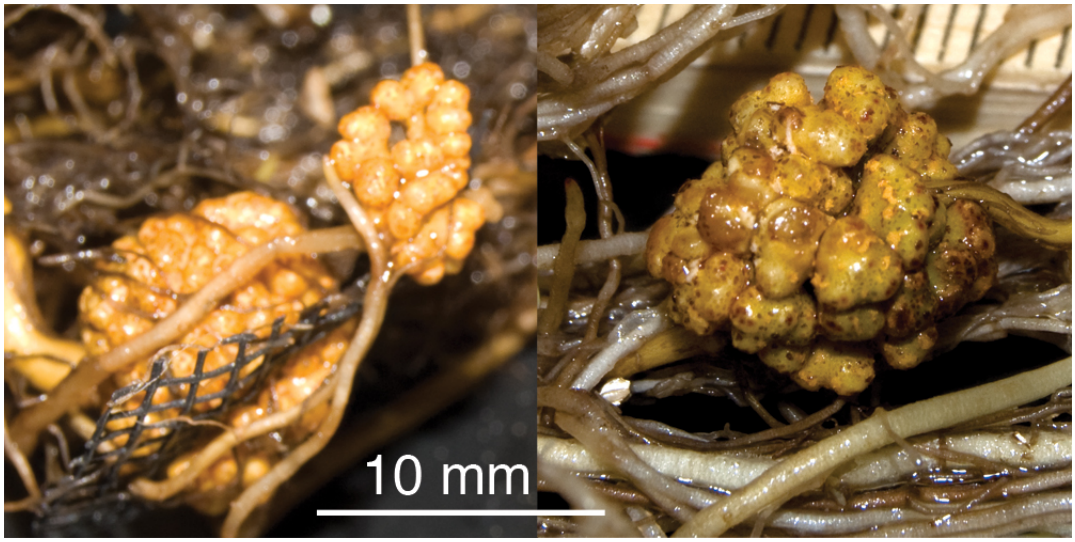

Figure 2.4: Active root nodules from *Alnus rubra* raised hypoxenically (left) or inoculated with *Burkholderia tropica* (right).

the length of the root, including root tips and branching points. Neither roots nor pseudonodules showed any acetylene reduction activity.

2.1.4 *Lupinus bicolor*

The hard seed coat and clay pelleting on the commercially-obtained *Lupinus bicolor* seed made it particularly difficult to sterilize. In initial germination tests, sterilized seeds showed persistently low germination and high contamination rates when plated on maltose agar. Even after selecting a sterilization technique that yielded acceptable germination levels on maltose, germination of the vermiculite-grown seeds was poor and inconsistent between treatment groups. Only 19% of 91 untreated tubes showed germination after 2 weeks, while 91 *Burkholderia*-treated tubes showed no germination at all until 5 weeks after planting. Once germinated, the seedlings grew very poorly and both treated and untreated plants remained chlorotic and stunted. At harvest time (14 weeks after planting), only 8 (inoculated) and 12 (control) tubes contained recoverable plants. Most plants were dead at harvest time, with only two living plants recovered for each group. No nodulation was observed, and the living plants did not reduce acetylene. Control and *Burkholderia*-treated plants showed no significant difference in shoot length (Fig. 2.1), plant fresh weight (Fig. 2.2), or root:shoot ratio (Fig. 2.3).

Figure 2.5: Pseudonodules on 20 week old *Cytisus scoparius* inoculated with *Burkholderia tropica*. No nitrogenase activity was observed.

2.1.5 *Macroptilium atropurpureum*

These plants, refreshingly, germinated quite well. 22 of 25 pots in the control group, and all 25 inoculated pots, contained at least one living plant when harvested at 10 weeks, for a total of 91 control and 89 inoculated plants. Most plants suffered moderate foliar damage from greenhouse infestation of white flies, and most plants were mildly chlorotic. Inoculated plants were significantly taller ($t = 2.9$, $p < 0.001$; Fig. 2.1) than control plants, but did not differ in plant weight (Fig. 2.2) or root:shoot ratio (Fig. 2.3). No nodules were observed, and randomly selected root samples showed no acetylene reduction activity.

2.2 Isolation of root endophytes from *Cytisus scoparius*

The few individuals from the November planting of *Cytisus scoparius* that survived to harvest time were of interest because they appeared to be less than entirely nitrogen-starved (Fig. 2.7); their weight and stem length suggested that they had found some minimal nitrogen source. In order to determine whether this N might come from

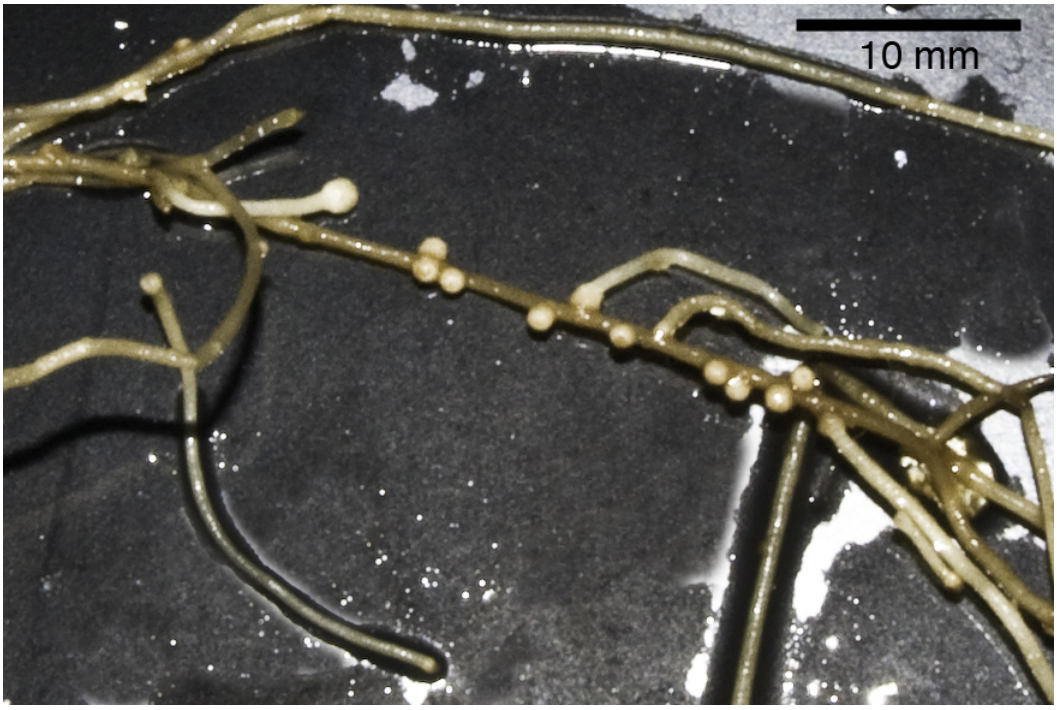

Figure 2.6: Pseudonodules on roots of 14 week old *Lupinus arboreus* inoculated with *Burkholderia*. No nitrogenase activity was observed.

associative endophytes, crushed root extracts from three *Cytisus scoparius* plants in each group were grown on LB at 30 °C and colonies of differing morphologies were replated separately. A total of 11 putatively pure cultures (6 inoculated, 5 control) were obtained (Tables 2.1 and 2.2).

To test these isolates for nitrogenase activity, nitrogen-starved liquid cultures were exposed to 10% acetylene under low-oxygen conditions and samples of the headspace gas were checked for ethylene production. Due to time and material constraints, the assay was performed only once using only one sample of each isolate. Three cultures from inoculated plants and three cultures from control plants exhibited good growth in liquid Nfb (Table 2.3), while the remaining cultures formed only very small, stringy, yellowish floating cell masses that did not form a pellet when centrifuged. Neither the well-grown nor the recalcitrant cultures reduced a detectable amount of acetylene over the course of 90 minutes.

To further characterize the isolates, samples of all 11 cultures were Gram-stained and examined under a light microscope at up to 1000x magnification (Tables 2.1 and 2.2). Strains "2+","3+","5+", and "2−" were found to be impure cultures of two or more distinct cell types. Of the six isolates (4+,5+,6+,2−,3−,5−) that grew well in Nfb, all but 5+ were dominated by Gram-negative cells. Strains 1+ and 1− were

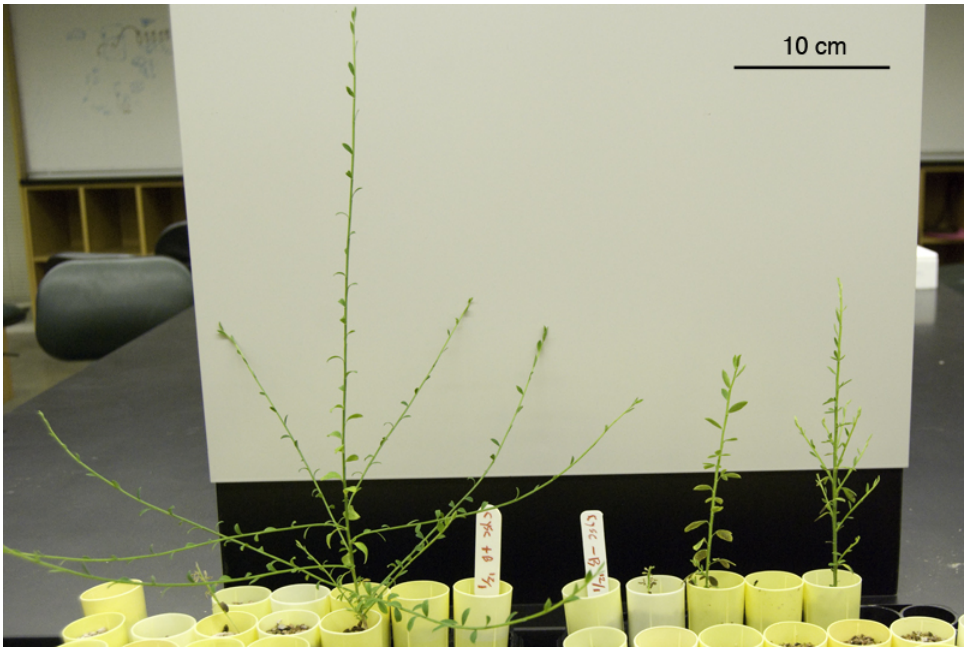

Figure 2.7: 20 week old *Cytisus scoparius* inoculated with *Burkholderia tropica* (left) or hypoxenically grown (right).

indistinguishable in both cell morphology and in colony growth on LB medium, and were hypothesized to represent the same strain.

Strain 1+/1− grown on LB plates for 3–4 days showed an abundance of small clearings with the characteristic appearance of bacteriophage plaques. A liquid culture of strain 1 was co-inoculated with phage recovered from a plaque and examined by Gram-staining after 1 day. Only a few intact strain 1 cells were visible among extensive lysis debris, idicating that a high concentration of phage was present in the sample.

Table 2.1: Characteristics of bacterial strains isolated from *Burkholderia*-treated *Cytisus scoparius* roots.

Strain no.	Plant no.	Colony dia. (mm)[a]	Colony color	Colony texture	Cell size (μm)	Gram stain	Cell shape
1+	1+, 3+	10	Clear	Granular	1 × 5–10	G+	Straight to slightly curved rods, usually in end-to-end strings of 4–10 cells
2+	2+	2–5	White to yellow	Crusty bullseye with fringed edges	0.5 × 2	G−	Straight to slightly curved rods
					1 × 2–4	G+	Tapered rods
					0.8 × 1	G+	Ovoid
					0.7 × 2–3	G+	Straight to slightly curved rods
3+	3+	1–5	White to cream	Crusty/granular, faint bullseye	0.8 × 2–3	G+	Straight rods
					1 × 1.5	G−	"Bubble": pink oval ring with clear center
					1.2 × 4	G−	Solid pink "rice grain"
4+	1+	1	Cream to yellow	Round with smooth margins	0.5 × 1–3	G−	Slightly curved rods
5+	2+	> 10	Cream turning yellow	"Cauliflower" blobs at margin, turning crusty when yellow	1 × 5	G+	Slightly curved rods
					0.5	G+	Cocci
					1 × 4–6	G−	Thin string-like rods
6+	2+	1	Yellow	Very mucousy, readily spreading into a film. Edges indistinct, colorless	0.4 × 1–2	G−	Straight rods

[a]Colony characteristics were observed after 2 days growing on LB agar at 30 °C.

Table 2.2: Characteristics of bacterial strains isolated from hypoxenically grown *Cytisus scoparius* roots.

Strain no.	Plant no.	Colony dia. (mm)[a]	Colony color	Colony texture	Cell size (µm)	Gram stain	Cell shape
1−	1−, 4−, 5−	Same characteristics as strain 1+ (Table 2.1)					
2−	5−	—	Cream	Extremely mucoid. No colonies; forms one smooth mass in plate	0.6 × 1–4	G−	Curved rods in 2 × 2–6 µm capsules
					0.5 × 1–1.5	G+	Rods
3−	1−	2	Yellow	Mucoid, smooth margins, readily spreads into film	0.5 × 1–1.5	G−	Straight to slightly curved rods with rounded corners
4−	4−	—	White	Flat mucoid film, no separate colonies	0.7 × 3–4	G−	Curved rods
					1 × 3	G−	"bubbles" with dark spots at poles
5−	4−, 1−	2	Cream	Round with smooth margins	0.5 × 1–1.5	G−	Straight to slightly curved rods with rounded corners

[a]Colony characteristics were observed after 2 days growing on LB agar at 30 °C.

Table 2.3: Growth of *Cytisus scoparius* root bacterial isolates in liquid culture.

Strain	NfbN[a]	Nfb[b]	A_{400}[c]
1+	−	−	0.0031
2+	−	−	−0.0059
3+	−	−	0.0056
4+	++	++	0.3694
5+	+	±	0.0041
6+	++	++	0.1814
1−	−	−	0.0073
2−	+++	++	0.1534
3−	++	++	0.2489
4−	−	−	0.0090
5−	+++	++	0.1045

[a]Overnight cultures grown at 30 °C in nitrogen-free broth supplemented with 13.7 mM NH_4NO_3. −: No growth. ±: Barely turbid. + to +++: Turbid to extremely turbid.

[b]Cells from 50 mL NfbN after overnight starvation in 25 mL nitrogen-free broth. Symbols as for NfbN.

[c]Absorbance at 400 nm. Measured 90 min after inoculating 10 mL Nfb with 40 µL concentrated N-starved cells in Nfb. Grown at 30 °C under 2% O_2 and 10% acetylene.

2.3 Nodulation genes

After experimenting with several sets of amplification conditions, a 45 °C annealing temperature was found to give relatively clean products from two of the four *nod* primer pairs in *B. tropica* Aa1 (Figure 2.8). Pair A produced a single 550 bp band (c. 500 bp expected). Pair C produced a single 850 bp band (c. 1 kb expected). Pair D produced 2–3 variable products usually featuring one strong band near 900 to 1500 bp and a weak band near 500 bp (c. 500 bp expected). Pair B produced no product in *Burkholderia* when annealed at 45 °C, and showed nonspecific amplification when annealed at 40 °C.

To demonstrate that the amplified fragments were actually *nod* genes, the same amplification was performed on the classic model nodulator *Rhizobium* GR234 as a positive control, and on *Pseudomonas fluorescens*, a well-characterized non-nodulator, as a negative control. Surprisingly, none of the primer pairs amplified any product from *Rhizobium* Fig 2.9). The same batch of cell extracts were used successfully to produce good products when amplified with two different sets of 16S primers (Fig. 2.10), indicating that the template DNA was not defective.

Even more surprisingly, primers for *nodB* produced a single, clean c. 600-bp band

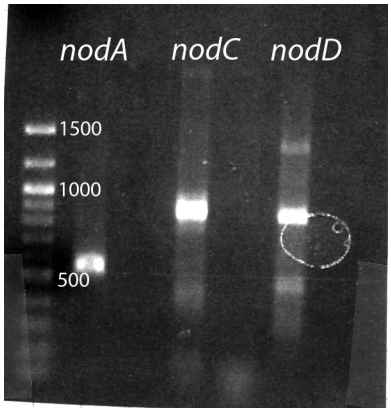

Figure 2.8: PCR products from *Burkholderia* DNA used for cloning of *nodACD*. Bands were visualized on 1% agarose using SYBR green.

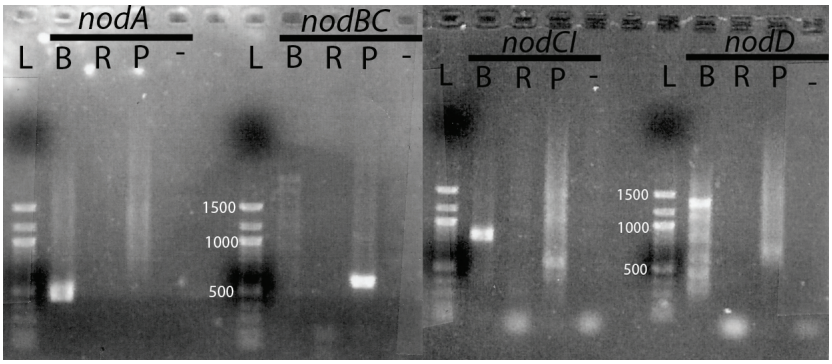

Figure 2.9: PCR products obtained using primers for the core nodulation genes. Bands were visualized on 1% agarose using SYBR green. L: 100 bp ladder. B: *Burkholderia tropica* Aa1. R: *Rhizobium* GR234. P: *Pseudomonas fluorescens*. −: no-DNA control.

in the non-nodulating *Pseudomonas fluorescens* (Fig. 2.9), despite having consistently failed to produce any usable product in either of the suspected nodulators. Further research in the GenBank database revealed that *Pseudomonas fluorescens* encodes locus YP_347747, a polysaccharide deacetylase that is annotated as a member of the NodB family.

The PCR products shown in Fig. 2.8 were ligated into a plasmid vector and cloned into competent *E. coli* in order to obtain sequence data. Due to time constraints, plasmid extractions were not completed and no sequence data was available for these gene fragments.

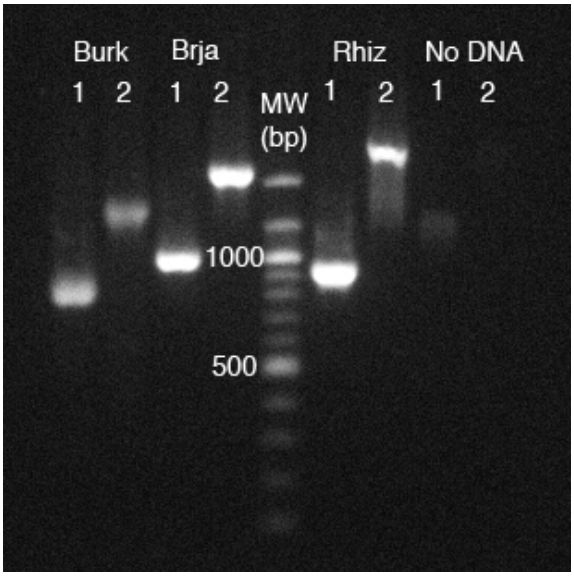

Figure 2.10: 16S PCR products from three bacterial strains. Bands were visualized on 1% agarose using SYBR green. MW: 100 bp ladder. Burk: *Burkholderia tropica* Aa1. Brja: *Bradyrhizobium japonicim* 122DES. Rhiz: *Rhizobium* GR234. 1: primers SDBact0515aA19/SDBact1371aS20. 2: primers 7f/1329r.

Chapter 3

Discussion

3.1 Nodulation

The foremost goal of this study was to ask whether *Burkholderia tropica* Aa1 is capable of nodulating any Oregon legumes. No evidence was found to support such capabilities. The only active nodules observed, those on the early-planted *Alnus rubra*, were probably formed by *Frankia* contaminants that either survived seed treatment or were introduced in the greenhouse; the visual appearance of the nodules did not differ between inoculated and untreated plants, and was like that of a typical wild-type *Alnus*/*Frankia* nodule (Fig. 2.4). Since only one nodule was observed in the inoculated group, it is not possible to make any statistical comparisons of nodule characteristics, but nodule weight (about 2–3% of total plant weight) also appeared to be similar between groups. It is tempting to ask whether *Burkholderia tropica* affects the number of nodules or the number of *Frankia*-nodulated plants per group, as was reported for *Burkholderia cepacia* by Knowlton and Dawson (1983), but since a fair test of nodulation by *Burkholderia* acting alone required that *Frankia* be actively excluded, this experiment was not the place to make such a comparison.

Nodulation and nitrogen fixation are different phenomena that can occur separately as well as together. The presence of pseudonodules on a given plant, even if no nitrogenase activity is detected, can indicate that the plant and the microsymbiont are engaging in successful signal exchange. In this study, both *Cytisus scoparius* and *Lupinus arboreus* were found to form pseudonodules. However, in both species inoculated and untreated plants were pseudonodulated to a roughly equal degree, indicating that the observed structures were not a response to stimulation by *Burkholderia*. This is backed up by the observation that there were no nodule-like structures at all on *Lupinus bicolor* or on *Macroptilium atropurpureum*, a wide-spectrum host that has

often been used as a shibboleth for the nodulation capabilities of β-rhizobia (Barrett and Parker, 2005; Bottomley et al., 1994; Elliott et al., 2007; Zakhia et al., 2006).

It is interesting to note that although all *Alnus rubra* seeds were given the same sterilization treatment, only one pot and none of the 100 growth tubes from each group developed nodules. Optimal oxygen concentrations for actinorhizal nodules are quite near normal atmosperic levels (Tjepkema et al., 1986); tube-grown alders were bottom-watered, leaving the base of each tube continuously flooded and presumably hypoxic. Since all nodules found in the potted alders were located at the extreme base of the pot, flooded conditions at the base of the growth tubes may have discouraged *Frankia* colonization. On the other hand, only the bottom 2–3 cm of each tube were submerged, and the vermiculite growth matrix was quite well-drained everywhere above the water line; presumably any *Frankia* present in the the root system would have been able to nodulate successfully near the surface of the tube.

3.2 Plant growth

Inoculation with *Burkholderia* produced a small stimulation of shoot length in *Macroptilium atropurpureum* (Fig. 2.1). *Cytisus scoparius* exhibited both increased plant weight and increased root:shoot ratio (Figs. 2.2 and 2.3). Increased plant weight and shoot length are hypothesized to indicate increased nitrogen supply, probably supplied by associative, non-nodulating *Burkholderia* that was fixing or scavenging small amounts of N. The observed stimulation was statistically significant but was not large, and there was no accompanying change in plant greenness—plants from both groups retained the general appearance of being "real, real hungry." Plants under nutrient limitations, especially low N, usually increase biomass allocation to the root zone in an effort to find more nutrients (Wilson, 1988). The increase in root:shoot ratio seen in *Cytisus scoparius* is consistent with the notion that any bacterial N fertilization effect was only enough to whet their appetites.

There are many ways in addition to nitrogen nutrition that a plant-microbe interaction can affect plant status. Bacteria may encourage plant growth by production of phytohormones. For instance, diazotrophs seem to produce remarkably high levels of indole-3-acetic acid (IAA) (Fuentes-Ramirez et al., 1993). Root bacteria can also enhance the uptake of nutrients other than N, either directly as through production of siderophores (iron-chelating agents) or indirectly by encouraging the colonization of the plant by nutrient-sharing mycorrhizal fungi (Vial et al., 2007; Will and Sylvia, 1990), but these factors are unlikely to bear heavily on this experiment because it

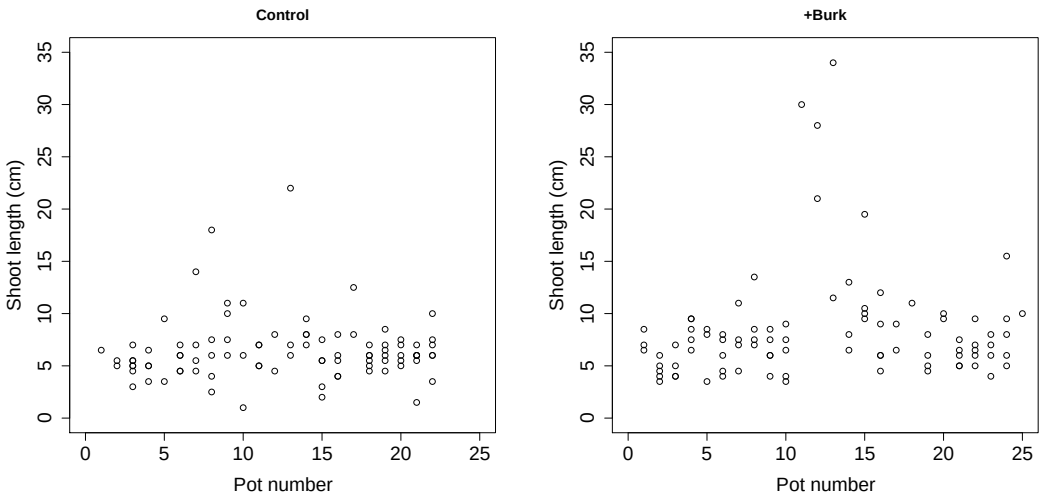

Figure 3.1: *Macroptilium atropurpureum* shows longer shoot lengths for midbench pots in both control (left) and *Burkholderia*-inoculated (right) groups.

was conducted under afungal conditions using adequately chelated nutrient solutions.

In both the inoculated and the control groups of *Macroptilium atropurpureum*, the pots containing the tallest plants came from the middle rows of the bench, possibly because of slight differences in light level (Figure 3.1). Since the effect was similar between groups, this is not likely to bias the observed results, but if this study were to be repeated it would be advisable to include regular rearrangement of the pots in order to avoid microsite differences.

The plants that were found to show significant differences between treatments were also the plants that were planted in February and harvested after 10–11 weeks rather than the 14–23 week harvest times for plants potted in November and December. This suggests the possibility that *Burkholderia* may have an early growth-promoting effect which dissipated as the plants grew larger. If this were the case, a greenhouse trial might disguise effects that would be stronger in an ecological setting where early seedling success provides strong competitive advantages. An alternate and less exciting explanation could be that the fall inoculations were not effective at establishing a resident *Burkholderia* population. Due to experimenter error, several of the November and December plantings were inoculated with *Burkholderia* cultures as much as 5 days old. *Burkholderia* does not form endospores, making it likely that these extremely old cultures would have contained only a few viable cells per mL compared to the dose provided by an equivalent quantity of a fresh overnight culture.

3.3 *Cytisus scoparius* root isolates

None of 11 bacterial strains recovered from *Cytisus scoparius* roots showed a clear match with *Burkholderia tropica* Aa1 for both colony structure and cell shape. Strain 3− is a possible exception; it has small yellow colonies with smooth margins, and the cells are roughly the expected size, but the resemblance is not striking. The lack of nitrogenase activity in liquid culture, using conditions reported by Cahill (2003) to yield on the order of 30 nmol ethylene per sample from *Burkholderia* Aa1, further reinforces the conclusion that the isolates obtained most likely did not include *Burkholderia*.

Although all strains failed to show any acetylene reduction activity, 5 strains were apparently able to obtain sufficient N for good growth in nitrogen-free media. Time and supply constraints limited the ARA experiments to one meaurement of one sample per strain, with the six poorly-growing strains measured at extremely low population densities if they were alive at all. It is impossible to say whether further optimization of the protocol would have found conditions that induced nitrogenase activity in any of these strains. Furthermore, the isolates obtained are expected to be a very small subset of the bacteria present in the root and there is no particular reason to expect the diazotrophs to be culturable, so the lack of N-fixing isolates does not provide strong evidence for their absence from the *Cytisus scoparius* roots. A cautionary tale can be gleaned from Benhizia et al. (2004), who reported finding nodules of *Hedysarum* from which they could only culture γ-proteobacteria and therefore argued that they had identified the first known γ-rhizobia. After a more careful analysis using 16S PCR directly on the root nodules, they reported that these nodules were primarily inhabited by α-rhizobia after all.

Strain 1+/1−, the bacterial strain most commonly isolated from *Cytisus scoparius* roots, was a Gram-positive rod occurring in long end-to-end chains and forming large, colorless, rough-textured colonies on LB agar. It was also found to carry a population of bacteriophage which could be isolated from plaques and used to re-infect cells of strain 1+/1−. The significance of the phage in the *Cytisus scoparius*/microbe interaction is unknown. Bacteriophage have been previously reported in other endophytic bacteria such as *Azospirillum* (Boyer et al., 2008), as well as in *Burkholderia cepacia* (Hens et al., 2005).

3.4 *nod* genes

PCR products apparently corresponding to three of the four "core" *nod* genes common to all known rhizobia were detected in *Burkholderia* Aa1. *nodA* and *nodC* primers produced single bands near the sizes expected (550 and 850 bp; Fig. 2.9), suggesting that the observed product was likely to contain the expected region. If the products were sequenced and found to match those of previously reported *Burkholderia nod* genes, this would provide strong proof that *nodAC* are functional in *B. tropica*.

Oddly, none of the four primers produced good products when tested on high-quality cell extracts from the well-studied model nodulator *Rhizobium* GR234. This finding is extremely hard to account for and needs to be investigated more closely before relying on the PCR data.

nodB gave no product unless run with an extremely low annealing temperature, in which case nonspecific amplification was observed. This may indicate that *Burkholderia tropica* has no functional copy of *nodB*, which would be consistent with the observed lack of nodulation by *Burkholderia*. The observed 600-bp band in the "negative control" *Pseudomonas fluorescens* is surprising; although *P. fluorescens* has many unusual talents, including bioaccumulation of yttria and production of plastics (Appanna et al., 2001; Jiang et al., 2008), it and all other *Pseudomonas* species have not been convincingly shown to form root nodules, and a cursory database search revealed no published nodulation genes from *P. fluorescens*. However, a more thorough examination of the NCBI protein database reveals that *P. fluorescens* encodes locus YP_347747, a polysaccharide deacetylase that is annotated as a member of the NodB family. This finding suggests that the *nodB* primer set should be able to amplify a true *nodB* gene if it were present, and provides additional support for the possibility of a missing or defective *nodB* locus in *Burkholderia tropica*.

The band for *nodD* showed different sizes under different cycler conditions, reducing confidence that it was producing a single product. Unlike the other three primer sets used, this one has not been previously reported to amplify *nod* genes in β-rhizobia. However, it is reported to amplify an intragenic region of *nodD* that should be highly conserved among rhizobia (Rivas et al., 2002). After finding the product to be variable and often much larger than the expected size (484 bp in *Devosia*), the primers were checked against other *nodD* sequences, and particularly against the *Sinorhizobium meliloti nodD* gene (AE007238) positions 31 to 53 and 585 to 568, which Rivas et al. (2002) say was used to design the primers. The sequence and the primers were found to disagree at three bases; the published

primer pair is 5′-CTCGTCGCGCTCGACGCATTGA-3′ / 5′-TGCCCCATGGAC-ATGTA-3′, while the corresponding sequence matching the *S. meliloti* sequence is 5′-CTCGTCGCGCTCGACGCACTGA-3′ / 5′-TGCCCCATCGACATATA-3′. It would be instructive to redo the *Burkholderia tropica* PCR using an altered *nodD* primer set.

3.5 Conclusion

Although it is an important endophytic nitrogen source for dune grasses, *Burkholderia tropica* does not seem to be capable of inducing nodule formation on any of five commonly nodulated plant hosts when they are grown under hypoxenic, N-limited greenhouse conditions. Inoculation with *B. tropica* results in a mild and possibly transient enhancement of plant growth but does not seem to appreciably change the nitrogen status of the plants. *B. tropica* may lack an active *nodB* gene, which would disable a critical deacetylase activity and prevent the successful biosynthesis of Nod signaling factors. Such a finding would be sufficient to account for the oberved lack of nodulation, but cannot be shown conclusively with the current evidence. Future work in this system should examine the nod genes more closely, perhaps by Southern blotting in addition to further PCR-based work, in order to determine conclusively whether each of the core *nod* genes, and *nodB* in particular, is present and functional.

Appendix A

Nutrient solutions for plants and bacteria

N-free plant nutrients

Solution 1: Macronutrients

1.58	μM	K_2SO_4
2.0	μM	$MgSO_4$
0.57	μM	KH_2PO_4
0.43	μM	K_2HPO_4
6.0	μM	$CaSO_4$
0.5	μM	$CaCl_2$

Solution 2: Micronutients

(Make up 1000x stock. Concentrations given are for 1x.)

3.7	μM	H_3BO_3
0.72	μM	$MnSO_4$
0.76	μM	$ZnSO_4$
0.31	μM	$CuSO_4$
0.10	μM	Na_2MoO_4
0.17	μM	$CoCl_2$
0.17	μM	$NiCl_2$

Solution 3: Iron

Make 1000x stock by adding 16.7 g Sequestrene 138 Fe per liter of DI H_2O.
(Sequestrene = FeEDDHA from Ciba-Geigy, Greensboro NC 27409)

Nfb: Nitrogen-free broth

5.00	g	D,L-malic acid
0.50	g	K_2HPO_4
0.20	g	$MgSO_4 \cdot 7H_2O$
0.10	g	NaCl
0.02	g	$CaCl_2 \cdot 2H_2O$
2.00	mL	Minor element solution
4.00	mL	Bromthymol blue, 0.5% w/v in 0.2 M KOH

Dissolve in 800 mL DI water, adjust pH to 6.8 with KOH. Bring volume to 1 L with DI water, autoclave 20 minutes. Add 1.00 mL vitamin solution after cooling. For NfbN, add 1.1 g NH_4NO_3 per liter.

Minor element solution

0.40	g	$CuSO_4 \cdot 5H_2O$
0.12	g	$ZnSO_4 \cdot 7H_2O$
1.40	g	H_3BO_3
1.00	g	$NaMoO_4 \cdot 2H_2O$
1.50	g	$MnSO_4 \cdot H_2O$

Dissolve in 800 mL DI water, then bring volume up to 1 L.
(Or use 1000x plant micronutrient stock instead)

Vitamin solution

10	mg	Biotin
20	mg	Pyridoxol-HCL
100	mL	DI water

Dissolve all ingredients, then filter-sterilize through an 0.2-micron filter. Freeze 1-mL aliquots, let media cool before adding to avoid breakdown of heat-labile vitamins.

YM: Yeast-mannitol broth

0.5	g	K_2HPO_4
0.2	g	$MgSO_4 \cdot 7H_2O$
0.1	g	NaCl
10.0	g	Mannitol
0.4	g	Yeast extract

Dissolve in 800 mL DI water. Adjust pH to 6.8, bring volume to 1 L. For solid media, add 15 g agar. Autoclave.

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