The Quantitation of *Bradyrhizobium japonicum* Communities and the Isolation of Bacteriocin-Producing *Bradyrhizobium* Strains from Manitoban Soil

by

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Abstract

Bradyrhizobium japonicum is a Gram negative α-proteobacteria capable of reducing atmospheric nitrogen to ammonia while in a symbiotic relationship with soybean. This symbiosis manifests itself as nodules formed on the roots of soybean plants. A well-nodulated plant can derive all the nitrogen necessary for growth from this association. One problem that is often encountered is not having sufficient numbers of the correct strains of rhizobia present as the seed is germinating which prevent effective nodules to develop in a timely manner. Currently, the quantification of *Bradyrhizobium* in soil relies heavily on culture-based assays which are time-consuming, labour-intensive, and lacks the ability to differentiate between strains of *B. japonicum*. In this study, we developed a rapid and sensitive real-time qPCR based assay for the quantification of *B. japonicum*. This assay is able to differentiate between strains of *B. japonicum*. Using this assay, we showed the differences in the composition of *B. japonicum* community in fields utilizing narrow (15 inches) and wide (30 inches) row spacing. We showed that the composition of *B. japonicum* community in soil is affected by the population density of host plants. We also isolated *B. japonicum* strain FN1 in Manitoba. FN1 produces substances with bacteriocin-like characteristics that inhibit the growth of multiple other strains of *Bradyrhizobium*. In this study, we were able to identify a gene that is responsible for the production of the bacteriocin-like substance that inhibits the growth of the strain SR-16. Furthermore, we showed that the ability of FN1 to produce bacteriocin-like substance provide a competitive advantage for nodule occupancy over SR-16. This is the first study in *B. japonicum* that shows that the ability to produce bacteriocin-like substance is related to the competitiveness for nodule occupancy.

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List of Abbreviations

ABC = ATP-binding cassette

AntiSMASH = Antibiotics and secondary metabolites analysis shell

ATP = Adenosine triphosphate

ATCC = American Type Culture Collection

bp = Base pair

BAGEL = BActeriocin GEnome mining tooL

BLAST = Basic Local Alignment Search Tool

Cfu = Colony forming unit

DNA = Deoxyribonucleic acid

kb = kilobase

Km = Kanamycin

Nm = Neomycin

PCR = Polymerase chain reaction

RT-PCR = Real-time PCR

RTX = Repeats in toxin

Sm = Streptomycin

USDA = United States Department of Agriculture

QPCR = Quantitative PCR

TFX = Trifolitoxin

YEM = Yeast extract mannitol

Chapter 1

Literature Review

1.1. History of Soybean Cultivation

1.1.1. Soybean Cultivation around the World

Soybean (*Glycine Max*) was first domesticated in the eastern half of Northern China during the 11th century BCE (Hymowitz 1970). Most early documentation on soybean cultivation and its use for human consumption originated from China and Japan (Shurtleff and Aoyagi 2014). It wasn't until much later that soybean was cultivated outside of Asia. Soybean was introduced to Europe around the 1700's, and was grown in the Netherlands, France, Germany, and England (Shurtleff and Aoyagi 2014).

Soybean was first cultivated in the United States of America in 1851, where it was known as "Japan pea" for several decades (Hymowitz 1987). Soybean was introduced to Illinois by Benjamin Franklin Edwards after he obtained the seeds as a gift from a shipwrecked Japanese person in San Francisco (Hymowitz 1970). Cultivation of soybean was carried out by John H. Lea, a horticulturist in Illinois (Ernst 1854; Hymowitz 1987). He subsequently gave the soybean seeds to J. R. Jackson and A. H. Ernst who planted them in Iowa and Ohio respectively (Ernst 1854; Hymowitz 1987).

The earliest known documentation of soybean cultivation in Brazil is in 1882 by Gustavo D'utra (Shurtleff and Aoyagi 2014). He described the cultivation of soybean in Latin America, as well as describing the utilization of soyfoods (products derived from soybean) for human consumption by the Japanese (Shurtleff and Aoyagi 2014).

Cultivation of soybean in Oceania and New Zealand is known to have occurred as early as 1883 (Shurtleff and Aoyagi 2014). The initial cultivation of soybean was the southwest coast of the North Island of New Zealand (Shurtleff and Aoyagi 2014).

1.1.2. Soybean Cultivation in Canada

The introduction of soyfood products in Canada (such as soya sauce) took place at 1831 (Shurtleff and Aoyagi 2010). Evidence suggests that soybeans may have been cultivated in Canada as early as 1855 (Shurtleff and Aoyagi 2010). Soybean was cultivated at Ohio, U.S.A and the seeds were disseminated to Texas through Canada (Shurtleff and Aoyagi 2010).

In 1881, soybean was cultivated at the Ontario Agricultural College (Brown 1882). W. Brown, a professor of Agriculture, and a farm superintendent at the Ontario Agricultural College, reported that the crops grew well and produced a good yield (Brown 1882). In 1893, the early yellow variety of soybean was reported to be cultivated in Ontario (Shurtleff and Aoyagi 2010; Shurtleff and Aoyagi 2013). The early yellow seeds were introduced by Charles A. Zavitz of the Ontario Agricultural College. These seeds were from Kansas, together with 3 other varieties: edamame, kiyusuke daizu, and large yellow (Shurtleff and Aoyagi 2010; Shurtleff and Aoyagi 2013; Zavitz 1894). It was subsequently reported that the early yellow variety gave better results compared to other varieties that he obtained from Kansas (Zavitz 1894). Charles A. Zavitz also dedicated his life to research on soybeans suited to Ontario (Zavitz 1894; Zavitz 1899; Zavitz 1908). He was later known as the pioneer of soybean in Canada due to his lifetime research on soybean (Shurtleff and Aoyagi 2010).

The first commercial cultivation of soybean in Canada occurred in Ontario in 1922 (Campbell and Squirrel 1922). Canada started exporting soybeans to United Kingdom in 1954 (Shurtleff and Aoyagi 2010). In 1970, the production of soybean reached a record of 10 million tonnes and Canada started exporting soybeans to Japan in 1972 (Shurtleff and Aoyagi 2010).

Canada is a world leader in the export of premium quality food-grade soybean to pacific-rim countries such as Japan, Malaysia and Singapore (Shurtleff and Aoyagi 2010). Canada is also the 7th largest soybean producing country in the world, producing 6,050,000 tonnes in 2015 (USDA, 2016).

The three largest soybean-producing provinces are Ontario, Manitoba, and Quebec. As of 2016, Ontario remained as the main soybean-producing province in Canada (Statistics Canada, 2016). On the other hand, commercial scale production of soybean in Manitoba was not able to flourish in 1970's due to the absence of a high-yielding cultivar that was well-adapted for the short growing season in the prairies (Shurtleff and Aoyagi 2010). The development of new cultivars suitable to the prairies led to the restarting of commercial scale production of soybean in Manitoba in the early 1990's. By 2014, Manitoba replaced Quebec as the second largest soybean-producing province in Canada.

1.1.3. Soybean Cultivation in Manitoba

Soybean was first cultivated in Manitoba in 1898 (Bedford 1899). In 1917, W. Southworth of Manitoba Agricultural College, currently known as University of Manitoba, developed an early maturing variety of soybean called "Manitoba Brown" (Shurtleff and Aoyagi 2010). This was crucial in circumventing the short prairie growing season (Shurtleff and Aoyagi 2010). By 1941, southern Manitoba became one of the major soybean producing regions in Canada together with Ontario, southern Quebec, and British Columbia (Shurtleff and Aoyagi 2010).

In 1966, two new varieties of soybean, "Altona" and "Portage", were developed at the University of Manitoba (Stefansson 1966a, b). When comparisons were made to the other early

maturing varieties, it was found that the Altona variety had an improved yield as well as an increased resistance to phytophthora root rot (Stefansson 1966a, b).

Despite of the development of early maturing soybean cultivars in Manitoba (Manitoba Brown, Altona, and Portage), commercial production of soybean did not flourish (Shurtleff and Aoyagi 2010, Stefansson 1966a, b). The idea of commercial scale production of soybean was abandoned by most growers in Manitoba by the early 1970's due to the low return from growing soybean as a crop (Shurtleff and Aoyagi 2010). A major contributing reason was that the early maturing varieties did not provide consistently high yields as observed in the late maturing varieties, which unfortunately were not well adapted to the Northern Prairies (Shurtleff and Aoyagi 2010). In addition, growers had more interest in other crops that required less management and provided a higher cash return (Shurtleff and Aoyagi 2010).

Commercial scale production of soybean in Manitoba did not start again until the early 1980's (Tsukamoto 1981). This was mostly due to the improved early maturing varieties: Maple Presto and McCall that were able to produce consistently high yields (Tsukamoto 1982). At this time, commercial scale production of soybean in western Canada was limited to Manitoba due to the lack of a market for the crop in Alberta and Saskatchewan (Tsukamoto 1982).

Commercial scale production of soybean has continued to flourish in Manitoba since the 1990's (Shurtleff and Aoyagi 2010). In the early 2000's, Manitoba was the third major soybean-producing province in Canada behind Ontario (where the majority of Canadian soybeans are still produced) and Quebec (Statistics Canada, 2005). In 2014, Manitoba reported a record of 1.1 million tonnes of soybean produced from 1.3 million seeded acres. This placed Manitoba as the second major soybean-producing province in Canada; replacing Quebec which reported 898,000

tonnes soybean produced from 852,500 acres that were seeded that year (Statistics Canada, 2014).

1.2. Overview of Soybean Utilization

1.2.1. Soybean Seeds Overview

The chemical composition of soybean seed is approximately 40% protein, 20% oil, 35% carbohydrates, and 5% ash (Chen et al. 2012). Due to its high protein and oil content, soybeans have been utilized for manufacturing of food products intended for human consumption. In addition, soybeans have also been utilized as animal feed and the manufacturing of other non-edible products thus, making it an agro-economically important crop.

1.2.2. Soybean, Soyfoods, and Soy-Derived Products

Soybean has been used in the production of soyfoods to supply protein to the human diet for a long time in Asia, especially in China. Since soybean domestication first took place in China, it is not surprising that a lot of well-known soyfoods such as tofu, soy milk, and fermented soy products (soy sauce, fermented soy paste, etc.) originated in China (Shurtleff and Aoyagi 2010). A notable exception is tempeh. Tempeh is a fermented soy steak that originated in Indonesia and has become more known in North America (Shurtleff and Aoyagi 2011).

The introduction of soyfood to the Continent of Europe took place earlier than the cultivation of soybean. The export of Japanese soy sauce from Japan to Europe was mentioned in a record by merchants of Dutch East India Company in 1647 (Shurtleff and Aoyagi 2014). In addition, there were of reports other soyfoods such as tofu and miso in the early 1600's (Shurtleff and Aoyagi 2014).

Currently, soyfood and food products containing soy can be found all over the world. These products are derived from soybean and contain high levels of protein that can be utilized as substitutes for animal protein in the human diet (Chen et al. 2012). Soy milk, and products derived from it, also contain no lactose and are essential in the production of milk product substitutes for individuals that are lactose intolerant. In addition, soyfoods such as tofu, tempeh, and soy milk have been gaining popularity as health foods due to studies that have suggested that they have positive effects on human health (Chen et al. 2012; Qin et al. 2006; Wanezaki et al. 2015).

Soybeans have also been utilized in the production of oil due to their high oil content. Soybean oil is used in the production of edible products such as cooking oil, and non-edible products such as soaps, printing oil, biodiesel, and many more (Chen et al. 2012; Roy et al. 2007; Shurtleff and Aoyagi 2010).

1.3. *Bradyrhizobium japonicum* and Agronomy

1.3.1. Overview of *B. japonicum*

Bradyrhizobium japonicum is a Gram-negative, rod-shaped, nitrogen-fixing bacterium. *B. japonicum* may exist either as a free-living soil bacterium or as a symbiont. As a free-living soil bacterium, *B. japonicum* is able to grow chemoautotrophically utilizing H_2 and CO_2 as electron donors. It was also shown that the sequenced strain, *B. japonicum* USDA110, is capable of utilizing thiosulfate as an electron donor and CO_2 as a carbon source (Hanus et al 1979; Kaneko et al. 2002; Lepo et al 1980; Masuda et al. 2010). Recently, a reclassification of *B. japonicum* group Ia which includes the strain USDA110 to *Bradyrhizobium diazoefficiens* sp. nov has been proposed (Delamuta et al. 2013).

B. japonicum has the ability to establish symbiosis with soybean plants (Franck et al. 2008; Hanus et al. 1979; Lorite et al. 2000). This symbiosis manifests itself as nodules formed on the roots of the soybean plants. Within the nodule, the bacteria are released into a plant derived, membrane enclosed compartment. While in this state, they are termed bacteroids (Fischer 1994). The plant host controls the diffusion of oxygen and supplies the bacteroids with a source of reduced carbon (Fischer 1994). In return, the bacteria reduce atmospheric nitrogen which is supplied to the host plant (Hunt and Layzell 1993; Fischer 1994).

1.3.2. The Importance of *B. japonicum* in Agronomy

Previous studies showed that inoculation of legume fields with a compatible *Rhizobium* strain has led to an increased yield in a cost effective manner (Caldwell 1969; Solomon et al. 2012). A study utilizing an Italian agro-ecosystem also demonstrated that an inoculated soybean field produced more than twice the yield as that of the field utilizing a chemical fertilizer (Panzieri et al. 2000).

The utilization of a *Rhizobium* inoculant on a legume crop leads to the decreased use of chemical fertilizer, specifically reduced nitrogen that is derived from the Haber-Bosch process. In turn, this alleviates the need for excessive use of fertilizer on farm fields which can result in high nutrient run-off leading to the deterioration of surface and ground water. It was also shown that the inoculated field requires less non-renewable resources when compared to the field dependent on the application of industrially derived chemical fertilizer (Panzieri et al. 2000). Taken together, these results demonstrate the importance of a good Bradyrhizobial inoculum for the efficiency and sustainability of soybean cultivation.

1.4. The Quantification of *B. japonicum* in Soil

1.4.1. The Traditional Approaches for Quantifying *B. japonicum* in Soil

The traditional approach in quantifying *B. japonicum* in soil is through the **M**ost **P**robable **N**umber method (MPN). The MPN method is a classic bacteriological technique that can be applied to a number of different bacteria. Essentially, the number of bacteria present is estimated by diluting the original sample until no bacterial growth can be detected in any of the replicates (Oblinger and Koburger 1975).

For the quantification of rhizobia present in an environmental sample a plant infection MPN assay can be utilized. In this method the appropriate host plant serves as a selective agent for the correct Rhizobium species (Woomer et al. 1988). For quantification of *B. japonicum*, soybean plants are used as the appropriate host plant. The MPN method then utilizes the sum of the nodulated plants, or the pattern of nodulated and non-nodulated units to derive a population estimate (Brockwell 1963; Fisher and Yates 1963; Woomer et al. 1988).

Another method used to quantify *B. japonicum* is by selection utilizing laboratory growth media containing selective agents. Currently there is a variety of selective agents used for this methodology including antibiotic, dyes, and metabolic inhibitors (Danso et al. 1973; Thompson and Vincent 1967; Van Schreven 1970). Examples that have been utilized are: penicillin, pentachloronitrobenzene (PCNB), brilliant green, sodium azide, and crystal violet (Pattison and Skinner 1974). In addition, heavy metals have also been used as a selection agent (Tong and Sadowsky 1994). The use of heavy metals as an indicator is based on the principle that rhizobia tend to be more tolerant to heavy metals (Kinkle et al. 1987).

Quantitative immunofluorescence has also been used for the quantification of rhizobia in soil (Postma et al. 1998; Schmidt et al. 1968; Tong and Sadowsky 1994). In this method,

detection of the strain of interest is achieved by utilizing a specific antibody against that particular strain (Schmidt et al. 1968).

1.4.2. The Limitations of the Traditional Approaches of *B. japonicum* Quantification

Although these assays have the ability to provide an estimate of the population of *B. japonicum*, each of the assays has their own limitations. For example, the plant infection MPN method is very time consuming (Tong and Sadowsky 1994). It takes approximately 4-6 weeks before a result can be obtained and this method is also based on the assumption that a single bacterium is capable of forming a nodule (Tong and Sadowsky 1994). In addition, this method is sensitive to the host species and the host plants used (Woomer et al. 1988).

The quantitative immunofluorescence method tends to overestimate the number of bacteria in samples (Tong and Sadowsky 1994). The assay can be affected by the difference in the level of dispersal and flocculation of bacteria that occurs because of the type of soil. In addition, the non-specific uptake of dye by the soil particles as well as the availability and the nature of the antibodies used can affect quantification (Schmidt et al. 1968; Tong and Sadowsky 1994).

1.4.3. The overview of Real-Time PCR

Real-time PCR is a method that is used to simultaneously amplify, detect, and quantify a target DNA segment of interest from a particular sample (Bustin 2000; Lie and Petropoulos 1998; Walker 2001). This method utilizes fluorogenic probes that are used for continuous monitoring of the amplification progress (Walker 2001). The amplification progress is monitored in a closed tube in real time using either laser or a camera (Bustin 2000; Higuchi et al. 1992; Higuchi et al. 1993; Walker 2001). This bypasses the need to use a post-PCR processing method

such as gel electrophoresis for the detection of the product since detection occurs during each PCR cycle (Lie and Petropoulos 1998).

There are both direct and indirect methods of generating fluorescence for monitoring the progress of amplicon replication (Walker 2001). In an indirect assay, the fluorescence is generated during the primer extension stage of the PCR reaction. One such example is the Taqman system (Heid et al. 1996). The Taqman probes utilize the intrinsic 5' exonuclease activity of Taq DNA polymerase to digest a DNA probe annealing to a specific DNA sequence (Heid et al. 1996; Holland et al. 1991; Walker 2001). This probe consists of a quencher and reporter fluorochromes separated by a specific DNA sequence (designed to anneal to the specific region target DNA molecule) (Walker 2001). When intact, fluorescence is quenched due to the proximity between the reporter fluorochrome and the quencher (Walker 2001). During primer extension, 5' exonuclease activity of Taq DNA polymerase degrades the probe, releasing the fluorochorome. The loss of quenching allows for the observation of fluorescence which is directly correlated with the amplification of the target DNA (Holland et al. 1991; Walker 2001).

In contrast, direct assays utilize the incorporation of either fluorescent molecules or fluorescence interference probes to the amplified product (Walker 2001). Hairpin probes, such as those used in Scorpion and Sunset primers, are fluorescent interference probes (Nazarenko et al. 1997; Thelwell et al. 2000; Whitcombe et al. 1999). Each probe consists of a fluorochrome and a quencher which are kept in close proximity by a hairpin structure. When this probe binds to a specific target, the hairpin structure is disrupted and the fluorescence quenching is alleviated.

Another common direct detection method involves the utilization of the fluorescent dye SYBR green (Walker 2001). In this method fluorescence results from the binding of SYBR green to the double stranded DNA that is produced (Walker 2001). Therefore, the more double

stranded DNA present as the result of PCR amplification reaction, the more fluorescence will be detected. An advantage of this system is that since SYBR green will bind to any double-stranded DNA products, this method does not require a specific probe (Walker 2001).

1.4.4. The Advantage of Real-Time PCR-Based Assays for the Quantification of Bacteria

The introduction of real-time PCR has allowed for rapid, more sensitive, and less-labour intensive assays for the quantification of bacteria (Cho et al. 2015; Josefsen et al. 2010; Liu et al. 2006; Sakamoto et al. 2001). Quantitative real-time PCR methods allow for bypass of culture-based detection methods which tend to be time consuming (Cho et al. 2015; Josefsen et al. 2010). The ability to rapidly identify a pathogen is very important for the detection of diseases. For example, the procedure for the culture-based detection of *Campylobacter* can take as long as 5-6 days (Fitzgerald et al. 2008; Josefsen et al. 2010). In addition, real-time PCR-based assays are more robust and can detect non-growing bacteria. For example, *Campylobacter* can enter into a "viable but non-culturable" state in which it can still cause disease but not be readily detectable by a culture based assay (Josefsen et al. 2010; Rollins and Colwell 1986).

Real-time PCR-based quantification has also been proven to be very useful in the detection of plant pathogens such as *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*) which causes bacterial ring rot in potato plants (Cho et al. 2015). The very highly contagious nature of this disease has led many countries to implement a quarantine program with a zero-tolerance policy (Cho et al. 2015; Gutbrod 1987). In order to control the spread of this disease, a method to rapidly detect and quantify *Cms* cells is crucial since this pathogen multiplies very rapidly (Cho et al. 2015).

One method to diagnose *Cms* involves the isolation of pathogen, gram-staining, the usage of selective medium, and the analysis of biochemical characteristics (Cho et al. 2015; Delacruz et al. 1992). Unfortunately, these methods require several weeks before a result can be obtained (Cho et al. 2015). Other methods such as immunofluorescent-antibody staining (IFA), enzyme-linked immunosorbent assay (ELISA), and conventional PCR provide a more rapid means for the detection of *Cms* (Cho et al. 2015; Deboer and Wieczorek 1984; Deboer et al. 1988; Hu et al. 1995). However, the inability of these methods to differentiate between other closely related bacterial strains has caused problems in the diagnosis of *Cms* (Cho et al. 2015).

Recently, a real-time PCR based assay for quantification of *Cms* was developed (Cho et al. 2015). This assay bypasses the need for utilizing the time-consuming isolation-based methods and is very sensitive to the presence of *Cms*. It was shown that this real-time PCR based quantification method had a detection limit of 1.47×10^{2} copies/µl of cloned-amplified target DNA, or 5 fg of genomic DNA, or a 10^{-6} dilution of culture at an OD_{600} of 0.12 (Cho et al. 2015).

1.5. Nitrogen Problem, Rhizobia and Legume Symbiosis, and Competition Problem

1.5.1. Nitrogen Problem

Nitrogen gas (N_2) occupies the majority of the Earth's atmosphere, approximately 78% by volume. N_2 is relatively non-reactive due to the presence of a strong triple bond. Despite its great abundance on earth, N_2 is not bioavailable. Humans fulfill all of their nutritional nitrogen required by obtaining nitrogen either directly or indirectly from the plants (Vance 2001). However, the growth of most plants is critically limited by the presence of bioavailable nitrogen (Graham and Vance 2000; Smil 1999; Socolow 1999; Vance 2001). In order to overcome the

lack of bioavailable nitrogen to the plants, the utilization of fertilizer has become a common practice since 1945 (Vance 2001).

The nitrogen problem does not only revolve around the lack of bioavailable nitrogen for the plants, but also the depletion of fossil fuels and the cost required for the production of nitrogen fertilizer, and the excessive use of nitrogen fertilizer in intensive farming (Vance 2001). Currently, commercial nitrogen fertilizer is readily available. The majority of this commercial nitrogen fertilizer production is achieved through Haber-Bosch process which requires an extensive use of fossil fuels (Galloway et al. 1995).

Unfortunately, the availability of commercial fertilizer has led to the excessive use of nitrogen fertilizer in agriculture. In addition, crops are unable to efficiently recover all of the nitrogen in the soil. For example, nitrogen recovery by grain crops ranges from 35% to 75% with an average near 50% (Smil 1999; Socolow 1999). These two factors contribute to the large residual quantity of nitrogen in soil which lead to a wide variety of environmental and health problems (Galloway et al. 1995).

The nitrification and denitrification of soil nitrogen by microbes are the major sources of NO_x and N_2O from agricultural soils (Socolow 1999). N_2O acts as a greenhouse gas due to its long residence time and absorption of infrared (IR) radiation (Vance 2001). On the other hand, NO_x, which is toxic to plants, also contributes to the depletion of stratospheric ozone (Vance 2001).

Nitrogen fertilizer not recovered by the plants also has the ability to pollute the groundwater pools through run-off and leaching (Vance 2001). This can lead to an increase in the concentration of NO_3^- in drinking water (Galloway et al. 1995; Smil 1999; Vance 2001). An excess of NO_3^- in drinking water (at the concentrations above 10 mg NO3-N L^{-1}) has been

implicated in methemoglobin anemia in infants and young children (Smil 1999, Galloway et al. 1995). Furthermore, the pollution of surface water by NO_3^- has been associated with eutrophication and hypoxia in aquatic ecosystems (Galloway et al. 1995).

1.5.2. Overview of *Rhizobium*-Legume Symbiosis and Symbiotic Nitrogen Fixation

Despite the abundance of N_2 in the atmosphere, plant growth is often limited by the availability of biologically active forms of nitrogen such as nitrates and ammonia, in the soil (Oldroyd et al. 2011). Legumes are able to overcome this problem by entering into a symbiotic relationship with rhizobia (Oldroyd et al. 2011). Through this association, legumes can derive all the nitrogen necessary for growth.

In this symbiotic relationship, rhizobia reduce atmospheric nitrogen to ammonia, supplying it to the host plant (Udvardi and Poole 2013). In return, the host plant provides rhizobia with reduced carbon from photosynthesis (Udvardi and Poole 2013). The energy required for symbiotic nitrogen fixation comes from the sun via photosynthesis in the plants (Udvardi and Poole 2013). Approximately 6 grams of photosynthetic carbon is required to fix one gram of nitrogen (Udvardi and Poole 2013; Vance and Heichel 1991). In addition, rhizobia also rely on the host plant for all other elements required for metabolism in this association (Udvardi and Poole 2013).

The *Rhizobium*-legume symbiosis manifests itself as nodules form on the roots of the plants. The formation of these nodules requires two developmental processes: nodule organogenesis and bacterial infection (Oldroyd et al. 2011). To ensure nodule organogenesis at the site of bacterial infection, these 2 processes must be coordinated in temporal and spatial fashion (Oldroyd and Downie 2008; Oldroyd et al. 2011).

The first step of nodule formation involves the attachment of the correct strains of *Rhizobium* to the roots and root hairs of the host plant (Downie 2010). An important factor in this step requires the close proximity of the correct strains of rhizobia to the roots and root hairs (Downie 2010). This is essential in ensuring the supply of nutrients which will enable these strains to grow and eventually allowing the build-up of biofilm via the bacteria that are attached directly to the roots and root hairs (Downie 2010).

The next step involves the plant recognition of specific lipochito-oligosaccharides called Nod factors that are secreted by rhizobia (Ivanov et al. 2012; Oldroyd and Downie 2008). The presence of Nod factors induces the formation of root hair curling, which is when the root hair curls tightly back on itself, entrapping the bacteria (Downie 2010; Gage 2004). This process is an important step for rhizobial infections in legumes (Downie 2010). This is also the reason why the bacteria directly attached to the root-hairs are in the best position to infect the plants (Downie 2010).

From the site of the curled root hair, the formation of the infection thread is initiated (Oldroyd et al. 2011). The infection process is a clonal event in which the bacteria that successfully infect the legumes are derived from a single cell or a microcolony which then multiply to very high numbers in the plant (Downie 2010). The infection threads progress into the inner cortex where a series of cell divisions result in the formation of the nodule primordium (Oldroyd et al. 2011). The further process of cell division will then result in the formation of nodule meristem (Oldroyd 2011).

In the nodules, infecting rhizobia further differentiate into bacteroids which fix N_2 to ammonia; a process which involves the enzyme nitrogenase (Oldroyd 2011; Udvardi and Poole 2013). Since nitrogenase is irreversibly inactivated by oxygen, the nitrogen reduction process

utilizing this enzyme requires an anoxic or nearly anoxic environment (Gage 2004). In an oxic environment, the inactivation of nitrogenase is prevented by sequestering it in differentiated cells with specific morphological and biochemical characteristics that limit exposure to oxygen (Gage 2004). In *Rhizobium*-legume symbioses, the nodules' outer cell layers provide a barrier to gaseous diffusion, creating a microaerobic environment for the nitrogen-fixing bacteroids (Gage 2004). The high respiration rates of bacteroids and plant mitochondria, that causes oxygen to be consumed as fast as it can enter the nodules also helps creating this microaerobic environment (Udvardi and Poole 2013). Furthermore, the plant haemoglobin also binds oxygen in the cytoplasm with high affinity and rapidly delivers it to both plant mitochondria and bacteroids (Udvardi and Poole 2013). These three different aspects help to maintain a steady-state concentration of free oxygen in the infected zones of legume nodules to tens of nanomolar (Udvardi and Poole 2013; Kuzma et al. 1993).

1.5.3. Rhizobium Competition Problem

A problem that is often encountered in establishing an efficient *Rhizobium*-legume symbiosis is having sufficient numbers of the correct rhizobia species present as the seed is germinating, allowing for effective nodules to develop in a timely manner. This problem is usually circumvented by the application of inocula of rhizobia that is supplied by seed companies in various forms and applied either directly to the seed, or to the field at the time of planting.

However, it has been reported that inoculum strains often fail to compete with indigenous soil rhizobia (Triplett and Sadowsky 1992). This problem persists even when the level of the inoculum strain far exceeds the number of indigenous rhizobia in the soil (Weaver and Frederick 1974a, b). This situation is known as the "*Rhizobium* competition problem" (Triplett and

Sadowsky 1992). There are several factors involved in the competitiveness for nodule occupancy such as motility, cell-surface characteristics, speed of nodulation and auto-regulatory response, symbiotic effectiveness, and antibiosis (Triplett and Sadowsky 1992).

The competitiveness for nodule occupancy in rhizobia is affected by the cell-surface characteristics. It was shown that a non-mucoid mutant of *Sinorhizobium fredii* USDA 208 was more competitive for nodulation of its host plant, soybean (Zdor and Pueppke 1991). In addition, the inability to produce exopolysaccharide (EPS) by *B. japonicum* resulted in strains that were less competitive for nodule occupancy (Bhagwat et al. 1991).

Motility was also shown to be important in the competitiveness for nodule occupancy in rhizobia. In *Sinorhizobium meliloti*, the non-motile strains regardless of the presence of the flagella were shown to be less competitive for nodule occupancy compared to the wild type strain (Ames and Bergman 1981). Interestingly, these mutants are identical in growth rate and nodule occupation compared to the wild type strain (Ames and Bergman 1981). Similar data were also shown for *R. leguminosarum* bv. *trifolii* TA1 in which the non-motile mutant strain was less competitive compared to the wild type despite the identical growth rate (Mellor et al. 1987).

The early infection of legume roots induces an autoregulatory response in the host plant that limits the number of infections that produce mature nodules (Triplett and Sadowsky 1992). This was shown by several studies that utilized a split-root system (Kosslak et al. 1983; Kosslak and Bohlool 1984; Sargent et al. 1987; Triplett and Sadowsky 1992). In this system, seedlings were treated such that there were roughly two symmetrical root systems that were spatially separated and inoculated at different time points (Kosslak and Bohlool 1984, Sargent et al. 1987). It was shown that nodulation was supressed on the root system that was inoculated at a later time

point. In addition, the magnitude of suppression of nodulation was increased when the time interval for inoculation was increased (Kosslak et al. 1983; Kosslak and Bohlool 1984; Sargent et al. 1987; Triplett and Sadowsky 1992).

Mutations in the *nod* and *nif* gene regions affect nodule formation and nitrogen fixation respectively (Hahn and Hennecke 1988; Sargent et al. 1987; Triplett and Sadowsky 1992). Although these genes grossly affect the symbiotic process, they also affect the competitiveness for nodule occupancy in a more subtle manner. Strains carrying mutations in the *nod* gene region were shown to be unable to stimulate the autoregulatory response, displayed slower infection rates, were impaired in nodule development, and had decreased nodulation competitiveness (Hahn and Hennecke 1988; Sargent et al. 1987; Triplett and Sadowsky 1992). In contrast, when present in multiple copies, *nifA* has the ability to improve the competitiveness for nodule occupancy (Triplett and Sadowsky 1992).

Some strains of *Rhizobium* produce antibiotics that inhibit the growth of other strains. The most characterized antibiotic production in rhizobia is the bacteriocin trifolitoxin (TFX) which is produced by *Rhizobium leguminosarum* bv. *trifolii* strain T24 (Triplett 1988; Triplett and Barta 1987). TFX targets a wide range of rhizobial strains and its presence has been shown to improve the competitiveness for nodule occupancy (Robleto et al. 1998; Triplett 1990; Triplett et al. 1994).

1.6. Bacteriocins

1.6.1. Overview of Bacteriocin

Bacteriocins are toxins produced by both Gram-negative and Gram-positive bacteria, and archaea (Riley and Wertz 2002; Torreblanca et al. 1989; Subramanian and Smith 2015;

Torreblanca et al. 1994). In general, these are narrow spectrum toxins, targeting only the strains related to the producer strains (Riley and Wertz 2002, Zeth 2012). However, broad spectrum bacteriocins capable of targeting isolates of a different species are also present (Collins et al. 2016; Rea et al. 2011; Subramanian and Smith 2015). The bacteriocin family is diverse and consists of proteins with different sizes, microbial targets, modes of action, and immunity mechanisms (Riley and Wertz 2002).

1.6.2. Bacteriocins Produced by Gram-negative Bacteria

Colicins produced by *Escherichia coli* are the most extensively studied bacteriocins of Gram-negative bacteria (Cascales et al. 2007; Ghazaryan et al. 2014). Colicins are exclusively encoded on the colicinogenic plasmid, pCOL (Cascales et al. 2007). The gene cluster responsible for the production of colicin consists of a colicin gene, an immunity gene, and a lysis gene (Cascales et al. 2007; Ghazaryan et al. 2014). The colicin gene, encoded by *cxa*, is responsible for the production of the toxin (Ghazaryan et al. 2014). The immunity gene, encoded by *cxi*, is responsible for the production of a protein that binds to the toxin and inactivates it, conferring the immunity to the producer cell (Ghazaryan et al. 2014). The lysis gene encodes a protein responsible for the release of colicin through the lysis of the producer cell (Cascales et al. 2007; Ghazaryan et al. 2014; Riley and Wertz 2002).

Colicin production is mediated by the SOS regulon and is mainly produced as a stress response (Butala et al. 2008). The toxin encoded by the colicin gene contains a surface receptor domain that binds to a specific region of lipopolysaccharide (LPS) of the target cells (Johnson et al. 2014). Colicins have two primary modes of action, either by forming a pore in the cell

membrane of the target cells, or as nuclease targeting DNA, rRNA, and tRNA (Cascales et al. 2007; Riley and Wertz 2002).

Other gram negative bacteria also produce bacteriocins with similarities to colicin (Kim et al. 2014). One such example is the chromosomally-encoded pyocin of *Pseudomonas aeruginosa* (Michel-Briand and Baysse 2002). More than 90% of *P. aeruginosa* strains are capable of producing pyocins and each strain is capable of producing more than one type of pyocins (Michel Briand and Baysse 2002). Similar to colicin, pyocin binds to the target cell through a specific cell-surface receptor (Kim et al. 2014).

1.6.3. The Bacteriocins Produced by Gram-Positive Bacteria

Bacteriocins from Gram-positive bacteria are more diverse than those produced by Gram-negative bacteria (Jack et al. 1995). There are two main differences between the bacteriocins produced by Gram-positive and Gram-negative bacteria. First, the production of toxins in Gram-positive bacteria is not always lethal to the producers unlike in Gram-negative bacteria (Riley and Wertz 2002). This may be due to the fact that some Gram-positive bacteria utilize a bacteriocin specific transport system or the *sec*-dependent export pathway for the release of bacteriocin (Riley and Wertz 2002). The second difference is in the regulation of the bacteriocin encoding genes. Gram-negative bacteria chiefly rely on host regulatory networks, whereas bacteriocin encoding genes in Gram-positive bacteria seem to have evolved bacteriocin-specific regulation (Jack et al. 1995; Riley and Wertz 2002).

Lactic acid bacteria (LAB) produce many different types of bacteriocins. These have been divided into four classes (Abee et al. 1995; Klaenhammer 1993). Class I bacteriocin are called lantibiotics. These bacteriocins contain amino acids such as lanthionine and B-

methyllanthionine that are formed by post-translational modifications (Guder et al. 2000; Klaenhammer 1988; Riley and Wertz 2002). Lantibiotics are further divided into subgroups A and B based on their structural features and modes of action (Jung 1991). Type A lantibiotics range between 21 to 38 amino acids, and are involved in depolarization of the cytoplasmic membrane of target cells (Jack et al. 1995; Riley and Wertz 2002). A well-studied example of the type A lantibiotics is nisin. Lantibiotics belonging to subgroup B tend to be larger than 19 amino acids (Brötz et al. 1995). In addition, they generally have a more globular secondary structure (Brötz et al. 1995). An example of a type B lantibiotic is mercasidin. Mercasidin has been shown to interfere with cell wall biosynthesis of target cells (Brötz et al. 1995).

Class II LAB bacteriocins are heat-stable peptides with a size ranging between 30 to 60 amino acids and do not contain lanthionine (Riley and Wertz 2002). This class is also divided into several subgroups. The largest subgroup, class IIa bacteriocins, are *Listeria*-active peptides containing the conserved amino terminal sequence YGNGVXaaC (Klaenhammer 1993). The bacteriocins that belong to this class act by forming pores in the membranes of target cells (Riley and Wertz 2002). Some examples of Class IIa bacteriocins are sakacin A and leucocin A (Hastings et al. 1991; Klaenhammer 1993). Class IIb bacteriocins also act by forming pores on the membranes of target cells (Riley and Wertz 2002). The difference between class IIa and class IIb bacteriocins is that the latter consists of two proteinaceous peptides that are necessary for biological activity (Klaenhammer 1993). Some examples of class IIb bacteriocins include lacticin F and lactococcin G (Muriana and Klaenhammer 1991; Nissen-meyer et al. 1992). Class IIc bacteriocins such as lactococcin B are *sec*-dependent, thiol-activated peptides that require reduced cysteine residues for activity (Klaenhammer 1993; Riley and Wertz 2002; Venema et al. 1993).

Class III bacteriocins consist of large heat-labile proteins larger than 30 kDa (Riley and Wertz 2002; Klaenhammer 1993). An example of bacteriocins belonging to this group is lactacin B (Barefoot and Klaenhammer 1984).

To date, class IV bacteriocins are the least studied group of LAB bacteriocins. Class IV bacteriocins consist of proteins requiring additional chemical moieties such as a lipids or carbohydrates for activity (Riley and Wertz 2002; Klaenhammer 1993). An example of Class IV bacteriocins is sublancin that is produced by *Bacillus subtilis* 168 (Oman et al. 2011). This bacteriocin contains an S-linked glycopeptide containing a glucose attached to a cysteine residue (Oman et al. 2011).

Most of Gram-positive bacteriocins have a wide spectrum of activity (Jack et al. 1995). For example, whereas lactococcins A, B, and M have a relatively narrow spectrum that only targets *Lactococcus*, the lantibiotics nissin A and mutacin B-Ny266, affect other Gram-positive bacteria belonging to different genera such as *Actinomyces*, *Bacillus*, *Clostridium*, *Corynebacterium*, *Enterococcus*, *Gardnerella*, *Lactococcus*, *Listeria*, *Micrococcus*, *Mycobacterium*, *Propionibacterium*, *Streptococcus*, and *Staphylococcus* (Mota-Meira et al. 2000; Ross et al. 1999). In addition, these wide-spectrum bacteriocins are also able to target some Gram-negative bacteria such as those belonging to the genus *Campylobacter*, *Haemophilus*, *Helicobacter*, and *Neisseria* (Mota-Meira et al. 2000).

1.6.4. The Bacteriocins Produced by Archaea

The bacteriocins produced by archaea are known as archaeocins (Besse et al. 2015). The most studied archaeocins so far are halocins produced by halobacteria (Meseguer and Rodriguez-valera 1985; Rodriguez-Valera et al. 1982; Torreblanca et al. 1994; Torreblanca et al. 1989). One

example is halocin H4 (HalH4) which is the first high molecular mass halocin to be studied (Cheung et al. 1997). Originally isolated from *Haloferax mediterranei* R4 (ATCC33500), HalH4 is ribosomally synthesized as a 359 amino acid precursor. It contains a highly cationic 39.6 kDa N-terminal leader region polypeptide composed of a 46 amino acids, which is subsequently cleaved yielding a 313 amino acid mature protein (Meseguer and Rodriguez-Valera 1985; Rodriguez-Valera et al. 1982). The proposed mechanism of action for HalH4 is through the disruption of ion-gradients across the membrane of target cells leading to cell lysis (Besse et al. 2015).

Another example is halocin S8 (HalS8). HalS8 was the first characterized low molecular mass halocin (Besse et al. 2015). HalS8 was purified from an uncharacterized haloarchaeal strain S8a isolated from Great Salt Lake in Utah, USA (Price and Shand 2000). This halocin contains 36 amino acids (3.6 kDA) and is relatively hydrophobic (Price and Shand 2000). Currently, nothing is known regarding the mechanism of action for HalS8.

Crenarchea, such as the ones belong to the genus *Sulfolobus* produce archaeocins known as sulfolobicins (Besse et al. 2015; Ellen et al. 2011). Sulfolobicins are a novel class of antimicrobial toxins with no detectable homology with other proteins (Ellen et al 2011). Currently, there is very little known regarding archaeocins belonging to this group (Ellen et al. 2011; O'Connor and Shand 2002; Prangishvili et al. 2000).

1.6.5. The Application of Bacteriocins

Many bacteriocins have been applied in the food industry to prevent the outgrowth of harmful human pathogens. Nisin, the first bacteriocin to be isolated and approved for use in foods, was originally used to prevent the outgrowth of *Clostridium botulinum* in cheese spreads

(Chung et al. 1989). Since then, nisin has been widely used worldwide as food preservative (Riley and Wertz 2002).

Another example is the utilization of bacteriocins to prevent an outbreak of listeriosis caused by the human pathogen *Listeria monocytogenes* (Riley and Wertz 2002). Many class IIa bacteriocins from Gram-positive bacteria are active against *L. monocytogenes* and have been utilized for this purpose (Riley and Wertz 2002, Klaenhammer 1993).

Some studies have also shown promising results using bacteriocins against nosocomial, antibiotic-resistant human pathogens (Dobson et al. 2012). Mersacidin, a bacteriocin produced by *Bacillus sp*. strain HIL Y85, was shown to be effective in eradicating methicillin-resistant *Staphylococcus aureus* (MRSA) infection in mouse rhinitis model (Kruszewska et al. 2004). In addition, lacticin 3147 (produced by *Lactococcus lactis* DPC3147) and thuricin CD (produced by *Bacillus thuringiensis* DPC6431) were shown to be inhibitory against *Clostridium difficile* (Rea et al. 2011; Rea et al. 2010; Rupnik et al. 2009). Also, in contrast to conventional antibiotics, thuricin CD did not cause a major alteration to the microbiome of the gastrointestinal tract (Rea et al. 2011).

Current studies utilizing halocins and sulfolobicins to inhibit the growth of bacteria such as *E. coli*, *Staphylococcus aureus*, *Bacillus megaterium*, *Pseudomonas aeruginosa*) and fungi (*Saccharomyces cerevisiae*) have been unsuccessful (Besse et al. 2015). This suggests that archaeocins have little significance for application against human pathogens.

1.6.6. Rhizobiocins

Strains belonging to the genera *Bradyrhizobium*, *Rhizobium*, and *Sinorhizobium* have been shown to produce bacteriocins (Oresnik et al. 1999; Roslycky 1967; Triplett and Barta

1987). It has been suggested that these should be referred to as rhizobiocins (Oresnik et al 1999). Rhizobiocins have been categorized as small, medium, and large based on their presumptive sizes and diffusion characteristics (Hirsch 1979; Oresnik et al. 1999; Schwinghamer and Brockwell 1978). Small rhizobiocins are heat-labile and soluble in chloroform (Hirsch 1979; Van Brussel et al. 1985). They have molecular masses of less than 2000 daltons and were shown to be acylated homoserine lactone compounds related to those associated with quorum sensing (Gray et al. 1996; Hirsch 1979; Schripsema et al. 1996; Van Brussel et al. 1985). Large rhizobiocins have been shown to bear resemblance to defective bacteriophages (Hirsch 1979; Lotz and Mayer 1972; Schwinghamer and Brockwell 1978). Not much is known about medium rhizobiocins. They are found to be produced by *R. leguminosarum*, but they are not as commonly produced as the small rhizobiocins (Oresnik et al. 1999).

Rhizobium leguminosarum 248 produces a medium rhizobiocin with molecular mass of 100 kDa (Oresnik et al. 1999). This bacteriocin has similarities to RTX toxins such as hemolysin and leukotoxin, which are calcium-dependent cytolysins (Oresnik et al. 1999; Welch 1991). This bacteriocin was shown to contain the nonapeptide sequence L/I/F-X-G-G-X-G-N/D-D-X found in the RTX (for repeat in toxin) proteins (Oresnik et al. 1999). The authors also showed that the bacteriocin produced was able to bind calcium like other RTX proteins (Oresnik et al. 1999). In addition, the presence of calcium increased the potency of bacteriocin (Oresnik et al. 1999). Using isogenic mutants, it was shown that this bacteriocin could affect the strain's competitiveness for nodule occupancy (Oresnik et al. 1999).

The best characterized and described bacteriocin in rhizobia is trifolotoxin. Trifolitoxin is a peptide that was initially classed as a bacteriocin produced by *Rhizobium leguminosarum* bv. *trifolii* strain T24 (Breil et al. 1996; Breil et al. 1993; Scupham et al. 2002; Scupham and Triplett

2006; Triplett 1988, 1990; Triplett and Barta 1987). *R. leguminosarum* bv. *trifolii* strain T24 was shown to be more competitive for nodule occupancy due to its ability to produce trifolitoxin (TFX) (Triplett and Barta 1987). TFX was argued to not be a true bacteriocin due to the fact that it targets not only the related strains of *R. leguminosarum*, but also the strains of the closely related genera such as *Agrobacterium*, *Brucella*, *Mycoplana*, *Ochrobactrum*, *Phyllobacterium*, *Rhodobacter*, *Rhodopseudomonas*, *Rhodospirillum*, and *Rhizobium* (Triplett et al. 1994). However, TFX is very similar to the lantibiotics nisin A and mutacin B-Ny266 that are produced by Gram-positive bacteria, with respect to its ability to target strains belonging to different genera.

It was shown that strains of *Agrobacterium*, *Brucella*, *Phyllobacterium*, *Rhizobium*, and *Rhodospirillum* were able to produce TFX if the operon responsible for the production was introduced on a plasmid (Triplett et al. 1994). In *R. etli*, the ability to produce TFX significantly increased the competitiveness for nodule occupancy (Robleto et al. 1998). In addition, a strain of *Agrobacterium* with the ability to produce TFX has an enhanced ability to control crown gall disease in *Nicotiana glauca* compared to a commercial strain (Herlache and Triplett 2002).

1.6.7. The Production of Bacteriocins in Bradyrhizobia

The production of bacteriocins in *B. japonicum* was first reported in 1967 (Roslycky 1967). In this study, an agar overlay containing an indicator (bacteriocin-sensitive) strain was poured over macrocolonies of putative bacteriocin producers (Roslycky 1967). From this study, it was shown that 13 out of the 27 strains tested produced a zone of inhibition (Roslycky 1967).

In 1978, it was reported that 58 out of 66 strains of *B. japonicum* used in a study were found to produce bacteriocins (Gross and Vidaver 1978). Despite their inability to inhibit the growth of the indicator rhizobia strains used, these bacteriocin producers were able to inhibit the

growth of *Corynebacterium nebraskense*, a causal agent of Goss's bacterial wilt and blight of corn (Gross and Vidaver 1978; Vidaver 1977). This data suggest that bacteriocin-producing strains of *B. japonicum* can be utilized as biocontrol agents against bacterial phytopathogens. In fact, similar data were reported more than 20 years later when TFX was shown to be able to control crown gall disease in *Nicotiana glauca* (Herlache and Triplett 2002).

Previous studies also showed that the ability to produce bacteriocins (TFX in particular), increase the competitiveness for nodule occupancy in rhizobia (Triplett et al. 1994; Robleto et al. 1998; Triplett and Barta 1987). However, an attempt to demonstrate the positive relationship between the ability of *B. japonicum* strains to produce bacteriocin and the competitiveness for nodule occupancy has not been successful (Gross and Vidaver 1978). In addition, an attempt to utilize TFX to increase the competitiveness of *B. japonicum* strains for nodule occupancy was also unsuccessful. It was found that although the transfer of a plasmid containing the genes necessary for the TFX production and resistance into *B. japonicum* was possible, it did not result in a strain that was capable of producing TFX (Triplett and Barta 1987). Furthermore, the strains of *Bradyrhizobium* were not sensitive to TFX (Triplett and Barta 1987; Triplett 1988). Since then, there has been no attempt at increasing the competitiveness of inoculum strains of *B. japonicum* by using bacteriocins. In fact, there has been little to no study done of bacteriocin production in *Bradyrhizobium* since this time.

1.7. Thesis Objectives

With the near logarithmic rise in the amount of soybean that is being seeded in Manitoba there were questions that were being raised by stakeholder groups like the Manitoba Pulse Growers Association (now the Manitoba Pulse and Soybean Growers Association). In particular,

since there had been neither extensive cultivation of soybean nor the application of inoculum, there were very basic questions such as how long does it take for an indigenous population of *B. japonicum* to develop? And more specifically, how does a grower know if it is practical to buy inoculum when they buy seed? Related to this, are questions of whether there are indigenous strains of *B. japonicum* in Manitoba, and if there are, could they contain determinants that could be used to affect competition for nodule occupancy? These general questions were used to formulate specific objectives; namely the development of a qRT-PCR assay to enumerate *B. japonicum* from field soil as well as the isolation and characterization of *B. japonicum* strains that are indigenous to Manitoba. Each is described briefly below.

1.7.1. The Development of Real-Time PCR-Based Assay for Rapid Quantification of *B. japonicum* in soil

The symbiosis between *B. japonicum* and a soybean plant manifests itself as nodules that form on the roots of the host plant. A soybean plant can fulfill its entire nitrogen requirement given that the plant forms symbiosis with the correct strain of *B. japonicum*. However, this is not always the case due to the presence of indigenous strains that are able to form nodules, but not as efficient at fixing nitrogen (Singleton and Tavares 1986). Therefore, a rapid, and precise, method of quantification of *B. japonicum* that allows a soybean producer to know whether they will benefit from buying inoculum for their crop can be as beneficial as a soil test to determine the levels of nutrients in the soil to ensure healthy plant growth. One of the objectives of this study is to develop a rapid and sensitive real-time PCR-based assay with the ability to differentiate between strains of *B. japonicum*. As a proof of principle, the developed assay will be utilized to quantify *B. japonicum* in field soils.

1.7.2. Identification of the Genes that are Involved in the Production of Bacteriocins in *B. japonicum*

The production of bacteriocins in rhizobia has been shown to increase competitiveness for nodule occupancy. Most of the studies have been done on strains of *R. leguminosarum*. One example is the production of RTX-like toxin in *R. leguminosarum* 248 that was shown to increase the competitiveness of the bacteriocin producing strain (Oresnik et al. 1999). Another example is trifolitoxin (TFX) that is produced by *R. leguminosarum* bv. *trifolii* strain T24 (Triplett 1988). The ability to produce TFX increases the competitiveness of *R. leguminosarum* strains for nodule occupancy (Triplett 1988). Furthermore, it has been shown that the ability to produce TFX can be utilized to increase competitiveness for nodule occupancy in a *Rhizobium* belonging to a different species (Robleto et al. 1998).

B. japonicum strains have also been shown to produce bacteriocins and bacteriocin-like substances (Gross and Vidaver 1978; Roslycky 1967). However, there has been little to no study on the role of the production and utilization of bacteriocins produced by *B. japonicum*. The objective of this study was to isolate *B. japonicum* strains from agricultural soils in Manitoba and to identify if any of these strains produce bacteriocins. Candidate strains that have bacteriocin-like phenotypes was sequenced and an analysis of the genome was undertaken to identify putative genes that are responsible for inhibiting the growth of indicator strains. Candidate genes were mutated to determine if any are responsible for the bacteriocin-like phenotype, and if this phenotype can be correlated with an increased competitive phenotype.

Chapter 2

The Development of a Real-Time PCR-Based Assay for the Rapid Quantification of *B. japonicum* in the Environment

2.1. Introduction

Soybean (*Glycine max*) is a widely grown legume crop that forms a symbiotic relationship with *Bradyrhizobium japonicum*. This symbiosis manifests itself as nitrogen fixing nodules formed on the roots of the soybean plants. A well-nodulated plant can derive all the nitrogen necessary for growth from this association. This is important because the availability of nitrogen limits plant growth in field conditions hence, affecting the yield.

A problem that is often encountered is having sufficient numbers of the correct Rhizobia species present as the seed is germinating so that effective nodules can develop in a timely manner. This is usually circumvented by the application of inocula of Rhizobia that is supplied by seed companies in various forms and applied either directly to the seed, or to the field at the time of planting. The use of a *Rhizobium* inoculant on a legume crop leads to the decreased use of fertilizer. This in turn will prevent the excessive use of fertilizer on farm fields that results in high nutrient run-off that can lead to the deterioration of surface and ground water.

Using inoculant also provides growers with economic benefit. In 2015, United States Department of Agriculture (USDA) estimated that utilizing fertilizer cost growers US$34/acre or C$45/acre (McBride 2016). In contrast, the cost of utilizing inoculant ranges between C$3/acre (liquid inoculant) to C$11/acre (granular inoculant) which is approximately 4-15 times less expensive than fertilizer (Arnason 2015).

Although soybean was introduced to the Canadian prairies as a crop relatively early (Bedford 1899), and that some of the early maturing cultivars were developed in Manitoba (Shurtleff and Aoyagi 2010; Stefansson 1966a; Stefansson 1966b), soybean did not flourish and commercial scale production of soybean was abandoned by the early 1970s (Shurtleff and Aoyagi 2010). Soybean has since been reintroduced and production has increased steadily over

the past seven years from 520,000 acres to 1.6 million acres (Bekkering 2014; Statistics Canada, 2016). Because of this recent increase in soybean production, fields in Manitoba do not have large indigenous populations of *B. japonicum* that are capable of entering into a nitrogen fixing association.

A question that has arisen due to the massive increase in soybean cultivation is whether fields need to be continually inoculated. Current guidelines for the Canadian northern Plains (Southern Manitoba and Saskatchewan) suggest double inoculation (inoculum on seed and in furrow), especially on fields that have not been seeded and inoculated with *B. japonicum*. The reasoning for the advice is that *B. japonicum* populations are not thought to have become part of the established microbial community.

Currently, it is also unknown whether row spacing and plant population affect *B. japonicum* populations in soil. Previous studies showed that row spacing and plant population affect soybean yield (De Bruin and Pedersen 2008; Thompson et al. 2015). However, there has been no study that correlates these factors to *B. japonicum* community in soil to our knowledge. In Manitoba, both wide and narrow row spacing strategies are utilized. Since *B. japonicum* is crucial for soybean growth and yield, it is very important to understand which row spacing strategy can better sustain *B. japonicum* populations hence, allowing the community to establish itself in Manitoba.

The ability to quantitate *B. japonicum* still relies on the culture-based methods or immunofluorescence based- assays (Danso et al. 1973; Graham 1969; Kinkle et al. 1987; Pattison and Skinner 1974; Schmidt et al. 1968; Thompson and Vincent 1967; Tong and Sadowsky 1994; Top et al. 1990; Van Schreven 1970). Despite the ability to provide a precise estimation of the number of *B. japonicum* in the soil, the culture-based methods often fall short

in their ability to differentiate between the strains of *B. japonicum* (Tong and Sadowsky 1994). In addition, these methods are time-consuming and require several weeks before a result can be obtained (Tong and Sadowsky 1994).

To address these problems we set out to develop a rapid, sensitive method to quantify *B. japonicum* using a culture independent real-time PCR-based assay. We show that this assay can quantitate *B. japonicum* from field soil. The utility of this assay to address agronomic problems is demonstrated by addressing the affect that row spacing may have on the *B. japonicum* population over a field season.

2.2. Materials and Methods

2.2.1. Bacterial Strains, Media, and Growth Conditions

Bacterial strains that were used in this study are *B. japonicum* USDA110 (Kaneko et al. 2002), *Sinorhizobium meliloti* strain Rm1021 (Meade and Signer 1977), as well as *R. leguminosarum* bv. *trifolii* strain Rlt100 (also known as W14-2) (Baldani et al. 1992; Oresnik et al. 1998). *B. japonicum* USDA110 is a commercial soybean inoculum strain. *S. meliloti* strain Rm1021 and *R. leguminosarum* form symbiosis with alfalfa and sweet clover respectively. *B. japonicum* strains were grown at 30°C in yeast extract mannitol (YEM) medium (Vincent 1970), whereas *S. meliloti* and *R. leguminosarum* were routinely grown on either YEM, Luria-Bertani (LB), or tryptone yeast extract (TY) medium (Vincent 1970; Beringer 1974; Sambrook et al. 1989).

2.2.2. Primer Design and PCR conditions

The conditions used for PCR are listed in A2.1. Primers were designed using the Primer-BLAST software that is freely accessible via NCBI (National Centre for Biotechnology Information). Briefly, using the standard program parameters, various regions that we thought would be of interest were identified. The program scans the region against all the deposited sequences within the NCBI database and suggests primers that should be specific. The region we investigated was the 16S rDNA. Total bacterial populations were determined as previously described (Fierer et al. 2012).

2.2.3. Quantification of *B. japonicum* Utilizing Real-Time Quantitative PCR

To quantify *B. japonicum*, different dilutions were made from a pure culture of *B. japonicum* with a known concentration. DNA samples were obtained from different dilutions and utilized in qPCR as standards to allow for quantification of *B. japonicum* from unknown samples. The concentration of *B. japonicum*/gram of soil from each sample was calculated based on the amount soil that was used for DNA extraction. The conditions utilized for real-time qPCR are listed in A2.2

2.2.4. Soil Samples.

Soil samples (2009, 2011, and 2012) were kindly provided by Bruce Brolley (Manitoba Agriculture). Samples were collected during the summer of 2008. Soil samples (~ 160g) were collected into paper sample bags and kept at 4 ^{o}C until they were delivered to the lab (typically between 2-12 hours). Once in the lab, the soils were either immediately extracted, stored at -80 ^{o}C, or in some cases left at 4 ^{o}C.

2.2.5. DNA Extraction

DNA extractions were carried out using MO BIO's PowerSoil^{TM} DNA isolation kit. The kits were used as directed by the manufacturer. Extractions were performed using 0.25 g of soil and the resulting DNA was resuspended in 100 µl of sterile ddH_20. DNA samples were routinely quantified using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific). PCR and qPCR reactions were carried out using standard conditions.

2.2.6. Field Study

Soil samples were taken from a field study that was conducted during the 2013 growing season at the Ian N. Morrison Research Station in Carman, Manitoba, Canada (49.50 ^{0}N, 98.03 ^{0}W) on plots that had not previously been inoculated with *B. japonicum*. The study was designed to evaluate soybean yield loss caused by volunteer canola (volunteers are the plants that are not deliberately planted). However, since the study also included variable row spacing as a variable, soil samples were collected and assayed for *B. japonicum*.

The study used a split-plot randomized complete block design with four replications. Soybean row spacings (19 cm and 76 cm) were considered main plots and volunteer canola densities (0 seeds m^{-2}, 20 seeds m^{-2}, 80 seeds m^{-2}, and 320 seeds m^{-2}) were the subplots. Soybean (23-10 RY, Monsanto), was seeded into cultivated wheat stubble from the previous growing season at a target density of 432,000 plants ha^{-1} (70 kg seed ha^{-1}). The soybean was inoculated with TagTeam granular inoculant (Novozymes BioAg, Saskatoon, SK.; minimum guarantee 1.0 x 10^5 cfu *Penicillium bilaii* g^{-1} and 1.0 x 10^8 viable cells *Bradyrhizobium japonicum* g^{-1}) and was banded with the seed at a rate of 6.67 kg ha^{-1}. To keep the *B. japonicum* population density the same in wide- and narrow-row soybean, inoculant was applied in 19 cm rows in both soybean

row spacings. However, inter-row inoculant was surface applied in the wide-row soybean treatment to avoid soil disturbance.

Four sample dates were conducted in the 2013 growing season (June 05 (VE), July 02 (V1), July 26 (R3) and September 04 (R8)) while one sample date was conducted in the spring of 2014 (May 21) before planting. In each sub-plot, two core samples totaling 61.32 cm^2, were taken to a depth of 12.5 cm midway between two adjacent soybean rows. Both core samples were bulked and transferred on ice to a refrigerator (4 °C).

Mixed model analysis in SAS 9.3 was used to conduct an ANOVA on *B. japonicum* population and total bacterial community (cfu g^{-1} FW soil) data with sample date as a repeated measure (Littell et al. 1998). Soybean row spacing, sample date, and volunteer canola density were considered fixed effects while experimental replication and volunteer canola density nested within row spacing was considered a random effect. Residuals were tested for normality, and data were corrected for heteroscedasticity when necessary to meet the assumptions of ANOVA. Lund's test was used to remove outliers (Lund 1975), and in the case of the total bacterial community data, the square root transformation was used to meet the assumption of normality. Means were separated based on Fisher's protected LSD ($\alpha = 0.05$) using the pdmix 800 macro (Saxton 1998).

2.2.7. Plant Growth Chamber Assays

Soybean seeds were sterilized using the method previously described with few modifications (Wacek and Brill 1975). Briefly, the seeds were washed in 1.2% sodium hypochlorite for approximately ten minutes. This was followed by thoroughly rinsing the seeds with 10 volumes of sterile water, followed by treating the seeds with 10 μM hydrochloric acid (HCl) for ten minutes. The seeds were finally washed with 10 volumes of sterile water and

germinated on sterile water agar (1.3% w/v) at room temperature, in the dark for 4-7 days. Germinated soybeans were planted in sterilized Magenta jar assemblies containing a mixture of sand, vermiculite, soaked with an equal volume of Jensen's medium as previously described (Oresnik et al. 1999).

2.3 Results

2.3.1. J16S Primer Set Appears to be Specific for *B. japonicum*

Primers used for qPCR are ideally situated such that the amplification product would be between 80 and 250 bps in length. To design our primers we chose to target the 16S rDNA region of *B. japonicum*. Alignment of the 16S rDNA sequence highlighted two regions 943-989 and 1162-1226 that when aligned against other *Rhizobium* 16S sequences, appeared to be specific for *B. japonicum*. Three primers were designed such that 2 different amplification products could be generated. These were: Primer 1 (left) Tm = 60^{o}C**,** 5'AGGACCGGTCGCAGAGAT 3'; Primer 2 (right) Tm = 60^{o}C 5'CCATTGTAGCACGTGTGTAGC 3'; and Primer 3 (right) Tm = 60^{o}C 5'CACCGGCAGTCTCCTAGAG 3. These primers were used in standard PCR reactions with *B. japonicum*, *R. leguminosarum*, and *S. meliloti* genomic DNA. Amplification products were only seen with *B. japonicum* genomic DNA. Further testing of these primers showed that primer 1 and 2 gave the greatest specificity with a complete absence of non-specific amplification products from both *S. meliloti* and *R. leguminosarum* (Figure 1). This primer pair was designated as J16S.

2.3.2. J16S Primers can Quantitatively Amplify *B. japonicum* from Field Samples

Our initial trials using the J16S primer pair with DNA that was extracted from soil samples taken from fields with various histories of growing soybean that were inoculated with commercial inoculants demonstrated that the J16S primer pair could amplify products using qPCR. The data did not show any correlation with respect to the number of years soybean had been grown and the titre of *B. japonicum* we could detect (data not shown). To systematically test our ability to quantitate *B. japonicum* in field soil, a sample of soil was spiked with a known quantity of *B. japonicum* USDA110. The DNA was assayed with the J16S primer set as well as with primers that were designed to *bll5079*; a gene that is specific for USDA110 (Gupta and Mok 2007). The data clearly show that following extraction we were able to account for 75 ± 25% (n=3) of the added *B. japonicum* cells (Figure 2). Estimates of the level of detection based on the amount of sample extracted as well as the assay volume suggest that our assay could detect between 50-100 *B. japonicum* cells per assay. This translates to an approximate soil titer of 10^4 *B. japonicum* cfu/g.

2.3.3. Row Spacing Affects *B. japonicum* Population in the Field

To utilize the assay in real conditions it was apparent that protocols for sampling would need to be standardized. At the same time we were intrigued whether the assay could be utilized to answer agronomic questions that could be affected by the population of *B. japonicum* that was present. Row spacing of soybean in the field can have multiple implications at all levels of production.

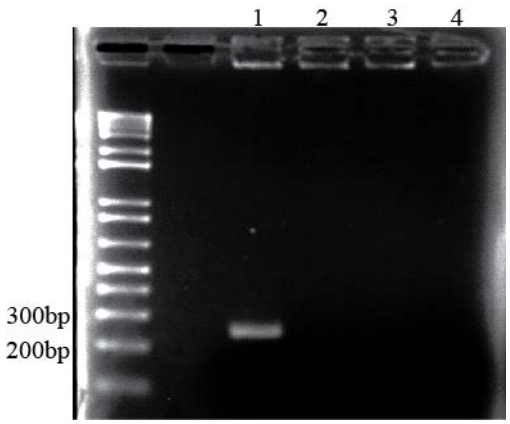

Figure 1. J16S primers can differentiate between *B. japonicum* USDA110 and other rhizobia. The figure shows the result of PCR amplification utilizing the colonies of *B. japonicum* USDA110, *Sinorhizobium meliloti* Rm1021, and *Rhizobium leguminosarum* bv. *trifolii* Rlt100 (lane 1, 2, and 3 respectively). Negative control is shown in lane 4.

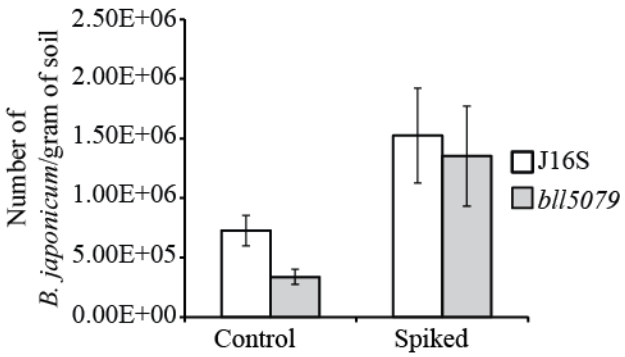

Figure 2. The specific primers designed can precisely quantify *B. japonicum* in soil. DNA samples were obtained from soil which was spiked with *B. japonicum* USDA110 and control soil (not spiked). J16S and *bll5079* primers were used for quantification utilizing real-time qPCR. Error bars represent standard deviation (n=3). Recovery percentage = (The difference in the number *B. japonicum*/gram of soil in spiked and control samples ÷ The number of *B. japonicum* in spiked soil) x 100%.

Previous work showed that microbial community composition in the environment can change due to factors such as soil depth, the presence of herbicides, also chemical and nutrient profiles (Xu et al. 2013; Risal et al. 2010; Chaudhry et al. 2012; Deng et al. 2012; Fierer et al. 2012; Jönemann et al. 2012; Stein et al. 2005). For example, the relative abundance of *Bacteriodetes* changes relative to the depth of soil with greater abundance in the surface horizons relative to deeper horizons (Eilers et al. 2012). The effect on depth and proximity to a soybean plant on the number of *B. japonicum* is currently not known. Since these data can be critical to reproducibility the effect of depth and distance from a plant were investigated.

During the 2012 field season, soil samples were obtained from two soybean fields (Froebe and Pritchard) that utilized either a 15 or 30 inch row spacing. Soil samples were collected to a depth of 4 inches, and divided on the basis of depth (0-1 inch, 1-2 inches, 2-3 inches, and 3-4 inches). These samples were taken based on the distance from the plants (1 or 5 inches from the plants). Although there were some fluctuations in the numbers, the results showed that there was no significant change of *B. japonicum* population with respect to depth of sampling (Figure 3). When the data were analyzed with respect to row spacing it was found that proportion of *B. japonicum* found in the fields utilizing 15 inch row spacing was nearly twice that of samples that were taken from fields with 30 inch row spacing, suggesting that row spacing had an effect on the *B. japonicum* population (Figure 4).

2.3.4. Plant Density Affects *B. japonicum* Population in Controlled Conditions

The row spacing data (Figure 4), suggested that plant density may affect the population of *B. japonicum*. Alternately, it could be argued that fields employing narrow row spacing receive twice the inoculum of a field employing wide row spacing. To directly test the

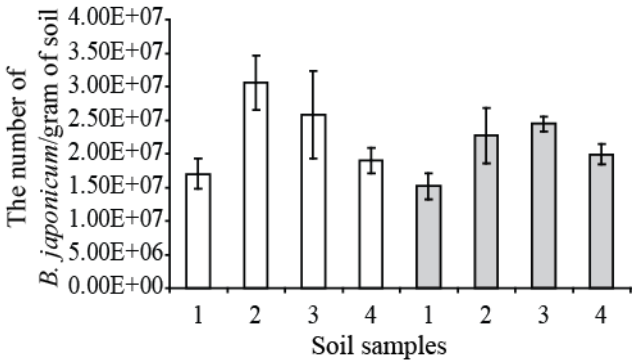

Figure 3. There is no significant difference in the composition of *B. japonicum* community between the soil samples. White columns and grey columns represent soil samples which were obtained 1 inch away from the plants and 5 inches away from the plants respectively. Soil samples were also taken from a depth; 0-1 inch, 1; 1-2 inches, 2; 2-3 inches, 3; and 3-4 inches, 4. Error bars represent standard deviation (n=3).

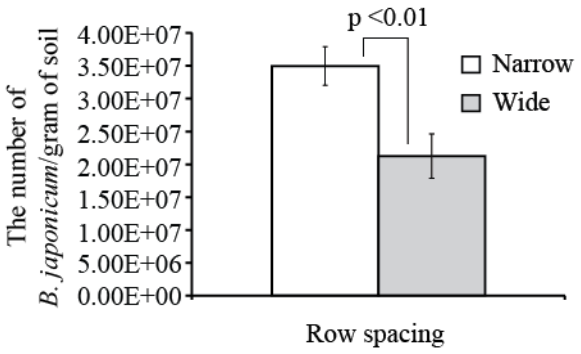

Figure 4. The field utilizing narrow row spacing has more *B. japonicum*. The data are represented as the number of *B. japonicum*/gram of soil. Error bar represents standard deviation (n=18).

hypothesis that plant density affected the *B. japonicum* population, soil samples were used as a source of inoculum for Magenta jar assemblies that contained one, two, or four plants.

The data showed that there was no significant difference in the amount or proportion of *B. japonicum* in the jars containing 1 plant compared to the control (Figure 5). However, there was a significant increase in the number of *B. japonicum* in the jar grown with 2 and 4 soybean plants (Figure 5). This data are consistent with the hypothesis that the field effects are due to the presence of soybean plants rather than being caused by excess inoculum.

2.3.5. Row Spacing Effects are Correlated with Plant Development

In an effort to replicate the row spacing effects that we had observed during the 2012 growing season, the opportunity to sample from a randomized block experiment that also had row spacing as a variable became available. Samples were taken from plots at intervals over the 2013 growing season as well as the following spring.

The data show that following inoculation the number of *B. japonicum* increases following planting as the plant grows (Figure 6). At the mid-August sampling the populations of *B. japonicum* declined in from both the plots that had wide or narrow row spacing. However, the number of *B. japonicum* detected recovers prior to harvest and finally declines to levels below those measured 6 days following inoculation (Figure 6). Of note, the only sample points where the number of *B. japonicum* was greater in the narrow row spacing than the wide row spacing was during the August sampling.

The total bacterial population was determined using a primer pair optimized to amplify bacteria. Statistical analysis of all samples collected over the field season were analyzed and the results show that although the number and proportion of the *B. japonicum* were significantly

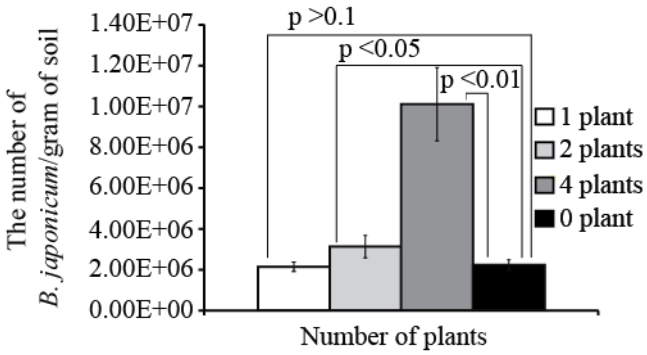

Figure 5. The presence of soybean plants has a positive impact on *B. japonicum* community. A different number of soybean plants (1, 2 or 4 plants) were grown in each Leonard jar assembly (Leonard 1943; Trung and Yoshida 1983). Each jar was inoculated with the same amount of *B. japonicum* (10^6 cfu). Error bar represents standard deviation (n=3). Student's t-test was performed to determine the significance between the jars that contain 1, 2, or 4 plants and the control jars.

different from the August sampling, the total bacterial population was statistically larger in field plots that utilized a narrow row spacing regime (Figure 6).

2.4. Discussion

This study was initiated to develop a tool for determining the number of *B. japonicum* in agricultural field soil. We have shown that using a portion of the 16S rDNA we were able to design primers that were specific to *B. japonicum* and suitable for use with qRT-PCR that did not cross react with other common Rhizobia (Figure 1), and could be used in a quantitative manner (Figure 2). Moreover, these primers were able to detect *B. japonicum* from samples taken from fields and allowed the development of a sampling protocol to determine how it would be best to sample fields (Figure 3).

To demonstrate the versatility of the protocol, the effect of row spacing was investigated using soil that was sampled from soybean fields in Southern Manitoba that employed either wide or narrow row spacing. The data strongly suggested that row spacing could significantly affect *B. japonicum* populations. We found that fields utilizing a 15 inch row spacing contained about twice the number of *B. japonicum* when compared to fields utilizing 30 inch row spacing. This data can be interpreted either that fields with narrower row spacing received greater numbers of inoculum at seeding, or that the host plant was positively influencing the *B. japonicum* population.

To address this in a controlled fashion, field soil was used to inoculate pots containing an increasing number of plants grown in a growth chamber. The results showed that after 4 weeks, there was positive correlation between the number of plants in a defined space when compared to the overall number of *B. japonicum*. The data support the hypothesis that the presence of plants

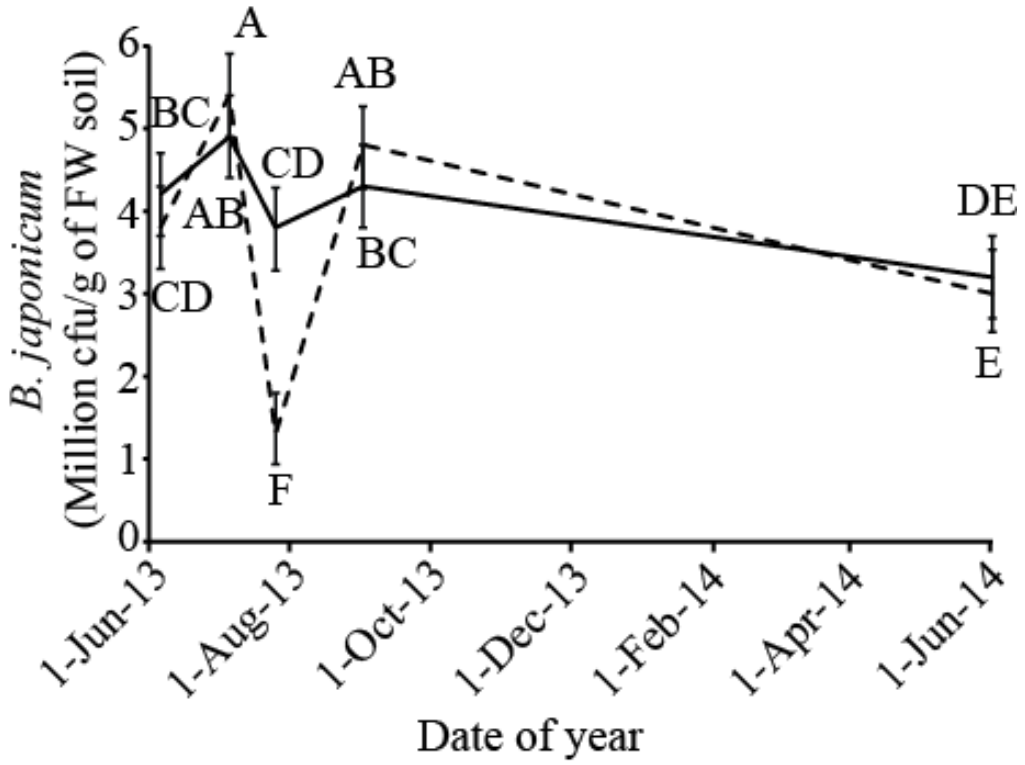

Figure 6. The effect of different row spacing strategies on *B. japonicum* population. *B. japonicum* population (Million cfu g^{-1} FW soil) in the inter-row spaces of soybean seeded at narrow and wide-row spacings indicated by dashed and solid line respectively ($P < 0.0001$). Error bars indicate +/- one standard error of the mean. Different letters indicate significant differences as determined by Fisher's protected LSD ($\alpha = 0.05$).

supports the growth of the *B. japonicum* community in soil. A possible explanation for this observation is due to the fact that the plant roots secrete a variety of compounds such as sugars and amino acids (Barbour et al. 1991; Graham 1991; Liao et al. 2012; Timotiwu and Sakurai 2002). This suggests an increased number of plants present in a given area may secrete or make available more nutrients that are usable by the *B. japonicum* that are present in that area. The data also correlate well with previous work that showed increased yields were obtained from soybean fields utilizing narrow row spacing (Thompson et al. 2015; De Bruin and Pedersen 2007). Taken together this suggests that a more efficient nodulation by the correct strains of *B. japonicum* may be one of the contributing factors to the higher yield that was seen on the soybean field utilizing narrow row spacing.

In an effort to replicate this phenomenon, we were able to sample from field plots that utilized a robust experimental design. The data followed the ontogeny of the *B. japonicum* from 6 days following inoculation through to harvest, as well as sampling following overwintering. The data showed that *B. japonicum* populations showed some fluctuations over the year and returned to levels that were very close to basal levels following an over wintering (Figure 6). Of interest, the greatest fluctuation of *B. japonicum* that was detected was from the early August sampling. These data correlated very well with the *ad hoc* sampling that was carried out during protocol development (Figure 5). In this case, samples were collected because the crop had reached the stage of fruit set and pod fill; a time when the host plant undergoes a major reallocation of resources. Our data suggest that this reallocation of resources affects the plant-associated population of *B. japonicum*. Of note, when these samples were quantitated using primers specific for eubacteria, the data showed that the total number of all bacteria were also greater from fields using a narrower row spacing regime.

In this work we show that qRT-PCR can be used to quantitate *B. japonicum* on the basis of DNA extracted from field samples. Over all of our enumerations, we found that we were able to detect a "basal population" of *B. japonicum* of around 10^5 *B. japonicum*/gram of soil. Although it appears as a population of *B. japonicum* based on our primers, uninoculated soils that have never been seeded and inoculated with *B. japonicum* do not harbor nodulating *B. japonicum* that we were able to detect using nodule trapping experiments. This may suggest that non-symbiotic *B. japonicum* may be present. We note that there is a precedent for the presence of non-symbiotic rhizobia that has been previously reported (Hollowell et al. 2016; Laguerre et al. 1993; Sullivan et al. 1996).

Our values from inoculated fields ranged from between 10^6-10^7 *B. japonicum*/gram soil. The protocol clearly has the sensitivity to detect population fluctuations through a growing season (Figure 6). What we do not currently know is what level of *B. japonicum* must be present in a field prior to seeding soybean to allow the ability to advise a grower that the addition of inoculum is not necessary. Ongoing work is being carried out to determine if these primers can be used to make these types of decisions.

Chapter 3

Isolation and Genome Sequencing of a *Bradyrhizobium japonicum* Strain Capable of Producing Bacteriocin-like Activity

3.1 Introduction

The study of bacteriocins and their mechanisms has been carried out in a number of different bacteria (Cascales et al. 2007; Ghazaryan et al. 2014; Jack et al. 1995; Jung 1991; Michel-Briand and Baysse 2002). Bacteriocin or bacteriocin-like phenotypes have been described for a number of Rhizobia (Oresnik et al. 1999; Roslycky 1967; Triplett and Barta 1987). However, the number of bacteriocins that have been characterized for Rhizobia are very few. Previous work has shown that strains of *B. japonicum* are capable of producing bacteriocins and bacteriocin-like substances (Robleto et al. 1998; Roslycky 1967). It is of note that a bacteriocin-like substance from *B. japonicum* capable of inhibiting the growth of *Corynebacterium nebraskense*, a causal agent of Goss's bacterial wilt and corn blight, has been previously described (Gross and Vidaver 1978, Vidaver 1977). Despite this result, there have been no further studies that focus on the utilization of bacteriocins for biocontrol. In fact, literature searches show that there has been little to no study of bacteriocin production in *Bradyrhizobium* since the late 70's.

Since soybean is a relatively new crop in Manitoba, it is common practice to inoculate soybean with inoculant strains that are provided to the farmer by the seed provider. At this time, there is no evidence to suggest that the *B. japonicum* inoculums that are provided are not competitive. This is presumably due to the low indigenous population of *B. japonicum* that are found in Manitoba soil.

This study was initiated to explore the hypothesis that bacteriocin production can be a determinant for competition for nodule occupancy. We hypothesize that there are strains of *B. japonicum* present in Manitoba and these strains might produce bacteriocin or bacteriocin-like compounds that could be used to enhance their ability to compete for nodule occupancy.

The aims of this chapter are two-fold. The first is to demonstrate that there are strains of *B. japonicum* that can produce compounds that inhibit other *Bradyrhizobia*. The second is to isolate a local strain with these inhibition capabilities that can be further studied at a genetic level to determine potential genes that could be responsible for producing bacteriocins.

3.2 Materials and Methods

3.2.1. Bacterial Strains, Media, and Growth Conditions

Bacterial strains used in this work are listed in Table 1. *B. japonicum* strains were grown at 30°C in yeast extract mannitol (YEM) medium (Vincent 1970). When required, Congo Red was added as an indicator dye for *Bradyrhizobium* at a concentration of 0.025 g/L.

3.2.2. Isolating *Bradyrhizobium* Strains from Soil

B. japonicum strains were isolated from soil by the use of nodule trapping experiments. Essentially field soil was used to grow soybean seeds that were surface sterilized and bacteria were isolated from nodules that developed. For these experiments, soybean seeds were surface sterilized by gently washing the seeds in 1.2% sodium hypochlorite (20% v/v bleach) for approximately five minutes. The seeds were then rinsed with 10 volumes of sterile distilled water, and then soaked (about a minute) in sterile 10 μM hydrochloric acid (HCl). Following the HCl treatment, the seeds were washed with 10 volumes of sterile water. The seeds were then sprouted in the dark for 4-7 days on 0.4% water agar plates at room temperature.

To isolate nodule bacteria, nodules were picked after about 4 weeks and surface sterilized. Surface sterilization of nodules was carried out essentially as previously described (Somasegaran and Hoben 1994). The nodules were first immersed in 95% ethanol for 5-10 seconds. The ethanol was then decanted and the nodules were soaked for about 2-4 minutes in approximately

Table 1. The list of strains and plasmids utilized for studying the production of bacteriocins in *B. japonicum*

Rhizobium strain	Genotype or phenotype	Reference or source
USDA110	Wild type	Kaneko et al. 2002
FN1	*B. japonicum* strain isolated in Manitoba	This work
BRJ16	Soybean-nodulating rhizobia in Manitoba	This work
USDA442	*B. japonicum*	ATCC
F8	*B. japonicum*	Lab collection
SR-10	Soybean-nodulating rhizobia isolated in Pakistan	This work
SR-11	Soybean-nodulating rhizobia isolated in Pakistan	This work
SR-12	Soybean-nodulating rhizobia isolated in Pakistan	This work
SR-14	Soybean-nodulating rhizobia isolated in Pakistan	This work
SR-15	Soybean-nodulating rhizobia isolated in Pakistan	This work
SR-16	Soybean nodulating strain of *B. yuanmingense* isolated in Pakistan	This work

2-3% sodium hypochlorite. Finally, the hypochlorite was removed and the nodules were washed 5 times with sterile distilled water (to similar volume of the total volume of nodules). The nodules were then transferred to a sterile eppendorf tube containing 100 µL of sterile water and crushed using a sterile inoculating stick. An aliquot of the suspension was then streaked onto YEM agar plates containing 0.025 g/L of Congo Red to facilitate the identification of *Bradyrhizobium*. Bacteria were single colony-purified three times.

3.2.3. Bacteriocin Assays

The bacteriocin assays were performed as previously described (Oresnik et al. 1999) with few modifications. The indicator strains were suspended in YEM containing 0.6% agar to obtain a concentration of approximately 10,000 - 100,000 colony forming unit (cfu)/plate (approximately 400-4,000 cfu/ml). A colony of the bacteria to be tested for bacteriocin production (tester strain) was stabbed using an inoculating stick into a plate containing the indicator strain (the strain that is potentially susceptible to the bacteriocins produced by tester strains). The plates were then incubated for 1 week in 30°C. Although the zones of clearing were often evident at this time, it was found that if the plates were routinely left at room temperature for about 1-2 weeks, zones of inhibition were often much clearer. Zones were scored at both 1 week and then rechecked about a week later. The positive results for bacteriocin production are visible as the zones of clearance surrounding the stab-inoculated culture (Figure 7).

3.2.4. The generation of FN1 draft genome.

DNA from strain FN1 was isolated using a Qiagen whole genome isolation kit. Approximately 1-2 µg was sent to the Next Generation Sequencing platform at the Manitoba

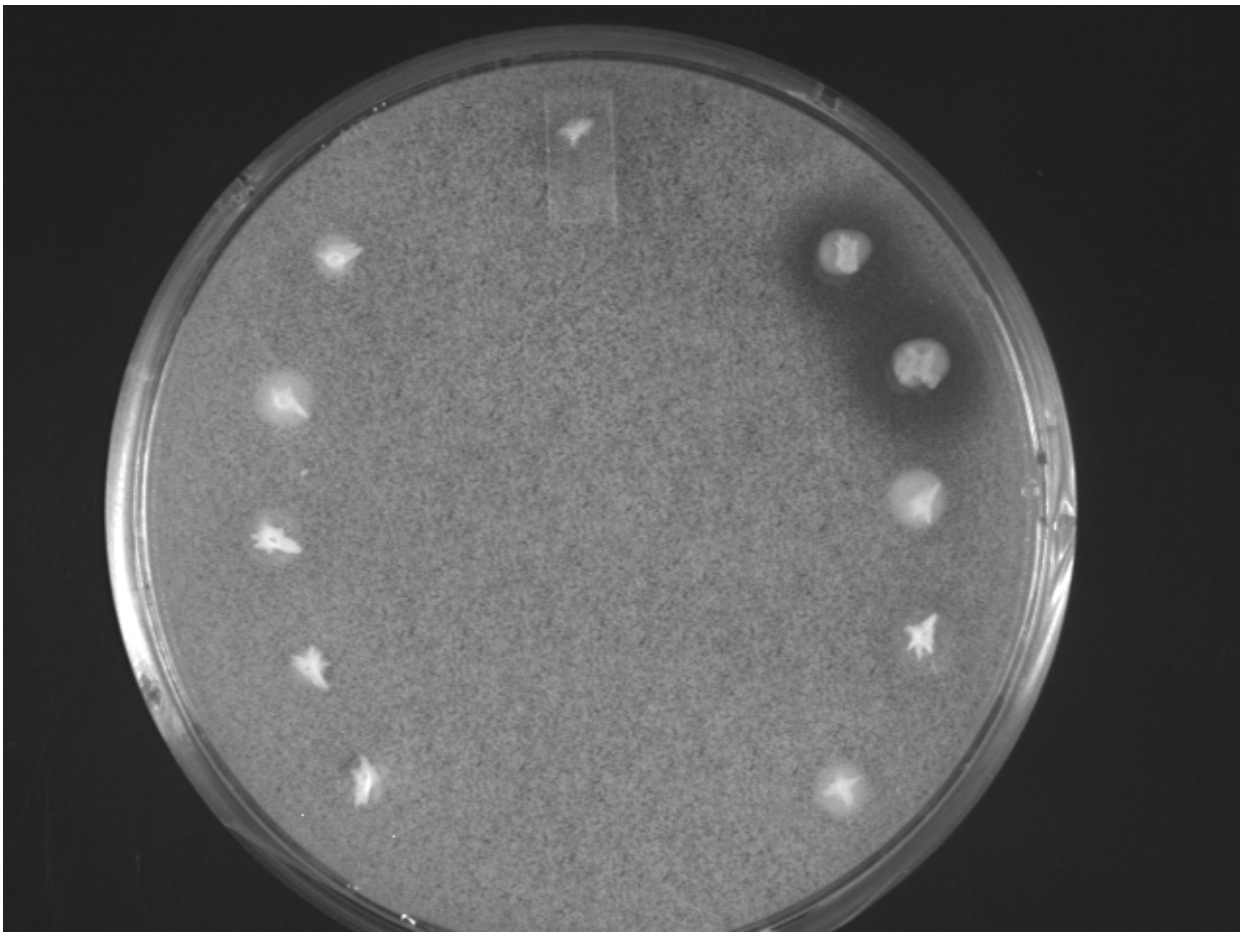

Figure 7. A bacteriocin assay. The plate contains solid medium seeded with an indicator strain. The strains to be tested were inoculated by stab-culture method. The zone of clearance that forms around the stab-culture indicates production of putative bacteriocins.

Institute of Child Health for library construction and sequencing utilizing Illumina MiSeq technologies. A draft genome was generated utilizing Illumina paired-end reads sequencing method. The genome was assembled utilizing optimized-VELVET (Zerbino and Birney 2008), and submitted to the Joint Genome Institute's Integrated Microbial Genomes Expert Review (IMG ER) annotation pipeline for gene annotation (Markowitz et al. 2006). This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession JGCL00000000. The version described in this thesis is version JGCL01000000 (Kohlmeier et al 2015).

3.2.5. The Identification of the Putative Bacteriocin Biosynthetic Genes of *B. japonicum* FN1

The identification of genes that may be involved in the biosynthesis of bacteriocins in FN1 was carried out using a workflow that combined, AntiSMASH, (Blin et al. 2013; Medema et al. 2011), maintained at http://antismash.secondarymetabolites.org, BLASTP (Altshcul et al. 1990; Boratyn et al. 2013, Johnson et al. 2008), as well as orthologous neighbourhood analysis (Mavromatis et al. 2009), that is maintained by IMG-JGI (http://img.jgi.doe.gov) (figure 8). Briefly, the genome (nucleotide) sequence of FN1 in FASTA format was used as query for antiSMASH. The conceptually translated amino acid sequences of biosynthetic genes within the identified "bacteriocin clusters" were used as queries for a BLASTP search against the FN1 genome that is available in IMG-JGI. Since we had previously determined that USDA110 was not inhibited by FN1, the top hits were then analysed for the presence of orthologs in *B. japonicum* USDA110. Only the genes that did not have orthologs in USDA110 were chosen as potential candidates.

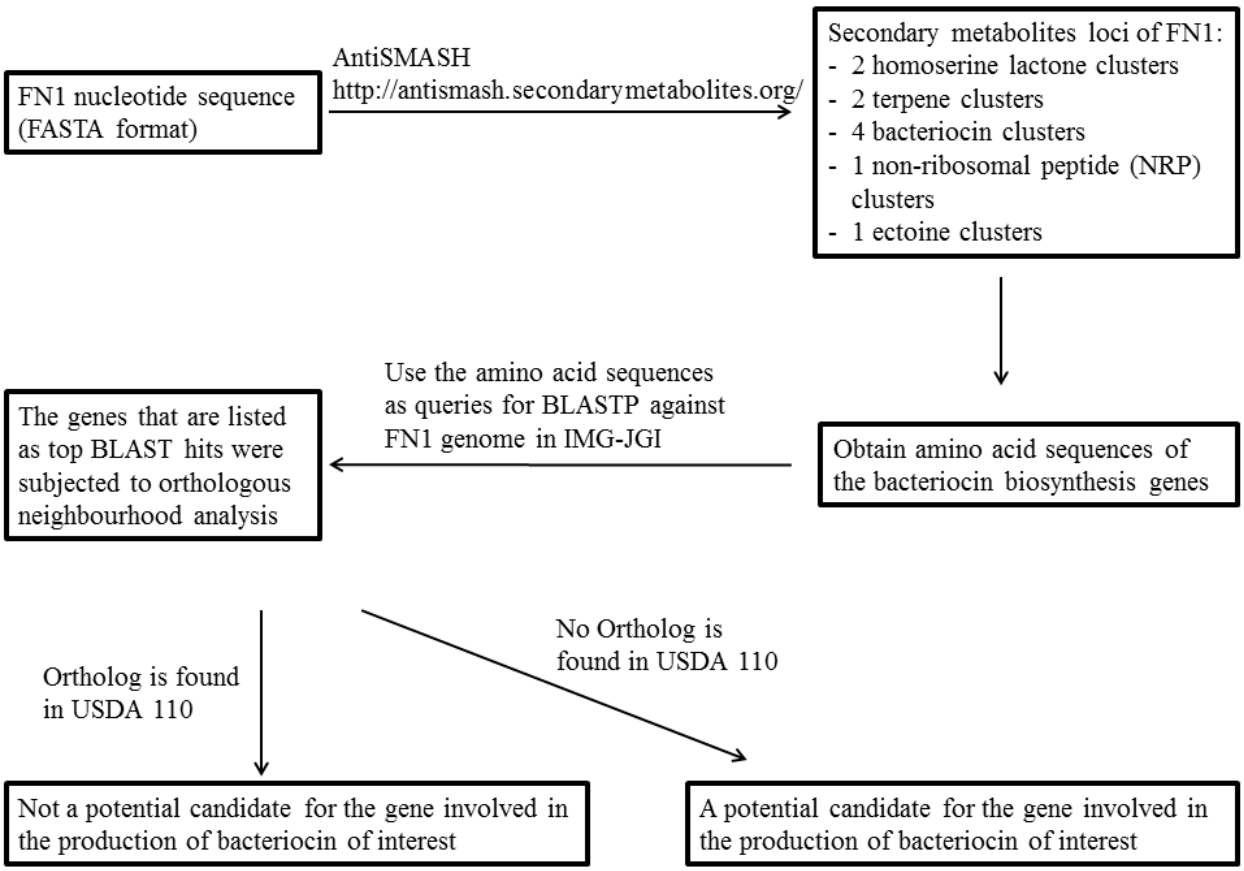

Figure 8. The workflow for identifying bacteriocin genes in FN1 utilizing antiSMASH.

3.3 Results

3.3.1. Isolation of Bradyrhizobia from Manitoba Fields

To find Bradyrhizobia, samples of field soils were routinely screened for the presence of Rhizobia capable of nodulating soybean. Field soil that was used generally did not produce nodules on soybean plants grown in growth chambers unless they had been previously inoculated with *B. japonicum*. Two unique strains were isolated from nodule trapping experiments: FN1 and BRJ16. Once single colony purified, these strains were confirmed for their ability to form nodules on soybean plants upon re-inoculation. To identify the bacteria, genomic DNA was isolated and used as a template for PCR reactions utilizing the V3-V4 region of the 16S rDNA region. The results of the sequencing suggested that both strains were *Bradyrhizobium sp*. Whereas the nearest match for FN1 was *B. japonicum*, BRJ16 was tentatively identified as *B. yuanmingense* (Figure 9).

3.3.2. The Strains of *Bradyrhizobium* Produce Multiple Different Bacteriocin-Like Substances

In preliminary experiments we had found that a number of strains that were originally isolated in Pakistan (labelled SR-10-16) worked well as indicator strains (A. Ali and I. Oresnik, personal communication). To test the isolated strains (FN1 and BRJ16) for the capability of producing bacteriocin-like zones of inhibition they were tested against these Bradyrhizobia. In addition, a number of Bradyrhizobia that were in the laboratory culture collection were also tested for the ability to produce zones of clearing against the tester strains (Table 2).

A.

Score	Expect	Identities	Gaps	Strand
749 bits(405)	0.0	417/422(99%)	3/422(0%)	Plus/Minus

```
FN1        2 CGTC-TTATCTTCCCGCACAAAAGAGCTTTACAACCCTAGGGC--ACATCACTCACGCGG 58
             |||| |||||||||||||||||||||||||||||||||||||   |||||||||||||||
E109 1605237 CGTCATTATCTTCCCGCACAAAAGAGCTTTACAACCCTAGGGCCTTCATCACTCACGCGG 1605178

FN1       59 CATGGCTGGATCAGGGTTGCCCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGA 118
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
E109 1605177 CATGGCTGGATCAGGGTTGCCCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGA 1605118

FN1      119 GTTTGGGCCGTGTCTCAGTCCCAATGTGGCTGATCATCCTCTCAGACCAGCTACTGATCG 178
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
E109 1605117 GTTTGGGCCGTGTCTCAGTCCCAATGTGGCTGATCATCCTCTCAGACCAGCTACTGATCG 1605058

FN1      179 TCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCAGACGCGGGCCGATCTTTCGGC 238
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
E109 1605057 TCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCAGACGCGGGCCGATCTTTCGGC 1604998

FN1      239 GATAAATCTTTCCCCGTAAGGGCTTATCCGGTATTAGCACAAGTTTCCCTGTGTTGTTCC 298
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
E109 1604997 GATAAATCTTTCCCCGTAAGGGCTTATCCGGTATTAGCACAAGTTTCCCTGTGTTGTTCC 1604938

FN1      299 GAACCAAAAGGTACGTTCCCACGCGTTACTCACCCGTCTGCCGCTGATGTATTGCTACGC 358
             |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
E109 1604937 GAACCAAAAGGTACGTTCCCACGCGTTACTCACCCGTCTGCCGCTGACGTATTGCTACGC 1604878

FN1      359 CCGCTCGACTTGCATGTGTTAAGCCTGCCGCCAGCGTTCGCTCTGAGCCAGGATCAAACT 418
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
E109 1604877 CCGCTCGACTTGCATGTGTTAAGCCTGCCGCCAGCGTTCGCTCTGAGCCAGGATCAAACT 1604818

FN1      419 CT 420
             ||
E109 1604817 CT 160481
```

B.

Score	Expect	Identities	Gaps	Strand
662 bits(358)	0.0	367/371(99%)	2/371(0%)	Plus/Minus

```
BRJ16              2 TCACTCACGCGGCATGGCTGGATCAGGGTTG-TCCCATTGTCCAATATTCCCCACTGCTG 60
                     |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
B. yuanmingense  370 TCACTCACGCGGCATGGCTGGATCAGGGTTGCTCCCATTGTCCAATATTCCCCACTGCTG 311

BRJ16             61 CCTCCCGTAGGAGTTTGGGCCGTGTCTCAGTCCCAATGTGGCTGATCATCCTCTCAGACC 120
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B. yuanmingense  310 CCTCCCGTAGGAGTTTGGGCCGTGTCTCAGTCCCAATGTGGCTGATCATCCTCTCAGACC 251

BRJ16            121 AGCTACTGATCGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCAGACGCGGGC 180
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B. yuanmingense  250 AGCTACTGATCGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCAGACGCGGGC 191

BRJ16            181 CGATCTTTCGGCGATAAATCTTTCCCCGTAAGGGCTTATCCGGTATTAGCACAAGTTTCC 240
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B. yuanmingense  190 CGATCTTTCGGCGATAAATCTTTCCCCGTAAGGGCTTATCCGGTATTAGCACAAGTTTCC 131

BRJ16            241 CTGTGTTGTTCCGAACCAAAAGGTACGTTCCCACGCGTTACTCACCCGTCTGCCGCTGAT 300
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B. yuanmingense  130 CTGTGTTGTTCCGAACCAAAAGGTACGTTCCCACGCGTTACTCACCCGTCTGCCGCTGAC 71

BRJ16            301 TTTATTGCTACGCCCGCTCGACTTGCATGTGTTAAGCCTGCCGCCAGCGTTCGCTCTGAG 360
                      | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
B. yuanmingense   70 GT-ATTGCTACGCCCGCTCGACTTGCATGTGTTAAGCCTGCCGCCAGCGTTCGCTCTGAG 12

BRJ16            361 CCAGGATCAAA 371
                     ||||||||||||
B. yuanmingense   11 CCAGGATCAAA 1
```

Figure 9. The identification of soybean-nodulating rhizobia isolated from Manitoban soil. The partial sequence of V3 region of 16S rDNA of FN1 (A) and BRJ-16 (B) as queries for BLASTN. FN1 and BRJ16 were identified as *B. japonicum* strain E109 and *B. yuanmingense* respectively.

Testing each of the strains for resistance or production showed that except for USDA110, all strains were able to produce zones of inhibition against some strains (Table 2). The zones of inhibition could be grouped into at least 5 different groups. Strains FN1, USDA442, and F8 all produced zones of inhibition against SR-10, SR-11, SR-15 and SR-16 (table 2). BRJ16 produced zones of clearing against SR-11, SR-12, and SR-14 (table 2), whereas SR-16 was able to produce zones of clearing against SR-14 and SR-15 (table 2).

Zones of inhibition to SR-10, 11, 15, 16 occurred around FN1, USDA442, and F8. However, SR-16 also produced a zone of inhibition against SR15, suggesting that mechanism of inhibition of SR15 is not the same as that associated with SR10, 11, and 16. Similarly BRJ16 was able to produce a zone of inhibition against SR11, suggesting that this was also distinct. Finally, only BRJ-16 was able to produce zones of inhibition against SR-12 and SR13. Taken together, this suggests that there are at least 5 groups of molecule that are responsible for these zones of inhibition consisting of; inhibition of SR-10 and SR-16, inhibition of SR11, inhibition of SR-12, inhibition of SR-14, and inhibition of SR15 (Table 2).

3.3.3. The Generation of the Whole-Genome Sequence of Strain FN1

The genome of *B. japonicum* strain FN1 was sequenced utilizing the Next Generation Sequencing platform at the Manitoba Institute of Child Health using Illumina MiSeq technologies (Kohlmeier et al. 2015). Two successful runs both yielded 8,402,786 sequences, all of which were paired 150-bp reads with an average library insert size of 957 bp (Kohlmeier et al. 2015). Data output was assembled via Optimized-Velvet (Zerbino and Birney 2008) into two formats; one consisting of 141 contigs and the other further organized into 87 scaffolds. Both data sets were submitted to the Joint Genome Institute's (JGI) Integrated Microbial Genomes-Expert Review (IMG ER) platform (Markowitz et al. 2009) for annotation. The genome consists

Table 2. The identification of bacteriocin-producing rhizobia

Strains	SR-10	SR-11	SR-12	SR-14	SR-15	SR-16
USDA110	-	-	-	-	-	-
FN1	+	+	-	-	+	+
BRJ16	-	+	+	+	-	-
USDA442	+	+	-	-	+	+
F8	+	+	-	-	+	+
SR-16	-	-	-	+	+	-

The indicator strains are listed in columns while the strains tested for the ability to produce putative bacteriocins are listed in rows. Positive result indicates the formation of zone of clearance surrounding the tester strain.

of 9,138,496 bp, with a GC count of 64%, and has 8,613 coding sequences (Table 3). It contains a symbiosis island, housing the nodulation and nitrogen fixation genes. Genes encoding the enzymes involved in the Calvin-Benson-Bassham cycle as well as genes associated with hydrogen uptake were also detected. This suggests that FN1 is capable of chemolithoautotrophic growth using H_2 as an electron donor and CO_2 as a source of carbon. In addition, characteristic genes of the Embden-Meyerhoff-Parnas pathway, the Entner-Doudoroff pathway, Pentose Phosphate pathway as well those involved in poly-hydoroxy alkanoate production were present. These features are consistent with the published genomes (Kaneko et al. 2002).

3.3.4. The Identification of the Putative Bacteriocin Genes in *B. japonicum* FN1

AntiSMASH analysis of the FN1 genome identified 2 clusters putatively involved in homoserine lactone production, 2 gene clusters putatively involved in the synthesis of terpene like compounds, 4 clusters putatively involved in bacteriocin production, 1 cluster involved in non-ribosomal peptide production, and 1 cluster involved in ectoine production (Figure 8).
A BLASTP search of FN1 using the biosynthetic genes followed by an orthologous neighbour search yielded 7 genes as putative candidate genes (Table 4). Based on the annotation one of these genes *bjfn_5173*, was predicted to encode an uncharacterized conserved protein and two of these genes, *bjfn_05747* and *bjfn_0566*, were predicted to by encode “hypothetical proteins. The other putative candidates were predicted to encode proteins that were either in the alkylhydroperoxidase family (*bjfn_04428* and *bjfn_01204*) or could encode carboxymuconolactone decarboxylases (*bjfn_02461* and *bjfn_02861*) (Table 5). Except for *bjfn_01204*, each of the candidate genes appears to be gene arrangements that suggest they may be part of an operon (Figure 10).

Table 3. Statistics for the whole genome sequencing of FN1

Statistics	Results
Sequence generated	8,402,786 sequences
Contigs	141 contigs
Scaffolds	87 scaffolds
Base pair	9,138,496 base pairs
GC content	64%
Coding sequence	8,613 coding sequences

Table 4. The list of the putative bacteriocin genes in FN1 identified utilizing AntiSMASH

Genes	IMG product name
bjfn1_05173	Uncharacterized protein conserved in bacteria
bjfn1_05747	Hypothetical protein
bjfn1_04428	Alkylhydroperoxidase family enzyme
bjfn1_05166	Hypothetical protein
bjfn1_01204	Alkylhydroperoxidase family enzyme
bjfn1_02461	4-carboxymuconolactone decarboxylase
bjfn1_02861	4-carboxymuconolactone decarboxylase

Table 5. The list of the putative bacteriocin genes

Putative bacteriocin genes	Conserved Domain Database (CDD) annotation	E value
bjfn1_05173	Hypothetical protein	4.00e-141
bjfn1_05747	Carboxymuconolactone decarboxylase family	1.47e-13
bjfn1_04428	Carboxymuconolactone decarboxylase family	1.35e-12
bjfn1_05166	Uncharacterized peroxidase-related enzyme	2.43e-09
bjfn1_01204	Carboxymuconolactone decarboxylase family	1.22e-08
bjfn1_02461	Uncharacterized conserved protein (Function unknown)	7.39e-05
bjfn1_02861	Uncharacterized conserved protein (Function unknown)	8.77e-04

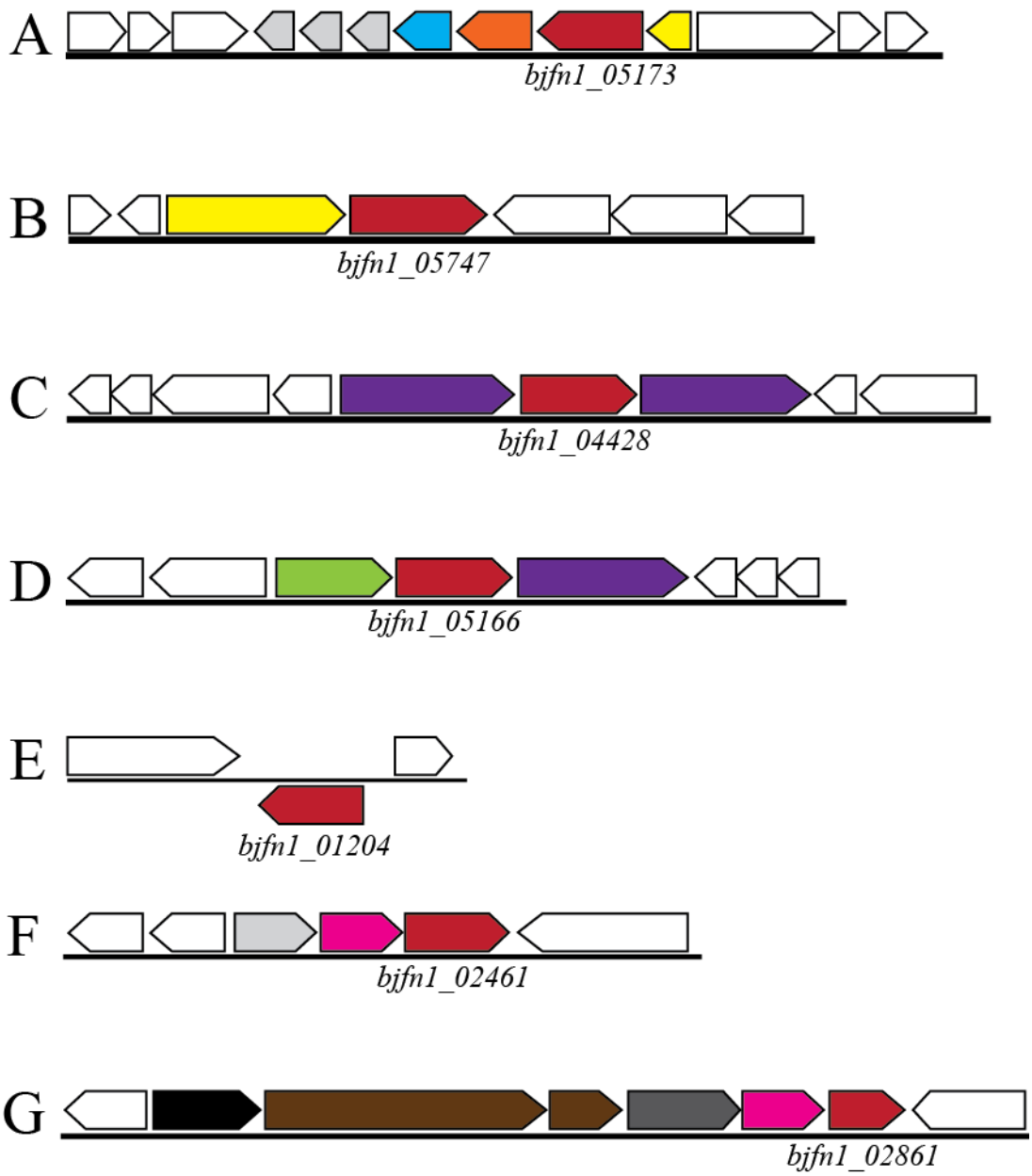

Figure 10. Genetic context of genes identified as being involved in bacteriocin-like activity. Genes are represented as coloured boxes arrow heads. The arrowheads signify the direction of transcription. Loci arrangements are depicted on the based on the IMG-JGI Neighbourhood Viewer. Colors are used to indicate different annotated gene products: red, target gene identified; light grey, hypothetical protein; yellow, membrane protein; orange, DNA-binding-domain-containing protein; blue, oxidoreductase; purple, dehydrogenase; green, transcriptional regulator;

magenta, decarboxylase; dark grey, protein related to antibiotic resistance; brown, protein related to the production of flagella; black, chaperonin. Boxes colored in white are used to indicate genes flanking the loci region of interest.

In an effort to provide additional information on how these encoded proteins might be involved in producing compounds that could be bacteriocin-like each of the conceptual amino acid sequences was searched using the conserved domains database (CCD). This analysis corroborated what was found using the IMG annotation with the notable exception that this analysis showed that *bjfn_01204* was also related to the carboxymuconolactone decarboxylase family.

3.4. Discussion

Bacteriocins and the genes encoding the proteins necessary for bacteriocin activity are poorly characterized within the Rhizobiaceae. The more recent work was centered either on the non-ribosomal protein trifolotoxin (Triplett 1988; Triplett and Barta 1987), or what have been termed rhizobiocins (Oresnik et al. 1999). Although work on bacteriocins in *Bradyrhizobium japonicum* was pursued nearly forty years ago (Roslycky 1967), work in this area has not progressed.

In this chapter two Bradyrhizobia strains were isolated. One of which appeared to be a *Bradyrhizobium japonicum*, whereas the second appeared to be a *Bradyrhizobium yuanmingense*. Both of these strains had bacteriocin like activity (Table 2). What is striking is the diversity of bacteriocin-like activity that can be demonstrated (Table 2). At present, none of the determinants that are involved in the growth inhibition activities are known. In an attempt to identify some of these determinants FN1 was chosen for further study because it was isolated from soil in Manitoba, it exhibited bacteriocin activity, and finally that it was less fastidious to grow in culture than BRJ-16.

Two separate strategies were used to try to identify regions involved in bacteriocin production. The first, Tn*5* mutagenesis, and the second, whole genome sequencing followed by bioinformatic analysis. Although Tn*5* mutagenesis followed by phenotype screening is continually carried out in both *R. leguminosarum* and *S. meliloti* in the lab, attempts at utilizing this strategy with FN1 were limited by the yield of 10 colonies/10^8 recipients plated. The whole genome approach followed by an antiSMASH analysis did produce a short list of candidate genes that could be targeted for site directed mutagenesis to determine if they affect the bacteriocin activity profile of FN1.

It is noteworthy that if the IMG product naming and the conserved domain database searches are taken together that 5 of the candidate genes were predicted to encode 4-carboxymuconolactone decarboxylases which is also related to a class of alkylhydroperoxidases. It seems that the assumptions that were made by targeting 'bacteriocin biosynthetic genes" from the antiSMASH analysis appears to have a strong bias towards carboxymuconolactone decarboxylases. This family of proteins is predicted to cleave carbon-carbon bonds, and it seems to be involved in the catabolism of aromatic compounds such as protochatechuate/catechol (Ornston and Stanier 1966). In *Legionella pneumophila*, a carboxymuconolactone encoded by *lpg0406* has been implicated in antioxidant defense has been recently crystalized (Chen et al. 2015). Although there are some indications of how these enzymes work, the association of this type of activity has not been previously associated with bacteriocin production.

Chapter 4

The Identification of the Genes that are Involved in the Production of Bacteriocin-like Substances in *Bradyrhizobium japonicum*

4.1 Introduction

Bradyrhizobium japonicum can exist either as a saprophytic free-living bacteria, or it can enter into a symbiotic relationship with soybean (Franck et al. 2008; Hanus et al. 1979; Lorite et al. 2000; Stacey 1995). The end result of the interaction as a root nodule that is capable of reducing atmospheric nitrogen to ammonia and supplying it to the host plant (Hunt and Layzell 1993; Fischer 1994). Since it can interact symbiotically, *B. japonicum* has been routinely supplied to farmers to use as an inoculum and thus decrease the amount of nitrogen that needs to be added to the soil to ensure yields.

The efficacy of an inoculum is often compromised by its inability to compete with indigenous bacteria that are less effective at providing fixed nitrogen to the plant (Triplett 1988; Triplett and Sadowsky 1992). This has been termed the *Rhizobium* competition problem (Triplett and Sadowsky 1992). Nodulation is a relatively complicated process that involves a signal exchange, the colonizing of a curled root hair, propagation down an infection thread, and finally release into plant cells where the bacteria can become a nitrogen fixing bacteroid. Since the process is a multifaceted progression, there are many biotic, as well as abiotic events that can impact the ability of a strain to be competitive (Kosslak and Bohlool 1985; Kosslak et al. 1983; Triplett and Sadowsky 1992). One such aspect that has been investigated in *R. leguminosarum* is the ability to produce bacteriocins (Robleto et al. 1998; Triplett et al. 1994; Triplett 1990; Triplett 1988; Triplett and Barta 1987).

Bacteriocins can be defined as narrow spectrum antibiotics that are produced by bacteria that affect only closely related species. The production of bacteriocins by *Rhizobium* was first described for *Rhizobium trifolii* strains (Triplett 1988; Triplett and Barta 1987). Subsequent work has shown that bacteriocins from *R. leguminosarum* can bestow a competitive advantage to the

bacteria both in laboratory culture medium as well as when competing for nodule occupancy (Oresnik et al. 1999; Triplett 1990). Although bacteriocin producing strains of *B. japonicum* have been reported (Roslycky 1967), these strains are no longer available and a systematic study to determine if bacteriocins can play a role in addressing the Rhizobium competition problem have never been evaluated. To this end, we isolated a *B. japonicum* strain from field soil in Manitoba, showed that it has bacteriocin activity, generated a draft genome, and identified genes that are putatively involved in bacteriocin biosynthesis (Chapter 3).

The objectives of this chapter are: 1) construct mutations in genes that have been implicated in bacteriocin biosynthesis, 2) to determine if any of these mutations affect any of the bacteriocin-like activities of FN1, and if they do, do they play a role in the strains' overall competitiveness and its ability to compete for nodule occupancy?

4.2 Materials and Methods

4.2.1. Bacterial Strains, Media, and Growth Conditions

Bacterial strains and plasmids used and generated in this work are listed in Table 6. *B. japonicum* strains were grown at 30°C in yeast extract mannitol (YEM) medium (Vincent 1970). *E. coli* strains were grown at 37^{o}C in LB (Sambrook et al. 1989). When required, *B. japonicum* and *E. coli* were grown with the following antibiotics: streptomycin (Sm) 200 μg/mL; Kanamycin (Km) 20 μg/mL; Neomycin (Nm) 400 μg/mL.

4.2.2. Genetic Techniques and DNA Manipulations

Triparental matings for plasmid transfer between strains was carried out as previously

Table 6. The list of strains and plasmids utilized for studying the production of bacteriocins in *B. japonicum*

Strain or plasmid	Genotype or phenotype	Reference or source
Bradyrhizobium		
BRJ-24	FN1, Sm^R	
BRJ-34	BRJ-24, *bjfn1_05166* :: pKAN, Kan^R	This work
BRJ-40	BRJ-24, *bjfn1_05747* :: pKAN, Kan^R	This work
BRJ-44	BRJ-24, *bjfn1_04428* :: pKAN, Kan^R	This work
BRJ-48	BRJ-24, *bjfn1_01204* :: pKAN, Kan^R	This work
E. coli		
DH5α	λ⁻ ϕ80d*lacZ* M15 (*lacZYA-argF*)*U169 recA1endA1 hsdR17* (*rk- mk-*) *supE-44 thi-1 gyrA relA1*	(Hanahan 1983)
MT616	MT607 (pRK600)	(Finan et al. 1985)
Plasmids		
pRK7813	Broad-host-range vector, Tc^R	(Jones and Gutterson 1987)
pKAN	Suicide vector containing kanamycin resistance cassette	(Pickering and Oresnik 2008)
pHY128	pKAN/*bjfn1_05166*	This work
pHY131	pKAN/*bjfn1_05747*	This work
pHY133	pKAN/*bjfn1_04428*	This work
pHY135	pKAN/*bjfn1_01204*	This work
pHY139	pKAN/*bjfn1_05173*	This work
pHY141	pKAN/*bjfn1_02461*	This work
pHY142	pKAN/*bjfn1_02861*	This work
pMK31	pRK7813/*bjfn1_01204*, Tc^R	This work

described using MT616 as a helper strain (Finan et al. 1985). Isolation of a streptomycin resistant variant of FN1was carried out by plating late log phase cultures of FN1 onto YEM plates containing 200 μg/mL streptomycin. Colonies that arose were single colony purified three times and tested for their ability to form nodules as well as the original isolate FN1. The final isolate that was subsequently used was denoted as BRJ-24 (Table 6).

Standard techniques were used for DNA isolations, polymerase chain reactions (PCR), restriction enzyme digests, gel isolations, ligations, transformations, and agarose gel electrophoresis (Sambrook et al. 1989).

4.2.3. Construction and Verification of Single-Cross-Over Mutations in a *B. japonicum* FN1

To construct strains of *B. japonicum* FN1 carrying mutations in putative bacteriocin genes, approximately 400 bp internal fragments for each of the putative bacteriocin genes was generated using open reading frame specific primers. These primers were designed such that they contained restriction sites (*Sac*II or *Sma*I) to facilitate cloning of the PCR fragment. The primer pairs were designed such that the resulting products could be cloned as *Sac*II and *Sma*I fragments (Table 7). The resulting products were cloned into the suicide vector, pKan (Pickering and Oresnik 2008), and transformed into DH5αλpir. Each of the resulting constructs were verified by nucleotide sequencing and then mobilized to BRJ-24 (Sm resistant variant of FN1) using MT616 (Figure 11). Single cross-over recombinants were selected on YEM containing Sm and Nm.

The constructs were verified utilizing 3 different groups of primers (Table 8). The first group of primers were designed to anneal to the regions approximately 100 bp upstream or

Table 7. The primers utilized for gene knock-out mutation utilizing pKAN suicide vector

Primer	Sequence 5'-3'	Target gene	Tm (°C)
KAN05173F	ATAT**CCGCGG**TCAACCAAGACAG ACCTGCG	*bjfn1_05173*	60.25
KAN05173R	ATAT**CCCGGG**ACCATGCCAGATGT TCGGAG	*bjfn1_05173*	60.11
KAN05747F	ATAT**CCGCGG**CACCGGGATTTTGT GCGAG	*bjfn1_05747*	59.50
KAN05747R	ATAT**CCCGGG**AGGTCGATTTCAAA GGTCTCCA	*bjfn1_05747*	59.36
KAN04428F	ATAT**CCGCGG**ATGTGAAGATCGG CAGGGAG	*bjfn1_04428*	59.53
KAN04428R	ATAT**CCCGGG**ACTGCTGGAGTACC TCTTTGTAA	*bjfn1_04428*	59.10
KAN05166F	ATAT**CCGCGG**ATTCGATCCTGCAC AAGCAGA	*bjfn1_05166*	60.07
KAN05166R	ATAT**CCCGGG**TAGAAACCGAGTG CGACGAT	*bjfn1_05166*	59.19
KAN03821F	ATAT**CCGCGG**GGACTTGGCAATG ACCTGCT	*bjfn1_03821*	60.61
KAN03821R	ATAT**CCCGGG**CAACTCGTCATCGC CGTTAC	*bjfn1_03821*	59.36
KAN01204F	ATAT**CCGCGG**ATCCGGGTCCACTT TCCCTG	*bjfn1_01204*	61.56
KAN01204R	ATAT**CCCGGG**CACAGCAGCATGA TCTCAAGG	*bjfn1_01204*	59.33
KAN02461F	ATAT**CCGCGG**AGTTGTCGCAGGAT TACGTGT	*bjfn1_02461*	60
KAN02461R	ATAT**CCCGGG**TTCGATCTGTCCGT CACCTG	*bjfn1_02461*	59.47
KAN02861F	ATAT**CCGCGG**GTGGAATGGTTCGC GCACG	*bjfn1_02861*	62.65
KAN02861R	ATAT**CCCGGG**CTTGAACGGCTCCT GCTTGT	*bjfn1_02861*	60.89

The primers were designed to have either a *Sac*II (CCGCGG) or *Sma*I (CCCGGG) restriction sites and ATAT overhangs as enzyme attachment sites

Table 8. The Primers utilized for sequencing *bjfn1_01204* knock-out mutant of BRJ-24

Primer	Sequence 5'-3'	Tm (°C)
CKAN01204F	GGAGATCCAGGCGCAGTTC	60.52
CKAN01204R	GCTGCCTTCTCCTCCAGC	60.12
PKO5OUTA	GCTTAGTACGTGAAACATGAGAGC	59.97
PKO5OUTB	AGTACGTGAAACATGAGAGCTTAGT	60.05
PKO5OUTC	AGAGCTTAGTACGTGAAACATGAGA	59.82
PKO3OUTA	GGCAAAATCCTGTATATCGTGCG	60.36
PKO3OUTB	CCTGTATATCGTGCGAAAAAGGAT	59.25
PKO3OUTC	GCGAAAAAGGATGGATATACCGAA	59.25
KAN5OUTA	GGTGATGCTGCCAACTTACTG	59.53
KAN5OUTB	CAAAGTGCGTCGGGTGATG	59.50
KAN5OUTC	GGGTGATGCTGCCAACTTACT	60.61
KAN3OUTA	TGACTACCGGAAGCAGTGTG	59.68
KAN3OUTB	GACTACCGGAAGCAGTGTGA	59.40
KAN3OUTC	AGTGTGACCGTGTGCTTCTC	60.25

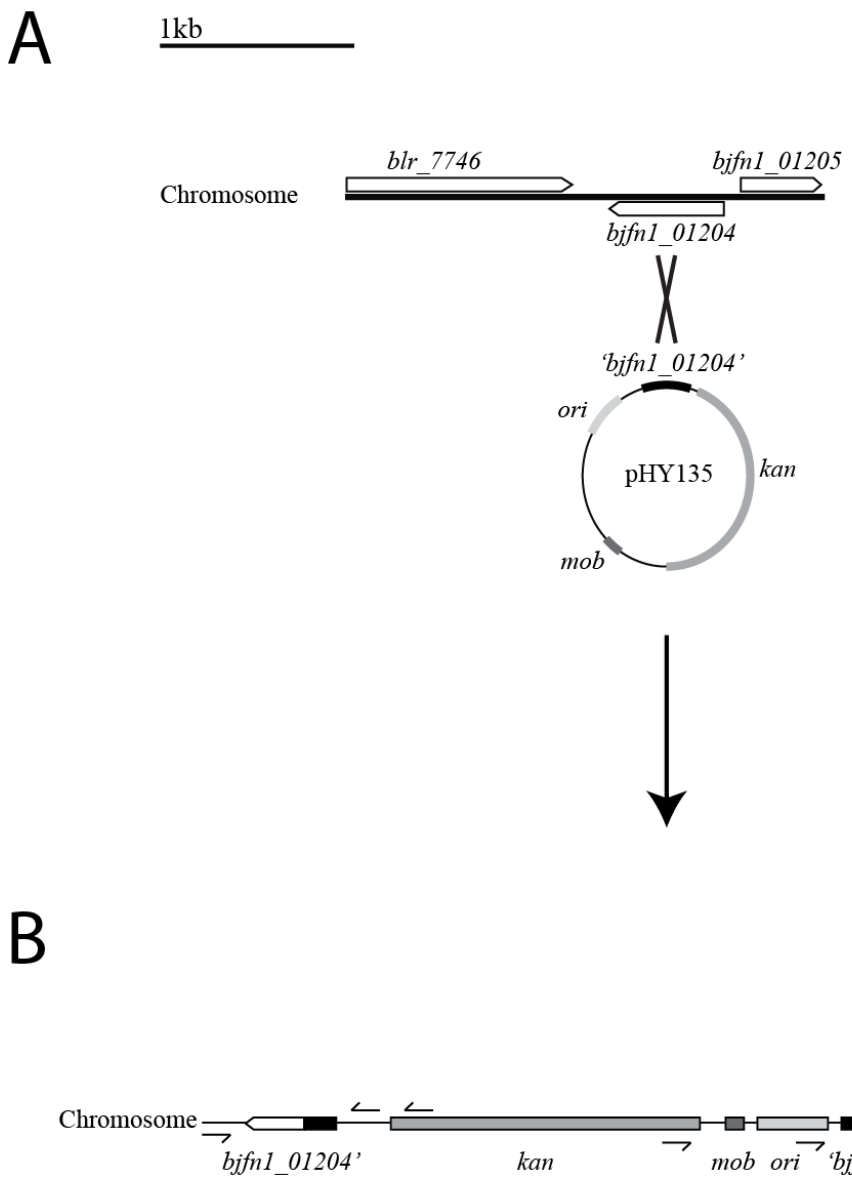

Figure 11. The gene knock-out mutation of *bjfn1_01204* utilizing pKAN. The figure shows the locus containing *bjfn1_01204* and pKAN/pHY135 (Figure 11a) and the locus after pKAN integration into the chromosome (Figure 11b). The single-headed arrows in Figure 11b shows the approximate annealing locations of the primers utilized for confirming the mutation through sequencing.

downstream of the internal fragment used for the gene knock-out method. Another group of primers were designed to anneal to either the 5' end or 3' end of Km resistance cassette. The last group of primers were designed to anneal to the MCS of pKAN outside of the restriction sites used for cloning the internal fragment of *bjfn1_01204*. For sequencing, a primer annealing to the outside of the internal fragment used for mutation was paired correctly with one that anneals to Km resistance cassette or pKan region close to the restriction sites used for cloning (Figure 11).

4.2.4. Bacteriocin Assays

Bacteriocin assays were carried out essentially as described in Chapter 3.

4.2.5. Competition Assays

Competition assays were used to determine if mutant strains were differentially competitive than the isogenic wild-type. These assays were carried out either in a broth culture medium, or as a competition for nodule occupancy.

4.2.5a. Broth Co-Culture Competition Assay

To carry out these assays, strains to be competed were pre-grown in YEM. The culture densities were measured (OD_{600}). The value of the optical densities was used to approximate how much of each strain should be used to achieve a desired starting ratio. The starting OD_{600} of a competition was approximately 0.05. Samples were taken at inoculation, appropriately diluted, and plated onto YEM agar plates. The resultant colonies were screened on the appropriate selective plates to determine the initial starting ratio. Following 7 days after inoculation aliquots

of the co-inoculated broth were plated and screened to determine the final ratio in the broth culture.

4.2.5b. Competition for Nodule Occupancy

Competition for nodule occupancy experiments were carried out essentially as previously described (Geddes et al. 2014, Poysti et al. 2007). Surface sterilized soybean seeds were germinated on 0.4% agar plates in the dark. The germinated seeds then transferred aseptically to sterile Leonard jar assemblies (Trung and Yoshida 1983) containing 250 mL Jensen's medium (one seedling/assembly). After approximately 1 week, the seeds were co-inoculated with 1:1 ratio of wild-type and mutant.

For co-inoculation, cultures were first grown in YEM and the optical densities were adjusted to the same OD_{600}. The diluted cultures were then mixed together and diluted 1/100 in sterile distilled water. The actual inoculation ratio was determined by plating an aliquot of this mixed inoculum and screening the resultant colonies for the appropriate antibiotic resistances. The rest of the inoculum (10 ml) was used to inoculate the seedling.

After approximately 4 weeks, the soybean plants were harvested and the nodules were removed, surface sterilized (described in Chapter 3), and finally homogenized with a sterile inoculating stick in an eppendorf tube containing 100 μL sterile water. The resulting suspension was spotted (approximately 10 μL) onto YEM containing the appropriate antibiotics. Differences between the inoculation ratio and the observed nodule occupancy ratio were used to assess the ability of each strain to compete against the wild-type. The data from these experiments was evaluated using Student’s t-test for significance.

4.3. Results

4.3.1. Construction of Mutants in BRJ-24

B. japonicum, strain FN1, was shown to produce zones of inhibition around a number of tester strains of *B. japonicum*. By sequencing the genome, a number of genes were identified as putatively being involved in bacteriocin biosynthesis by using a combination of AntiSMASH and orthologous neighbourhood analysis (Table 4). It was hypothesized that if any of these genes were involved in bacteriocin production, the loss of the gene would lead to the loss of a zone of inhibition.

Primers targeted to amplify internal fragments of the seven genes (*bjfn1_05173*, *bjfn1_05747*, *bjfn1_04428*, *bjfn1_05166*, *bjfn1_01204*, *bjfn1_02461*, and *bjfn1_02861*) identified in Chapter 3 (Table 4) were designed and used to generate PCR fragments. These fragments were cloned into the vector pKan yielding the series of plasmids (pHY128, pHY131, pHY133, and pHY135). These vectors were mobilized into BRJ-24 using *E. coli* strain MT616. Following 2 days of incubation the mating spots were plated on YEM medium containing both streptomycin and neomycin. The selection plates typically contained between 10-100 colonies/plate. Assuming that between 10^9 and 10^8 *B. japonicum* were present in the mating spot, and that plasmid transfer occurred at unity over the 48 hour incubation, this suggests that the frequency of finding single cross-over mutations was in the range of 10^{-7} to 10^{-8}. With 3 of these crosses (those targeting *bjfn1_05173*, *bjfn1_02461*, and *bjfn1_02861*), no putative single cross-over mutations were found. After repeated unsuccessful attempts, these were not pursued. Following confirmation by the generation and sequencing of PCR fragments utilizing the appropriate primers (Table 8), it was found that mutations in *bjfn1_05166*, *bjfn1_05747*, *bjfn1_04428*, and *bjfn1_01204* were constructed. These were named BRJ-34, BRJ-40,

BRJ-44, and BRJ-48 respectively (Table 6).

4.3.2. *bjfn1_01204* Plays a Role in Affecting SR-16

To determine if any of the mutations affected bacteriocin production, bacteriocin assay was utilized to screen for the inability of mutant strains to inhibit the growth of other closely related strains of Bradyrhizobia. FN1 has been shown to produce zones of inhibition against SR-10, SR-11, SR-15, and SR-16 (Table 2). Based on the pattern of inhibition/resistance of other Bradyrhizobia, these zones of inhibition have the potential to be caused by 4 mechanisms (Table 2). The results show when BRJ34, BRJ40, BRJ44, and BRJ48 were tested against strains SR-10, 11, 15, and 16 that, these mutants still inhibited SR-10, SR-11, and SR-15. However, SR-16 was no longer inhibited by BRJ48 (Table 9).

BRJ48 carries a mutation in *bjfn1_01204*. This gene appears to be a single open reading frame; divergently transcribed from *bjfn1_01205*, and convergently transcribed with *bjfn1_01203*. This gene order suggests that the constructed mutation affects only *bjfn1_01204*. To determine if the inability to inhibit SR-16 was due to the loss *bjfn_01204*, the entire annotated open reading frame was amplified using the primers: forward primer (Tm = 60°C) 5'ATATAAGCTTGGAGATGCATGCATGTCCCAGGCCGCGCC3' containing HindIII site and reverse primer (Tm=62°C) 5'ATATGAATTCTTAGACCGCCGGAAACCGC3' containing EcoR1 restriction site. The amplification product was cloned into pRK7813 such that the ATG of *bjfn1_01204* was preceded by a ribosome binding site and this construct would be expressed for the p_{lac} promoter in pRK7813. This construction yielded pMK31 which was subsequently conjugated into BRJ48. The results show that the introduction of pMK31 into BRJ48 was

Table 9. Bacteriocin assays utilizing bacteriocin gene knock-out mutants

Strain	SR-10	SR-11	SR-15	SR-16
BRJ24 (FN1, Sm^R)	+	+	+	+
BRJ34 (BRJ24, *bjfn1_05166*::pKan)	+	+	+	+
BRJ40 (BRJ24, *bjfn1_05747*::pKan)	+	+	+	+
BRJ44 (BRJ24, *bjfn1_04428*::pKan)	+	+	+	+
BRJ48 (BRJ24, *bjfn1_01204*::pKan)	+	+	+	-

Tester strains and the indicator strains (utilized to create a lawn) are shown in rows and columns respectively. Positive result is indicated by the formation of zone of clearance that forms around the tester strain.

sufficient to restore the ability to produce a zone of inhibition when tested against SR-16. The introduction of pMK31 into BRJ48 did not affect the zones of inhibition against any of the other tester strains. Together, these data strongly suggest that *bjfn1_01204* is involved in the production of a zone of inhibition against SR-16 (Table 10).

4.3.3. *bjfn1_01204* does not Alter the Ability of Other Rhizobia to Affect the Growth of *B. japonicum* Tester Strains

The transfer of the *R. leguminosarum* rhizobiocin encoded on the large plasmid pRL1JI from strain 248 was capable of bestowing bacteriocin-like activity to *S. meliloti* strain Rm1021 (Oresnik et al. 1999). To determine if *bjfn1_01204* was capable of conferring the ability to have a heterologous host produce a similar zone of inhibition, pMK31 was conjugated into a series of *R. leguminosarum* strains as well as a *S. meliloti* strain that was in the laboratory culture collection. The data show that the introduction of pMK31 did not change the inhibition profile of strain 248, 3841, VF39 or Rm1021 (Table 11). Somewhat surprisingly it was found that all of these strains were capable of inhibiting the indicator strains in the absence of the plasmid. This finding was not further characterized.

4.3.4 The Ability of FN1 to Outcompete SR-16 in Broth Culture is not Dependent upon *bjfn1_01204*

Our data showed that FN1 produced a zone of inhibition when grown on YEM agar plates seeded with SR-16 (Table 2). The same phenotype was also present with the streptomycin resistant variant BRJ24 (Table 9). Extending the characterization of this inhibition

Table 10. The complementation of the *bjfn1_01204* mutant

Strain	SR-10	SR-11	SR-15	SR-16
BRJ24	+	+	+	+
BRJ48	+	+	nd	-
BRJ48/ pRK7813	+	+	nd	-
BRJ48/ pMK31	+	+	nd	+

SR-16 was utilized as an indicator strain. The tester strains used for stab culture are shown in rows. BRJ48/pRK7813 is the empty vector control. Positive result indicates the formation of zone clearance around the tester strains (nd = not determined).

Table 11. The complementation of other strains of Rhizobia with *bjfn1_01204* gene

Strain	SR-10	SR-11	SR-15	SR-16
S. meliloti Rm1021	+	+	nd	-
R. leguminosarum 248	-	-	nd	-
R. leguminosarum 3481	-	-	nd	-
R. leguminosarum VF39	+	-	nd	-
S. meliloti Rm1021/pMK31	+	+	nd	-
R. leguminosarum 248/pMK31	-	-	nd	-
R. leguminosarum 3481/pMK31	-	-	nd	-
R. leguminosarum VF39/pMK31	+	-	nd	-
R. leguminosarum 248/pRK7813	-	-	nd	-
R. leguminosarum 3481/pRK7813	-	-	nd	-
B. japonicum BRJ48	+	+	nd	-

The indicator strains and the tester strain (stab culture) are shown on columns and rows respectively. Positive result indicates the presence of zone of clearance forming around the tester strain (nd = not determined).

it was found that if BRJ24 was also highly competitive in co-culture assays that included SR-16. Since BRJ48 did not form zones of inhibition against SR-16, we wished to determine if *bjfn1_01204* played a role in allowing BRJ24 to be more competitive.

BRJ24 or BRJ48 were co-cultured with SR-16 in YEM and the ratios of the strains were determined at the time of inoculation and following 7 days of incubation. In both cases, BRJ24 and BRJ48 were the dominant strain making up 96.7±3.2% and 74.6±2.9% of the cultures respectively (Figure 12). In both cases, these ratios were significantly different from the inoculation ratio, suggesting that *bjfn1_01204* does not play a significant role in allowing BRJ24 to be more competitive than SR-16 in broth cultures of YEM.

4.3.5. Absence of *bjfn1_01204* Affects the Ability of BRJ24 to Compete for Nodule Occupancy

Preliminary experiments suggested that BRJ24 was a more competitive for nodule occupancy than SR-16. It was hypothesized that the ability to produce bacteriocin-like zones of inhibition was correlated with the competition for nodule occupancy observations. To test this hypothesis, soybean plants were grown under controlled conditions and inoculated with a co-culture of either BRJ-24 and SR-16 or BRJ-48 and SR-16.

The results show that when approximately equal numbers of BRJ24 and SR-16 were inoculated onto soybean plants, 88 ± 6% of the nodules were occupied by BRJ24 (Figure 13). However, when BRJ48 (carrying a mutation in *bjfn1_01204*) was inoculated in equal numbers with SR-16, 50 ± 2% of the nodules were occupied by BRJ48 (Figure 13). Whereas the frequency of nodule occupancy of BRJ24 was significantly different from the inoculation ratio, the nodule occupancy ratio of BRJ48 was not (Figure 13). Taken together, the data are consistent

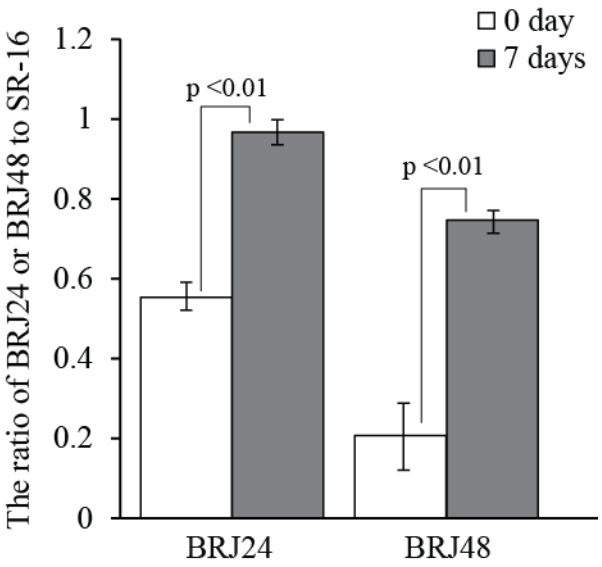

Figure 12. Competition assays in broth culture. Competition assays of tester strains, BRJ24 or BRJ48 against the bacteriocin-sensitive strain, SR-16 were done in YEM broth culture. The results are shown as the total ratio of either BRJ24 or BRJ48 in the culture. Error bar represents standard deviation (n=3). Student's t-test was performed to determine the significance between the data.

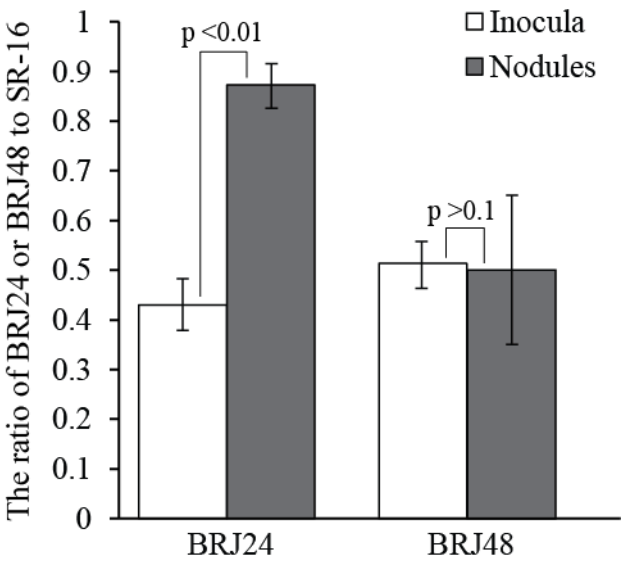

Figure 13. Nodule competition assays. Nodule competition assays of BRJ24 or BRJ48 against the bacteriocin sensitive strain, SR-16. The ratios of bacteria used in the inocula used and in the nodules (after 4 weeks of incubation) were obtained. The results were shown as the total ratio of either BRJ24 or BRJ48. Error bar represents standard deviation (n=3). Student's t-test was performed to determine the significance between the data.

with the hypothesis that the ability to produce a bacteriocin-like zone of inhibition against SR-16 is correlated with the ability to be more competitive for nodule occupancy under controlled conditions.

4.4. Discussion

In this work we identify a *bjfn1_01204* as a gene that encodes a product that is involved in the production of a bacteriocin-like zone of inhibition against *B. japonicum* strain SR-16. The loss of this gene did not show a dramatic effect at allowing the FN1 derivative to compete against SR-16 in culture medium, however, the effect on competition for nodule occupancy was pronounced (Figure 12 and 13). The transfer of this gene into other Rhizobia, did not seem to confer any changes in their ability to inhibit any of the tester strains (Table 11).

The gene *bjfn1_01204* is predicted to encode a putative muconolactone decarboxylase. Muconolactonase decarboxylase activities have been shown to be involved in catabolic pathways that are involved in the breakdown of compounds such as protocatechuates and catechols (Ornston 1966). For example, in *B. japonicum* USDA110 4-carboxymuconolactone decarboxylase is involved in degradation of phenolic compound 4-hydroxybenzoate and hydroaromatic compounds quinate and shikimate to tricarboxylic acid intermediates (Lorite et al. 1998). It was also shown that the degradation of these compounds can serve both to provide cellular nutrition as well as to detoxify the growth medium (Lorite et al. 1998). Although our initial assumptions were that *bjfn1_01204* encoded an enzyme that would be involved in the biosynthesis of a secreted compound, we cannot ignore the hypothesis that the loss of the bioactivity we were assaying was not due the modification of a metabolite in a catabolic manner

that is subsequently released outside of the cell. A further in-depth analysis is required to understand the metabolic pathways that involved *bjfn1_01204*.

It was unexpected that BRJ48 lost its advantage in competition for nodule occupany against SR-16, while its ability to compete in YEM broth medium was unaffected. It is noteworthy that the ability of FN1 to inhibit SR-16 was first assayed on YEM agar (Table 2). The differential observations (agar vs broth vs nodule occupancy) for inhibition appear complicated. A direct comparison of the growth conditions between semi-defined bacteriological medium, versus growth in a Leonard jar assembly (Trung and Yoshida 1983) where nutrition is being affected by the host plant are clearly very different. Therefore, the direct correlation of the loss of bacteriocin-like activity and the loss of the ability to compete for nodule occupancy should be made cautiously. Similarly, the physiological differences of bacteria growing in broth as opposed to the solid matrix that is provided by agar is relatively unexplored. However, the differences between planktonic growth and biofilm growth are documented within the literature (Booth et al. 2011; Heffernan et al. 2009). Taking all of this into account the differential observations should not be unexpected.

The goal of this work was to explore if bacteriocin activity could affect competition for nodule occupancy. The finding of a gene that was identified as encoding a protein that is putatively involved in secondary metabolism and affects competition for nodule occupancy suggests that this is an area that could be a potentially promising area of research. The finding of this gene poses more questions than it answers. For example, what is the molecular role of this gene? Is this gene differentially expressed with respect to environment? Does the ultimate "bacteriocin" work by increasing the ability of FN1 to compete, or does it work to directly inhibit SR-16? The answering of these types of questions will add a great deal of fundamental

knowledge that can be used in a translational manner to improve the production of commercial soybean inocula.

Chapter 5

General Conclusions, Outstanding Questions, and Future Work

5.1. Thesis General Conclusions

Soybean has become a major crop in Manitoba over the last decade. The work presented in this thesis was funded by two related grants to carry out studies with *B. japonicum* in Manitoba fields. The first grant funded work to develop a qPCR assay to determine *B. japonicum* populations. The second was to determine if there were indigenous populations of *B. japonicum* in Manitoba that could produce bacteriocin, and if there were, could bacteriocins be used to make inoculum strains more competitive.

In this study, we were able to develop a real-time qPCR-based assay for rapid and sensitive quantification of *B. japonicum* in soil. We showed that this quantification method is capable of differentiating between different common Rhizobia. Using this assay we showed that there is no significant difference in the composition of *B. japonicum* in soil samples obtained from depth of 0-4 inches and 0-5 inches away from the plant. Instead, a higher number of *B. japonicum* was detected in the field utilizing narrow (15 inches) row spacing as opposed to the one utilizing wide (30 inches) row spacing. Utilizing this quantification method, we were able to show that the presence of soybean plants has a positive impact on the *B. japonicum* community, and that the primers could be used in a seasonal study.

In Chapter 3, a strain of *B. japonicum*, FN1, was isolated from Manitoban soil that had bacteriocin-like activities against multiple strains of *Bradyrhizobium*. A draft genome sequence was generated and putative genes that could be involved in bacteriocin biosynthesis were identified. In Chapter 4 mutations were made in 4 of the 7 identified genes. One of these genes was found to be responsible for the production of one such molecule. We also showed that the ability to produce this particular molecule provides the isolated strain with an advantage in the competitiveness for nodule occupancy. To our knowledge, this is the first study utilizing a

Bradyrhizobium-soybean model to show that the ability to produce bacteriocin provides an advantage in the competition for nodule occupancy.

The reasoning for developing a qPCR based assay to determine the *B. japonicum* population was very much driven by an articulated need from the Manitoba Pulse Growers Association (now the Manitoba Pulse and Soybean Growers Association) in 2008. The primers developed in this thesis are robust and perform well using real world samples. They have been used to monitor populations over an entire field season. The primers are now being tested for efficacy to determine if they can be moved from a proof of principal assay to being used to inform farmers whether they will need to buy inoculum for their crops.

It is important to realize that the original conception and first preliminary experiments for this work were conceived at time when the cost of genome sequencing was relatively high. An estimated cost of a human genome was approximately $10 million in 2007. With the advent of Next Generation Sequencing technologies the estimated costs of the same genome have been estimated to have dropped to less than $10,000 (Barba et al 2014). With respect to microbial sequencing, the cost of a genome is often in the range of less than $2,000. The impact of this ability to sequence cheaply does ultimately limit the use of the primers developed here to ask questions of an ecological perspective. However, the ability to provide “yes/no” type answers with respect to whether inoculum should be applied to soybean may still be possible.

There are a number of studies that these primers might be able to provide practical information that can be useful to soybean growers that are described below (sections 5.2.1, 5.2.2, and 5.2.3). Thinking into the future, if these studies are carried out, it would also be prudent to use Next Generation Sequencing technologies to supplement the findings with 16S microbial community profiling.

In this study, we were able to find a bacteriocin-producing strain of *B. japonicum* and correlate the bacteriocin-producing ability to its competitiveness for nodule occupancy. This provides proof in principal that bacteriocins can be used as tools to bias rhizosphere interactions. This finding poses both applied as well as fundamental questions. The exact mechanism of action as well as the nature of the inhibiting compound is currently unknown (section 5.2.5, and 5.5.6). The possibility of producing co-culture inocula as well determining the effect of bacteriocin producing bacteria on the rhizosphere microbial ecology are also relatively unexplored (Section 5.2.4).

5.2. Outstanding Questions and Future Works

5.2.1. The Community of *B. japonicum* from Deeper Soil and Further Distance Relative to the Plants

A previous study showed that the relative abundance of *Bacteriodetes* changes relative to the depth of soil (Eilers et. al. 2012). The study showed that *Bacteriodetes* were more abundant in near surface horizons as compared to the deeper depths (Eilers et al. 2012). Utilizing real-time qPCR-based assay, we quantified *B. japonicum* in the soil samples obtained from either 0 inch or 5 inches away from the plants and from the depth of 0-4 inches. Contrary to the study on *Bacteriodetes*, our data showed that there was no significant difference in the composition of the *B. japonicum* community in these samples. However, it is not known whether a significant difference would be observed if samples were to be collected from soil deeper than 4 inches. Furthermore, it is not known if there will be a significant difference in *B. japonicum* community if soil samples were to be obtained from a distance further than 5 inches away from the plants. My hypothesis is that there will be less *B. japonicum* in soil samples further from the plants. This

is due to the fact that there will be less concentration of the nutrients secreted by the plants accessible to *B. japonicum*.

5.2.2. The Effect of Weather on the Community of *B. japonicum* in Manitoba

An established *B. japonicum* community in soil is important to achieve sustainable soybean cultivation without relying on the overuse of nitrogen fertilizers. Commercial scale soybean cultivation in Manitoba started approximately 20 years ago. Currently, Manitoba is the second largest soybean-producing province in Canada. However, it is unknown whether Manitoba has an established community of *B. japonicum* in soil. This subject is very important since Manitoba experiences one of the coldest winters in Canada. The temperature during a winter in Manitoba can drop as low as -40°C. Using the developed real-time qPCR-based assay, we would be able to analyse the effect of the changes in the temperature due to the changing season on *B. japonicum* communities in Manitoban soil.

5.2.3. The Effect of Chemical Interventions on the community of *B. japonicum* in the soil

Previous studies showed that the chemical and nutrient profiles affect the composition of microbial community in an environment (Li et al. 2013; Xu et al. 2013; Chaudhry et al. 2012; Deng et al. 2012; Fierer et al. 2012; Jünemann et al. 2012; Stein et al. 2004). Currently, there have been few studies done on how different chemical interventions in agriculture affect *B. japonicum* in soil. With real-time PCR-based quantification assays, we can study the effect that different herbicides, pesticides, and chemical fertilizers have on *B. japonicum* community in soil. Furthermore, this real-time qPCR based assay can be utilized to quantify a specific strain of *Bradyrhizobium* by changing the primers used for quantification.

5.2.4. Use of FN1 as a co-inoculum

In contrast to USDA110, FN1 which was isolated in Manitoba has the ability to produce bacteriocins (Table 2). However, USDA110 growth was not affected by the presence of FN1 during bacteriocin assays. This suggests FN1 does not produce any bacteriocin capable of inhibiting USDA110. This indicates that a co-inoculum consisting of USDA110 and FN1 may be beneficial in ensuring an efficient nodulation in agricultural settings. This is because both of these strains are capable of nodulating soybean and fixing nitrogen efficiently during a symbiotic relationship. In addition, the ability of FN1 to produce bacteriocin may affect overall soil communities. The role of the rhizosphere community in affecting competition for nodule occupancy is currently poorly understood. The use of a bacteriocin producing strain such as FN1 might provide a model that can be used to address these types of microbial ecology questions.

5.2.5. The Ability to Produce Bacteriocins and the Competitiveness for Nodule Occupancy

In this study we showed that FN1 was more competitive for nodule occupancy when compared to the bacteriocin sensitive strain SR-16. FN1 also showed the ability to inhibit the growth of BRJ16 in a bacteriocin assay (Table 2). This is an interesting result since BRJ16 is a soybean-nodulating strain that was isolated from Manitoban soil as opposed to SR-16 which was isolated from Pakistani soil. Since our study showed that the bacteriocin-sensitive strain is less competitive for nodule occupancy, this suggests that FN1 may be more competitive than BRJ16 for nodule occupancy. A fundamental question that underlies this relationship is how does bacteriocin inhibit SR-16? Work to elucidate this can give physiological insights that will further fundamental knowledge regarding cell growth and division.

5.2.6. In Depth Analysis of *bjfn1_01204* and Its Gene Product

In this study, we showed that *bjfn1_01204* of FN1 was responsible for the production of a molecule that inhibits the growth of SR-16 hence, providing FN1 with a competitive advantage in nodule occupancy over SR-16. Currently, there is very little known regarding *bjfn1_01204* and its gene products. A further study is needed to understand how this particular gene is regulated including the growth phase in which this gene is expressed. More analysis on the gene product of *bjfn1_01204* is also needed to determine its structure, mode of action, and the mechanism that is utilized for secreting this particular molecule. It is also unknown whether this particular molecule is subjected to post-translational modification processes.

5.2.7. Further Analysis of the Bacteriocins Produced by Different Strains of *B. japonicum*

In this study we were able to identify different strains of *Bradyrhizobium* capable of producing bacteriocins. We were also able to show that the production of bacteriocins was related to the competitiveness for nodule occupancy. However, it is unknown how the presence of bacteriocins affects the soil microbiome.

The identification of different bacteriocins has been limited by the number of indicator strains that were utilized. Some bacteriocins have a narrow spectrum, while others have the ability to target bacteria belonging to different species, and even different genera. In addition, our studies showed that a strain of *Bradyrhizobium* has the ability to produce multiple bacteriocins (Table 2). Utilizing metagenomic studies, we would be able to identify which bacteria are affected by the introduction of a bacteriocin-producing strain. Therefore, we would be able to study the production of bacteriocins in a strain more robustly.

Strains of *B. japonicum* are able to produce bacteriocins targeting the growth of bacterial phytopathogens (Gross and Vidaver 1978, Vidaver 1977). This suggests that bacteriocin-producing strains of *Bradyrhizobium* can be utilized as biocontrol agents against soybean bacterial phytopathogens such as *Pseudomonas syringae* and *Xanthomonas axonopodis* pv. *Glycines*. Since previous studies showed that bacteriocins of *B. japonicum* inhibits the growth of *C. nebraskense* which is a phytopathogen of corn (Gross and Vidaver 1978, Vidaver 1977), this suggests that bacteriocin-producing strains of *Bradyrhizobium* can be utilized as a biocontrol agent against the phytopathogens of plants other than soybean. However, further studies are required to identify strains of Bradyrhizobia capable of producing bacteriocins that inhibit the growth of different phytopathogens.

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Appendix

A1. Media

A1.1 Yeast Extract Manitol (YEM)

To prepare 1 L of medium:

- Yeast extract - 1 gram

- Mannitol - 10 gram

- Dipotassium phosphate (K_2HPO_4) - 0.5 gram

- Magnesium sulphate ($MgSO_4$) - 0.2 gram

- Sodium chloride (NaCl) - 0.1 gram

- Milli-Q water - to 1 L

- *Congo red - 0.025 gram

- ** Agar

* Optional (Congo red is a selective agent for rhizobia).

** Only for preparing solid media. The concentration of 4% agar are used to prepare solid YEM medium.

After dissolving all of the components in milli-Q water, the pH is adjusted to 6.8±0.2.

A1.2. Jensen's Medium

To prepare 1 L of Jensen's medium:

- Dicalcium phosphate ($CaHPO_4$) - 1 gram

- Dipotassium phosphate (KH_2PO_4) - 0.2 gram

- Magnesium sulphate ($MgSO_4$) - 0.2 gram

- Sodium chloride (NaCl) - 0.2 gram

- Ferric chloride ($FeCl_3$) - 0.1 gram

- *1000X trace elements solution - 1 mL

- Milli-Q water - To 1 L

After dissolving all of the components in milli-Q water, the pH is adjusted to 7.

*See A1.3.

A1.3. 1000X Trace Elements Solution for Jensen's Medium

To prepare 1 L of 1000X trace elements solution:

- Boric acid (H_3BO_3) - 1 gram

- Zinc sulphate ($ZnSO_4$) - 1 gram

- Cupric sulphate ($CuSO_4$) - 0.5 gram

- Manganese (II) chloride ($MnCl_2$) - 0.5 gram

- Sodium molybdate ($NaMoO_4$) - 1 gram

- EDTA (ethylenediaminetetraacetic acid) - 10 gram

- NaFeEDTA (sodium iron ethylenediaminetetraacetic acid) - 2 gram

- Biotin - 0.4 gram

- Milli-Q water - To 1 L

After combining all of the components in milli-Q water, the pH is adjusted to 7 (otherwise EDTA will not dissolve).

A2. Conditions for PCR and Real-Time QPCR

A2.1. PCR Conditions

1. Initial melting, 95°C for 1 minutes
2. Melting, 95°C for 15 seconds
3. Annealing, 56-60°C for 30 seconds
4. Elongation, 72°C, the length for this step is 1 minutes/kbp product size
5. Repeat step 2-5 for 35 cycles
6. Final elongation, 72°C for 5 minutes

A2.2. Real-Time QPCR Conditions

Cepheid SmartCycler®II was utilized for real-time qPCR

Stage 1:

Temperature = 95°C

Time = 150 seconds

Optics = Off

Stage 2: 2-temperature cycle:

1. Degree/second = NA

   Temperature = 95°C

   Time = 15 seconds

   Optics = Off

2. Degree/second = NA

   Temperature = 58°C

   Time = 120 seconds

   Optics = Off

Repeat for 40 cycles

Stage 3: melt curve:

Starting temperature: 58°C

End temperature: 95°C

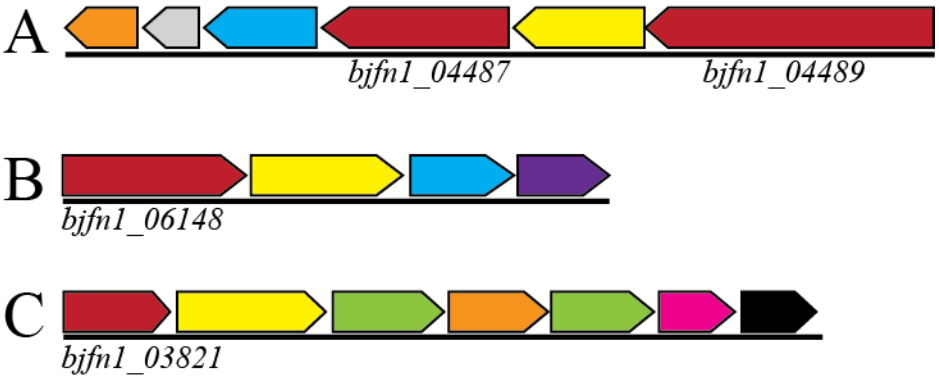
Optics = Ch1

Degree/second = 0.2°C/second

A3. Other Data

A3.1. RTX-Like Genes in FN1

One of the best characterized Rhizobiocins are the RTX-like bacteriocins in *Rhizobium leguminosarum* strain 248. A preliminary analysis of the FN1 genome showed that 4 such RTX-like genes exist. These four genes were found in three different operons (see below). Only *bjfn1_06148* was successfully targeted. However, the resulting mutant was not affected in its ability to inhibit the growth of the indicator strains sensitive to the presence of FN1 in bacteriocin assays.



The loci for the genes that are homologs of RTX-like toxin genes of *R. leguminosarum* 248. Genes are represented as coloured boxes arrow heads. The arrowheads signify the direction of transcription. Loci arrangements are depicted on the based on the IMG-JGI Neighbourhood Viewer. Colors are used to indicate different annotated gene products: red, target gene identified; light grey, hypothetical protein; yellow, ATP binding cassette protein; orange, transcriptional regulator; blue, HlyD-type protein; purple, transporter; light green, membrane protein; magenta, reductase; black, isomerase.