How rhizobia adapt to the nodule environment

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Keywords: rhizobium-legume symbiosis, nitrogen fixation, root nodule, microaerobiosis, growth arrest, metabolism, modelling, host sanctioning

Running title: Physiological changes in bacteroids

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Abstract

Rhizobia are a phylogenetically diverse group of soil bacteria that engage in mutualistic interactions with legume plants. Although specifics of the symbioses differ between strains and plants, all symbioses ultimately result in the formation of specialized root nodule organs which host the nitrogen-fixing microsymbionts called bacteroids. Inside nodules, bacteroids encounter unique conditions that necessitate global reprogramming of physiological processes and rerouting of their metabolism. Decades of research have addressed these questions using genetics, omics approaches, and more recently computational modelling. Here we discuss the common adaptations of rhizobia to the nodule environment that define the core principles of bacteroid functioning. All bacteroids are growth-arrested and perform energy-intensive nitrogen fixation fueled by plant-provided C_4-dicarboxylates at nanomolar oxygen levels. At the same time, bacteroids are subject to host control and sanctioning that ultimately determine their fitness and have fundamental importance for the evolution of a stable mutualistic relationship.

Infection process

Plants are dependent on nutrients such as phosphorus, nitrogen, and sulfur to be present in a bioavailable form, which often limits plant growth. To facilitate nutrient uptake, mutualistic symbioses between plants and root-associated microorganisms have evolved. The symbiosis with arbuscular mycorrhiza (AM) originates back to the Devonian period more than 400 million years ago (1). The same signaling pathway that facilitated AM symbioses has since been adopted for other symbionts and is shared amongst all intracellular root symbionts (2, 3). This includes various root nodule symbioses (4, 5) that evolved around 110 million years ago (6), where the microsymbionts are either actinomycetes or rhizobia. These diazotrophic bacteria fix atmospheric N_2 gas into bioavailable ammonia. Biological nitrogen fixation accounts for approximately 50% of the bioavailable nitrogen on earth (7) and is of great importance for sustainable agriculture and protection of susceptible ecosystems from leached fertilizers (8, 9).

The lack of genetic tools for the microsymbiont has hampered the investigation of actinorhizal symbioses (10), making rhizobium-legume symbioses the most researched and best understood interactions so far (11). The symbioses between rhizobia (belonging to α- and β-proteobacteria (12)) and legumes (Fabaceae) exist with a variety of different infection mechanisms and nodule morphologies (13-15). Although bacterial entry via cracks in the root epidermis and Nod factor-independent infection processes exist (16-19), the best-studied mechanism is root invasion through root hairs via infection threads (ITs). Most major crop legumes and many model plants such as *Medicago* spp., *Pisum sativum* (pea), *Lotus japonicus*, *Phaseolus vulgaris* (bean), and *Glycine max* (soybean) follow this type of infection (20-26). Soil-dwelling rhizobia sense flavonoids exuded by the plant (27) and compatible flavonoids trigger the production of Nod factors (lipochito-oligosaccharides; for reviews see (28, 29)). Nod-factor signaling leads to the induction of nodule primordia in the root cortex and causes the root hairs to curl their tips, resulting in the characteristic shape called 'shepherd's crook'. Bacteria attached to root hairs may be entrapped by this curling and form a microcolony from which they enter the root hair via an IT that is formed by inverted growth of

77 the plant cell wall and membrane. Since the microcolony is derived from one or very few founder
78 cells, ITs are a major bottleneck for bacteria during invasion (30, 31).

79 Inside ITs, bacteria are thought to move mainly by cell division and potentially some gliding motility
80 (32, 33). Eventually, the ITs reach the preformed nodule primordia and rhizobia are taken up into
81 individual plant cells by endocytosis, enclosed by a symbiosome membrane of plant origin. There
82 they multiply until the plant cell is densely packed with bacteria, which differentiate into their
83 nitrogen-fixing form called bacteroids. Nodules usually have one of two distinctive morphologies,
84 depending on the host plant (34, 35): (i) determinate nodules, lacking a persistent meristem,
85 therefore ceasing to grow at some point and forming spherical nodules or (ii) indeterminate nodules
86 with a persistent meristem, resulting in indefinite growth, branching or lobe formation of the nodule,
87 giving rise to elongated or irregular shapes. The detailed infection process and differences between
88 determinate and indeterminate nodules have been extensively discussed elsewhere (36-39).

89 **Competitiveness**

90 Host plants can influence the composition of the root microbiome (40, 41), but rhizobia must
91 compete with various other bacteria for root colonization and with other compatible rhizobial strains
92 for nodulation. Competitiveness is a complex trait: different abiotic factors such as soil pH (42-44) or
93 nutrient availability (45) have an impact on the outcome of symbioses. Biotic factors also affect
94 competitiveness, for example the host-symbiont pairing (46-48), plant- or strain-intrinsic factors such
95 as type VI secretion systems (49, 50), exopolysaccharide production (51), and catabolic capacity in
96 rhizobia for various substrates such as *myo*-inositol (52-54), glycerol (55), arabinose, protocatechuate
97 (56), rhamnose (57), homoserine (58), or erythritol (59). During the transition from a soil-dwelling
98 bacterium to a nitrogen-fixing bacteroid, rhizobia encounter drastically changing conditions, but it
99 has not always been addressed at which stage of symbiosis formation a mutant strain is affected.
100 Competitiveness in the rhizosphere is largely determined by metabolic functions and motility (48).
101 Transcriptomics and mutant studies have shown that *Rhizobium leguminosarum* depends on a
102 variety of substrates such as C_4-dicarboxylates, C_2-organic acids, and aromatic compounds for growth

in the rhizosphere. However, sugar and sugar alcohol transport systems were similarly induced, showing that metabolism in the rhizosphere is highly complex (60). It is well documented that strains and mutants can be competitive in rhizosphere colonization but nonetheless lack competitiveness for nodulation (52, 61, 62), highlighting the need for changing adaptations throughout the infection process.

INSeq (insertion sequencing) experiments using saturated random transposon mutant libraries identified essential genes in *R. leguminosarum* during four distinct stages of the symbiosis: rhizosphere growth, root attachment, nodule bacteria (viable bacteria within nodules), and differentiated bacteroids (63). In these experiments, bacteria were under selective pressure by pea plants, and mutants lacking competitiveness were lost at the affected and subsequent stages. Interestingly, while several genes were associated with competitiveness in the rhizosphere and root attachment, they were not necessarily involved in competitive nodulation, further indicating that persistence on the root surface is a distinct trait. In total, 390 genes were associated with competitive nodulation. Among these, 146 were already needed in the rhizosphere, 33 in the root-attached stage, and 211 in ITs and pre-differentiated bacteroids. Functions related to competitive nodulation from the rhizosphere onwards include LPS and EPS biosynthesis, the NtrBC system and the σ^{54} factor RpoN, and the stringent response. LPS and EPS modifications have a known role in evading the plant immune response during IT formation (64-70) and the stringent response has been shown to be involved at multiple stages during the *Sinorhizobium-Medicago* symbiosis (71-73). During later infection stages, allophanate, aldehyde, erythritol metabolism, and glycogen synthesis play important roles. Interestingly, in agreement with previous screens (48), almost all mutations causing auxotrophies lacked competitiveness, indicating that *de novo* synthesis of essential building blocks is crucial even when they are exogenously present in root exudates (74, 75).

The large number of genes associated with nodulation found outside the symbiotic plasmids in *R. leguminosarum* (63) is consistent with the findings of the chimeric *Ralstonia solanacearum*/*Cupriavidus taiwanensis-Mimosa pudica* experimental evolution of a symbiosis (76). Although the initial chimera was unable to form nodules, nodulation was achieved and

competitiveness gradually increased with repeated inoculation cycles on *M. pudica* seedlings. Strikingly, the ability to nodulate was directly linked with competitiveness (77). After initial infection was achieved by loss of pathogenicity, a major improvement in competitiveness was seen when additional mutations in global transcriptional regulators enhanced intracellular colonization and persistence (78, 79), further highlighting that the ability to competitively nodulate legume plants heavily relies on global rewiring of the bacterial gene expression outside the acquired classical symbiosis genes (*nod*, *nif*, *fix* etc.) found on mobile symbiotic plasmids or islands (80-84).

Notably, competitiveness for initial nodulation (but not persistence) is not affected by a strain's efficiency in nitrogen fixation (85). Under field conditions, indigenous rhizobia with inferior fixation capacity may outcompete the "elite" rhizobial strains inoculated on the legume seeds, resulting in suboptimal yields (86, 87). The dependence on environmental parameters and overall complexity based on symbiotic and general-function genes (88) makes competitive strains with high nitrogen fixation efficiency difficult to identify. A recently published barcode and P_{nifH}-driven sfGFP-based toolkit allows for simultaneous high-throughput analysis of a strain's competitiveness and nitrogen fixation rate (89). It enabled identification of highly effective strains by measuring fixation rates of individual strains in single nodules. This approach facilitates parallelized inoculation of multiple strains and simultaneous assessment of their nodulation competitiveness and nitrogen fixation ability.

Adaptations to nitrogen fixation

Root nodules are *de novo* plant organs that share root- and stem-typical traits (90) and are only formed in the presence of compatible rhizobia. Their specialized function – to host intracellular bacteria for nitrogen fixation – makes the conditions inside nodules unique. Nodules are immunocompromised compartments with an immune response that differs from the rest of the plant, but they are also confined and insulated from the root, allowing them to host large numbers of intracellular bacteria (91). Despite some distinguishing properties specific to different rhizobia and their plant hosts, all rhizobium-legume symbioses share some universal characteristics. With the goal

of highlighting these core changes defining the transition from free-living bacteria to nitrogen-fixing bacteroids, we performed a meta-analysis of 18 transcriptome and one proteome datasets published in 13 different studies on various symbioses (92-104) (**Table 1**). All genes/proteins were classified according to the Clusters of Orthologous Genes (COG) system (105) to enable interspecies comparisons.

Overall, most of the subsystems contained more downregulated than upregulated genes, indicating a reduced physiological complexity in bacteroids (**Fig. 1**). Notable exceptions to the general downregulation were 'energy production and conversion' and 'inorganic ion transport and metabolism' (**Table S1**). Upregulated features in these subsystems comprised nitrogenase components (*nif* genes) and electron transfer proteins (including *fix* genes) such as ferredoxins, which act as electron donors for nitrogenase (106, 107) (**Table S2**). Free-living diazotrophs such as *Klebsiella* regulate *nif* gene expression in response to nitrogen starvation and low oxygen concentration (108), whereas the nitrogen status in many bacteroids plays either no or only a minor role in *nif* gene activation, which is mainly induced by low oxygen concentrations (see **Adaptations to microaerobic conditions**). All rhizobia regulate *nif* and *fix* gene expression using a similar set of oxygen-responsive pathways (FixLJ or hFixL FxkR, FixK/Fnr, and NifA). The pathways differ in their sensitivity to oxygen (109), leading to a staggered activation and thus gradual adaptation of the invading rhizobia to the microoxic nodule environment (110). However, each species uses them in a slightly different way and the interconnections between the pathways differ as well, a topic that has been discussed elsewhere (111-115). In addition to *nif* genes, expression of iron and/or molybdenum transporters was increased across the various datasets, consistent with the requirement for these metals as cofactors of the nitrogenase enzyme (116).

A general upregulation was further found for chaperones, indicating a response to stress factors in nodules and re-structuring of the bacteroid proteome. This agrees with the established importance of chaperones in bacteroids. Nodules formed by a *clpB* mutant of *Mesorhizobium ciceri* contained only few bacteroids (117) and GroESL chaperones were found to be essential for symbiosis (118, 119) and to interact with nodule-specific cysteine-rich (NCR) peptides (120) (see **Adaptations to a non-**

growing state). Further examples of stress responses include glutathione *S*-transferases, which were consistently upregulated in bacteroids. Mutants unable to produce glutathione, an important antioxidant molecule, showed reduced symbiotic efficiency (121, 122). Similarly, enzymes involved in detoxification of reactive oxygen species, such as superoxide dismutases and catalases, were commonly upregulated in agreement with the presence of reactive oxygen species due to auto-oxidation of leghemoglobin and ferredoxin (123).

Adaptations to microaerobic conditions

The iron-sulfur clusters of the nitrogenase enzyme are highly susceptible to molecular oxygen (124) and the nodule cortex therefore contains an oxygen diffusion barrier. This limits the amount of free oxygen within the nodule, creating a microoxic environment of around 11 nM free oxygen (112, 125). For comparison, the freely diffused oxygen concentration in water at atmospheric oxygen levels is around 255 µM. Nitrogen fixation is an energy-intensive process, and energy in biological systems is most efficiently generated by respiratory chains with molecular oxygen as the final electron acceptor (126). To circumvent this apparent paradox, where oxygen is needed to energize nitrogen fixation and nitrogenase is sensitive to oxygen, rhizobia possess high-affinity terminal oxidases that allow respiration at low oxygen concentrations. Rhizobia can usually express a complex set of different respiratory oxygen reductases, reflecting their diverse lifestyles (127-130). Biochemical and genetic data show that a cytochrome *c* oxidase, encoded by *fixNOQP*, is a high affinity cbb_3-type oxidase responsible for respiration under microaerobic conditions in rhizobia. Biochemical assays revealed a K_m value of 4-7 nM for oxygen bound to membranes of anaerobically grown bradyrhizobia, corresponding to the cbb_3-type cytochrome oxidase FixNOQP (131). These values are compatible with the low oxygen concentration in nodules. Mutants of *fixNOQP* in *Bradyrhizobium japonicum* had only marginal nitrogenase activity (132, 133), whereas multiple copies of *fixNOQP* are present in the genome of *Mesorhizobium loti*, *Sinorhizobium meliloti*, *Rhizobium etli*, and *R. leguminosarum*. In *S. meliloti* and *R. leguminosarum*, the two copies are functionally redundant, but only one copy is functional during symbiosis in *R. etli* (134-136). Mutation of the (single copy) cbb_3-type oxidase in

Azorhizobium caulinodans resulted in only 50% reduction of nitrogenase activity in symbiosis (137). The remaining activity could be abolished by creating a double mutant with a *bd*-type quinol oxidase. This type of oxidase is crucial for nitrogen fixation in the free-living diazotrophs *Klebsiella oxytoca* (138) and *Azotobacter vinelandii* (139). Recently, genome sequences of β-rhizobia showed that *Paraburkholderia* strains lack *fixNOQP* but contain *bd*-type quinol oxidase genes. However, functional studies are missing (140). In accordance with the described adaptations to the microaerobic nodule environment, several cytochromes and high-affinity cytochrome oxidases showed upregulation in our meta-analysis. Interestingly, subunits of the ATP synthase were mostly downregulated, indicating that the overall energy demand in bacteroids is lower compared to exponentially growing cells.

Nodules have a characteristic red color due to accumulation of leghemoglobins. These plant-derived proteins are found exclusively in the plant cytoplasm and provide buffering capacity for free oxygen as well as facilitating diffusion of oxygen towards symbiosomes (141-144). Mutation or silencing of leghemoglobin biosynthesis resulted in inefficient symbioses (145, 146). The free oxygen concentration was however only marginally higher in mutant compared to wild-type nodules. Although there was a slight increase in oxygen available for respiration, the ATP/ADP ratio dropped compared to the wild-type (146). Thus, the main function of leghemoglobins is not to keep free oxygen concentrations low (which is achieved by the oxygen diffusion barrier), but rather to enhance oxygen supply to symbiosomes, which is limiting *because* of the diffusion barrier. In this function, leghemoglobins are equivalent to myoglobins in animals.

Adaptations to a non-growing state

In addition to changes induced by microoxic conditions, bacteroids are characterized by their non-growing state. Growth arrest in free-living bacteria is usually induced by unfavorable conditions, such as starvation, oxygen depletion, or antibiotic substances. Accordingly, the main adaptations to such conditions focus on persistence, i.e. maintaining low levels of proton motive force, synthesizing integral cell building blocks, slowing metabolism, and catabolizing nonessential endogenous compounds, which enables bacteria to stay viable for months or years (147).

Commonly downregulated traits in the analyzed datasets that are shared with growth-arrested cells (92) include translation (ribosomal proteins), cell envelope biogenesis (outer membrane proteins, peptidoglycan and exopolysaccharide synthesis), intracellular trafficking, cell cycle control and DNA replication, signal transduction, motility, and the F0F1-type ATPase (**Table S3**). The ribosomal silencing factor RsfS, which inhibits the assembly of the 30S and 50S ribosomal subunits (148, 149), was generally upregulated, indicating additional downregulation of protein biosynthesis. Furthermore, ClpA, a subunit of the ClpAP protease, known for its role in cell cycle control and stationary phase adaptation (150-152), but also potentially part of FixK regulation (153), was commonly upregulated in bacteroids. These findings suggest that a major adaptation to the nodule environment for bacteroids is related to growth arrest. Strikingly, the experimental evolution experiments described above found mutations in *efpR* to be associated with adaptation to intracellular colonization (78). EfpR was found to act as a positive regulator of several 30S ribosomal protein subunits and as a negative regulator of the adapter protein ClpS (78), which redirects protein aggregates to ClpAP (154), mimicking the bacteroid growth arrest adaptation.

In contrast to free-living growth-arrested bacteria however, bacteroids are not nutrient-starved, are well-adapted for respiration in microoxic conditions, and retain high levels of metabolic activity to sustain nitrogen fixation. It thus remains unknown how and why bacteroids stop dividing. Some legumes induce terminal differentiation and chromosomal endoreduplication in bacteroids, meaning the bacteroid is unable to dedifferentiate into a free-living bacterium (155, 156). Generally, hosts with determinate nodules (e.g. *Lotus*, soybean, bean) harbor bacteroids that are similar to free-living bacteria and differentiation is not terminal (U-morphotype) (157). Legumes of the inverted repeat-lacking clade and some *Aeschynomene* spp. contain enlarged and elongated bacteroids (E-morphotype) that sometimes branch and become Y- or irregular-shaped. Many legumes of the Dalbergioid cluster (e.g. peanut, *Aeschynomene* spp.) cause bacteroids to become enlarged and spherical (S-morphotype). Rhizobia capable of nodulating different host plants will adopt the bacteroid morphotype of the respective host (31, 158, 159). Enlargement of the bacteroids in case of E- and S-morphotypes is accompanied by endoreduplication. This differentiation leads to up to 24C

(chromosomes) in E-morphotype *S. meliloti* bacteroids in symbiosis with *Medicago* (155). Similarly, *Bradyrhizobium* sp. ORS285 bacteroids reach 7C in *Aeschynomene afraspera* (E-morphotype), and 16C in *Aeschynomene indica* (S-morphotype) (160).

The key factor for bacterial endoreduplication are NCR peptides. These consist of 60-90 amino acids and display similarities with plant defensins (161, 162). NCR peptides are abundant in hosts harboring swollen bacteroids (around 700 are known in *Medicago truncatula*), but evolved independently in different clades (155, 156, 163). They are exclusively expressed in bacteroid-containing cells and are targeted to the bacteroid (164). Plant mutants with defects in the NCR peptide secretory pathway form ineffective nodules (164-166). Some NCR peptides have antimicrobial activities (167, 168) and rhizobia with increased sensitivity to NCR peptides form ineffective symbioses because they are rapidly killed after release from ITs into plant cells (160, 169-172). At the right dosage however, NCR peptides will promote endoreduplication even in free-living cultures (173), although the mechanisms by which they interfere with the bacterial cell cycle are not yet fully understood (167). The mode of action of some NCR peptides has been identified, for example inhibition of FtsZ (the structural component of the Z-ring involved in cell division) and ribosomal proteins (120). Moreover, rhizobia are successively exposed to different NCR peptides, suggesting they act in an orchestrated manner, controlling various steps of rhizobial physiology (173-175). NCR peptide-mediated endoreduplication and terminal differentiation might contribute to the growth-arrested phenotype of bacteroids. However, NCR peptides are absent in hosts harboring unswollen (U-morphotype) bacteroids (176) and endoreduplication and swelling of bacteroids only happen after bacteroids stop dividing (159), indicating the presence of other mechanisms yet to be discovered.

Metabolic adaptations

As a result of the adaptations required for symbiotic nitrogen fixation, bacteroid metabolism is unique in several respects: there is a strong downregulation of most biosynthetic functions compared to growth in the free-living state, while specialized enzymes for nitrogen fixation are highly induced (98, 177). In contrast to free-living bacteria in stationary phase, bacteroid metabolism is

289 highly active due to the energy requirement of nitrogen fixation despite the microoxic environment.
290 In addition, bacteroid metabolism is closely interdependent with the plant host because of
291 bidirectional nutrient exchanges between the symbiotic partners (**Fig. 2**) (116, 178).
292 An important implication of nutrient provision by the plant is that downregulation in biosynthetic
293 pathways can occur either because a compound is only required in low quantities in bacteroids or
294 because it is provided by the plant. Multiple auxotrophic rhizobial mutants are known which have a
295 Fix^+ phenotype, indicating plants provide the respective compounds to their bacteroids (179). A
296 striking illustration of this concept is symbiotic auxotrophy: mutants lacking the general amino acid
297 permeases Aap and Bra in *R. leguminosarum* were severely impaired in their symbiotic efficiency,
298 indicating a requirement for plant supply of amino acids (180). While free-living rhizobia are capable
299 of synthesizing all proteinogenic amino acids, a downregulation in branched-chain amino acid
300 synthesis occurs in bacteroids, making them dependent on the supply of branched-chain amino acids
301 by their host plant (181, 182). A similar downregulation was observed in our meta-analysis for serine
302 biosynthesis. Indeed, a serine auxotrophic mutant of *R. etli* was fully proficient in symbiosis (183). Yet
303 cysteine biosynthesis, which requires serine as a precursor, was one of the few upregulated
304 pathways for amino acid biosynthesis. This upregulation is likely related to production of iron-sulfur
305 clusters for the nitrogenase enzyme, which starts with cysteine as a sulfur donor (184). It is therefore
306 possible that plant hosts supply serine to bacteroids to assist with cysteine biosynthesis for
307 nitrogenase assembly. Experimental evidence also supports provision of amino acids by the plant
308 host that are non-essential for bacteroid functioning. In pea, enzymes catabolizing γ-aminobutyric
309 acid (GABA) are highly induced in bacteroids and labelling studies indicated that GABA is supplied by
310 the plant, but mutants of *R. leguminosarum* unable to catabolize GABA differentiated into functional
311 bacteroids (185).
312 Apart from amino acids, a different example of plant-derived metabolites essential for nitrogenase
313 activity is homocitrate, a component of the iron-molybdenum cofactor. In contrast to free-living
314 diazotrophs, most symbiotic rhizobia are unable to synthesize homocitrate, and a homocitrate

synthase mutant (Fen1) of *L. japonicus* was incapable of forming effective nodules (186). These observations illustrate the intricate control that plants exercise over their bacterial symbiont.

Legumes provide bacteroids with C_4-dicarboxylates as the main carbon source, primarily malate and succinate (187-189). Catabolism of C_4-dicarboxylates requires their conversion to both oxaloacetate and acetyl-CoA, which are condensed to form citrate in the first step of the tricarboxylic acid (TCA) cycle. There are two pathways for generating acetyl-CoA from TCA cycle intermediates: (i) malate is oxidized to pyruvate by malic enzyme (ME) or (ii) phosphoenolpyruvate carboxykinase (PCK) converts oxaloacetate into phosphoenolpyruvate, which is converted into pyruvate by pyruvate kinase (PK). In both cases, acetyl-CoA is obtained from pyruvate via the pyruvate dehydrogenase complex. While all bacteroids are required to convert TCA cycle intermediates into acetyl-CoA, the synthesis routes differ. Bacteroids of *S. meliloti* and *A. caulinodans* exclusively use ME (190, 191) whereas *Sinorhizobium fredii* and *R. leguminosarum* utilize both ME and PCK/PK (191, 192). In addition, activity of PCK is important for bacteroids to sustain gluconeogenesis, which is essential for production of precursors for various metabolites (63, 193). The TCA cycle is the main pathway for dicarboxylate metabolism in bacteroids, and at least some TCA cycle enzymes were upregulated in the transcriptome/proteome datasets across different rhizobial species. However, studies with individual mutants have produced contradictory evidence on which parts of the TCA cycle are required for nitrogen fixation. Mutants in different TCA cycle enzymes were Fix^- in *S meliloti*, *S. fredii*, *Rhizobium tropici* and *R. leguminosarum* (194-198), but several TCA cycle mutants in *B. japonicum* still formed an efficient symbiosis (199-202). This may be explained by flux through anaplerotic reactions or the existence of uncharacterized isozymes (203). For example, an aconitase mutant of *B. japonicum* retained almost 30% of aconitase activity (201). Additional mutant studies with careful evaluation of both enzyme activities and redirection of metabolic fluxes are therefore required to elucidate the essentiality of flux through different parts of the TCA cycle.

Dicarboxylate catabolism in the TCA cycle generates large amounts of reduced electron carriers (NADH and $FADH_2$) required for ATP synthesis and nitrogen fixation (116, 178). Due to the low

341 oxygen levels in nodules, flux through the TCA cycle may become disadvantageous because oxidation
342 of electron carriers in the respiratory chain is restricted, and accumulation of reducing equivalents
343 inhibits pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (204-206). Bacteroids could
344 therefore channel carbon into polymers, such as poly-hydroxybutyrate (PHB), glycogen, and lipids
345 (106, 207-209). Accumulation of these polymers is observed in bacteroids, although the nature of the
346 compounds differs between symbioses (106, 210). In *B. japonicum*, the regulator of PHB synthesis,
347 PhaR, has been shown to repress various gene targets, notably $FixK_2$, a transcriptional regulator for
348 adaptation to microoxic conditions (211, 212). This suggests a certain degree of coordination
349 between PHB metabolism and symbiotic adaptation, at least in some rhizobial strains.

350 A fundamental requirement for rhizobium-legume symbioses is the secretion of fixed nitrogen to the
351 plant instead of its assimilation by the bacteroid. Ammonia is the main secretion product, but
352 secretion of alanine and aspartate has also been reported (213-216). The glutamine synthetase-
353 glutamate oxoglutarate aminotransferase (GS-GOGAT) system, which assimilates ammonia into
354 glutamate in free-living rhizobia, appears to be largely downregulated in bacteroids (116, 217).
355 Rhizobia usually possess two glutamine synthetases (GSI/GlnA and GSII/GlnII), with some strains
356 encoding a third enzyme (GSIII/GlnT). In general, only GSI activity is present at low levels in
357 bacteroids and deletion of any of the GS genes does not impair the symbiosis (217, 218). Mutant
358 studies have further shown GOGAT to be dispensable in *S. meliloti* (219, 220), *R. etli* (221) and
359 *R. leguminosarum* (222). Overall, these results indicate no need for ammonia assimilation by the
360 bacteroid, which may be due to amino acid provision by the plant or reduced protein biosynthesis as
361 a result of growth arrest.
362 A recent study showed that the nitrogen-related phosphotransferase system in *R. leguminosarum*
363 controls central carbon metabolism in response to carbon and nitrogen availability, and mutation of
364 this system caused an ineffective symbiosis (223). This highlights the complex interconnection
365 between carbon and nitrogen metabolism in bacteroids, which must be finely tuned to enable a

functional symbiosis. The complex regulatory systems underlying rhizobial nitrogen metabolism have been comprehensively discussed elsewhere (179, 217, 224).

Modelling bacteroid metabolism

Bacteroid metabolism is difficult to investigate experimentally due to the challenge of measuring nutrient exchange with the plant host, the multiple lifestyle adaptations required during symbiosis formation (63), and the fragile nature of isolated bacteroids (178). In addition to various *omics* techniques, constraint-based metabolic models have therefore become a popular tool for investigating rhizobium-legume symbioses (225). Metabolic models are knowledge bases of the reactions catalyzed by enzymes encoded in an organism's genome. By imposing an objective function and assuming steady state of all metabolic reactions, flux distributions are calculated using Flux Balance Analysis (FBA) (226, 227). Models with different scopes have been reconstructed for various rhizobial species (**Table S4**) and studies have progressed from describing bacteroid metabolism (228-231) to integration of bacterial and plant models, as well as models of nodule zone-specific metabolism (232, 233). Rhizobial genomes are large and may include secondary replicons encoding niche-specific functions (60, 234). Metabolic models specific to the nitrogen-fixing state must therefore be constrained using transcriptome and proteome datasets (228) and/or appropriate objective functions (231, 234). Since direct measurement of reaction rates and exchange fluxes with the plant is infeasible, FBA studies have mostly used objective functions composed of generally accepted features of bacteroid metabolism, such as nitrogen fixation, amino acid secretion, and production of carbon polymers (229, 230, 235).

All models published to date are consistent with experimental evidence for use of the TCA cycle and oxidative phosphorylation as the main pathways fueling bacteroid metabolism. Similar to the disparities in experimental results, operation of both a full and partial TCA cycle during nitrogen fixation have been predicted. This may be due to strain-specific differences as well as the choice of constraints, in particular carbon and oxygen uptake rates. Synthesis of carbon polymers as well as amino acid exchange with the plant have also been investigated *in silico*. An increase in symbiotic

392 efficiency was predicted for deletion of glycogen or PHB synthesis in *R. etli* (229), which agrees with
393 the enhanced symbiotic properties of a glycogen synthase mutant of *R. tropici* (236), but not with the
394 phenotypes of mutants for PHB and glycogen synthases in *R. leguminosarum* and *S. meliloti* (207,
395 209). Most, but not all, models assume extensive amino acid cycling between plant and bacteroid.
396 This is based on early experimental studies suggesting glutamate supply by the plant (180), which
397 was however later shown to be non-essential for bacteroids (181). With regard to amino acids
398 secreted by the bacteroid, alanine has been predicted to be the main nitrogenous compound
399 produced at low oxygen levels, since it serves as a sink for both carbon and electrons (232).
400 A recent study of an integrated model for plant and bacteroid metabolism further addressed the
401 question of why C_4-dicarboxylates are supplied to bacteroids instead of sucrose, which is abundant in
402 nodules. Rhizobia differentiating into bacteroids were predicted to catabolize sugars (233), which is
403 supported by studies of mutants in dicarboxylate transport, which are able to nodulate and
404 differentiate into bacteroids, but fail to fix nitrogen (187, 188). Modelling results in (233) indicated
405 that sucrose catabolism in fully differentiated bacteroids would achieve higher yields of fixed
406 nitrogen and proton supply by the plant is required to support catabolism of C_4-dicarboxylates.
407 Provision of C_4-dicarboxylates was further predicted to be driven by oxygen limitation of the plant
408 mitochondria (233). Experimental evidence has been found for oxygen limitation of both plant (106)
409 and bacteroid (237, 238) fraction, highlighting this as an outstanding question for future studies.
410 It is important to note that carbon polymer synthesis and amino acid secretion are commonly
411 included in the bacteroid objective function in addition to ammonia export. Since flux distributions
412 calculated by FBA must produce all compounds that are part of the objective function, carbon and
413 nitrogen allocation in metabolic models are constrained by forcing the stoichiometric production of
414 these compounds. This was circumvented in (233) by an integrated model of plant and bacteroid
415 metabolism and optimization for plant biomass production. In future studies, the application of
416 comprehensive and unbiased modelling approaches, such as elementary flux mode enumeration
417 (239), may be insightful to improve and expand our current understanding of bacteroid metabolism.

Apart from direct characterization of bacteroid metabolism, the underlying gene-protein-reaction relationships in metabolic models are useful for guiding targeted investigation of gene essentiality. Seeing as symbiosis formation is a multi-step process requiring various metabolic capabilities at different stages (63), it is difficult to distinguish between genes essential for infection and nitrogen fixation itself. Discrepancies between experimental and *in silico* gene essentiality can thus unravel metabolic requirements at different stages of symbiosis formation.

Plant control and metabolic sanctioning

Both computational and experimental studies have demonstrated how bacteroid metabolism can be modulated in response to nutrient supply by the plant, in particular carbon and oxygen (240, 241). Plant control over bacteroid metabolism is essential due to the high fitness cost of nodule formation and maintenance of bacteroids. The plant host must therefore be capable of monitoring symbiotic performance and responding accordingly, which becomes even more important when considering that efficiency of the symbiont cannot be evaluated prior to nodulation (85, 242). Mathematical models demonstrated that substantial investment in nitrogen fixation will only be advantageous for rhizobia if the plant adjusts its resource allocation according to symbiotic performance (243). To maximize their fitness, reproductive bacteroids can divert some of the plant-supplied carbon into polymers and catabolize those after de-differentiation into the free-living state. For non-reproductive bacteroids, the undifferentiated population in the nodule benefits from the carbon supply and will be released into the soil after nodule senescence (244). Thus, the rhizobium-legume symbioses seem prone to selecting for cheaters which fix no or little nitrogen. In fact, field isolates vary greatly in their nitrogen fixation efficiency (89). Consequently, legumes must have means to identify and punish inefficient strains to maximize nitrogen return for their carbon investment. It is well established that Fix^- strains do not persist within mature nodules (245) and when nodules are co-inhabited with a fixing strain, the Fix^- strain is sanctioned rapidly in a cell-autonomous way (246). Sanctioning results in a fitness increase of good symbionts since higher numbers will be released from nodules. Under experimental conditions, efficient symbionts will outcompete Fix^- strains in a few plant generations

(247, 248) since plants readily select the most efficient strain (249) even when the initial cell numbers are lower compared to the less efficient strain. Experimental data on carbon and oxygen supply to cheating strains (85, 240, 241) show that plants sense and integrate nitrogen output from bacteroids and readjust carbon investment (250) or apply oxygen sanctioning in response to poorly performing strains.

Final conclusions and future perspectives

The bacteroid stage in the rhizobial life cycle features a unique combination of traits. It is characterized by a non-growing state in an oxygen-depleted environment. Despite the downregulation of many biosynthetic pathways, bacteroids maintain a highly active and specialized metabolism driven by catabolism of C_4-dicarboxylates. Rhizobia are equipped with specialized functions to cope with this environment, and global adjustments of cellular and metabolic processes must take place to ensure a successful transition into nitrogen-fixing bacteroids. Failure to do so is punished by the host plant and results in reduced fitness of a rhizobial strain. Host plants impose control over their symbionts at multiple levels. Nutrient supply, mostly in the form of malate and succinate, can be reduced, oxygen supply limited, or in some cases plants can target bacteroids directly with antimicrobial peptides to force them into terminal differentiation.

Despite major advances in our knowledge, the complex interactions in rhizobium-legume symbioses retain many unanswered questions. Modulation of the plant immune response by both the host and the symbiont to accommodate thousands of intracellular prokaryotes per cell, what it shares with the better understood immune response during infection, and its interplay with host sanctioning and pathogens remain exciting research areas despite substantial published work (245, 251-253). Similarly, our understanding of nutrient supply to bacteroids beyond malate and branched-chain amino acids is fragmented, obscuring the stoichiometry of metabolic fluxes that drive nitrogen fixation. The rhizobium-legume symbioses are prime examples for biological systems whose complexity necessitate the combination of experimental and computational approaches. *In silico*

modelling is a rapidly advancing field that will greatly contribute to our understanding of the symbioses and aid in advancing towards engineering synthetic symbioses.

Acknowledgments

This work was supported by the Swiss National Science Foundation (PostDoc.Mobility Fellowship number 183901 to RL) and the Biotechnology and Biological Sciences Research Council (BBSRC) [grant number BB/M011224/1]. CCMS is supported by the Clarendon Fund (Oxford University Press) and the Keble College De Breyne Scholarship.

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Tables

Table 1. Datasets used in the meta-analysis.

Species (rhizobium strain – plant host)	Reference dataset	Bacteroids terminally differentiated	Reference
Rhizobium leguminosarum bv. *viciae* 3841 – *Pisum sativum*	Bacteria grown in MM (mid-exponential phase)	Yes	(98)
Rhizobium leguminosarum bv. *viciae* 3841 – *Vicia cracca*	Bacteria grown in MM (mid-exponential phase)	Yes	(98)

Rhizobium leguminosarum bv. *viciae* A34 – *Pisum sativum*	Bacteria grown in MM (mid- to late exponential phase)	Yes	(99)
Rhizobium leguminosarum bv. *phaseoli* 4292 – *Phaseolus vulgaris*	Bacteria grown in MM (mid- to late exponential phase)	No	(99)
Rhizobium etli CFN42 – *Phaseolus vulgaris*	Bacteria grown in MM (early exponential phase)	No	(92)
Rhizobium etli CFN42 – *Phaseolus vulgaris*	Bacteria grown in MM (stationary phase)	No	(92)
Sinorhizobium meliloti 2011 – *Medicago truncatula*	FIId: distal fraction of ZII, contains cells during infection and differentiation	Yes	(102)
Sinorhizobium meliloti 1021 – *Medicago truncatula*	Bacteria grown in CM (mid-exponential phase)	Yes	(95)
Sinorhizobium meliloti 1021 – *Medicago sativa* cv. Europe	Bacteria grown in MM (mid-exponential phase)	Yes	(104)
Sinorhizobium medicae – *Medicago truncatula*	FI: non-fixing zone of the nodule	Yes	(97)
Sinorhizobium sp. NGR234 - *Vigna unguiculata*	Bacteria grown in CM (mid-exponential phase)	No	(101)
Sinorhizobium sp. NGR234 – *Leucaena leucocephala*	Bacteria grown in CM (mid-exponential phase)	No	(101)
Bradyrhizobium sp. ORS285 – *Aeschynomene afraspera*	Bacteria grown in CM (mid-exponential phase)	Yes	(100)
Bradyrhizobium sp. ORS285 – *Aeschynomene indica*	Bacteria grown in CM (mid-exponential phase)	Yes	(100)
Bradyrhizobium japonicum	Bacteria grown in CM (mid-exponential phase)	No	(96)

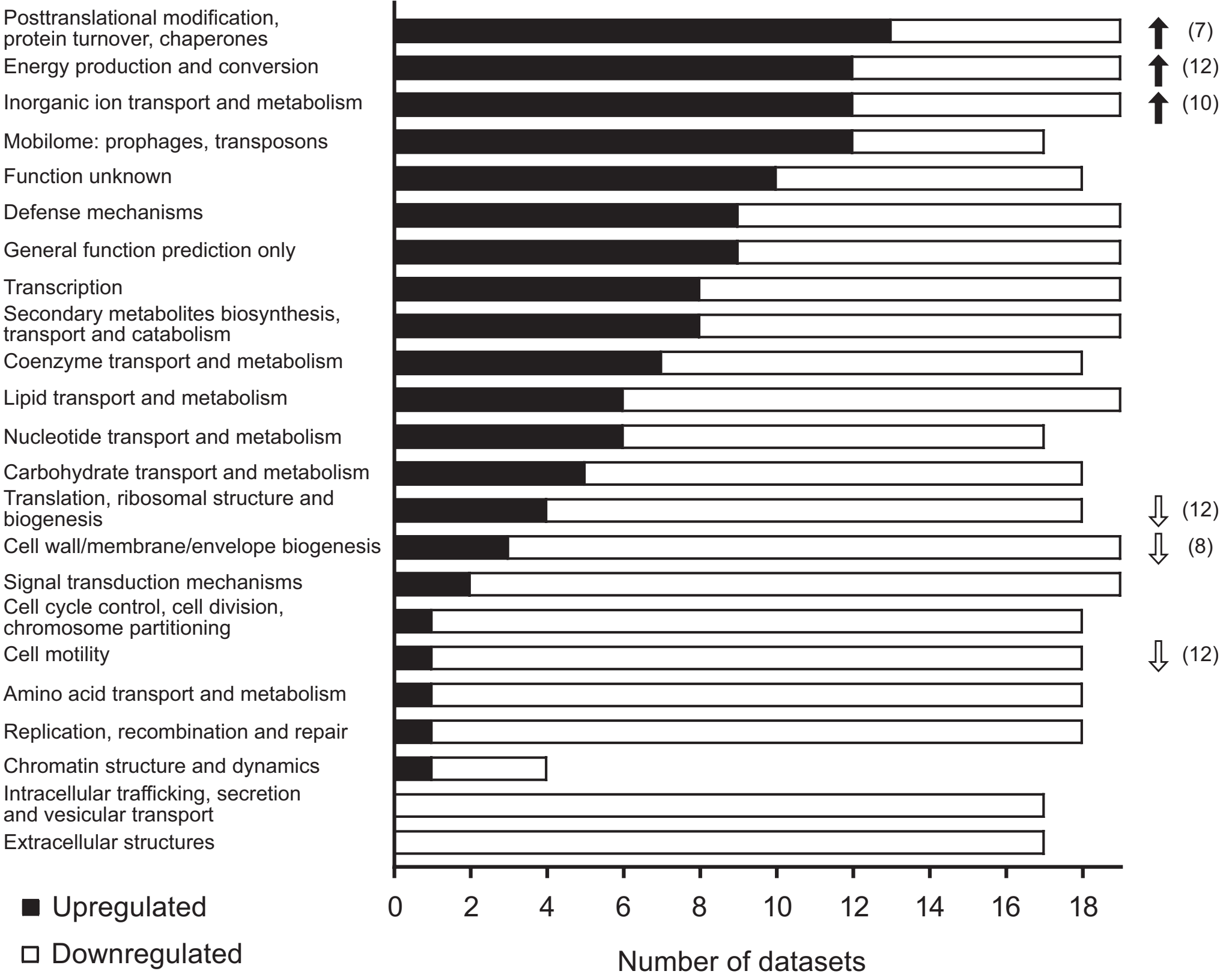
USDA110 – *Glycine max*			
Bradyrhizobium japonicum USDA110 – *Vigna unguiculata*	Bacteria grown in CM (mid-exponential phase)	No	(103)
Bradyrhizobium japonicum USDA110 – *Macroptilium atropurpureum*	Bacteria grown in CM (mid-exponential phase)	No	(103)
Azorhizobium caulinodans ORS571 – *Sesbania rostrata*	Bacteria grown in MM (mid- to late exponential phase)	No	(94)
Mesorhizobium haukuii 7653R – *Astragalus sinicus*	Bacteria grown in CM (late exponential phase)	Yes	(93)

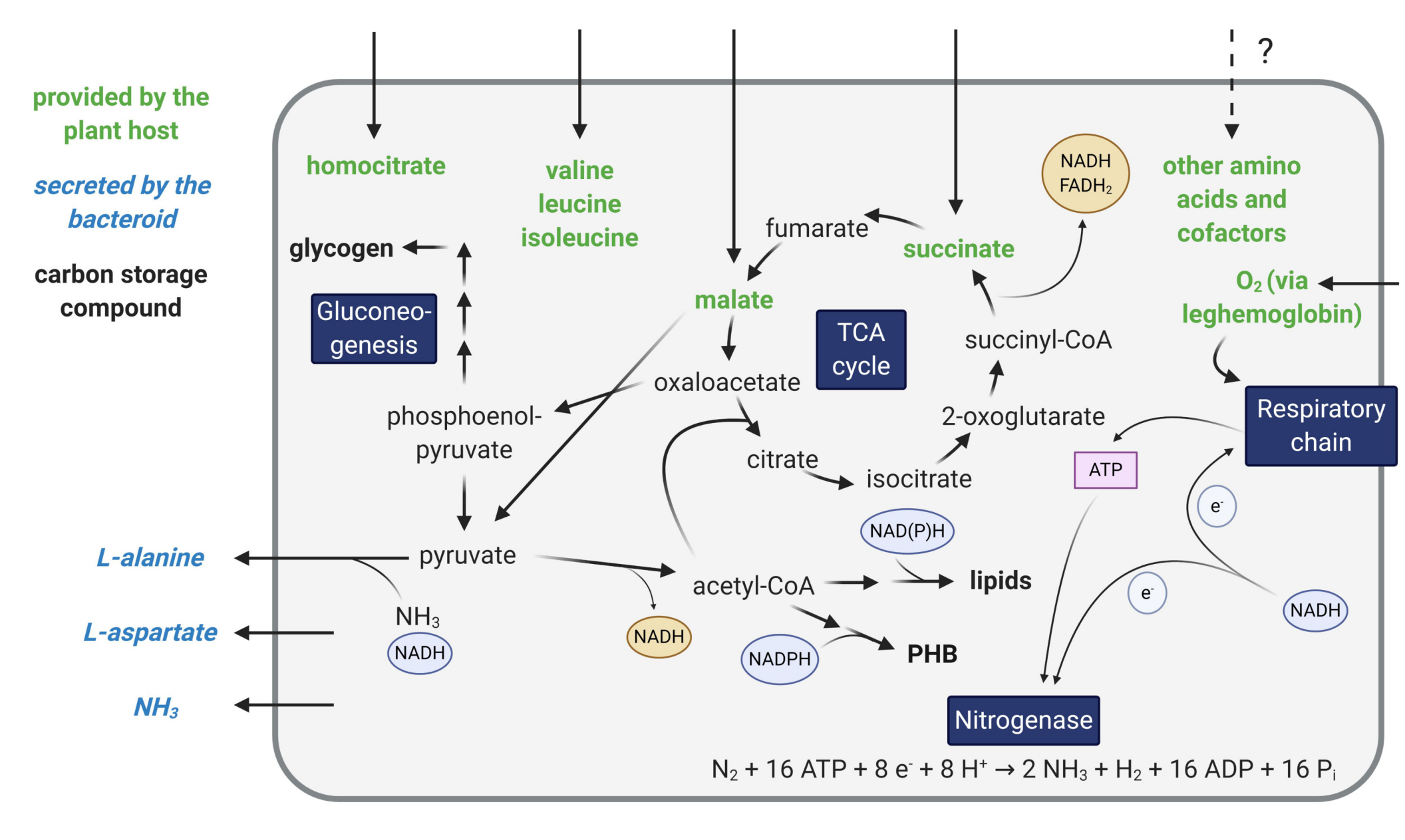
Transcriptome data were used in all cases apart from the *Sinorhizobium medicae* study, which is a proteome dataset. All datasets compare bacteroids with the reference condition described in the second column.
MM: minimal media; CM: complex media

Figure legends

Figure 1. Central features of bacteroids determined from genome-scale datasets. A meta-analysis of 18 transcriptome and one proteome dataset for bacteroids of various rhizobial strains was performed. The bar graph shows the number of datasets in which a COG category contained more upregulated than downregulated (black) or more downregulated than upregulated (white) genes/proteins. Where the total number is smaller than 19, some datasets contained either no differentially expressed genes/proteins or an equal number of up- and downregulated genes/proteins in this category. Arrows indicate the 3 categories that were most often significantly enriched within upregulated (black) or downregulated (white) genes/proteins, with numbers in brackets indicating the number of datasets for which significant up-/downregulation was observed. *P* values were determined with a hypergeometric test followed by Bonferroni multiple test correction and $P < 0.05$ was considered significant. For the same analysis separated according to terminally differentiated and not terminally differentiated bacteroids, see Fig. S1.

Figure 2. Central metabolic and transport reactions in bacteroids. Schematic map of major metabolic pathways and transport reactions in bacteroids as well as important flows of electrons and ATP. This figure was created with BioRender.com.


Posttranslational modification, protein turnover, chaperones
Energy production and conversion
Inorganic ion transport and metabolism
Mobilome: prophages, transposons
Function unknown
Defense mechanisms
General function prediction only
Transcription
Secondary metabolites biosynthesis, transport and catabolism
Coenzyme transport and metabolism
Lipid transport and metabolism
Nucleotide transport and metabolism
Carbohydrate transport and metabolism
Translation, ribosomal structure and biogenesis
Cell wall/membrane/envelope biogenesis
Signal transduction mechanisms
Cell cycle control, cell division, chromosome partitioning
Cell motility
Amino acid transport and metabolism
Replication, recombination and repair
Chromatin structure and dynamics
Intracellular trafficking, secretion and vesicular transport
Extracellular structures
(7)
(12)
(10)
(12)
(8)
(12)
Upregulated
Downregulated
0
2
4
6
8
10
12
14
16
18
Number of datasets


provided by the plant host
secreted by the bacteroid
carbon storage compound
homocitrate
valine
leucine
isoleucine
?
other amino acids and cofactors
NADH
FADH2
fumarate
succinate
glycogen
Gluconeo-genesis
malate
TCA cycle
succinyl-CoA
O2 (via leghemoglobin)
oxaloacetate
phosphoenol-pyruvate
2-oxoglutarate
Respiratory chain
citrate
isocitrate
ATP
e-
NAD(P)H
L-alanine
pyruvate
acetyl-CoA
lipids
e-
NH3
NADH
NADH
L-aspartate
NADPH
PHB
NH3
Nitrogenase
N2 + 16 ATP + 8 e- + 8 H+ → 2 NH3 + H2 + 16 ADP + 16 Pi