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Title: Multi-disciplinary approaches for studying rhizobium – legume symbioses

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ABSTRACT

The rhizobium-legume symbiosis is a major source of fixed nitrogen (ammonia) in the biosphere. The potential for this process to increase agricultural yield while reducing the reliance on nitrogen-based fertilizers has generated interest in understanding and manipulating this process. For decades, rhizobium research has benefited from the use of leading techniques from a very broad set of fields, including population genetics, molecular genetics, genomics, and systems biology. In this review, we summarize many of the research strategies that have been employed in the study of rhizobia and the unique knowledge gained from these diverse tools, with a focus on genome and systems-level approaches. We then describe ongoing synthetic biology approaches aimed at improving existing symbioses or engineering completely new symbiotic interactions. The review concludes with our perspective of the future directions and challenges of the field, with an emphasize on how the application of a multi-disciplinary approach and the development of new methods will be necessary to ensure successful biotechnological manipulation of the symbiosis.

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SYMBIOTIC NITROGEN FIXATION: CHALLENGES AND PROSPECTS

Biological nitrogen fixation (BNF) is an agriculturally and ecologically crucial process that is performed by prokaryotes in two ecological groups: i) BNF performed by free-living cells often in close association with plants, or ii) by rhizobia that fix nitrogen during an endosymbiotic relationship with legumes. The fixation of N_2 -gas into ammonia by root-nodule bacteria (rhizobia) is referred to as symbiotic nitrogen fixation (SNF), and it is a more efficient process in terms of supplying nitrogen to the plant. Phylogenetically, most rhizobia are α -proteobacteria, but some rhizobia are β -proteobacteria (Figure 1). The various genetic, biochemical, and evolutionary aspects of the symbiotic interaction have been reviewed over the years (Long 1996, Gage 2004, MacLean et al. 2007, Jones et al. 2007, Gibson et al. 2008, Oldroyd and Downie 2008, Masson-Boivin et al. 2009, Downie 2010, Oldroyd et al. 2011, Udvardi and Poole 2013, Haag et al. 2013, Remigi et al. 2016, Mus et al. 2016, Poole et al. 2018). In brief, the symbiosis is initiated following an exchange of signals between the roots of the plant and free-living rhizobia in root-proximal soil (the rhizosphere). As root nodule tissue develops (Figure 2), the rhizobia enter this specialized tissue through an extracellular infection thread; as these inwardly growing threads reach differentiated nodule cells, the bacteria become surrounded by a plant-derived membrane and taken up into the plant cytosol, where they are known as bacteroids. The nascent bacteroids then undergo major morphological and transcriptional changes, leading to active nitrogen fixation, which is the conversion of atmospheric N_2 into NH_3 for the plant.

It has been estimated that BNF contributes several teragrams of nitrogen (the equivalent of billions of USD of nitrogen based fertilizer) annually to global agricultural systems (de Vries et al., 2011; Herridge et al., 2008; Ladha et al., 2016). Rhizobial inoculants are inexpensive alternatives to industrial nitrogen fertilizers that can improve crop yields (Figure 2), resulting in

greater profits and potentially significant impacts on the livelihood of the community, especially for the world's poorest farmers (Bloem et al. 2009, Mutuma et al. 2014). BNF also has a substantial positive impact on the environment through reducing the application of nitrogen fertilizers. These fertilizers contribute to elevated atmospheric levels of the potent greenhouse gas nitrous oxide (Park et al. 2012), to toxic nitrate-laden water that contributes to algae blooms and eutrophication (Conley et al., 2009; Randall and Mulla, 2001; Ward, 2009), and to the depletion of fossil fuel as industrial ammonia synthesis by the Haber-Bosch process accounts for 1-2% of total human energy consumption (Erisman et al. 2008). These negative consequences are ameliorated by relying more on biological solutions to nitrogen generation for agricultural systems.

The benefits of SNF can be maximized in agricultural systems by two general strategies. The first is optimizing the amount of nitrogen fixed by rhizobial bio-inoculants in extant legume symbioses. This will involve not only increasing the rate of nitrogen fixation in the nodule, but also increasing the competitiveness of inoculant strains in the soil and rhizosphere. The second strategy is to engineer completely new nitrogen fixing symbioses with non-leguminous plants. Many of the world's staple crops, such as the cereals, cannot enter into a N_2 -fixing symbiosis with rhizobia (Charpentier and Oldroyd 2010, Oldroyd and Dixon 2014). Engineering synthetic symbioses with these crops therefore presents a monumental and challenging opportunity for exploiting SNF.

Successful implementation of either strategies requires an intricate understanding of both symbiotic partners, in terms of abiotic interactions, responses to complex microbial communities, symbiotic communication, developmental processes, and the metabolism that underpins the nitrogen fixation process. Below, we will explore leading-edge questions in rhizobial biology, and describe how a variety of sound experimental approaches have propelled our understanding of

symbiosis biology. The current synthetic biology approaches and future directions and challenges for engineering BNF for agricultural gains will be described, highlighting the need for multi-level studies of rhizobial biology to provide the necessary data to guide rational improvement of SNF through systems biology approaches.

RHIZOBIAL LIFE-HISTORY

Nitrogen fixing rhizobia have complex life-cycles (Figure 3) (Poole et al. 2018). Rhizobia are found as free-living bacteria in general soil environments and in the rhizosphere. These two environments provide unique nutritional and stress conditions (Hinsinger et al. 2009) that the rhizobia must effectively manage. Rhizobia must also compete against other microbial community members to establish a stable population (Smalla et al. 2001, Li et al. 2016). From the rhizosphere, the rhizobia can infect a compatible legume host, wherein growth conditions vary according to the stage of symbiotic development, from the acidic conditions in the pocket of a curled root hair (Hawkins et al. 2017), to linear growth along penetrating infections threads, to symbiotic differentiation after release into host cells. Each stage of free-living and symbiotic growth requires a unique set of genes and metabolic capabilities. Development of next-generation commercial inoculant strains must account for these and other biological properties, including growth in industrial liquid cultures and survival during desiccation (O'Callaghan 2016).

The rhizobial life-cycle does not occur in isolation. It is also necessary to account for social interactions between rhizobia and host plants, and between rhizobia and competing microbes (Checcucci et al. 2017). The rhizobia-host interaction involves multiple signal exchange events, as well as the massive exchange of metabolic resources. The initial events of infection thread formation and nodule primordium induction exhibit a high degree of cooperation between partners;

yet, the culminating developmental events leading to N fixation reveal hints of conflict and aspects of an evolutionary arms race (Sachs et al. 2018). For example, plant-produced nodule-specific cysteine-rich (NCR) peptides (Mergaert et al. 2003, Van de Velde et al. 2010) and corresponding bacterial NCR peptidases (Price et al. 2015) represent a late-stage dialogue in many legumes in which bacteroids improve their fitness at the cost of the host plant by resisting symbiotic enslavement (for reviews, refer to: (Kondorosi et al. 2013, Haag et al. 2013, Alunni and Gourion 2016, Pan and Wang 2017). As multiple rhizobial strains may inhabit the same or different nodules on a single plant (Hagen and Hamrick 1996, Checcucci et al. 2016), cheating behaviours have emerged in the rhizobia (Singleton and Tavares 1986, Sachs et al. 2010, Checcucci et al. 2016, Regus et al. 2017), while plants have been observed to limit cheating through sanctions and partner discrimination (Kiers et al. 2003, Kiers and Denison 2008, Heath and Tiffin 2009, Sachs et al. 2010, Quides et al. 2017, Westhoek et al. 2017, Daubech et al. 2017).

Rhizobia also interact with a diverse soil microbial community, where they must effectively compete by antagonism and scavenging of nutrients. This can involve, for example, the production of bacteriocins that have anti-bacterial activity against closely related taxa (Hirsch 1979, Wilson et al. 1998, Twelker et al. 1999, Oresnik et al. 1999). The native microbiome may also promote symbiotic development by a rhizobial inoculant; it was observed that soil isolated *Rhizobium fabae* can promote nodulation of *R. etli* in a quorum sensing dependent mechanism (Miao et al. 2018). Rhizobia also encounter numerous bacteriophages in nature that may influence the effectiveness of a rhizobial inoculant. Phages generally are virulent to only some strains of a species, and can be used to reduce the population density of a poor symbiont to allow the better symbiont to flourish (Evans et al. 1979, Hashem and Angle 1990). However, phages can also reduce the effectiveness of rhizobial inoculants (Mendum et al. 2001).

Nodulation efficiency and efficiency of nitrogen fixation are distinct phenotypes (Bourion et al. 2017). It is clear that understanding the diverse mechanisms underlying all stages of the rhizobial life-history, including competitive fitness in the rhizosphere and each stage of symbiotic development, will be imperative in designing and implementing improved rhizobia-based agricultural strategies. To this end, moving beyond artificial, sterile symbiotic assays in lab conditions will be necessary to generalize results to field conditions.

GENETIC MANIPULATIONS AND FUNCTIONAL GENOMICS

The majority of the community's knowledge of the core symbiotic processes comes from classical molecular genetic analyses involving forward- and reverse-genetic techniques, wherein single mutations are associated with defective phenotypes. Tn5-based transposon mutagenesis, coupled with plasmid-based complementation and DNA sequencing, played a foundational role in the discovery of the rhizobial *nod* and *nif* genes (Becker et al., 1993a; 1993b; 1993c; Buikema et al., 1983; Chua et al., 1985; Glucksmann et al., 1993; Jagadish and Szalay, 1984; Long et al., 1988; 1982; Swanson et al., 1987). Both suicide plasmids and broad host-range plasmids outfitted to autonomously replicate in rhizobia have permitted studies that rely on random transposon mutagenesis, targeted gene disruption, and complementation. Such plasmid-based gene manipulation systems continue to play a prominent role in genetic studies in rhizobia (Griffitts and Long 2008, Griffitts et al. 2008, Carlyon et al. 2010, VanYperen et al. 2015), and targeted genetic studies remain essential to complement and validate observations from systems- and genome-level analyses.

High-throughput functional genomic studies have become more common for cataloguing rhizobial gene functions. *Sinorhizobium meliloti* ORFeome (Schroeder et al. 2005) and fusion

(Cowie et al. 2006) libraries have been developed to facilitate functional and expression analyses in free-living and symbiotic states. A *S. meliloti* promoter library was developed and employed in the identification of genes involved in the early symbiotic process (Zhang and Cheng 2006). Similarly, a *Rhizobium leguminosarum* genome library was produced for use with *in vivo* expression technology (IVET), and used to identify genes expressed specifically in the legume rhizosphere (Barr et al. 2008).

Several groups have employed signature-tagged mutagenesis (STM) in the genome-scale identification of genes involved in symbiosis or competition for nodule occupancy. STM involves production insertion mutants using a library of transposons each containing a distinct tag. Mutants containing different transposons are then be pooled, passed through a selective regimen, and the relative abundance of each mutant in the output, relative to the input, can be measured using microarrays detecting the unique tags (Becker and Pobigaylo 2013). A STM library of *S. meliloti* was constructed (Pobigaylo et al. 2006), and insertions in nearly 9,600 strains were mapped to ~ 3,700 genes throughout the genome (Serrania et al. 2017). This STM library was employed in the identification of 38 new genes influencing symbiosis. (Pobigaylo et al. 2008). In further work, this library was used to show that ~ 2% of *S. meliloti* genes contribute to rhizosphere colonization (Salas et al. 2017). Screening of a *Mesorhizobium loti* STM library on host plants identified 13 Nod⁻ or Fix⁻ mutants as well as 33 mutants impaired in symbiotic competitiveness (Borjigin et al. 2011). Recently, a *Sinorhizobium fredii* STM library consisting of 25,500 mutants was reported, and an initial screen of 10% of these mutants identified four mutants that were impaired in symbiosis (Wang et al. 2016). STM has also been employed in *Rhizobium leguminosarum* bv. *viciae* (Garcia-Fraile et al. 2015), and a screen for genes relevant to rhizosphere colonization

revealed the importance of arabinose and protocatechuate catabolism specifically during rhizosphere growth (Garcia-Fraile et al. 2015).

Although STM facilitates much higher throughput than classical analyses, the more recently developed transposon sequencing (Tn-seq), also known as insertion sequencing (INseq), allows for a further increase in throughput and quantitative assessment of gene functions. In this technique, a saturation insertion mutagenesis is performed to produce a pool of colonies each containing a single insertion, and next-generation sequencing is employed to identify the position of hundreds of thousands of insertions throughout the genome. In this way, mutations contributing to fitness in a given environment can be mapped across an entire genome with a resolution of just several nucleotides (Chao et al. 2016). Example data is shown in Figure 4, illustrating the sensitivity of this method in identifying essential or condition-specific essential genes. Multiple research groups have recently adapted Tn-seq for use in the rhizobia. A mariner transposon was used to examine the core genome of *R. leguminosarum* bv. *viciae* (Perry and Yost 2014, Perry et al. 2016), as well as to investigate the effects of carbon source and O₂ levels, a key signal during symbiosis, on the fitness of gene disruptions (Wheatley et al. 2017). Similarly, a Tn5 derived transposon was used to interrogate the core genome of *S. meliloti*, and to inspect how chromosomal gene phenotypes differ when the extra-chromosomal replicons are removed (diCenzo et al. 2018). Additionally, a mariner transposon was used to investigate the genes contributing to sensitivity of *S. meliloti* to a NCR peptide, which plays a key role during rhizobial differentiation in the nodule (Arnold et al. 2017). Future use of this technique might prove valuable in the genome-scale elucidation of genes involved at various stages of the rhizobial life-style.

LESSONS FROM COMPARATIVE GENOMICS

Genetic variations present in natural rhizobium strains can be powerfully leveraged to understand SNF through the comparison of genomic features, a process known as comparative genomics. This approach has become increasingly feasible due to the ever-growing number of rhizobial species and strains with whole-genome sequences available through public repositories. These genome sequences have facilitated many high-quality comparative genomic analyses of rhizobia that have contributed to our understanding of symbiosis and plant growth promotion.

Multiple comparative genomic studies have led to the perhaps surprising observation that there is significant variation between rhizobia in terms of their genomic adaptations to symbiosis (González et al. 2003, Masson-Boivin et al. 2009, Black et al. 2012). Indeed, while there are many genes that appear specific to symbiotic nitrogen fixing bacteria (Young et al. 2006, Pini et al. 2011), there appears to be no gene unique to all bacteria capable of performing SNF (Amadou et al. 2008). Even the common *nodABC* genes required for Nod factor synthesis are not 100% conserved, as they are absent in certain *Bradyrhizobium* species capable of forming nodules in a Nod factor-independent fashion (Giraud et al. 2007). Other examples of symbiotic genes showing variable presence/absence are accessory *nod* genes involved in determining host specificity, the *fixNOPQ* genes encoding a cytochrome *cbb3* oxidase complex, as well as genes encoding polysaccharide biosynthesis and secretion systems (Black et al. 2012, Sugawara et al. 2013, De Meyer et al. 2016). In addition, the operon structure and genomic organization of symbiotic genes within rhizobial genomes differs among species (González et al. 2003, MacLean et al. 2007, Black et al. 2012, De Meyer et al. 2016). Overall, while many of the core symbiotic genes are highly conserved, the observed genomic variation reflects multiple mechanisms and adaptations for establishing an effective symbiosis, as discussed by (Masson-Boivin et al. 2009). Such diversity

is bound to complicate our understanding of the minimal set of genes necessary for establishing a symbiosis, and it suggests that strategies to optimize symbiotic interactions may be strain specific.

Despite the differences in the symbiotic gene clusters of distinct rhizobia, comparative genomics can help identify new symbiotically relevant genes. A comparative analysis of 163 rhizobial genomes identified 184 protein families putatively involved in optimizing the symbiotic process (Seshadri et al. 2015). In another study using a similar comparative genomic approach, 139 putative genes specific to symbiotic rhizobia were identified (Queiroux et al. 2012). Further characterization of 13 of these genes in *S. meliloti* showed that eight were expressed more highly by *S. meliloti* in the nodule than in free-living *S. meliloti* (Queiroux et al. 2012). Additionally, mutation of the one gene chosen for follow-up study was shown to increase nodule occupancy competitiveness (Queiroux et al. 2012). These results illustrate how comparative genomics coupled with experimental validation is a powerful approach to identify new candidate genes for improving rhizobial symbiotic efficiency.

Population genetic studies (i.e., the genetic variation present within a population) have uncovered high genetic variability in rhizobia, which reflected in the large, open pangenomes of many rhizobial species (González et al. 2010, Galardini et al. 2011, Tian et al. 2012, Galardini et al. 2013, Sugawara et al. 2013, Porter et al. 2017). This is demonstrated in Figure 5, which displays the distribution pattern of 17,494 genes found in at least one of the 20 included strains of *S. meliloti*. A clearer understanding of the eco-evolutionary drivers of this genomic variation constitutes an important research frontier in this field. The influence of soil properties, plant host, and other biotic factors including microbial communities, bacteriophages, plasmids, and other invasive DNA elements all surely play a role in the generation of rhizobial diversity (Bromfield et al. 1987, Harrison et al. 1989, Carelli et al. 2000, Silva et al. 2007, Talebi et al. 2008, Toro et al. 2018).

Understanding the relationships between these factors and the variable genome could potentially allow for the exploitation of the large genetic diversity of rhizobial species for developing elite rhizobial bioinoculants in precision agriculture (Checcucci et al. 2017).

LARGE-SCALE GENOME MANIPULATIONS

Several *Rhizobium*, *Sinorhizobium*, and *Mesorhizobium* derivatives have been produced that are ‘cured’ of one or more of their large extra-chromosomal replicons, revealing the influence of these replicons on numerous free-living phenotypes. These include the ability to catabolize a wide range of nutrient sources, growth and survival in soil environments (including the rhizosphere), biosynthesis of essential nutrients, production of polysaccharides, and motility (Barbour and Elkan 1989, Hynes et al. 1989, Hynes and McGregor 1990, Baldani et al. 1992, Brom et al. 1992, Moëne Loccoz 1995, Hu et al. 2007, García-de-los-Santos et al. 2008, Stasiak et al. 2014). Similarly, genome reduction studies have highlighted the importance of megaplasms to the symbiotic process, revealing that key symbiotic genes can be located on megaplasms, that optimal symbiotic performance often requires more than one megaplasmid, and that megaplasms can carry loci required for effective competition for nodule occupancy (Barbour and Elkan 1989, Hynes and McGregor 1990, Baldani et al. 1992, Brom et al. 1992, Hu et al. 2007, Barreto et al. 2012, Stasiak et al. 2014). In contrast, some rhizobial plasmids impair symbiotic development. Loss of a large *Mesorhizobium* plasmid can improve both nitrogen fixation and nodulation competitiveness (Pankhurst et al. 1986, Hu et al. 2007), while several large *S. meliloti* accessory plasmids may result in legume-host incompatibility (Crook et al. 2012, Price et al. 2015).

A more targeted approach is the production of a library of large-scale deletion mutations that cumulatively remove all of the extra-chromosomal replicon(s). We are aware of this approach

being employed with *S. meliloti* and *R. etli* (Landeta et al. 2011, Yurgel et al. 2013a, Milunovic et al. 2014). These libraries have been used to localize and identify novel symbiotic genes, as well as to screen for phenotypes such as carbon metabolic abilities, cytochrome c oxidase activity, soil growth, and osmotolerance (Dominguez-Ferreras et al. 2006, Landeta et al. 2011, Yurgel et al. 2013a, diCenzo et al. 2016a, Zamani et al. 2017).

In addition to genome reduction approaches, genome expansion studies have been used to evaluate the genetic basis of symbiosis. The large symbiotic plasmids of several *Rhizobium* spp. and *S. meliloti* have been transferred to related, non-symbiotic organisms such as *Agrobacterium* spp. and *Ensifer adhaerens* (Hooykaas et al. 1982, Wong et al. 1983, Truchet et al. 1984, Hirsch et al. 1984, Hynes et al. 1986, Finan et al. 1986, Martínez et al. 1987, Novikova and Safronova 1992, Abe et al. 1998, Rogel et al. 2001, Nakatsukasa et al. 2008). The primary conclusion of these studies is that poor, if any, symbiotic abilities are transferred to the recipient strain. This observation is perhaps surprising considering that natural lateral transfer of symbiotic plasmids and islands between related strains results in a gain of symbiotic abilities (Mozo et al. 1988, Laguerre 1992, Sullivan et al. 1995, Sullivan and Ronson 1998, Pérez Carrascal et al. 2016, Haskett et al. 2016). However, non-fixing outcomes after inter-species heterologous gene transfer may be due to an incomplete complement of symbiotic functions in the transconjugant strains, or due to incompatible alleles of a common gene (diCenzo et al. 2017c). To overcome these limitations, a *S. meliloti* strain lacking the pSymA and pSymB symbiotic replicons was constructed, which represents a 45% reduction of the genome (diCenzo et al. 2014). As expected, this strain is Nod⁻ Fix⁻, and re-introduction of all pSymA and pSymB genes fully restored symbiosis (diCenzo et al. 2016b). The chromosome-only strain, known to be permissive to SNF,

is being used for identification of the necessary and sufficient set of symbiotic genes on these replicons.

In a particularly notable case, inter-species transfer of a symbiotic plasmid was coupled with laboratory evolution in an ongoing attempt to yield a symbiosis-competent strain. The researchers established *Ralstonia solanacearum* (a plant pathogen) carrying the symbiotic plasmid of the rhizobium species *Cupriavidus taiwanensis* as the test system (Marchetti et al. 2010, Guan et al. 2013, Marchetti et al. 2014, Remigi et al. 2014, Marchetti et al. 2017, Capela et al. 2017). Although initially unable to form nodules, adaptive mutations, such as in the type III secretion system, were identified that allowed for nodulation to occur (Marchetti et al. 2010). Sequential passage of these nodulating strains led to the recovery of derivatives with improved nodulation capabilities, although not yet nitrogen fixation (Marchetti et al. 2017). Nevertheless, this long-term study lends insight into the genomic changes required for the evolution of a new rhizobial species, such as a need to rewire transcriptional networks and to limit induction of the legume immunity response (Marchetti et al. 2014, Capela et al. 2017).

In contrast to the inter-species plasmid transfers described above, intra-species transfer of megaplasmids in *S. meliloti* has remarkably yielded strains displaying plant host cultivar specific improvements in symbiotic effectiveness (Checcucci et al. 2018), suggesting that plasmid transfer is a viable method for ‘breeding’ improved inoculants. Collectively, these studies underscore the importance of subtle genetic interactions between replicons in bringing about efficient symbiotic nitrogen fixation.

TRANSCRIPTOMIC STUDIES OF RHIZOBIA

Transcriptomic studies, in the form of both microarrays and RNA deep-sequencing (RNA-seq) have been used to investigate the transcriptional responses to a broad range of genetic and environmental conditions, to elucidate the regulons of key transcriptional regulators, and to study RNA-based regulation (see Table 1 for a detailed list and description of rhizobium transcriptomic studies). Notable examples of studies of symbiotically relevant regulators include the FixL/FixJ/FixK system involved in activation of symbiotic genes in response to low oxygen (Bobik et al. 2006, Mesa et al. 2008), and NodD involved in induction of nodulation genes (Capela et al. 2005).

Transcriptomic studies have also probed early and late aspects of symbiotic development (Table 1). This has involved characterizing processes involved in rhizosphere colonization, such as growth in the presence of root exudates (Liu et al. 2017b, Klonowska et al. 2018), or during adaptation to various plant rhizosphere environments (Ramachandran et al. 2011). Several studies have examined the transcriptional consequences of flavonoids detection (Ampe et al. 2003, Barnett et al. 2004, Capela et al. 2005, Pérez-Montaña et al. 2016a, 2016b), a key initial step in the establishment of the symbiosis (reviewed by (Jiménez-Guerrero et al. 2017). In some species, flavonoid perception results in the induction of many genes (Pérez-Montaña et al. 2016b), and can influence a range of phenotypes including growth and biofilm formation (Spini et al. 2015). Differentiation within the nodule involves changes in the cell cycle, and studies have examined the transcriptional control of the cell cycle of *S. meliloti* and cell cycle dependent gene expression (De Nisco et al. 2014, Pini et al. 2015). Others have examined the transcriptional effects of plant produced NCR peptide application to free-living rhizobia, with the results revealing changes in cell cycle behaviour (Penterman et al. 2014) and changes associated with membrane depolarization (Tiricz et al. 2013). Moreover, the transcriptome of *S. meliloti* has been compared between cells

growing in oxic and microoxic conditions (Ampe et al. 2003, Becker et al. 2004, Bobik et al. 2006, Pessi et al. 2007), as oxygen limitation is a major trigger for symbiotic gene expression. These studies have confirmed that FixJ is the major regulator of genes differentially expressed under these conditions (Bobik et al. 2006), and that not all genes induced by oxygen limitation in free-living bacteria are expressed in the nodule (Becker et al. 2004).

Transcriptomic experiments have been carried out to evaluate gene regulatory events occurring in root nodules (Table 1). Some of these studies have monitored gene expression of both symbiotic partners (for example, (Barnett et al. 2004, Heath et al. 2012, Roux et al. 2014, Mitsch et al. 2018), or have focused on comparing free-living versus symbiotic bacterial cells (Ampe et al. 2003, Becker et al. 2004, Barnett et al. 2004, Pessi et al. 2007, Karunakaran et al. 2009). A general consensus from these studies is that the bacteria undergo dramatic transcriptional changes in the symbiotic state, with possibly a thousand genes differentially expressed. The majority of differentially expressed genes are down-regulated in the nodule, consistent with a general metabolic repression during SNF. Transcriptomics has also been used to decipher the role of specific genes, regulators, or metabolic processes in the symbiotic process (Table 1), such as the genes *fixJ* (Barnett et al. 2004) and *bacA* (Karunakaran et al. 2010), and carbon import (Mitsch et al. 2018). Others have examined the transcriptome differences of bacteroids displaying distinct morphologies (Lamouche et al. 2018). Additionally, researchers have examined how genetic variations in the plant or rhizobium partner influence the nodule transcriptome (Heath et al. 2012, Burghardt et al. 2017), as well as how the bacteroid transcriptome of the same strain differs depending on the plant hosts (Ampe et al. 2003, Karunakaran et al. 2009, Li et al. 2013). These studies highlight how variation in symbiotic compatibility can result in significant transcriptional alterations.

Researchers have developed imaginative approaches to tease apart the various stages of symbiotic development by either isolating RNA from nodules of different ages (Ampe et al. 2003, Capela et al. 2006) or by using plant or bacterial mutants that are blocked at various stages of the developmental process (Ampe et al. 2003, Tian et al. 2006, Starker et al. 2006, Capela et al. 2006, Maunoury et al. 2010). Others have used laser-capture microdissection to separately capture each zone of the nodule to determine the spatial expression patterns within nodules (Roux et al. 2014). Such studies have clearly demonstrated a cascade of transcriptional events occurring throughout symbiotic development. This is demonstrated in Figure 6, which displays the unique gene expression patterns of ten genes across four developmental stages. These results highlight the complexity of the differentiation process and the need for detailed studies of each developmental stage in addition to whole nodule analyses.

PROTEOME LEVEL STUDIES OF RHIZOBIA

Numerous proteomic studies of rhizobia have been prepared, and a detailed list of these studies are provided in Table 1. Large-scale proteomic studies have been used for purposes such as improving genome annotation, identifying the phosphorylated proteome, characterizing the functions of specific proteins and non-coding RNAs, and evaluating the cellular response to varying environmental conditions (Table 1). Additionally, comparative proteomic studies have been used in the identification of putative symbiotic genes (Gomes et al. 2012b). Several processes of more direct relevance to SNF have also been studied using proteomics (Table 1), such as the proteomic response to flavonoids (Guerreiro et al. 1997, Chen et al. 2000b, Hempel et al. 2009, da Silva Batista and Hungria 2012, Tolin et al. 2013, Arrigoni et al. 2013, Gao et al. 2015, Meneses et al. 2017), and to micro-aerobic or anaerobic conditions (Dainese-Hatt et al. 1999). The

secretome of rhizobia, with a particular focus on the proteins exported by type III secretion systems, has also been investigated (Saad et al. 2005, Rodrigues et al. 2007, Hempel et al. 2009, Okazaki et al. 2009).

Symbiotic nodule-associated rhizobia have also been the subject of proteomic studies (Table 1). Comparison of the proteomes of nitrogen-fixing rhizobia to that of their free-living counterparts have helped identify biochemical processes active during SNF (Djordjevic 2004, Sarma and Emerich 2005, 2006, Djordjevic et al. 2007, Delmotte et al. 2010, Resendis-Antonio et al. 2011, Tatsukami et al. 2013). Similarly, comparing the proteomes of bacteroids isolated from naturally effective and ineffective symbioses suggested pathways contributing to symbiotic efficiency (Cooper et al. 2018). The proteome of the peribacteroid space and peribacteroid membrane (Saalbach et al. 2002) as well as the bacteroid periplasm (Strodtman et al. 2017) have been determined, and the results suggest strong partitioning of proteins among these functionally distinct compartments (Strodtman et al. 2017). Furthermore, nodule proteomics has been used to examine the proteome during field growth conditions (Delmotte et al. 2010), differences between root and stem nodules (Delmotte et al. 2014), and NifA regulated proteins (Salazar et al. 2010). Studies have compared the proteomes of nodules of different maturities/ages (Delmotte et al. 2014, Nambu et al. 2015, Marx et al. 2016), and recently, proteomes of three zones from the same nodules were independently isolated and characterized (Ogden et al. 2017). These studies have revealed how the proteome changes during symbiotic development, and they complement and extend conclusions generated using transcriptomics. A more detailed review on the use of proteomics to characterize the symbiosis was recently published (Larrainzar and Wienkoop 2017).

METABOLITE ANALYSES

Metabolomics, the large-scale study of the metabolites of the cell or its environment, has been used to characterize several rhizobial processes. Several studies have examined how the intracellular metabolome of free-living rhizobia differs depending on nutritional or stress conditions, or as a result of genetic manipulations (see Table 1 for a detailed list of relevant studies). Additionally, lipidomics has been used to characterize the membrane lipid composition and the effects of phosphate or acid stress, as well as to identify proteins involved in membrane lipid metabolism (Table 1). A couple studies have also used a fluxomic approach (the study of the rates of reaction fluxes) to characterize central carbon metabolism during growth with either glucose (Fuhrer et al. 2005) or succinate (Terpolilli et al. 2016) as the sole source of carbon.

Metabolomics has been employed to characterize the metabolic shifts occurring during SNF (Table 1). Studies have compared the metabolomes of bacteroids to free-living rhizobia to identify biochemical processes that differ between these physiologically distinct cell populations (Resendis-Antonio et al. 2012, Vauclare et al. 2013), such as the role of lipogenesis in nitrogen fixing bacteroids (Terpolilli et al. 2016). Others have compared the metabolite profile of effective and non-effective nodules to identify metabolic processes specifically active during SNF (Ye et al. 2013, Gemperline et al. 2015). As with transcriptomics and proteomics, metabolomics of nodules of different age/maturity has been performed to examine the developmental progression (Lardi et al. 2016), and zone-specific metabolite analyses have been performed (Ogden et al. 2017). Metabolomics has further been employed to compare the metabolome of nodules from different plants containing the same rhizobium (Lardi et al. 2016). Furthermore, metabolomics has been used to characterize metabolism in root hairs during the early stages of symbiosis (Brechenmacher et al. 2010). Overall, such studies provide insights into the metabolic processes occurring during SNF, and how these processes differ depending on the partners involved.

IN SILICO METABOLIC MODELLING

Genome-scale metabolic network reconstruction refers to the process of building an *in silico* representation of the complete metabolism of the cell (Thiele and Palsson 2010). Reconstructions link metabolism to genetics by listing all reactions expected to be present in the cell together with the corresponding genes encoding the enzymes that catalyze the reaction; they can therefore serve as valuable knowledgebases of the metabolic properties of an organism. The reconstructions can then be used to simulate the metabolism of cells under defined environmental conditions, using mathematical approaches such as flux balance analysis (FBA) (Schellenberger et al. 2011). This approach can predict the metabolic fluxes through the reactions in a given environment, can simulate how environmental perturbations change the flux distributions, and can predict how deletion of one or more genes influences growth rate and flux distribution. As an example, Figures 7 and S1 displays the predicted distribution of flux through the central carbon metabolic pathways of *S. meliloti* when grown with glucose or succinate as a carbon source.

Metabolic modelling is a relatively new method in the study of rhizobia. The first rhizobial reconstruction was reported in 2007 for *R. etli* (Resendis-Antonio et al. 2007) followed by several refinements (Resendis-Antonio et al. 2011, 2012). In 2012, the first reconstruction for *S. meliloti* was reported (Zhao et al. 2012). These reconstructions were relatively small and were designed solely for the purpose of representing bacteroid metabolism. Simulations of flux distribution and analyses of genes essentiality during SNF generally displayed good correlation with experimental data (Resendis-Antonio et al. 2007, Zhao et al. 2012). Recently, genome-scale metabolic network reconstructions capable of representing both free-living and symbiotic metabolism were reported for *S. meliloti* (diCenzo et al. 2016a) and *Bradyrhizobium diazoefficiens* (Yang et al. 2017c).

Simulations with both of these models were consistent with transcriptomic data that indicates a major metabolic shift and general down-regulation, particularly in biosynthesis of cellular components, during symbiosis compared to free-living bacteria (diCenzo et al. 2016a, Yang et al. 2017c). A metabolic reconstruction for the legume *Medicago truncatula* has also been developed (Pfau et al. 2016).

We expect that metabolic reconstruction and constraint-based modelling will serve as a useful tool in the field of SNF, and it will provide an *in silico* method for examining the progression of symbiotic development, which can be experimentally difficult to dissect. Results of the simulations may then be used to guide experimental follow-up, and they may contribute to the identification of putative targets for biotechnological manipulation and experimental validation. The use of metabolic modelling will be facilitated by the wealth of systems-level data available for rhizobia, as summarized above. Several methods exist for the integration of transcriptomics, metabolomics, proteomics, and functional genomics (Tn-seq) data into modelling procedures (Fondi and Liò 2015, diCenzo et al. 2017a), and rhizobial models have been combined with each of these data sets (Resendis-Antonio et al. 2011, 2012, Yang et al. 2017c, diCenzo et al. 2018). This process results in the creation of high-quality, context-specific representations of cellular metabolism. However, incomplete knowledge of the metabolic exchange between the plant and bacterium may limit attempts to accurately represent SNF through a metabolic modelling approach.

SYNTHETIC BIOLOGY APPROACHES TO ENGINEERING THE SYMBIOSIS

Developing improved rhizobium bio-inoculants has been the topic of a few recent reviews and book chapters (Lupwayi et al. 2006, Archana 2010, Dwivedi et al. 2015, Checcucci et al.

2017), and has been of interest to the scientific community for decades (Paau 1991). In order for a rhizobial strain to serve as an effective agricultural inoculant, it must be able to i) fix large amounts of nitrogen, and ii) effectively outcompete the indigenous rhizobial population for nodule occupancy. Unfortunately, natural rhizobial strains employed in agricultural settings are often effective in only one of these two abilities; and yet, despite the many genetic and systems-level studies that shed light on this symbiosis, genetically engineered rhizobial inoculants are not available. Studies such as those described in this review provide a solid background for targeted attempts at improving the rate of nitrogen fixation and exchange of nitrogen during the symbiosis. Indeed, there are several reports in the literature supporting the feasibility of doing so (Paau 1991, Ramírez et al. 1999, van Dillewijn et al. 2001, Orikasa et al. 2010).

However, a strain capable of high rates of nitrogen fixation is of little use if it is unable to effectively establish a population in the soil microbiome and outcompete native rhizobia for nodule occupancy. The native rhizobial community that is often the most competitive for nodule occupancy tends to exhibit low nitrogen fixation rates (see for example (Cardoso et al. 2017a, Chibeba et al. 2017). Indeed, for decades the failure of rhizobia to improve crop yield has often been attributed to the poor competitive abilities of the inoculant and not due to poor nitrogen fixation abilities (Triplett and Sadowsky 1992, Streeter 1994, Ndungu et al. 2018). Improving this aspect of the rhizobial life-cycle may be more difficult as it is likely that the traits required for effective competition will vary across the geographical landscape (Checcucci et al. 2017). A genomic-based approach comparing the genomes of nodule isolated rhizobia with the soil rhizobium population, coupled with Tn-seq studies of highly competitive strains in non-sterile environments, may assist in the identification of genes contributing to high competitiveness for nodule occupancy.

Many studies have highlighted strong host-strain compatibility properties in SNF (see references below). Consequently, a “personalized” (i.e. cultivar-specific, environment-specific) selection of elite inoculant rhizobia should be considered. However, additional studies are necessary to identify the G (plant genotype) x E (environment) x M (root and soil microbiota) x R (rhizobium) interactions, and to sort out the relevant rhizobium functions of this multi-component interaction (Busby et al. 2017, Finkel et al. 2017, Pahua et al. 2018). For example, *M. truncatula* cultivar-specific NCR peptides can block symbiosis in a bacterial strain-specific manner (Wang et al. 2017, Yang et al. 2017b); many other examples of plant genes restricting symbiosis in a rhizobia strain dependent fashion exist in the literature (for example, (Fan et al. 2017, Yamaya-Ito et al. 2018). Others have shown that relatively small differences between rhizobial genomes can result in significant variations in symbiotic ability (Jozefkiewicz et al. 2017). Additionally, studies have highlighted rhizobial lineage-specific adaptations to symbiosis (Tian et al. 2012, Liu et al. 2017a), and have identified rhizobial genes blocking symbiosis in a plant cultivar specific manner (Crook et al. 2012, Price et al. 2015, Nguyen et al. 2017). Moreover, studies have demonstrated that both the environment (Ji et al. 2017) and microbiome (Jozefkiewicz et al. 2017) influence the symbiotic properties of rhizobia.

A major factor limiting our ability to make the most of SNF in agriculture is the inability of many staple crops, such as the cereals, of entering into a SNF relationship. As such, there is significant interest in engineering synthetic symbioses between these plants and rhizobia, and there have been a few recent reviews on this topic (Beatty and Good 2011, Rogers and Oldroyd 2014, Mus et al. 2016, Burén and Rubio 2018). As one can imagine, engineering new inter-kingdom interactions is daunting (Mus et al. 2016), and will require engineering a complex developmental pathway into the plant genome. Fortunately, legume-rhizobium symbioses makes use of the

common symbiosis signalling pathway that is conserved in plants (including cereals) that participate in mycorrhizal interactions (Oldroyd 2013). In the short term, however, a simpler option would be to improve the beneficial interaction between free-living “associative” bacterial nitrogen fixing bacteria and these crop plants (Geddes et al. 2015), even if these looser symbiotic arrangements transfer nitrogen to the plant less efficiently (Beatty and Good 2011).

An alternative to achieving effective nitrogen fixation in plants is to bypass the bacterial component altogether, and directly engineer plants to perform their own nitrogen fixation. Several review and perspective articles discuss this idea and its associated challenges (Beatty and Good 2011, Oldroyd and Dixon 2014, Rogers and Oldroyd 2014, Curatti and Rubio 2014). The main objective is to engineer plants that express a functional nitrogenase inside the mitochondrion or plastid, as these organelles may have the necessary conditions to support this oxygen-sensitive enzyme. Several recent advances have been made in this area that moves the community closer to reaching this goal. Using *Saccharomyces cerevisiae* as a model eukaryotic organism, methods have been developed to express functional NifB protein (Burén et al. 2017a), NifDK tetramers (Burén et al. 2017b), and a functional version of the oxygen labile NifH protein in mitochondria (López-Torrejón et al. 2016). Other groups were able to individually express each of the *Klebsiella pneumoniae* Nif proteins within the mitochondria of a plant species (Allen et al. 2017), as well as to express active NifH in a plant plastid (Ivleva et al. 2016). Separately, others have managed to re-wire the transcriptional regulatory network of the *nif* genes (Wang et al. 2013), and identify a minimal nitrogenase gene cluster (Yang et al. 2014). Additionally, it has been shown that the functioning of nitrogenase in free-living bacterial diazotrophs can be supported by the electron-transport chain proteins of plant organelles (Yang et al. 2017a). Each of these studies demonstrates the feasibility of this ambitious research avenue.

CONCLUSIONS AND PERSPECTIVE

Leading-edge techniques from an extensive range of disciplines have been applied to the study of rhizobia, facilitating the development of a strong multi-disciplinary understanding of their interactions with leguminous plant hosts (Table 2). Going forward, it will be valuable to integratively employ these methods in orchestrated multidisciplinary studies. A major challenge of the future will be the development of tools to study the spatio-temporal function of genes in the nodule and during earlier stages of symbiotic development. While transcriptomic, proteomic, and metabolic approaches can help provide insights into the unique developmental stages, genetic and cell biology tools are required to validate conclusions reached from these systems-level analyses. The inducible Cre/lox system by (Harrison et al. 2011) is a step in this direction, as are the *lux* bioreporters for the *in vivo* analysis of the spatial and temporal presence of plant metabolites (Pini et al. 2017). Similarly, the *S. meliloti* multiple gene-expression reporter strain for examining the role of plant genes in nodule development helps address this problem (Lang et al. 2018). Moreover, tools and experimental settings for the fast analysis of host preference in a GxExMxR interaction framework are necessary, such as the recently described select and resequence approach (Burghardt et al. 2018). The development of additional tools and their application to studying SNF are a necessity to develop the required knowledge to be able to effectively manipulate this process.

Our understanding of the intricacies of SNF have rapidly advanced over the last few decades, and the emergence of synthetic biology in recent years presents an unprecedented opportunity to engineer improved rhizobia and SNF relationships. However, despite the progress reviewed here, there is still a long road ahead to achieve the ambitious goal of completely replacing

nitrogen fertilizer with BNF, and we should continue to balance discovery-oriented and applied research efforts.

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SUPPLEMENTARY FILE DESCRIPTIONS

File S1. Contains Figure S1, and the methods for generating the data underlying Figures 1, 5, 7, and S1.

Figure S1. *In silico* predicted flux distributions and reaction essentialities. Example data obtained through metabolic modelling. Results from *in silico* metabolic modelling of *S. meliloti* metabolism using the iGD726 metabolic reconstruction are shown (diCenzo et al. 2018). Growth was simulated using (A) glucose or (B) succinate as the sole carbon source. The pathways represent central carbon metabolism, with the exception of the pentose phosphate pathway (for simplicity). The width of the line represents the amount of flux through the reaction; a doubling in the width corresponds to a ten-fold flux increase. Arrows indicate the direction of flux. Colours indicate if the reaction is essential (red) or non-essential (blue). Genes associated with reactions are based on the information present in iGD726, but they are not necessarily comprehensive. See the Supplementary Materials for details on the

modelling procedures used. A version without gene names is provided as Figure 8 in manuscript.

File S2. Contains an enlarged version of the phylogeny in Figure 1, which contains all taxa names. Also contains a Newick formatted file of the phylogeny, the corresponding annotation file, and a README file to describe the contents of the archive.

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SUPPLEMENTARY MATERIAL LEGENDS

File S1. Contains the supplementary methods and associated references, and Figure S1.

Figure S1. *In silico* predicted flux distributions and reaction essentialities. Example data obtained through metabolic modelling. Results from *in silico* metabolic modelling of *S. meliloti* metabolism using the iGD726 metabolic reconstruction are shown (diCenzo et al. 2018). Growth was simulated using (A) glucose or (B) succinate as the sole carbon source. The pathways represent central carbon metabolism, with the exception of the pentose phosphate pathway (for simplicity). The width of the line represents the amount of flux through the reaction; a doubling in the width corresponds to a ten-fold flux increase. Arrows indicate the direction of flux. Colours indicate if the reaction is essential (red) or non-essential (blue). Genes associated with reactions are based on the information present in iGD726, but they are not necessarily comprehensive. See the Supplementary Materials for details on the modelling procedures used. A version without gene names is provided as Figure 7 in manuscript.

File S2. Contains an enlarged version of the phylogeny of Figure 1, the Newick formatted phylogeny and associated annotation file of Figure 1, and a README file to describe the contents of the archive.

TABLES AND FIGURES

Table 1. Studies of rhizobial species employing -omics technologies.

Topic of the study	Organisms	Approaches	References
Characterization of proteins			
SyrM (Nod factor synthesis)	<i>S. meliloti</i>	Transcriptomics	(Barnett and Long 2015)
NodD (Nod factor synthesis)	<i>S. meliloti</i>	Transcriptomics	(Capela et al. 2005)
NoIR (Nod factor synthesis)	<i>S. meliloti</i>	Proteomics	(Chen et al. 2000a, 2005)
FixL/FixJ/FixK (nitrogen fixation)	<i>S. meliloti</i> ; <i>B. diazoefficiens</i>	Transcriptomics	(Bobik et al. 2006, Mesa et al. 2008)
CbrA (cell cycle and symbiosis)	<i>S. meliloti</i>	Transcriptomics	(Gibson et al. 2007)
CtrA (cell cycle)	<i>S. meliloti</i>	Transcriptomics	(Pini et al. 2015)
RosR (exopolysaccharide synthesis)	<i>R. leguminosarum</i> bv. <i>trifolii</i>	Transcriptomics	(Rachwał et al. 2015)
FeuP/FeuQ (osmoadaptation and symbiosis)	<i>S. meliloti</i>	Transcriptomics	(Griffitts et al. 2008)
Sigma factors (stress response and symbiosis)	<i>R. etli</i> ; <i>B. diazoefficiens</i>	Transcriptomics	(Martínez-Salazar et al. 2009, Stockwell et al. 2012)
Clr (cAMP dependent regulator, nodulation)	<i>S. meliloti</i>	Transcriptomics	(Krol et al. 2016, Zou et al. 2017)
PraR (quorum sensing and biofilms)	<i>R. leguminosarum</i> bv. <i>viciae</i>	Transcriptomics	(Frederix et al. 2014)
PckR (central carbon metabolism)	<i>S. meliloti</i>	Transcriptomics	(diCenzo et al. 2017b)
PhoB (phosphate limitation response)	<i>S. meliloti</i>	Transcriptomics	(Krol and Becker 2004, Yuan et al. 2006)
GlnD/GlnBK (nitrogen limitation response)	<i>S. meliloti</i>	Transcriptomics	(Yurgel et al. 2013b)
NtrY/NtrX (nitrogen metabolism)	<i>S. meliloti</i>	Transcriptomics	(Calatrava-Morales et al. 2017)
Tkt2/Tal (central carbon metabolism)	<i>S. meliloti</i>	Proteomics; metabolomics	(Hawkins et al. 2018)
OxyR (oxidative stress)	<i>S. meliloti</i>	Transcriptomics	(Lehman and Long 2018)
RelA/Rsh (stringent response)	<i>S. meliloti</i> ; <i>R. etli</i>	Transcriptomics	(Vercruysse et al. 2011, Krol and Becker 2011)
RirA (iron limitation)	<i>S. meliloti</i> ; <i>R. leguminosarum</i> bv. <i>viciae</i>	Transcriptomics; proteomics	(Todd et al. 2005, Chao et al. 2005)
LeuB (leucine biosynthesis)	<i>S. meliloti</i>	Metabolomics	(Barsch et al. 2004)
AniA (PHB accumulation)	<i>R. etli</i>	Proteomics	(Encarnación et al. 2002)
FadD (Fatty acid metabolism)	<i>S. meliloti</i>	Lipid analysis	(Nogales et al. 2010)
PHB cycle mutations	<i>S. meliloti</i>	Transcriptomics; proteomics	(Wang et al. 2007, D'Alessio et al. 2017)
Dme/Tme (Malic enzymes)	<i>S. meliloti</i>	Transcriptomics; metabolomics	(Zhang et al. 2016)
Membrane lipid biosynthetic proteins (Cfa, PlsC, PlcP, Pcs, Pmt, OlsA, OlsB, PssA, Psd, SqdB, BtaA)	<i>S. meliloti</i>	Lipidomics and lipid analysis	(de Rudder et al. 1999, Weissenmayer et al. 2000, Sohlenkamp et al. 2000, de Rudder et al. 2000, Weissenmayer et al. 2002, Sohlenkamp et al. 2004, Gao et al. 2004, López-Lara et al. 2005, Vences-Guzmán et al. 2008, Basconcillo et al. 2009a, 2009c, Zavaleta-Pastor et al. 2010, Pech-Canul et al. 2011)
Riboregulation			
Small RNAs (sRNA)	<i>S. meliloti</i>	Transcriptomics	(Ulvé et al. 2007, Schlüter et al. 2010)
Hfq (RNA binding protein)	<i>S. meliloti</i>	Transcriptomics; proteomics	(Barra-Bily et al. 2010, Torres-Quesada et al. 2010, Gao et al. 2010, Sobrero et al. 2012)
Hfq binding RNAs	<i>S. meliloti</i>	Transcriptomics	(Torres-Quesada et al. 2014)
YbeY (endoribonuclease)	<i>S. meliloti</i>	Transcriptomics	(Saramago et al. 2017)
AbrC1/AbrC2 (sRNAs, nutrient uptake)	<i>S. meliloti</i>	Proteomics	(Torres-Quesada et al. 2013)
MmgR (sRNA, PHB accumulation)	<i>S. meliloti</i>	Proteomics	(Lagares et al. 2017)
Environmental adaptation and stresses			
Growth in root hairs	<i>B. diazoefficiens</i>	Metabolomics	(Brechenmacher et al. 2010)

Oxic versus microoxic growth	<i>S. meliloti</i> ; <i>B. diazoefficiens</i>	Transcriptomics	(Ampe et al. 2003, Becker et al. 2004, Bobik et al. 2006, Pessi et al. 2007)
Aerobic, micro-aerobic, anaerobic growth	<i>B. diazoefficiens</i>	Proteomics	(Dainese-Hatt et al. 1999)
Rhizosphere adaptation	<i>R. leguminosarum</i> bv. <i>viciae</i>	Transcriptomics	(Ramachandran et al. 2011)
Growth in presence of root exudates	<i>B. diazoefficiens</i> ; <i>B. phytatum</i> ; <i>C. taiwanensis</i> ; <i>R. mesoamericanum</i>	Transcriptomics	(Liu et al. 2017b, Klonowska et al. 2018)
Seed endophytic growth	<i>R. phaseoli</i> ; <i>S. americanum</i>	Proteomics	(Peralta et al. 2016)
Stationary phase adaptation	<i>S. meliloti</i> ; <i>R. etli</i>	Proteomics	(Guerreiro et al. 1999, Meneses et al. 2010)
Phosphate limitation	<i>S. meliloti</i>	Lipidomics and lipid analysis	(Basconcillo et al. 2009a, 2009b, 2009c, Zavaleta-Pastor et al. 2010)
Biotin limitation	<i>S. meliloti</i>	Proteomics	(Heinz and Streit 2003)
Carbon limitation	<i>S. meliloti</i>	Proteomics	(Djordjevic et al. 2007)
Nitrogen limitation	<i>S. meliloti</i>	Proteomics	(Djordjevic et al. 2007)
Osmotic/salt stress	<i>S. meliloti</i> ; <i>M. loti</i> ; <i>M. alhagi</i> ; <i>R. etli</i> ; <i>S. sp. BL3</i> ; <i>B. spp.</i>	Transcriptomics; proteomics	(Jebbar et al. 2005, Shamseldin et al. 2006, Dominguez-Ferreras et al. 2006, Tanthanuch et al. 2010, Liu et al. 2014, Laranjo et al. 2017, Dong et al. 2017)
Oxidative stress	<i>B. diazoefficiens</i>	Transcriptomics	(Donati et al. 2011, Jeon et al. 2011)
Acid stress	<i>S. meliloti</i> ; <i>M. loti</i> ; <i>S. medicae</i>	Transcriptomics; proteomics; metabolomics; lipidomics	(Reeve et al. 2004, Basconcillo et al. 2009a, Hellweg et al. 2009, Laranjo et al. 2014, Draghi et al. 2016)
Heat stress	<i>M. loti</i> ; <i>R. tropici</i> ; <i>B. diazoefficiens</i>	Transcriptomics; proteomics	(Münchbach et al. 1999, Gomes et al. 2012a, Alexandre et al. 2014)
Cold stress	<i>M. sp. N33</i>	Transcriptomics; metabolomics	(Ghobakhlou et al. 2013, 2015)
Heavy metal stress	<i>S. meliloti</i> ; <i>M. metallidurans</i> ; <i>M. sp. STM 4661</i> ; <i>R. sp. VMA301</i> ; <i>R. sp. E20-8</i>	Transcriptomics; proteomics; metabolomics	(Mandal et al. 2009, Maynaud et al. 2013, Lu et al. 2017, Cardoso et al. 2017b)
Dessication stress	<i>B. diazoefficiens</i>	Transcriptomics	(Cytryn et al. 2007)
Herbicide exposure	<i>R. leguminosarum</i> bv. <i>viciae</i>	Metabolomics	(Bhat et al. 2014)
Cellular processes			
Flavonoid perception	<i>S. meliloti</i> ; <i>S. fredii</i> ; <i>R. tropici</i> ; <i>R. leguminosarum</i> bv. <i>trifolii</i> ; <i>R. leguminosarum</i> bv. <i>viciae</i> ; <i>R. etli</i> ; <i>B. liaoningense</i> ; <i>B. japonicum</i> ; <i>B. diazoefficiens</i>	Transcriptomics; proteomics	(Guerreiro et al. 1997, Chen et al. 2000b, Ampe et al. 2003, Barnett et al. 2004, Capela et al. 2005, Hempel et al. 2009, da Silva Batista and Hungria 2012, Tolin et al. 2013, Arrigoni et al. 2013, Gao et al. 2015, Pérez-Montaña et al. 2016a, 2016b, Meneses et al. 2017)
Nod factor production	<i>S. arboris</i>	Metabolite analysis	(Penttinen et al. 2013)
Cell cycle	<i>S. meliloti</i>	Transcriptomics	(De Nisco et al. 2014, Pini et al. 2015)
Response to NCR peptides	<i>S. meliloti</i>	Transcriptomics	(Tiricz et al. 2013, Penterman et al. 2014)
Secretome / Type III secretion	<i>B. diazoefficiens</i> ; <i>S. fredii</i> ; <i>B. elkanii</i>	Proteomics	(Saad et al. 2005, Rodrigues et al. 2007, Hempel et al. 2009, Okazaki et al. 2009)
Biofilm formation	<i>R. etli</i>	Transcriptomics	(Reyes-Pérez et al. 2016)
Quorum sensing	<i>S. fredii</i> ; <i>S. meliloti</i> ; <i>R. leguminosarum</i> bv. <i>viciae</i>	Transcriptomics; proteomics	(Chen et al. 2003, Gao et al. 2005, Cantero et al. 2006, Gao et al. 2007, Krysiak et al. 2014)
Motility	<i>S. meliloti</i>	Transcriptomics	(Nogales et al. 2010)
Aerobic vs fermentative growth	<i>R. etli</i>	Proteomics	(Encarnación et al. 2003)
Lectin perception	<i>B. diazoefficiens</i>	Proteomics	(Pérez-Giménez et al. 2012)
Catabolism of phenanthrene	<i>S. sp. C4</i>	Metabolomics	(Keum et al. 2008)
Symbiotic island transfer	<i>M. ciceri</i>	Transcriptomics	(Haskett et al. 2018)
Nodule analyses			
Integrated plant + bacterium analyses	<i>S. meliloti</i> - <i>M. truncatula</i> ; <i>S. meliloti</i> - <i>M. alba</i> ; <i>S. medicae</i> - <i>M. truncatula</i> ; <i>R. leguminosarum</i> bv. <i>viciae</i> - <i>P. sativum</i>	Transcriptomics; proteomics	(Natera et al. 2000, Barnett et al. 2004, Heath et al. 2012, Roux et al. 2014, Marx et al. 2016, Ogden et al. 2017, Mitsch et al. 2018)
Bacteroids vs free-living	<i>S. meliloti</i> - <i>M. truncatula</i> ; <i>S. meliloti</i> - <i>M. sativa</i> ; <i>S. meliloti</i> - <i>M. alba</i> ; <i>R.</i>	Transcriptomics; proteomics; metabolomics	(Ampe et al. 2003, Becker et al. 2004, Djordjevic 2004, Barnett et al. 2004, Sarma and Emerich 2006, Djordjevic et al. 2007, Pessi et al.

	<i>leguminosarum</i> bv. <i>viciae</i> - <i>P. sativum</i> ; <i>R. leguminosarum</i> bv. <i>viciae</i> - <i>V. cracca</i> ; <i>B. diazoefficiens</i> - <i>G. max</i> ; <i>M. loti</i> - <i>L. japonicus</i> ; <i>R. etli</i> - <i>P. vulgaris</i>		2007, Karunakaran et al. 2009, Resendis-Antonio et al. 2012, Vauclare et al. 2013, Tatsukami et al. 2013)
Nodules of varying maturity	<i>S. meliloti</i> - <i>M. truncatula</i> ; <i>S. meliloti</i> - <i>M. sativa</i> ; <i>B. sp.</i> ORS 278 - <i>A. indica</i> ; <i>M. loti</i> - <i>L. japonicus</i>	Transcriptomics; proteomics	(Ampe et al. 2003, Capela et al. 2006, Delmotte et al. 2014, Nambu et al. 2015, Lardi et al. 2016, Marx et al. 2016)
Plant/bacterium mutants	<i>S. meliloti</i> - <i>M. truncatula</i> ; <i>S. meliloti</i> - <i>M. sativa</i> ; <i>B. diazoefficiens</i> - <i>G. max</i>	Transcriptomics; metabolomics	(Tian et al. 2006, Starker et al. 2006, Capela et al. 2006, Maunoury et al. 2010, Ye et al. 2013, Gemperline et al. 2015, Lardi et al. 2016)
Nodule zones	<i>S. meliloti</i> - <i>M. truncatula</i> ; <i>S. medicae</i> - <i>M. truncatula</i>	Transcriptomics; proteomics; metabolomics	(Roux et al. 2014, Ogden et al. 2017)
Reference nodule proteome/transcriptome	<i>B. diazoefficiens</i> - <i>G. max</i> ; <i>R. etli</i> - <i>P. vulgaris</i>	Transcriptomics; proteomics	(Sarma and Emerich 2005, Delmotte et al. 2010, Resendis-Antonio et al. 2011)
Sulfenylated nodule proteome	<i>S. meliloti</i> - <i>M. truncatula</i>	Proteomics	(Oger et al. 2012)
Peribacteroid space/membrane proteome	<i>R. leguminosarum</i> bv. <i>viciae</i> - <i>P. sativum</i>	Proteomics	(Saalbach et al. 2002)
Bacteroid periplasm	<i>B. diazoefficiens</i> - <i>G. max</i>	Proteomics	(Strodtman et al. 2017)
FixJ in nodules	<i>S. meliloti</i> - <i>M. truncatula</i>	Transcriptomics	(Barnett et al. 2004)
BacA in nodules	<i>R. leguminosarum</i> bv. <i>viciae</i>	Transcriptomics	(Karunakaran et al. 2010)
NifA in nodules	<i>R. etli</i> - <i>P. vulgaris</i>	Proteomics	(Salazar et al. 2010)
C4-dicarboxylate transport	<i>R. leguminosarum</i> bv. <i>viciae</i> - <i>P. sativum</i>	Transcriptomics	(Mitsch et al. 2018)
Lipogenesis	<i>R. leguminosarum</i> bv. <i>viciae</i> - <i>P. sativum</i>	Metabolomics	(Terpolilli et al. 2016)
Effect of genetic variation	<i>S. meliloti</i> - <i>M. truncatula</i> ; <i>S. medicae</i> - <i>M. truncatula</i>	Transcriptomics	(Heath et al. 2012, Burghardt et al. 2017)
Effect of plant host	<i>S. meliloti</i> - <i>M. truncatula</i> ; <i>S. meliloti</i> - <i>M. sativa</i> ; <i>R. leguminosarum</i> bv. <i>viciae</i> - <i>P. sativum</i> ; <i>R. leguminosarum</i> bv. <i>viciae</i> - <i>V. cracca</i> ; <i>S. fredii</i> - <i>V. unguiculata</i> ; <i>S. fredii</i> - <i>L. leucocephala</i> ; <i>B. diazoefficiens</i> - <i>G. max</i> ; <i>B. diazoefficiens</i> - <i>V. radiata</i> ; <i>B. diazoefficiens</i> - <i>V. unguiculata</i> ; <i>B. diazoefficiens</i> - <i>M. atropurpureum</i>	Transcriptomics; metabolomics	(Ampe et al. 2003, Karunakaran et al. 2009, Li et al. 2013, Lardi et al. 2016)
Bacteroid morphology	<i>B. sp.</i> ORS285 - <i>Aeschynomene</i> spp.	Transcriptomics	(Lamouche et al. 2018)
Effect of field growth	<i>B. diazoefficiens</i> - <i>G. max</i>	Proteomics	(Delmotte et al. 2010)
Root versus stem nodules	<i>B. sp.</i> ORS278 - <i>A. indica</i>	Proteomics	(Delmotte et al. 2014)
Drought stress	<i>R. leguminosarum</i> - <i>P. sativum</i>	Proteomics	(Irrar et al. 2014)
Other			
Reference free-living proteome	<i>S. meliloti</i> ; <i>M. loti</i> ; <i>B. diazoefficiens</i> ; <i>B. japonicum</i> ; <i>R. tropici</i>	Proteomics	(Kajiwarra et al. 2003, Djordjevic et al. 2007, Batista et al. 2010, Gomes et al. 2012b, Kumar et al. 2013, Gomes et al. 2014)
Phosphorylated free-living proteome	<i>S. meliloti</i>	Proteomics	(Liu et al. 2015)
Effects of genome reduction	<i>S. meliloti</i> ; <i>R. leguminosarum</i> bv. <i>trifolii</i>	Transcriptomics; proteomics; metabolomics	(Guerreiro et al. 1998, Chen et al. 2000b, Fei et al. 2016)
Growth with different carbon sources	<i>S. meliloti</i> ; <i>R. leguminosarum</i> bv. <i>viciae</i>	Metabolomics; fluxomics	(Barsch et al. 2004, Fuhrer et al. 2005, Terpolilli et al. 2016)
Membrane lipid composition	<i>S. meliloti</i>	Lipidomics	(Basconcillo et al. 2009b)

Table 2. Summary of the fields covered in this review.

Field	Goal	Key lessons
Socio-microbiology	Study the interactions between rhizobia and other organisms that influence environmental growth and the symbiotic process.	Rhizobia interact with many organisms (eukaryotes, prokaryotes, viruses), influencing symbiotic efficiency and competition for nodule occupancy.
Genetics and molecular biology	Characterize the precise function of individual genes and their role in complex biological processes including symbiosis.	Laid the groundwork for modern studies with rhizobia, establishing the fundamentals of the symbiotic interactions.
High-throughput functional genomics	Perform genome-scale analyses to identify genomic elements that contribute to a particular process, growth in a specific environment, or symbiosis.	Elucidated countless loci of relevance to symbiosis or other phenotypes (e.g., rhizosphere growth), highlighting the complexity of these processes.
Large-scale genome manipulation	Generate massive addition or deletions in the genomic content of the cell, and characterize the phenotypic effects and symbiotic effects.	Genes related to symbiosis are spread throughout the genome, and gain of classical symbiotic genes is not necessary sufficient for symbiosis.
Genomics	Compare genome content between strains or species to identify loci likely contributing to a particular phenotype, such as symbiosis.	Rhizobia show massive genome variations that could be related to symbiotic efficiency, and it appears that no gene is common and specific to rhizobia.
Transcriptomics	Identify changes in gene expression across environments or during symbiosis to gain insights into important functions and regulatory mechanisms.	Major transcriptional changes occur during symbiosis, and there is a constant transcriptional rewiring throughout the developmental process.
Proteomics	Identify changes in protein levels across environments or during symbiosis to gain insights into important functions and regulatory mechanisms.	Have provided information into the biochemical pathways that are active during symbiosis, and the roles of proteins and ncRNAs in free-living cells.
Metabolite analysis	Identify changes in metabolite concentrations, or reaction fluxes, across environments or during symbiosis to gain insights into metabolism.	Has been used to identify metabolic pathways active in symbiosis, and has been instrumental in studying the genetics of membrane lipid biosynthesis.
Metabolic modelling	Use computer simulations to predict effect of genomic or environmental alterations on metabolism, and to integrate multi-level omics data.	Used to predict changes in metabolism during shifts in environments and during symbiosis, but application to rhizobia has so far been limited.
Synthetic biology	Engineer cells for the purpose of studying a biological process, or for engineering improved abilities, such as greater symbiotic efficiency.	Has demonstrated the feasibility of at least the initial steps of engineering improved rhizobial inoculants and of transferring BNF directly to plants.

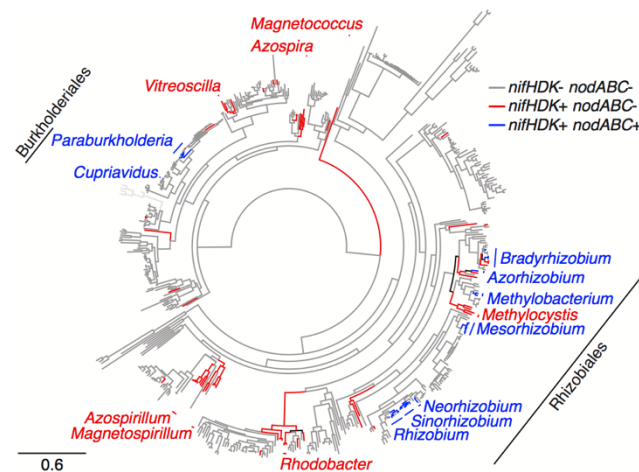


Figure 1. Phylogenetic distribution of biological nitrogen fixation. A maximum likelihood phylogeny of the α - and β -proteobacteria based on 23 highly conserved proteins (Frr, NusA, RplB, RplC, RplD, RplK, RplL, RplM, RplN, RplP, RplS, RplT, RpmA, RpoB, RpsB, RpsC, RpsE, RpsI, RpsJ, RpsK, RpsM, RpsS, Tsf). Species with at least one strain carrying the *nifHDK* nitrogen fixation genes are coloured red (sample genera are indicated), while species with at least one strain carrying the *nifHDK* nitrogen fixation genes and the *nodABC* nodulation genes are coloured blue (the genera and families are indicated). No strains have the nodulation genes but lack the nitrogen fixation genes. Branch lengths for the taxa that include the genera *Tremblaya*, *Hodgkinia*, and *Liberibacter* (light grey) were shortened for presentation. The complete phylogeny, including bootstrap values, is provided in File S2. See the Methods in File S1 for details on phylogeny construction and gene identification.

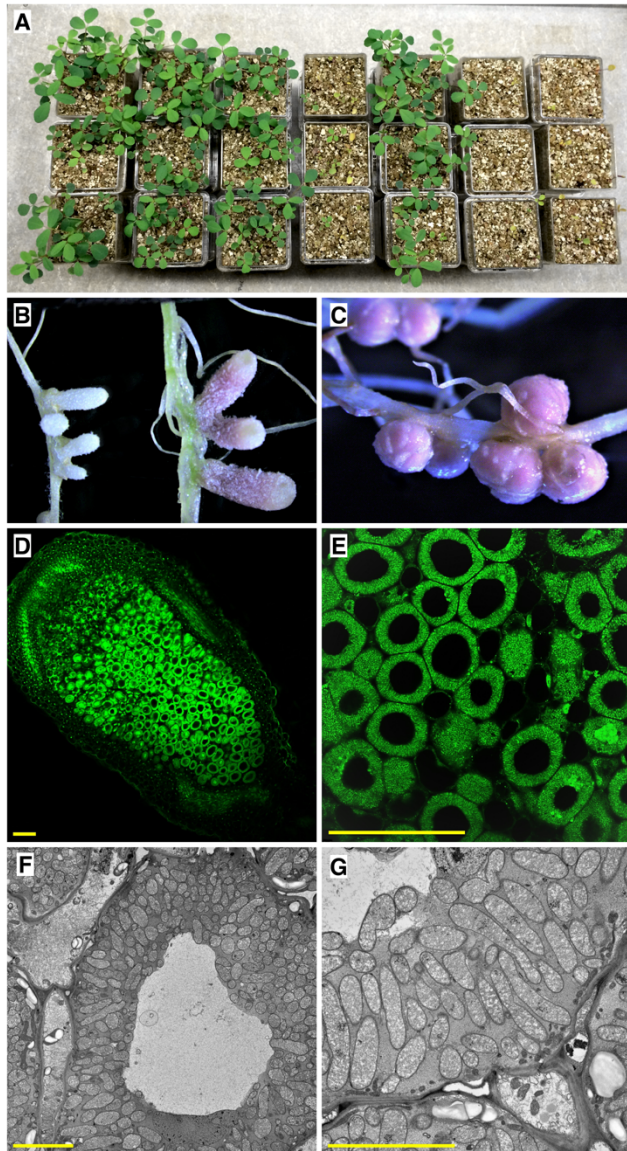


Figure 2. Visualizing the rhizobium – legume symbiosis. (A) A picture of *Medicago alba* (white sweet clover) plants grown in nitrogen free conditions. Plants in columns 1-3 and 5 are inoculated with nitrogen-fixing *S. meliloti*; plants in columns 4 and 6 are inoculated with a *S. meliloti* mutant unable to fix nitrogen; and plants in column 7 are not inoculated with *S. meliloti*. (B) *Medicago sativa* (alfalfa) root nodules, induced by *S. meliloti*. On the left are ineffective, white nodules containing a *S. meliloti* mutant unable to fix nitrogen; on the right are effective, pink nodules containing *S. meliloti* cells actively fixing nitrogen. (C) Effective, pink nodules of *Vigna unguiculata* (cowpea) containing *S.*

fredii NGR234 cells actively fixing nitrogen. (D, E) Confocal micrographs of alfalfa root nodules filled with *S. meliloti* cells. The nodule sections were stained with a fluorescent nucleic acid binding dye (Syto9); the green colour is indicative of the presence of *S. meliloti*. Scale bar represents 250 µm. (F, G) Transmission electron micrographs of alfalfa root nodules, showing plant cells filled with nitrogen fixing *S. meliloti* bacteroids. Scale bar represents 25 µm.

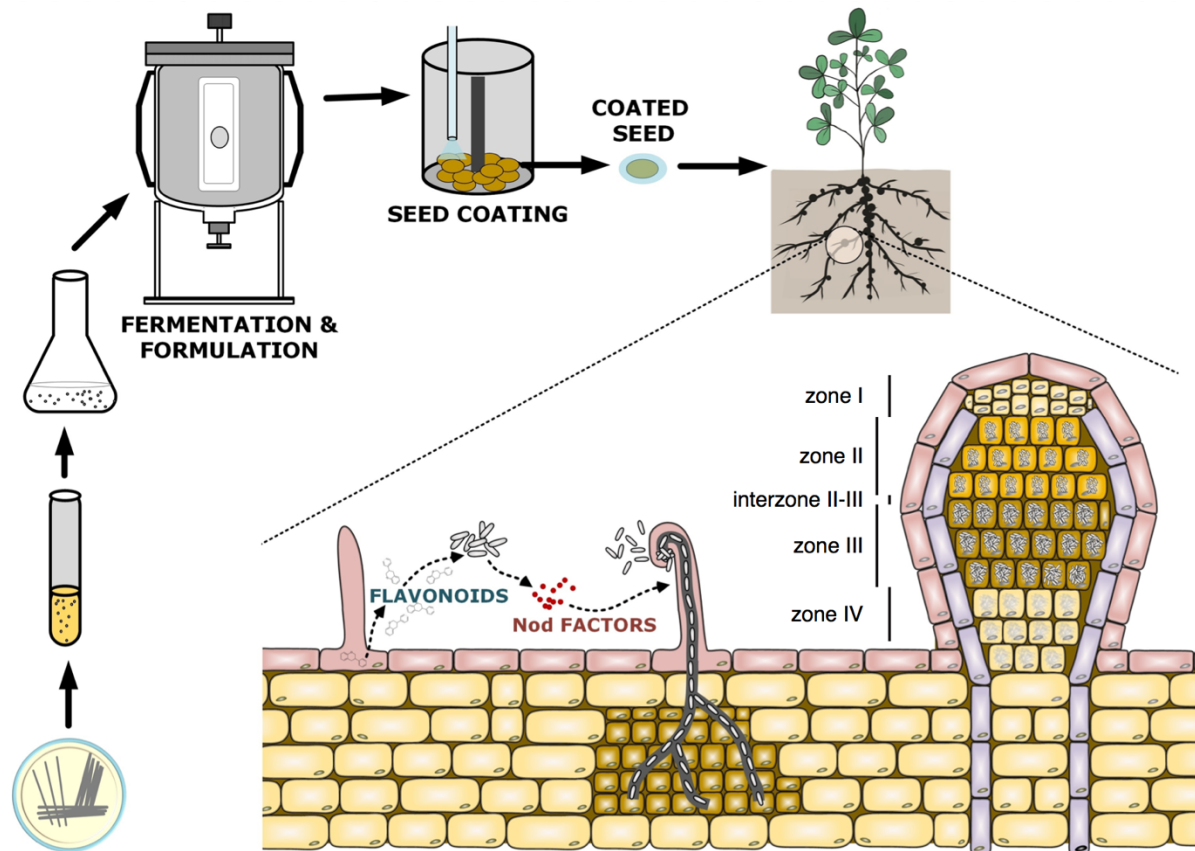


Figure 3. Schematic representation of the life-cycle of rhizobia. For inoculants, the life-cycle begins in an industrial environment, where the strain is grown in a fermenter. Inoculants are transported to the agricultural field, possibly as a seed coating. In the soil, the rhizobia must exploit nutrients, cope with the stresses, and compete with the indigenous microbiomes. The symbiotic interaction begins following an exchange of signals by the rhizobia and legume. Curling of a legume root hair traps a rhizobium cell, which then penetrates the cell wall of the root hair. The rhizobium proceeds to proliferates in a growing infection thread that progresses to the cortical cells. Here, the rhizobia are released into the cytoplasm of specialized cortical cells, resulting in them being enclosed in a plant derived membrane (peribacteroid membrane). The rhizobia and plant undergo a differentiation process that involves massive transcriptional and metabolic shifts, resulting in the formation of a nitrogen-fixing nodule. Five distinct developmental zones of the nodule are shown, but are not drawn to scale: zone I – apical meristem; zone II – infection and differentiation zone; interzone II-III – a small region between zone II and zone III; zone III – the nitrogen fixing zone; zone IV – the senescence zone found in mature nodules.

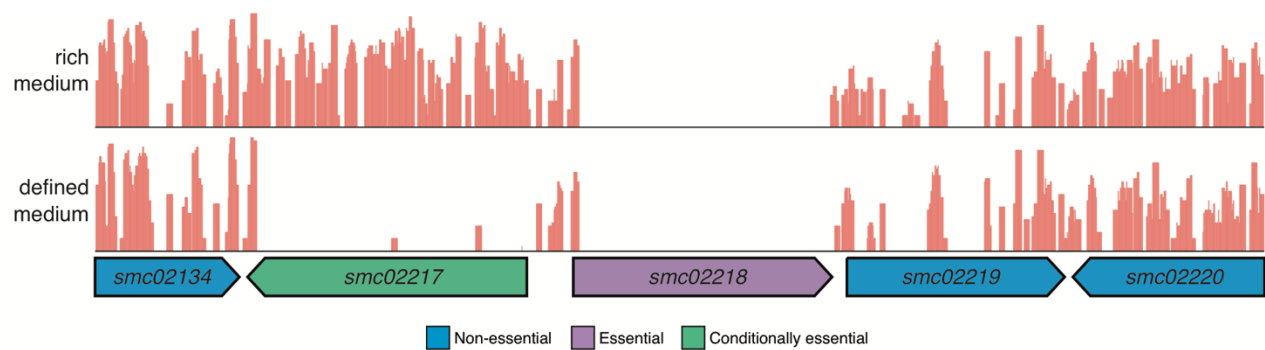


Figure 4. Example output of a Tn-seq study. A *S. meliloti* gene region illustrating the ability of Tn-seq to identify essential genes and environment-specific essential genes is shown. Published Tn-seq data (diCenzo et al. 2018) for *S. meliloti* grown in a nutritionally complex medium (rich medium) and a nutritionally defined medium (defined medium) were mapped to the *S. meliloti* chromosome. A five-gene region is shown. The location of the transposon insertions in the cell populations recovered from both conditions are indicated by the red bars, with the height of the bar representing the number of reads (log scale). Genes are colour coded based on their fitness classifications.

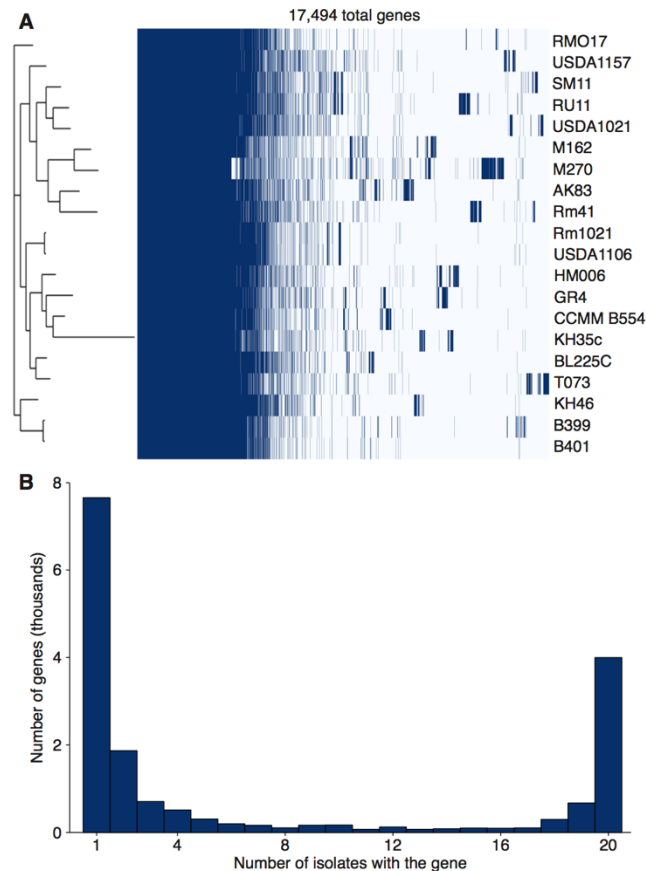


Figure 5. The *S. meliloti* pangenome. The pangenome of 20 *S. meliloti* strains with complete genomes was determined using Roary (Page et al. 2015). There was a median of 6,556 genes (standard deviation 362) per genome, and a total of 17,494 sets of orthologous proteins were detected of which 4,000 were present in all strains. **(A)** A heatmap showing gene presence (dark blue) or absence (light blue) in each of the 20 strains. A phylogeny built based on the 4,000 core genes is shown on the left, and the strain names are indicated on the right. **(B)** A histogram displaying the distribution of how many genomes each gene is found within. See the Methods in File S1 for details on pangenome construction.

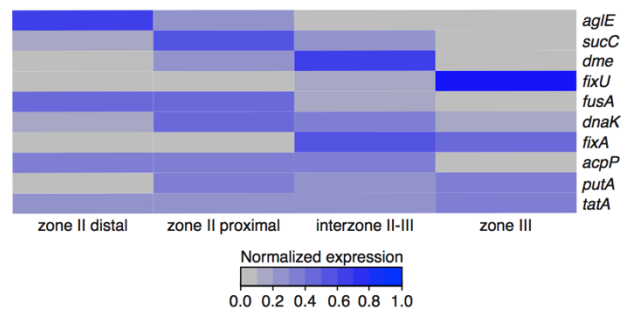


Figure 6. Expression patterns of select *S. meliloti* genes during symbiosis with *M. truncatula*.

The spatial expression patterns across a *M. truncatula* nodule of 10 *S. meliloti* genes, each displaying an unique pattern, is shown. Data was taken from the study of Roux *et al.* (Roux et al. 2014). The nodule zone is indicated along the bottom, and the gene names are given on the right. The plotted data is normalized across each zone; the darker the blue, the higher the expression of the gene in the indicated zone relative to other zones. For example, *aglE* is expressed strongly in the distal zone II but lowly in zone III, whereas *fixU* is primarily expressed in zone III. Colours provide no information on the expression of a gene compared to the other genes shown in the figure. Locations of each nodule zone are shown in Figure 3, with zone II distal and zone II proximal referring to the sections of zone II that are distal and proximal to the root, respectively.

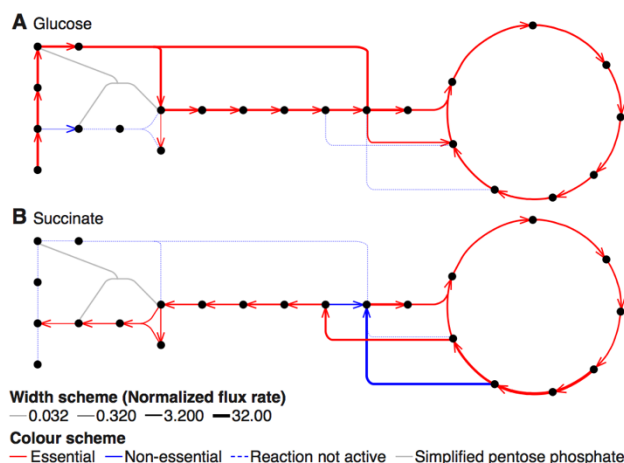


Figure 7. *In silico* predicted flux distributions and reaction essentialities. Example data obtained through metabolic modelling. Results from *in silico* metabolic modelling of *S. meliloti* metabolism using the iGD726 metabolic reconstruction are shown (diCenzo et al. 2018). Growth was simulated using (A) glucose or (B) succinate as the sole carbon source. The pathways represent central carbon metabolism, with the exception of the pentose phosphate pathway (for simplicity). The width of the line represents the amount of flux through the reaction; a doubling in the width corresponds to a ten-fold flux increase. Arrows indicate the direction of flux. Colours indicate if the reaction is essential (red) or non-essential (blue). See the Methods in File S1 for details on the modelling procedures used. A version with gene names is provided as Figure S1.