Multidisciplinary 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 emphasis 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.

Keywords: Rhizobia, nitrogen fixation, symbiosis, systems biology, multi-omics

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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.

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 could be ameliorated by relying more on biological solutions for supplying ammonia to 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, do not 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 strategy 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 and synthetic 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 and oxidative conditions in the pocket of a curled root hair (Santos et al. 2001, Hawkins et al. 2017), to linear growth along the oxidative penetrating infection threads (Santos et al. 2001), 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 industrial-scale growth 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 a high degree of cooperation between the partners, involving multiple signal exchange events as well as a massive exchange of metabolic resources (Oldroyd and Downie 2008, Oldroyd et al. 2011, Udvardi and Poole 2013).

Yet, hints of conflict and aspects of an evolutionary arms race can also be detected (Sachs et al. 2018). For example, plant immunity plays a key role in the infection process, which must be overcome by the rhizobia to establish an effective symbiosis (Gourion et al. 2015, Berrabah et al. 2018). Additionally, 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, Oresnik et al. 1999, Venter et al. 2001). The advantage of bacteriocin production is highlighted by the example of trifolitoxin, whose production by *Rhizobium* strains improves rhizosphere colonization and increases competitiveness for nodule occupancy (Triplett and Barta 1987, Robleto et al. 1997, Robleto et al. 1998). 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 they 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

Most of our knowledge about core symbiotic processes comes from classical molecular genetic analyses involving forward- and reverse-genetic techniques, wherein single mutations are associated with defective phenotypes. Tn*5*-based transposon mutagenesis, coupled with plasmid-based complementation and DNA sequencing, played a foundational role in the discovery of the rhizobial *nod* and *nif*/*fix* genes (Beringer et al. 1978, Rolfe et al. 1980, Meade et al. 1982, Jagadish and Szalay 1984, Chua et al. 1985, Swanson et al. 1987, Long et al. 1988, Becker et al. 1993a, 1993b, 1993c, Glucksmann et al. 1993). The availability of suitable suicide plasmids and broad host-range plasmids 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 (see for example: VanYperen et al. 2015,

177 Hood et al. 2017, Zamani et al. 2017, Ledermann et al. 2018, Wongdee et al. 2018), and targeted
178 genetic studies remain essential to complement and validate observations from systems- and
179 genome-level analyses.

180 High-throughput functional genomic studies have become more common for cataloguing
181 rhizobial gene functions (see Table 1 for a summary). *Sinorhizobium meliloti* ORFeome
182 (Schroeder et al. 2005) and fusion (Cowie et al. 2006) libraries have been developed to facilitate
183 functional and expression analyses in free-living and symbiotic states. *S. meliloti* libraries for use
184 with *in vivo* expression technology (IVET) have been produced and used to identify genes
185 functioning in early (Zhang and Cheng 2006) and late (Oke and Long 1999) symbiotic processes.
186 Similarly, a *Rhizobium leguminosarum* library for use with IVET was prepared and used to identify
187 genes expressed specifically in the legume rhizosphere (Barr et al. 2008).

188 Several groups have employed signature-tagged mutagenesis (STM) for genome-wide
189 identification of genes involved in symbiosis or competition for nodule occupancy. STM involves
190 generating insertion mutants using a library of transposons, with each transposon containing a
191 distinct sequence tag. Mutants containing different transposons are pooled and passed through a
192 selective regimen. The relative abundance of each mutant can be measured using microarrays that
193 detect each sequence tag, and these values can be compared for the library before and after the
194 selective regimen (Becker and Pobigaylo 2013). A STM library of *S. meliloti* was constructed
195 (Pobigaylo et al. 2006), and nearly 9,600 insertions were mapped to ~ 3,700 genes throughout the
196 genome (Serrania et al. 2017). This STM library was employed for the identification of 38 new
197 genes influencing symbiosis (Pobigaylo et al. 2008). In further work, this library was used to show
198 that ~ 2% of *S. meliloti* genes contribute to rhizosphere colonization (Salas et al. 2017). Screening
199 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 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 massively parallel transposon insertion sequencing (known as Tn-seq, or 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 are 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 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 O_2 levels, a key signal during symbiosis, on the fitness of gene disruptions (Wheatley et al. 2017). Similarly, a Tn*5* 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. 2018a). 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

223 differentiation in the nodule (Arnold et al. 2017). Future use of this technique might prove valuable
224 in the genome-scale elucidation of genes involved at various stages of the rhizobial life-style.
225
226 **LESSONS FROM COMPARATIVE GENOMICS**
227 Genetic variations present in natural rhizobium strains can be powerfully leveraged to
228 understand SNF through the comparison of global genetic features, a process known as
229 comparative genomics. This approach has become increasingly feasible due to the ever-growing
230 number of rhizobial species and strains with whole-genome sequences available through public
231 repositories. These genome sequences have facilitated many high-quality comparative genomic
232 analyses of rhizobia that have contributed to our understanding of symbiosis and plant growth
233 promotion.
234 Multiple comparative genomic studies have led to the perhaps surprising observation that
235 there is significant variation between rhizobia in terms of their genomic adaptations to symbiosis
236 (González et al. 2003, Masson-Boivin et al. 2009, Black et al. 2012). Indeed, while there are many
237 genes that appear specific to symbiotic nitrogen-fixing bacteria (Young et al. 2006, Pini et al.
238 2011), there appears to be no gene unique to all bacteria capable of performing SNF (Amadou et
239 al. 2008). Even the common *nodABC* genes required for Nod factor synthesis are not 100%
240 conserved, as they are absent in certain *Bradyrhizobium* species capable of forming nodules in a
241 Nod factor-independent fashion (Giraud et al. 2007). Other examples of symbiotic genes showing
242 variable presence/absence are accessory *nod* genes involved in determining host specificity, the
243 *fixNOPQ* genes encoding a cytochrome cbb3 oxidase complex, as well as genes encoding
244 polysaccharide biosynthesis and secretion systems (Black et al. 2012, Sugawara et al. 2013, De
245 Meyer et al. 2016). In addition, the operon structure and genomic organization of symbiotic genes

246 within rhizobial genomes differs among species (González et al. 2003, MacLean et al. 2007, Black
247 et al. 2012, De Meyer et al. 2016). Overall, while many of the core symbiotic genes are highly
248 conserved, the observed genomic variation reflects multiple mechanisms and adaptations for
249 establishing an effective symbiosis, as discussed by Masson-Boivin et al. (2009). Such diversity
250 is bound to complicate our understanding of the minimal set of genes necessary for establishing a
251 symbiosis, and it suggests that strategies to optimize symbiotic interactions may be strain specific.
252 Despite the differences in the symbiotic gene clusters of distinct rhizobia, comparative
253 genomics can help identify new symbiotically-relevant genes. A comparative analysis of 163
254 rhizobial genomes identified 184 protein families putatively involved in optimizing the symbiotic
255 process (Seshadri et al. 2015). In another study using a similar comparative genomic approach,
256 139 genes specific to symbiotic rhizobia were identified (Queiroux et al. 2012). Further
257 characterization of 13 of these genes in *S. meliloti* showed that eight were expressed more highly
258 in the nodule than in free-living cells (Queiroux et al. 2012). Additionally, mutation of the one
259 gene chosen for follow-up study was shown to increase competitiveness for nodule occupancy
260 (Queiroux et al. 2012). These results illustrate how comparative genomics coupled with
261 experimental validation is a powerful approach to identify new candidate genes for improving
262 rhizobial symbiotic efficiency.
263 Analysis of genetic variation present within a population (population genetics) has
264 uncovered high genetic variability in rhizobia, which is reflected in the large, open pangenomes of
265 many rhizobial species (González et al. 2010, Galardini et al. 2011, Tian et al. 2012, Galardini et
266 al. 2013, Sugawara et al. 2013, Porter et al. 2017). This is demonstrated in Figure 5, which displays
267 the distribution pattern of 17,494 genes found in at least one of the 20 included strains of *S. meliloti*.
268 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 these relationships 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 (Table 2), 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, Moënne-Loccoz and Weaver 1995a, 1995b, Hu et al. 2007, García-de-los-Santos et al. 2008, Stasiak et al. 2014). Similarly, genome reduction studies have highlighted the importance of megaplasmids and chromids to the symbiotic process, revealing that key symbiotic genes can be located on megaplasmids, that optimal symbiotic performance often requires more than one megaplasmid, and that megaplasmids 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

292 nitrogen fixation and nodulation competitiveness (Pankhurst et al. 1986, Hu et al. 2007), while
293 several large *S. meliloti* accessory plasmids may result in legume-host incompatibility (Crook et
294 al. 2012, Price et al. 2015).

295 A more targeted approach is the production of a library of large-scale deletion mutations
296 that cumulatively remove all of the extra-chromosomal replicon(s) (Table 2). We are aware of this
297 approach being employed with *S. meliloti* and *R. etli* (Landeta et al. 2011, Yurgel et al. 2013a,
298 Milunovic et al. 2014). These libraries have been used to localize and identify novel symbiotic
299 genes, as well as to screen for phenotypes such as carbon metabolic abilities, cytochrome c oxidase
300 activity, soil growth, and osmotolerance (Dominguez-Ferreras et al. 2006, Landeta et al. 2011,
301 Yurgel et al. 2013a, diCenzo et al. 2016a, Zamani et al. 2017).

302 In addition to genome reduction approaches, genome expansion studies have been used to
303 evaluate the genetic basis of symbiosis. The large symbiotic plasmids of several *Rhizobium* spp.
304 and *S. meliloti* have been transferred to related, non-symbiotic organisms such as *Agrobacterium*
305 spp. and *Ensifer adhaerens* (Hooykaas et al. 1982, Wong et al. 1983, Truchet et al. 1984, Hirsch
306 et al. 1984, Hynes et al. 1986, Finan et al. 1986, Martínez et al. 1987, Novikova and Safronova
307 1992, Abe et al. 1998, Rogel et al. 2001, Nakatsukasa et al. 2008). The primary conclusion of these
308 studies is that poor, if any, symbiotic abilities are transferred to the recipient strain. This
309 observation is perhaps surprising considering that natural lateral transfer of symbiotic plasmids
310 and islands between related strains results in a gain of symbiotic abilities (Mozo et al. 1988,
311 Laguerre 1992, Sullivan et al. 1995, Sullivan and Ronson 1998, Pérez Carrascal et al. 2016,
312 Nandasena et al. 2007). However, non-fixing outcomes after inter-species heterologous gene
313 transfer may be due to an incomplete complement of symbiotic functions in the transconjugant
314 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* 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. Similarly, intra-species transfer of symbiotic plasmids in *R. leguminosarum* resulted in increased nitrogen fixation efficiency (Brewin et al. 1980, DeJong

et al. 1981) and competitiveness for nodule occupancy (Brewin et al. 1983). Remarkably, *Mesorhizobium* strains with the same symbiotic island have widely varying symbiotic phenotypes, from non-effective to highly effective, and varying competitiveness for nodule occupancy (Nandasena et al. 2007, Haskett et al. 2016). Transferring the symbiotic islands from three of these strains to a common *Mesorhizobium* strain confirmed that the differences in symbiotic phenotypes were dependent on differences in the genetic backgrounds of the strains (Haskett et al. 2016). Collectively, these studies underscore the importance of functional interactions between symbiotic replicons/islands and other genomic regions in bringing about efficient symbiotic nitrogen fixation.

TRANSCRIPTOME STUDIES OF RHIZOBIA

Transcriptomic studies, in the form of both microarrays and RNA deep-sequencing (RNA-seq) have been used to investigate 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 3 for a detailed list and description of rhizobium transcriptomic studies). Notable 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 3). 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 flavonoid detection (Ampe et al. 2003, Barnett

et al. 2004, Capela et al. 2005, Pérez-Montaño 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-Montaño 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 3). 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

384 metabolic repression during SNF. Transcriptomics has also been used to decipher the role of
385 specific genes, regulators, or metabolic processes in symbiosis (Table 3), such as the genes *fixJ*
386 (Barnett et al. 2004) and *bacA* (Karunakaran et al. 2010), and carbon import (Mitsch et al. 2018).
387 Others have examined the transcriptome differences of bacteroids displaying distinct
388 morphologies (Lamouche et al. 2018). Additionally, researchers have examined how genetic
389 variations in the plant or rhizobium partner influence the nodule transcriptome (Heath et al. 2012,
390 Burghardt et al. 2017), as well as how the bacteroid transcriptome of the same strain differs
391 depending on the plant hosts (Ampe et al. 2003, Karunakaran et al. 2009, Li et al. 2013). These
392 studies highlight how variation in symbiotic compatibility can result in significant transcriptional
393 alterations.

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

PROTEOME STUDIES OF RHIZOBIA

Numerous proteomic studies of rhizobia have been prepared, and a detailed list of these studies are provided in Table 3. 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 3). 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 3), 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).

Nodule-associated rhizobia have also been the subject of proteomic studies (Table 3). 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 implicates 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

428 distinct compartments (Strodtman et al. 2017). Furthermore, nodule proteomics has been used to
429 examine the proteome during field growth conditions (Delmotte et al. 2010), to compare root-
430 nodules and stem-nodules (Delmotte et al. 2014), and to identify NifA-regulated proteins (Salazar
431 et al. 2010). Studies have compared the proteomes of nodules of different maturities/ages
432 (Delmotte et al. 2014, Nambu et al. 2015, Marx et al. 2016), and recently, proteomes of three zones
433 from the same nodules were independently isolated and evaluated (Ogden et al. 2017). These
434 studies have revealed how the proteome changes during symbiotic development, and they
435 complement and extend conclusions generated using transcriptomics. A more detailed review on
436 the use of proteomics to characterize the symbiosis was recently published (Larrainzar and
437 Wienkoop 2017).
438

439 **METABOLITE ANALYSES**

440 Metabolomics, the large-scale study of the metabolites of the cell or its environment, has
441 been used to characterize several rhizobial processes. Several studies have examined how the intra-
442 cellular metabolome of free-living rhizobia differs depending on nutritional or stress conditions,
443 or as a result of genetic manipulations (see Table 3 for a detailed list of relevant studies).
444 Additionally, lipidomics has been used to characterize the membrane-lipid composition and the
445 effects of phosphate or acid stress, as well as to identify proteins involved in membrane-lipid
446 metabolism (Table 3). Studies have also used a fluxomic approach (the study of the rates of
447 reaction fluxes) to characterize central carbon metabolism during growth with either glucose
448 (Fuhrer et al. 2005) or succinate (Terpolilli et al. 2016) as the sole source of carbon.

449 Metabolomics has been employed to characterize the metabolic shifts occurring during
450 SNF (Table 3). Studies have compared the metabolomes of bacteroids and 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 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 rhizobial strain (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 a valuable knowledge base 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 nutritional condition, can simulate how environmental perturbations change the flux distributions,

474 and can predict how deletion of one or more genes influences growth rate and flux distribution. As
475 an example, Figures 7 and S1 display the predicted distribution of flux through the central carbon
476 metabolic pathways of *S. meliloti* when grown with glucose or succinate as a carbon source.
477 Metabolic modelling is a relatively new method in the study of rhizobia. The first rhizobial
478 reconstruction was reported in 2007 for *R. etli* (Resendis-Antonio et al. 2007) followed by several
479 refinements (Resendis-Antonio et al. 2011, 2012). In 2012, the first reconstruction for *S. meliloti*
480 was reported (Zhao et al. 2012). These reconstructions were relatively small and were designed
481 solely for the purpose of representing bacteroid metabolism. Simulations of flux distribution and
482 analyses of genes essentiality during SNF generally displayed good correlation with experimental
483 data (Resendis-Antonio et al. 2007, Zhao et al. 2012). Recently, genome-scale metabolic network
484 reconstructions capable of representing both free-living and symbiotic metabolism were reported
485 for *S. meliloti* (diCenzo et al. 2016a) and *Bradyrhizobium diazoefficiens* (Yang et al. 2017c).
486 Simulations with both of these models were consistent with transcriptomic data that indicate a
487 major metabolic shift and general down-regulation, particularly in biosynthesis of cellular
488 components, during symbiosis compared to free-living bacteria (diCenzo et al. 2016a, Yang et al.
489 2017c). A metabolic reconstruction for the legume *Medicago truncatula* has also been developed
490 (Pfau et al. 2016).
491 We expect that metabolic reconstruction and constraint-based modelling will serve as a
492 useful tool in the field of SNF, and it will provide an *in silico* method for examining the progression
493 of symbiotic development, which can be experimentally difficult to dissect. Results of the
494 simulations may establish novel hypotheses amenable to experimental testing, and they may
495 contribute to the identification of putative targets for biotechnological manipulation and
496 experimental validation. Several methods have been developed 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. 2018a). This process results in the creation of high-quality, context-specific representations of cellular metabolism. However, current incomplete knowledge of the metabolic exchange between the plant and bacterium may still 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). Yet, despite the many genetic and systems-level studies that shed light on this symbiosis, genetically-engineered rhizobial inoculants are not available. 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, 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 yields 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). In strong support of this, it has been observed that the natural transfer of symbiotic islands from inoculant *Mesorhizobium* strains to the native *Mesorhizobium* population often, and rapidly, results in highly competitive strains with poor

nitrogen fixation capabilities (Sullivan et al. 1995, Nandasena et al. 2007). 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. At the same time, studies such as those described in this review provide a framework for targeted attempts at improving the rate of nitrogen fixation and exchange of nitrogen during the symbiosis. Reports over the last three decades support the feasibility of such an approach (Paau 1991, Ramírez et al. 1999, van Dillewijn et al. 2001, Orikasa et al. 2010).

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 rhizobial 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 rhizobial 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 (Jozefkowicz 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

542 that both the environment (Ji et al. 2017) and microbiome (Jozefkowicz et al. 2017) influence the
543 symbiotic properties of rhizobia.

544 A major factor limiting our ability to make the most of SNF in agriculture is the inability
545 of many staple crops, such as the cereals, of entering into a SNF relationship. As such, there is
546 significant interest in engineering synthetic symbioses between these plants and rhizobia, and there
547 have been a few recent reviews on this topic (Beatty and Good 2011, Rogers and Oldroyd 2014,
548 Mus et al. 2016, Burén and Rubio 2018). As one can imagine, engineering new inter-kingdom
549 interactions is daunting (Mus et al. 2016), and will require engineering a complex developmental
550 pathway into the plant genome. Fortunately, legume-rhizobium symbioses make use of the
551 common symbiosis signalling pathway that is conserved in plants (including cereals) that
552 participate in mycorrhizal interactions (Oldroyd 2013). In the short term, however, a simpler
553 option would be to improve the beneficial interaction between free-living "associative" nitrogen-
554 fixing bacteria and these crop plants (Geddes et al. 2015), even if these looser symbiotic
555 arrangements transfer nitrogen to the plant less efficiently (Beatty and Good 2011).

556 An alternative to achieving effective nitrogen fixation in plants is to bypass the bacterial
557 component altogether, and directly engineer plants to perform their own nitrogen fixation. Several
558 reviews and perspective articles discuss this idea and its associated challenges (Beatty and Good
559 2011, Oldroyd and Dixon 2014, Rogers and Oldroyd 2014, Curatti and Rubio 2014). The main
560 objective is to engineer plants that express a functional nitrogenase inside the mitochondrion or
561 plastid, as these organelles may have the necessary conditions to support this oxygen-sensitive
562 enzyme. Several recent advances have been made in this area that moves the community closer to
563 reaching this goal. Using *Saccharomyces cerevisiae* as a model eukaryote, methods have been
564 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). Others have re-wired the transcriptional regulatory network of the *nif* genes (Wang et al. 2013), and have identified 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 a wide 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 4). Going forward, it will be valuable to integratively employ these methods in orchestrated multi-disciplinary 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,

588 tools and experimental settings for the fast analysis of host preference in a GxExMxR interaction
589 framework are necessary, such as the recently described select and re-sequence approach
590 (Burghardt et al. 2018). The development of additional tools and their application to studying SNF
591 are a necessity to develop the required knowledge to be able to effectively manipulate this process.
592 Our understanding of the intricacies of SNF has rapidly advanced over the last few decades,
593 and the emergence of synthetic biology in recent years presents an unprecedented opportunity to
594 engineer improved rhizobia and SNF relationships. However, despite the progress reviewed here,
595 there is still a long road ahead to achieve the ambitious goal of completely replacing nitrogen
596 fertilizer with BNF, and we should continue to balance discovery-oriented and applied research
597 efforts.
598

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606

607 **SUPPLEMENTARY FILE DESCRIPTIONS**

608 **File S1.** Contains Figure S1, and the methods for generating the data underlying Figures 1, 5, 7,
609 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. 2018a). 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 the manuscript.

File S2a. An enlarged, high quality version of the phylogeny provided in Figure 1, including taxa names.

File S2b. The phylogeny of Figure 1 in Newick format, including bootstrap values and branch lengths.

File S2c. The annotation file for annotating the phylogeny of File S2b.

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FIGURE LEGENDS

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 enlarged phylogeny, including taxa names and bootstrap values, is provided as File S2. See the Methods in File S1 for details on phylogeny construction and gene identification (diCenzo et al. 2017c, diCenzo et al. 2018b).

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. Inoculant strains are typically grown at fermenter scale and used to coat seeds of compatible legumes. In the soil, the rhizobia must compete with the indigenous microbes and initiate symbiosis with an exchange of specific nodulation signals. Curling of a host root hair traps a rhizobium cell, allowing colonization to proceed in a growing infection thread that progresses inward to the cortical cells. Here, the rhizobia are released into the cytoplasm of specialized nodule cells, where they are 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 an indeterminate-type nodule are shown (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. We note that this figure shows an indeterminate nodule, that many of the features shown in this image are not universal, and that many types of nodulation exist; see Masson-Boivin et al. (2009) for a description of the differences.

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. 2018a) 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 was determined using Roary (Page et al. 2015). Strains possess a median of 6,556 genes per genome, with a total of 17,494 sets of orthologous proteins, 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 a unique pattern, is shown. Data were 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. 2018a). 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.

Table 1. Tools for functional genomics and genome manipulation of rhizobia, and rhizobium biosensor and reporter strains.

Functional genomic tools and resources		
Tool	**Description**	**References**
Marker-less gene deletion	Sample systems for the construction of marker-less gene deletions in diverse rhizobia	(Quandt and Hynes 1993, Ledermann et al. 2016)
Site-specific DNA integration	Site-specific gene integration in a neutral location	(Arango Pinedo and Gage 2009)
Cloning-free genome engineering	Cloning-free method for DNA insertion and deletion	(Döhlemann et al. 2016)
S. meliloti ORFeome	Plasmid-based ORF library representing all *S. meliloti* Rm1021 open reading frames	(Schroeder et al. 2005)
S. meliloti fusion libraries	Libraries of *S. meliloti* strains each with an integrated or replicative transcriptional reporter plasmid	(Cowie et al. 2006, Mauchline et al. 2006)
IVET* libraries	Libraries to identify expressed promoters in specific conditions (nodule, rhizosphere), in *S. meliloti*, and *R. leguminosarum*	(Oke and Long 1999, Zhang and Cheng 2006)
STM† libraries	Signature-tagged mutageneses libraries of *S. meliloti*, *S. fredii*, *R. leguminosarum*, and *M. loti*	(Pobigaylo et al. 2006, Borjigin et al. 2011, Garcia-Fraile et al. 2015, Wang et al. 2016)
Tn-seq / INseq transposons	Mariner and Tn*5* transposons for Tn-seq / INseq analyses	(Perry and Yost 2014, Arnold et al. 2017, diCenzo et al. 2018a)
Site-specific recombinases	Systems for using site specific recombinases (Flp/*FRT*, Cre/*lox*, ΦC31 integrase/*attB*/*attP*, lambda/*attB/attP*) for large-scale genome deletions, *in vivo* cloning, and gene insertion	(Chain et al. 2000, House et al. 2004, Landeta et al. 2011, Harrison et al. 2011, Heil et al. 2012, diCenzo et al. 2013, Yurgel et al. 2013a, Milunovic et al. 2014, diCenzo et al. 2016b, Döhlemann et al. 2016, diCenzo and Finan 2018)
Biosensors and reporter strains		
Description		**Reference**
Biosensors for measuring ecotoxicity and heavy metal presence based on constitutive *lux* expression and cell viability		(Paton et al. 1997, Chaudri 2000)
Biosensor for galactosides, based on promoter fusion to *gfp*		(Bringhurst et al. 2001)
Biosensors for sugars, dicarboxylates, and polyols based on the use of solute binding proteins of transporters and FRET¥		(Bourdès et al. 2012)
Biosensors for the *in vivo* mapping of the distribution of root exudate metabolites based on promoter fusions to *lux*		(Pini et al. 2017)
S. meliloti reporter strain to identify the stage of symbiosis in which plant mutants are blocked		(Lang et al. 2018)

* *In vivo* expression technology

† Signature-tagged mutagenesis

¥ Förster resonance energy transfer

Table 2. Studies reporting synthetic, large-scale reductions of the genomes of rhizobia

Organism	Description	References
S. meliloti Rm1021 and RmP110	Libraries of large-scale deletion mutants of the pSymA megaplasmid and pSymB chromid	(Charles and Finan 1991, Yurgel et al. 2013a, Milunovic et al. 2014)
S. meliloti Rm2011	Complete removal of one or both of the pSymA megaplasmid and pSymB chromid	(Oresnik et al. 2000, diCenzo et al. 2014)
S. meliloti Rm2011	Deletions of the pSymA megaplasmid	(Hynes et al. 1989)
S. meliloti MV11	Complete removal of four cryptic plasmids	(Hynes et al. 1989)
S. meliloti various	Complete removal large host-range determining plasmids	(Crook et al. 2012)
S. fredii NGR234	Complete removal of the symbiotic megaplasmid (pNGR234a)	(Morrison et al. 1983)
S. fredii USDA 206	Complete removal of one to three non-symbiotic plasmids	(Mathis et al. 1985, Barbour and Elkan 1989)
S. fredii GR64	Complete removal of one or both of the (mega)-plasmids, including the symbiotic plasmid	(Cervantes et al. 2011)
R. leguminosarum various	Complete removal or large deletions of many (mega)-plasmids, including symbiotic plasmids	(Hynes et al. 1989)
R. leguminosarum VF39	Complete removal of each of the six (mega)-plasmids	(Hynes and McGregor 1990)
R. leguminosarum bv. *trifolii* various	Complete removal of two to four of the (mega)-plasmids	(Baldani et al. 1992)
R. leguminosarum bv. *trifolii* W14-2	Complete removal of each of the four (mega)-plasmids and (mega)-plasmid combinations, including preparing a strain lacking plasmids	(Moënne-Loccoz et al. 1995)
R. leguminosarum bv. *trifolii* TA1	Complete removal of two megaplasmids, and deletions of the symbiotic megaplasmid	(Stasiak et al. 2014)
R. etli CFN42	Complete removal of five of the six (mega)-plasmids, deletions in the sixth, and combinations with up to five (mega)-plasmids removed in one strain	(Brom et al. 1992, 2000)
R. etli CFN42	Library of large-scale deletion mutants of the p42e chromid	(Landeta et al. 2011)
R. etli CFN42	Deletion of the symbiotic region of the symbiotic megaplasmid	(Romero et al. 1991)
R. tropici CIAT 899	Complete removal of three (mega)-plasmids including the symbiotic megaplasmid, with one to three plasmids removed per strain	(Barreto et al. 2012)
R. sp. P1	Complete removal of the symbiosis megaplasmid	(Sharma and Laxminarayana 1989)
M. loti NZP2213, NZP2037	Complete removal of the large non-symbiotic plasmid	(Chua et al. 1985)
M. loti R7A	Deletion of the large chromosomal symbiotic island	(Ramsay et al. 2006)
M. haukauii HN308SR, 7653R	Complete removal of plasmids	(Hu et al. 2008)

Table 3. Studies of rhizobial species employing -omics technologies.

Topic of the study	Organisms	Approaches	References
Characterization of proteins			
SyrM (Nod factor synthesis)	*S. meliloti*	Transcriptomics	(Barnett and Long 2015)
NodD (Nod factor synthesis)	*S. meliloti*	Transcriptomics	(Capela et al. 2005)
NolR (Nod factor synthesis)	*S. meliloti*	Proteomics	(Chen et al. 2000a, 2005)
FixL/FixJ/FixK (nitrogen fixation)	*S. meliloti*; *B. diazoefficiens*	Transcriptomics	(Bobik et al. 2006, Mesa et al. 2008)
CbrA (cell cycle and symbiosis)	*S. meliloti*	Transcriptomics	(Gibson et al. 2007)
CtrA (cell cycle)	*S. meliloti*	Transcriptomics	(Pini et al. 2015)
RosR (exopolysaccharide synthesis)	*R. leguminosarum* bv. *trifolii*	Transcriptomics	(Rachwał et al. 2015)
FeuP/FeuQ (osmoadaptation and symbiosis)	*S. meliloti*	Transcriptomics	(Griffitts et al. 2008)
ECF sigma factors (stress response and symbiosis)	*R. etli*; *B. diazoefficiens*	Transcriptomics	(Gourion et al. 2009, Martínez-Salazar et al. 2009, Stockwell et al. 2012)
Clr (cAMP dependent regulator, nodulation)	*S. meliloti*	Transcriptomics	(Krol et al. 2016, Zou et al. 2017)
PraR (quorum sensing and biofilms)	*R. leguminosarum* bv. *viciae*	Transcriptomics	(Frederix et al. 2014)
PckR (central carbon metabolism)	*S. meliloti*	Transcriptomics	(diCenzo et al. 2017b)
PhoB (phosphate limitation response)	*S. meliloti*	Transcriptomics	(Krol and Becker 2004, Yuan et al. 2006)
GlnD/GlnBK (nitrogen limitation response)	*S. meliloti*	Transcriptomics	(Yurgel et al. 2013b)
NtrY/NtrX (nitrogen metabolism)	*S. meliloti*	Transcriptomics	(Calatrava-Morales et al. 2017)
Tkt2/Tal (central carbon metabolism)	*S. meliloti*	Proteomics; metabolomics	(Hawkins et al. 2018)
OxyR (oxidative stress)	*S. meliloti*	Transcriptomics	(Lehman and Long 2018)
RelA/Rsh (stringent response)	*S. meliloti*; *R. etli*	Transcriptomics	(Vercruysse et al. 2011, Krol and Becker 2011)
RirA (iron limitation)	*S. meliloti*; *R. leguminosarum* bv. *viciae*	Transcriptomics; proteomics	(Todd et al. 2005, Chao et al. 2005)
LeuB (leucine biosynthesis)	*S. meliloti*	Metabolomics	(Barsch et al. 2004)
AniA (PHB accumulation)	*R. etli*	Proteomics	(Encarnación et al. 2002)
FadD (Fatty acid metabolism)	*S. meliloti*	Lipid analysis	(Nogales et al. 2010)
PHB cycle mutations	*S. meliloti*	Transcriptomics; proteomics	(Wang et al. 2007, D'Alessio et al. 2017)
Dme/Tme (Malic enzymes)	*S. meliloti*	Transcriptomics; metabolomics	(Zhang et al. 2016)
Membrane lipid biosynthetic proteins (Cfa, PlsC, PlcP, Pcs, Pmt, OlsA, OlsB, PssA, Psd, SqdB, BtaA)	*S. meliloti*	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)	*S. meliloti*	Transcriptomics	(Ulvé et al. 2007, Schlüter et al. 2010)
Hfq (RNA binding protein)	*S. meliloti*	Transcriptomics; proteomics	(Barra-Bily et al. 2010, Torres-Quesada et al. 2010, Gao et al. 2010, Sobrero et al. 2012)
Hfq binding RNAs	*S. meliloti*	Transcriptomics	(Torres-Quesada et al. 2014)
YbeY (endoribonuclease)	*S. meliloti*	Transcriptomics	(Saramago et al. 2017)
AbrC1/AbrC2 (sRNAs, nutrient uptake)	*S. meliloti*	Proteomics	(Torres-Quesada et al. 2013)
MmgR (sRNA, PHB accumulation)	*S. meliloti*	Proteomics	(Lagares et al. 2017)
Environmental adaptation and stresses			
Growth in root hairs	*B. diazoefficiens*	Metabolomics	(Brechenmacher et al. 2010)
Oxic versus microoxic growth	*S. meliloti; B. diazoefficiens*	Transcriptomics	(Ampe et al. 2003, Becker et al. 2004, Bobik et al. 2006, Pessi et al. 2007)

Aerobic, micro-aerobic, anaerobic growth	*B. diazoefficiens*	Proteomics	(Dainese-Hatt et al. 1999)
Rhizosphere adaptation	*R. leguminosarum bv. viciae*	Transcriptomics	(Ramachandran et al. 2011)
Growth in presence of root exudates	*B. diazoefficiens; B. phymatum; C. taiwanensis; R. mesoamericanum*	Transcriptomics	(Liu et al. 2017b, Klonowska et al. 2018)
Seed endophytic growth	*R. phaseoli*; *S. americanum*	Proteomics	(Peralta et al. 2016)
Stationary phase adaptation	*S. meliloti; R. etli*	Proteomics	(Guerreiro et al. 1999, Meneses et al. 2010)
Phosphate limitation	*S. meliloti*	Lipidomics and lipid analysis	(Basconcillo et al. 2009a, 2009b, 2009c, Zavaleta-Pastor et al. 2010)
Biotin limitation	*S. meliloti*	Proteomics	(Heinz and Streit 2003)
Carbon limitation	*S. meliloti*	Proteomics	(Djordjevic et al. 2007)
Nitrogen limitation	*S. meliloti*	Proteomics	(Djordjevic et al. 2007)
Osmotic/salt stress	*S. meliloti*; *M. loti*; *M. alhagi*; *R. etli*; *S.* sp. BL3; *B. spp*.	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	*B. diazoefficiens*	Transcriptomics	(Donati et al. 2011, Jeon et al. 2011)
Acid stress	*S. meliloti*; *M. loti*; *S. medicae*	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	*M. loti*; *R. tropici*; *B. diazoefficiens*	Transcriptomics; proteomics	(Münchbach et al. 1999, Gomes et al. 2012a, Alexandre et al. 2014)
Cold stress	*M.* sp. N33	Transcriptomics; metabolomics	(Ghobakhlou et al. 2013, 2015)
Heavy metal stress	*S. meliloti*; *M. metallidurans*; *M.* sp. STM 4661; *R.* sp. VMA301; *R.* sp. E20-8	Transcriptomics; proteomics; metabolomics	(Mandal et al. 2009, Maynaud et al. 2013, Lu et al. 2017, Cardoso et al. 2017b)
Dessication stress	*B. diazoefficiens*	Transcriptomics	(Cytryn et al. 2007)
Herbicide exposure	*R. leguminosarum bv. viciae*	Metabolomics	(Bhat et al. 2014)
Cellular processes			
Flavanoid perception	*S. meliloti*; *S. fredii*; *R. tropici*; *R. leguminosarum* bv. *trifolii*; *R. leguminosarum* bv. *viciae*; *R. etli*; *B. liaoningense*; *B. japonicum*; *B. diazoefficiens*	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-Montaño et al. 2016a, 2016b, Meneses et al. 2017)
Nod factor production	*S. arboris*	Metabolite analysis	(Penttinen et al. 2013)
Cell cycle	*S. meliloti*	Transcriptomics	(De Nisco et al. 2014, Pini et al. 2015)
Response to NCR peptides	*S. meliloti*	Transcriptomics	(Tiricz et al. 2013, Penterman et al. 2014)
Secretome / Type III secretion	*B. diazoefficiens*; *S. fredii*; *B. elkanii*	Proteomics	(Saad et al. 2005, Rodrigues et al. 2007, Hempel et al. 2009, Okazaki et al. 2009)
Biofilm formation	*R. etli*	Transcriptomics	(Reyes-Pérez et al. 2016)
Quorum sensing	*S. fredii*; *S. meliloti*; *R. leguminosarum* bv. *viciae*	Transcriptomics; proteomics	(Chen et al. 2003, Gao et al. 2005, Cantero et al. 2006, Gao et al. 2007, Krysciak et al. 2014)
Motility	*S. meliloti*	Transcriptomics	(Nogales et al. 2010)
Aerobic vs fermentative growth	*R. etli*	Proteomics	(Encarnación et al. 2003)
Lectin perception	*B. diazoefficiens*	Proteomics	(Pérez-Giménez et al. 2012)
Catabolism of phenanthrene	*S.* sp. C4	Metabolomics	(Keum et al. 2008)
Symbiotic island transfer	*M. ciceri*	Transcriptomics	(Haskett et al. 2018)
Nodule analyses			
Integrated plant + bacterium analyses	*S. meliloti* - *M. truncatula*; *S. meliloti* - *M. alba*; *S. medicae* - *M. truncatula*; *R. leguminosarum* bv. *viciae* - *P. sativum*	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	*S. meliloti* - *M. truncatula*; *S. meliloti* - *M. sativa*; *S. meliloti* - *M. alba*; *R. leguminosarum* bv. *viciae* - *P. sativum*; *R. leguminosarum* bv. *viciae* - *V. cracca;*	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. 2007, Karunakaran et al. 2009, Resendis-Antonio et al. 2012, Vauclare et al. 2013, Tatsukami et al. 2013)

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

Table 4. 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.

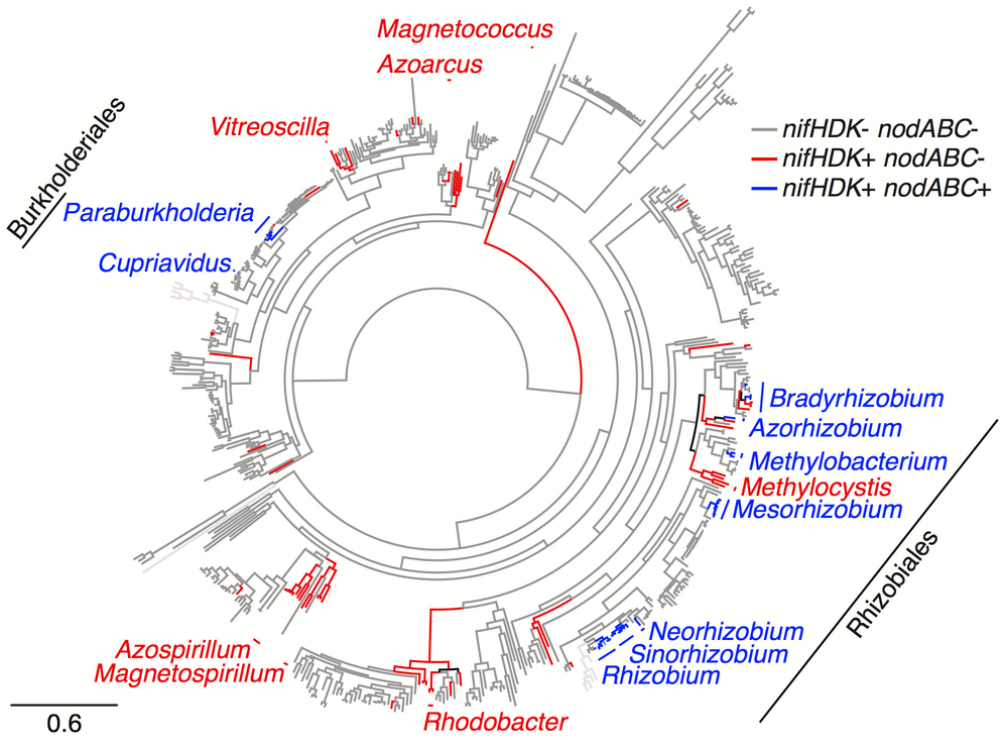

Figure 1. Phylogenetic distribution of biological nitrogen fixation. A maximum likelihood phylogeny of the α- and β-proteobacteria based on 23 highly conserved proteins (Frr, NusA, RplB, RplC, RplD, RplK, RplL, RplM, RplN, RplP, RplS, RplT, RpmA, RpoB, RpsB, RpsC, RpsE, RpsI, RpsJ, RpsK, RpsM, RpsS, 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 (diCenzo et al. 2017c, diCenzo et al. 2018b).

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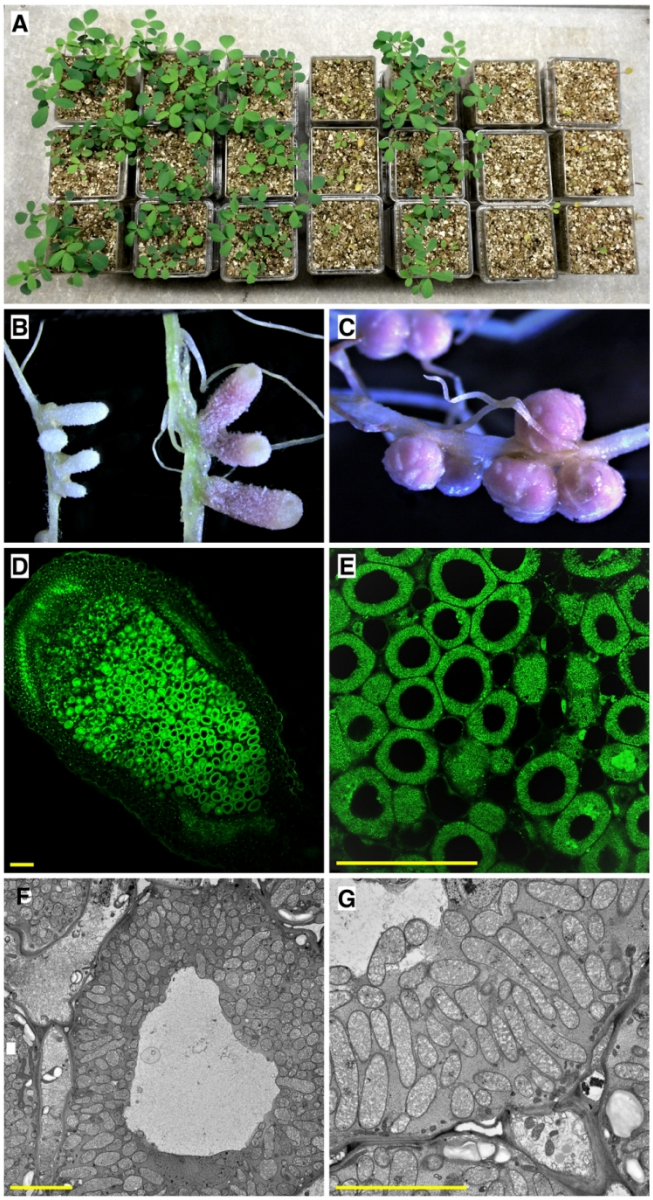

Figure 2. Visualizing the rhizobium – legume symbiosis. (A) A picture of Medicago alba (white sweet clover) plants grown in nitrogen free conditions. Plants in columns 1-3 and 5 are inoculated with nitrogen-fixing S. meliloti; plants in columns 4 and 6 are inoculated with a S. meliloti mutant unable 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.

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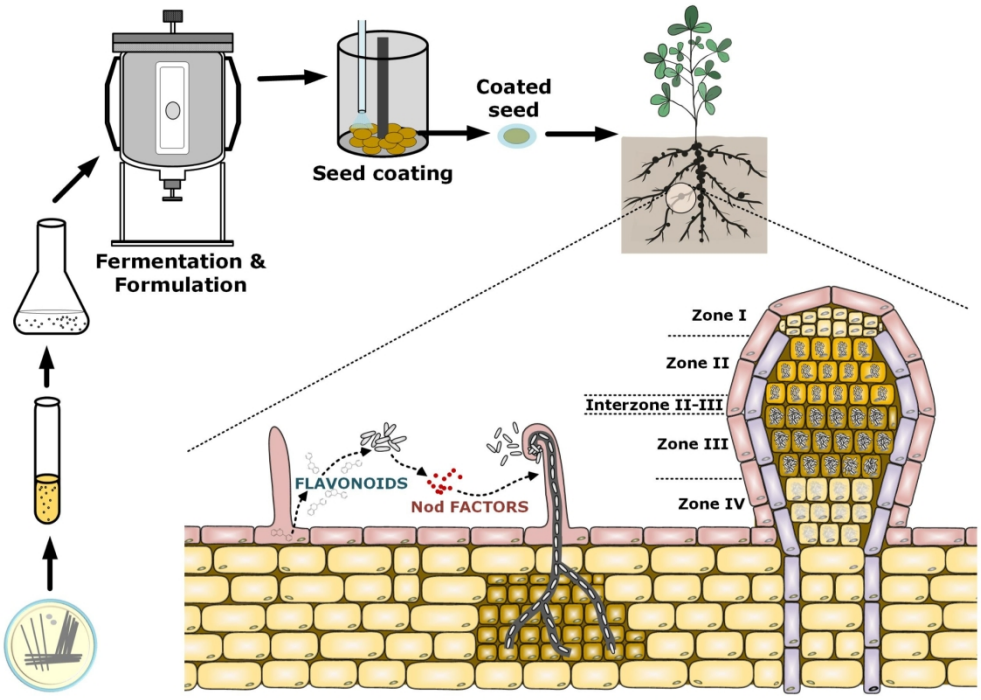

Figure 3. Schematic representation of the life-cycle of rhizobia. For inoculants, the life-cycle begins in an industrial environment, where the strain is grown in a fermenter. Inoculants are transported to the agricultural field, possibly as a seed coating. In the soil, the rhizobia must exploit nutrients, 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 they 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. This figure displays an indeterminate root nodule, formed through a Nod factor dependent mechanism and with infection proceeding through an infection thread. However, we note that none of these features are universal and that many types of nodulation exist; see Masson-Boivin et al. (2009) for a description of the differences.

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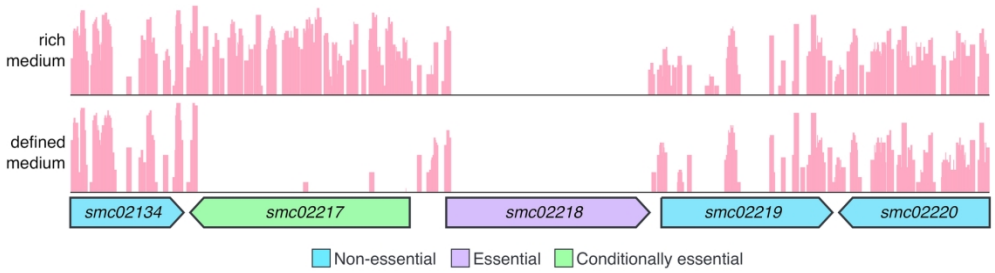

Figure 4. Example output of a Tn-seq study. A S. meliloti gene region illustrating the ability of Tn-seq to identify essential genes and environment-specific essential genes is shown. Published Tn-seq data (diCenzo et al. 2018a) for S. meliloti grown in a nutritionally complex medium (rich medium) and 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.

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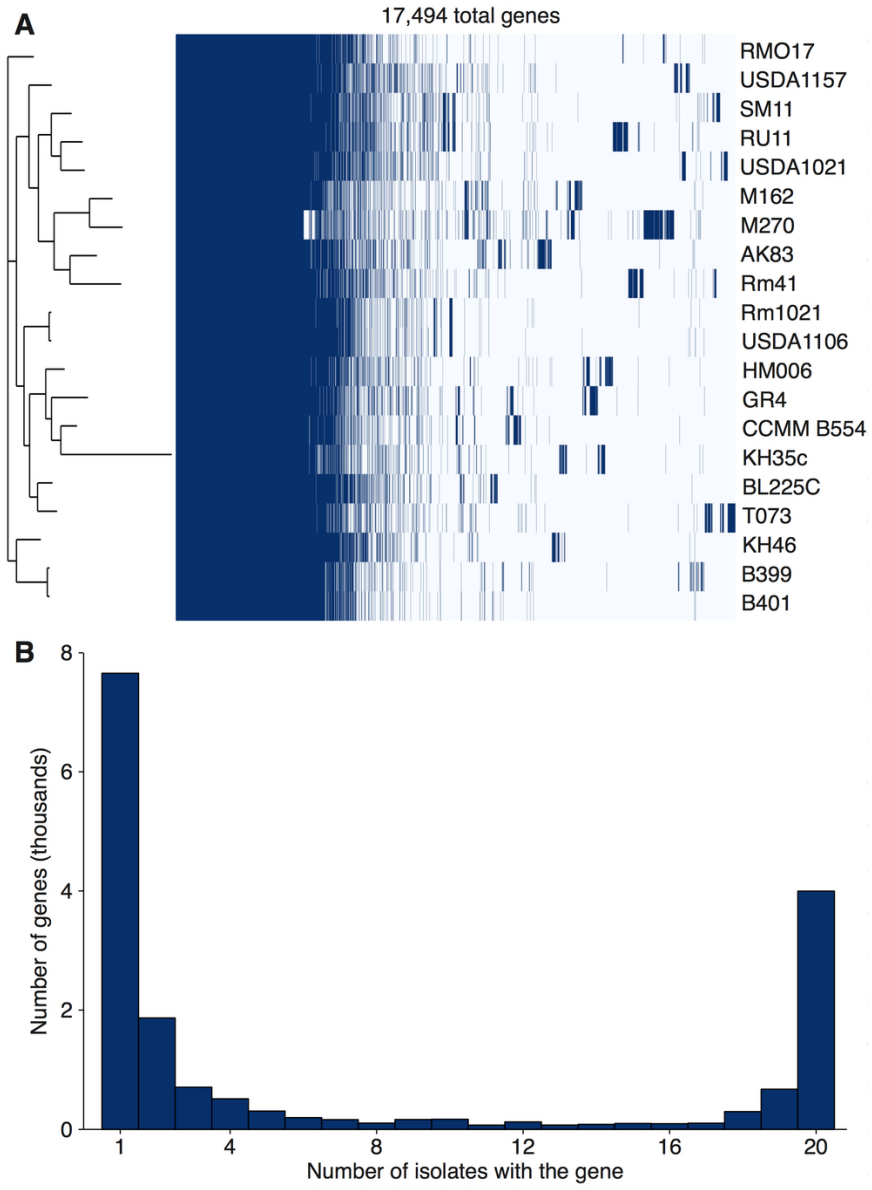

Figure 5. The S. meliloti pangenome. The pangenome of 20 S. meliloti strains with complete genomes was determined using Roary (Page et al. 2015). There was a median of 6,556 genes (standard deviation 362) per genome, and a total of 17,494 sets of orthologous proteins were detected of which 4,000 were 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.

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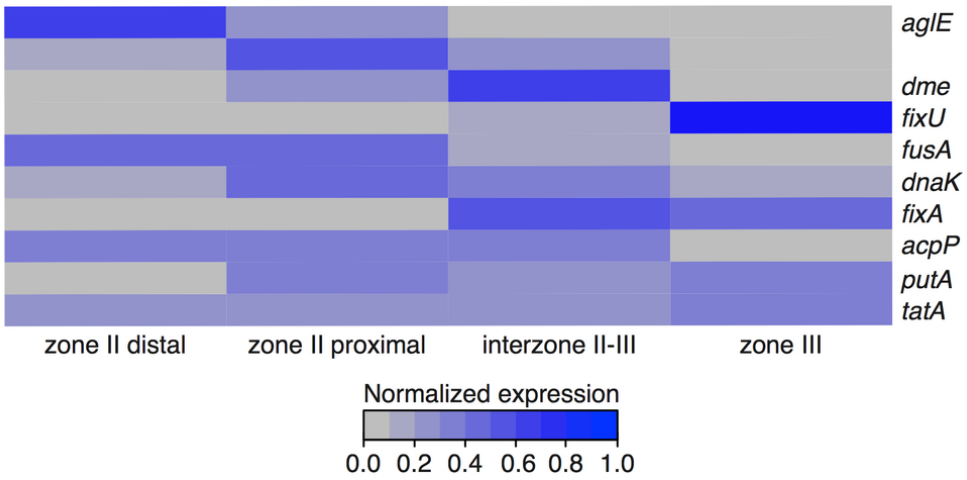

Figure 6. Expression patterns of select S. meliloti genes during symbiosis with M. truncatula. The spatial expression patterns across a M. truncatula nodule of 10 S. meliloti genes, each displaying a unique pattern, is shown. Data were taken from the study of Roux et al. (Roux et al. 2014). The nodule 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.

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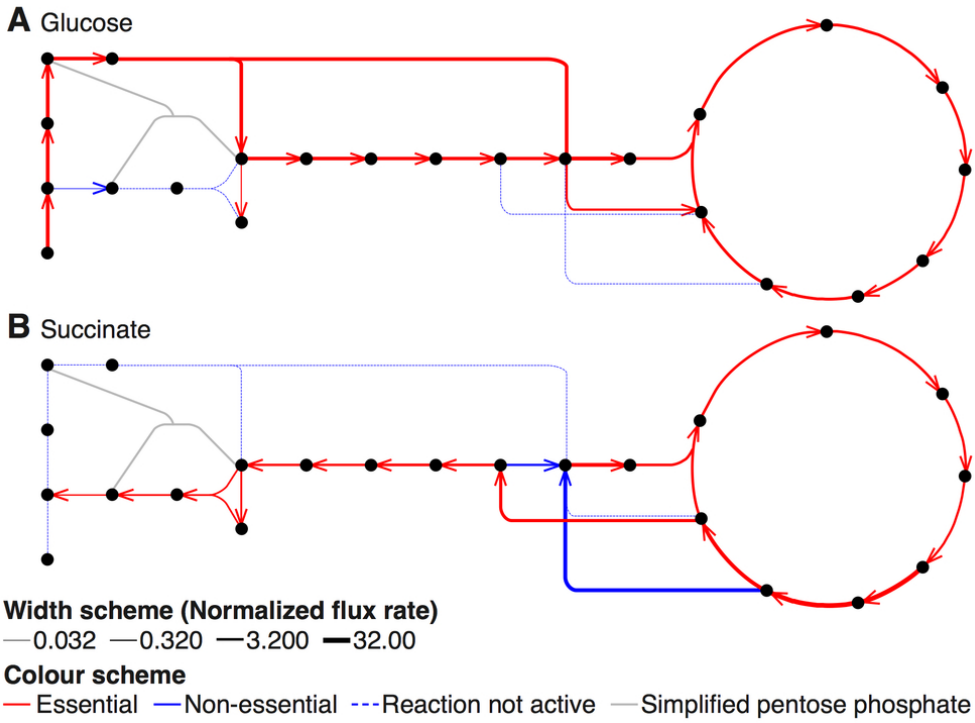

Figure 7. In silico predicted flux distributions and reaction essentialities. Example data obtained through metabolic modelling. Results from in silico metabolic modelling of S. meliloti metabolism using the iGD726 metabolic reconstruction are shown (diCenzo et al. 2018a). Growth was simulated using (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.

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