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MULTI-AUTHOR REVIEW

Rhizobial measures to evade host defense strategies and endogenous threats to persistent symbiotic nitrogen fixation: a focus on two legume-rhizobium model systems

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Abstract The establishment and maintenance of rhizobium–legume symbioses require a sequence of highly regulated and coordinated events between the organisms. Although the interaction is mutually beneficial under nitrogen-limited conditions, it can resemble a pathogenic infection at some stages. Some host legumes mount defense reactions, including the production of reactive oxygen species (ROS) and defensin-like antimicrobial compounds. To subvert these host defenses, the infecting rhizobial cells can use measures to passively protect themselves and actively modulate host functions. This review first describes the establishment and maintenance of active nodules, as well as the external and endogenous attack and threat stages. Next, recent studies of ROS scavenging enzymes, the BacA protein originally found in *Sinorhizobium meliloti*, and the type III/IV secretion systems are discussed, with a focus on two legume–rhizobium model systems.

Keywords Nitrogen-fixing mutualism · Host defense · Reactive oxygen species · Type III secretion system · Type IV secretion system · BacA protein

Introduction

Bacteria of the family Rhizobiaceae and compatible leguminous plants establish mutualistic relationships to exchange nitrogen and carbon fixed from the atmosphere. The establishment of symbiosis requires multistep reciprocal recognition with exchanges of signal molecules and complex developmental programs, which leads to the formation of nodules on the legume root and the differentiation of rhizobial cells into bacteroids [1]. Although the relationship is beneficial to both participants, it can resemble a pathogenic interaction in that the eukaryotic organism is chronically infected [2]. However, the host plant may suppress its defense mechanisms to maintain a successful symbiotic interaction [3]. Transcriptomic analyses have revealed that many defense- and stress-related genes are up-regulated in the legume host during the early stage of this interaction, but most are subsequently suppressed as the symbiosis proceeds [4, 5]. In addition, the rhizobial infection can be arrested by a mechanism similar to a hypersensitive reaction [6]. Furthermore, established bacteroids can be eliminated in some legume–host pairings by necrosis of the nodules [7, 8]. These indicate that the host plant can attack the infiltrating rhizobial cells. In addition to host threats, nitrogen-fixing bacteroids encounter reactive oxygen species (ROS) that are endogenously produced from ATP-producing respiratory oxidative phosphorylation and harmful to ROS-sensitive nitrogenases.

Rhizobia have developed mechanisms to survive exogenous (host-derived) and endogenous threats during symbiosis, from the initial contact to senescence. Some rhizobia passively protect bacterial cells or functions, whereas others actively interact with the host to reduce attack. This review focuses on proteins involved in ROS scavenging as a passive defense with an emphasis on two legume–rhizobium model systems: *Lotus–Mesorhizobium loti*, which forms determinate globular nodules without persisting meristems [9], and *Medicago–Sinorhizobium*

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meliloti, which forms indeterminate cylindrical nodules with persisting meristems [10]. This review also discusses the significance of the BacA protein, a bacterial factor essential for bacteroid development in *Sinorhizobium meliloti* [11], and the significance of the type III and type IV secretion systems, which inject proteins into the eukaryotic host [12]. Non-proteinaceous factors including lipochitooligosaccharides (LCOs), lipopolysaccharides (LPSs), and extracellular polysaccharides (EPSs) are also important for protection and evasion [1, 13, 14] but are not discussed in this review.

Stages of legume–rhizobium symbiotic processes

Encounter, attachment, and initial signal exchange

Because rhizobia can exist as saprophytes in the soil, the mutual recognition of two symbionts starts when the rhizobial cells perceive host-specific signal molecules (mostly phenolic flavonoid compounds) exuded to the rhizosphere by the legume roots (Fig. 1a). The signal molecules induce the bacterial regulatory protein NodD to activate transcription of several *nod* (nodulation) genes that participate in the synthesis of species- or strain-specific signal molecules [LCOs, also known as Nod factors (NFs)] [15]. Rhizobial cells attached to the tip of the emerging root hairs secrete NFs that trigger the nodulation developmental program, including root hair deformation and nodule primordia formation in the cortex (Fig. 1b). Because flavonoid compounds and NFs have specific chemical structures depending on their producers, the combinations of these signaling molecules are the primary determinants of the various host–rhizobium combinations. Hence, most rhizobial species can only establish symbiosis with a few host legumes [16].

Invasion of rhizobia into host cells and establishment of symbiosis

Rhizobial cells on a root hair tip are entrapped in the curled root hair, which is shaped similarly to a shepherd's crook, and form tubular structures known as infection threads, which contain rhizobial cells. The infection threads elongate inside the root hair, traverse multiple cell layers, ramify and reach the developing nodule primordia (Fig. 1c–f) [17]. The bacterial cells proliferate directionally to the front of the growing infection threads and invade the plant cytoplasm through an endocytosis-like mechanism that enables encapsulation of the engulfed bacteria within the plant plasma membrane. The bacterial cells enlarge, differentiate to bacteroids, and initiate nitrogen fixation in

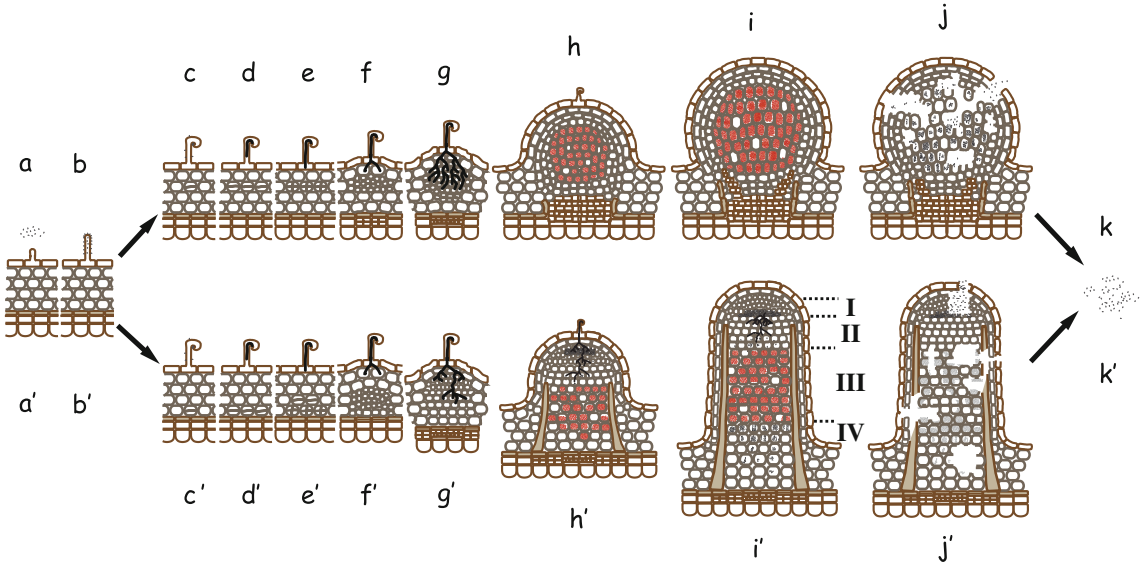

Fig. 1 The nodule developmental and decaying process. The processes for determinate (*upper*) and indeterminate (*lower*) nodules are shown. Saprophytic rhizobia exist in a rhizosphere (**a**). The rhizobia attach to root hairs (**b**), detect flavonoids from legumes and secrete NF to induce root hair deformation (**c**). The deformed root hair entraps the rhizobia and invaginates to form infection threads, which contain entrapped rhizobial cells (**d**). The infection thread elongates (**e**), ramifies, and penetrates the outer or inner cortex cell layers (**f**, **g**). The rhizobial cells are then enveloped in a plant-derived membrane and released as droplets into the plant cytosol (**h**). The released rhizobia differentiate to bacteroids and begin nitrogen fixation; hence, the droplets are called symbiosomes (**i**). After a period of nitrogen fixation, the nodule cells initiate senescence (**j**). Most bacteroids in determinate nodules and some undifferentiated rhizobial cells in indeterminate nodules return to a saprophytic lifestyle (**k**). Zones in mature indeterminate nodule are indicated by I–IV. *I* Meristematic zone; *II* invasion zone; *III* N_2-fixing zone; *IV* senescence zone

the capsules, which become organelle-like structures known as symbiosomes (Fig. 1g) [18–20]. As the bacteria differentiate, the host genomic DNA replicates in the invaded plant cells without mitosis, and the infected cells become large polyploid cells harboring thousands of symbiosomes [21]. The mature nodules actively fix nitrogen for a length of time that depends on environmental and plant developmental conditions and then enter senescence.

Indeterminate and determinate nodules

The origins of the plant cells that harbor symbiosomes and the fate of the bacteria are considerably different between indeterminate and determinate nodule types (Fig. 1). Indeterminate nodules originate from the nodule primordia formed in the inner root cortex next to the xylem pole (Table 1). Furthermore, these nodules possess a persistent meristem and elongate to become cylindrical so that a meristematic zone forms near the apex and successive zones form for rhizobial invasion, active nitrogen fixation, and senescence (Fig. 1h′–k′). Host cells in the nitrogen-fixation zone contain mature symbiosomes, which typically contain an enlarged, deformed bacteroid with low reproductive viability, while those in the senescence zone have decayed or disintegrating symbiosomes [21–24]. Consequently, an indeterminate nodule accommodates a heterologous population of rhizobial cells in various developmental states in distinct zones. In contrast, determinate nodules originate from the primordia formed in the outer or middle root cortex. In contrast to indeterminate nodules, determinate nodules do not have a persistent meristem and thus become globular (Fig. 1h–j), and the mature symbiosomes contain multiple bacteroids of normal size with high reproductive viability [25–27]. In a determinate nodule, the developmental stages of the host and rhizobial cells are relatively synchronized. Senescence of determinate nodules begins at the center of the nodule and extends to the periphery [28]. Rhizobial cells released from decaying and disintegrating nodules enter a saprophytic life cycle (Fig. 1k).

Phases of host-derived and endogenous threats to rhizobia

Observations of unsuccessful symbiosis by normal and mutant rhizobia and host legumes indicate that there are at least three major phases of threats to rhizobia [29, 30]. Each phase can be distinguished by nodulation efficiency, nitrogen-fixation capacity, duration and the symbiotic stage in which it takes place.

The initial phase threats appear to be related to the prevention of nodulation (few nodules phenotype). The threats at this phase occur just after rhizobial contact with the root hair or root surface and continue during the elongation of the infection threads (Fig. 2a). Contact between the rhizobial cells and the host root epidermis evokes innate immunity or basal defense responses similar to those that occur with pathogenic infections; however, the responses are transient and regulated during infection with compatible rhizobia, unlike the responses against pathogenic bacteria [4, 5]. The transient responses include the generation of ROS [31–33] and nitric oxide [34] and the expression of gene products similar to pathogen-related (PR) proteins [4, 5, 34, 35]. Some *S. meliloti* mutants with defective ROS scavenging enzymes have poor nodulation capacity on *Medicago sativa* [36, 37], indicating that the level of ROS produced by the host is tolerated by wild-type, but not mutant, *S. meliloti*. *Medicago* can use ROS and other mechanisms at this phase to interfere with the rhizobial infection process. The accumulation of phenolic compounds and PR proteins has been observed

Table 1 Representative differences between indeterminate and determinate nodule types

Property	Indeterminate	Determinate
Legume examples	*Medicago sativa*, *Pisum sativum*, *Astragalus sinicus*	*Glycine max*, *Phaseolus vulgaris*, *Lotus japonicus*
Normal nodule form	Cylindrical/branched	Spherical/globular
Site of initial cell division	Inner root cortex	Outer or middle cortex
Meristem type	Persistent meristem	No persistent meristem
Infection thread	Broad	Narrow
Infected cells	Highly vacuolated	Minimal vacuolation
Major bacteroid form	Enlarged, branched, one per symbiosome	Normal rod size, high viability, multiple per symbiosome
Poly-hydroxybutylate accumulation in bacteroid	Present	Absent
Bacteroid reproductivity	Low	High

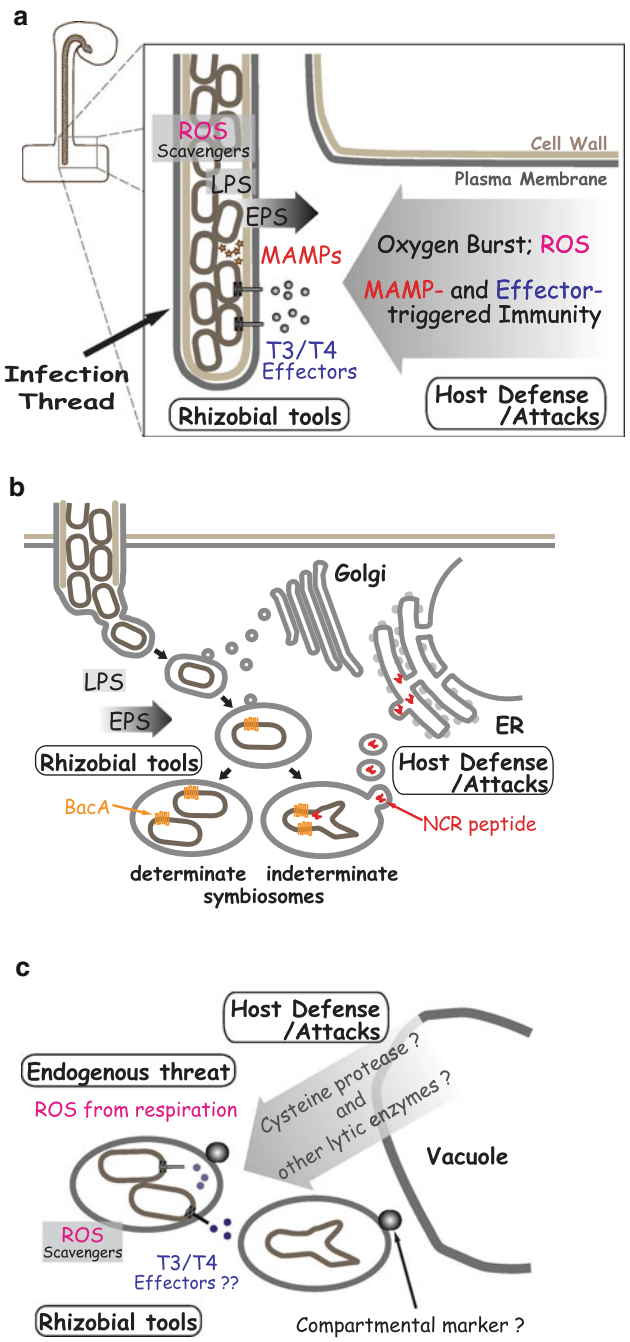

Fig. 2 Host-derived and endogenous threats to rhizobia and rhizobial counter measures at the different developmental stages. Host actions to infecting rhizobia and rhizobial counter measures in infection threads (**a**), in released droplets or young symbiosomes (**b**), and in mature symbiosomes (**c**)

in epidermal cells with aborted infection threads [6]. Therefore, attacks during this phase can be used by the host plant to eliminate excessive nodulation and related to a phenomenon known as autoregulation of nodulation [18].

The intermediate phase threats involve the reduction of intracellular niche formation by parasitic rhizobia (inactive empty nodules phenotype). The threats at this phase are mainly exerted during and just after the endocytosis-like invasion and are mediated by the drastic change in the bacterial environment within the intracellular encapsulated structure (Fig. 2b). Although the biochemical identities of these threats are not known, some are likely responsible for the aberrant phenotypes of the *S. meliloti bacA* and *lpsB* mutants, which are unable to form bacteroids [11, 38]. Intermediate phase threats may also be involved in the phenotypes of *S. meliloti* EPS mutants, which poorly form bacteria-filled infection threads and small nodules without bacteroids [39, 40]. Empty nodules can be formed by some EPS mutants that escape the initial phase threats but are unable to overcome the intermediate phase threats. While it is difficult to distinguish the passive and positive roles of EPS in the host defense mechanisms, a role for this signaling in the suppression of host defense genes is supported by the transcriptomic analysis of *M. truncatula* infected with *S. meliloti* [41].

Late (maintenance) phase threats are divided into two categories: those derived from the host attack of rhizobia established as mature nitrogen-fixing bacteroids and those derived from an endogenous threat from the co-existence of aerobic respiratory energy metabolism and highly oxygen-labile nitrogenase systems (Fig. 2c). An example of the former is the host response of *L. japonicus* to *Rhizobium etli*: although nodules formed by *Rhizobium etli* acquire the capability to fix nitrogen, they lose this capacity within 3 weeks of inoculation and enter early senescence [42]. A *L. japonicus* mycorrhiza-inducible phosphate transporter knockdown line exhibited nodule necrosis when its normal symbionts *M. loti* (rhizobium) and *Glomus mosseae* (mycorrhiza) were inoculated simultaneously, even though the line establishes normal nitrogen-fixing symbiosis when infected with *M. loti* alone [7]. The senescence process of *Medicago truncatula* has been studied in detail at the transcriptional level [43, 44] to confirm the involvement of various genes, including some encoding cysteine proteases that likely function in senescence in a number of legumes [28, 44–48]. A recent analysis of compartmental markers has demonstrated that the symbiosomes in *M. truncatula* change over the course of endocytotic formation to senescence [49]. Such alterations may determine whether each symbiosome persists or enters the lytic pathway, although the mechanism of the alteration is unknown. Although *G. max* is reported to sanction parasitic rhizobia [50], it has also been reported that some inefficient rhizobia, including *Rhizobium* sp. NGR234, persist for longer periods in *L. japonicus* nodules [8]. Thus, it is of interest to elucidate what enables the host to discriminate non-efficient from efficient rhizobia and how marker alterations are triggered.

ROS scavenging enzymes

ROS production occurs during all three phases of threats to rhizobia. Because ROS (especially H_2O_2) function not only as antimicrobial agents but also as signals for nodule organogenesis, regulation of ROS levels is required for successful symbiosis [51–53]. The ROS level during the initial phase must be low enough to allow the survival of compatible wild-type rhizobium with somewhat redundant ROS scavenging systems. The redundancy often masks some ROS detoxification enzymes, but some mutants with compromised systems have defective symbiotic capacities. *S. meliloti* has two monofunctional catalases encoded by *katA* and *katC*, a bifunctional catalase-peroxidase encoded by *katB* [36, 54, 55], a superoxide dismutase (SOD) encoded by *sodA* or *sodB* [37, 56, 57] and several uncharacterized ROS scavenging enzymes [14, 19]. *M. loti* has a monofunctional catalase encoded by *katE*, a bifunctional catalase-peroxidase encoded by *katG*, a SOD encoded by *sodA* and other enzymes [58].

Among the ROS scavenging enzymes, catalase genes have been most extensively characterized. Mutation of any of the three *kat* genes in *S. meliloti* does not affect H_2O_2 sensitivity, but a *katA katC* double mutant and a *katB katC* double mutant are deficient in nodule formation and nitrogen fixation [36, 55]. The *katA katC* double mutant can fix nitrogen, but has low nodulation efficiency [55] and sparsely distributed bacteroids, some of which are irregularly shaped [36]. The *katB katC* mutant has an even lower nodulation efficiency and is unable to form bacteroids and fix nitrogen [36]. Because these double mutants are capable of aerobic growth, *S. meliloti* likely requires both *katB* and *katC* to overcome the initial and intermediate phase attacks from the *Medicago* host before forming mature symbiosomes. In contrast, an *M. loti katE katG* double mutant forms a greater number of effective nodules, but they have a slightly lower nitrogen-fixing capacity than wild-type *M. loti* [59]. Because *M. loti katG* mutants are at least 30 times more vulnerable to exogenous H_2O_2 than wild-type *M. loti* and exhibit slower aerobic growth [59], it is possible that the initial and intermediate attacks from the *Lotus* host are not strong enough to prevent nodulation and formation of mature symbiosomes. In *M. loti*, *katE* contributes to survival during the stationary phase; a *katE* single mutant has decreased nitrogen-fixation capacity similar to the *katE katG* double mutant, while the *katG* single mutant has comparable capacity to wild-type *M. loti* [59]. This suggests that the monofunctional catalase KatE but not the bifunctional catalase-peroxidase KatG is required for continuing nitrogen fixation or to protect bacteroids from maintenance phase threats. In addition, the *S. meliloti katC* mutant, which lacks the monofunctional catalase similar to *M. loti* KatE, forms nodules with slightly decreased nitrogen-fixing capacity [55]. It is unclear why catalase disruption has different effects on nodulation of *S. meliloti* and *M. loti*, but the initial and intermediate phase threats to infecting rhizobia appear to be stronger in *Medicago* than *Lotus*.

The requirement of rhizobial ROS scavenging proteins to properly maintain nitrogen-fixation capacity is supported by studies examining *Rhizobium etli*, which nodulates the determinate host *Phaseolus vulgaris* [60, 61]. *R. etli* has only one catalase, a catalase-peroxidase encoded by *katG*, that is responsible for protection from exogenous H_2O_2 and survival during the stationary phase [60]. Like many rhizobia, this species encodes 2-Cys peroxiredoxin, a non-heme protein that catalyzes the reduction of H_2O_2 in the presence of thiol [62]. This peroxiredoxin is encoded by *prxS* and is expressed under symbiotic conditions [61]. *katG* and *prxS* single mutants both show a symbiotic phenotype similar to wild-type *R. etli*, however, a *katG prxS* double mutant forms nodules with a nitrogen-fixing capacity that is approximately 50% of the capacity of wild-type *R. etli* [61]. Notably, *S. meliloti* has another peroxiredoxin encoded by *nex1* that is primarily expressed in nodules [63].

The heterologous over-expression of a cyanobacterial flavodoxin in *S. meliloti* contributes to delayed nodule senescence without significant side effects, although its expression does not result in an increase of plant biomass [64]. As neither *S. meliloti* nor its host *Medicago* encodes flavodoxin, the excess of the FMN-containing electron transport protein may have functioned as a sink for ROS. If this strategy to express a ROS scavenging protein is successful, it would be worth to control endogenous enzymes at proper level. Monofunctional catalases, including *S. meliloti katC* and *M. loti katE*, might be good candidates for this strategy because they are exceptionally stable [65] and function in a wide pH range [66] to detoxify the membrane-permeable ROS H_2O_2.

SOD mutants of the same gene in different *S. meliloti* strains have been reported to have different symbiotic phenotypes. The disruption of *sodA* in *S. meliloti* strain Rm5000 results in fewer infection events, poor nodulation on *Medicago*, failure to differentiate into bacteroids and rapid senescence, all of which result in poor nitrogen fixation [37]. In contrast, disruption of the homologous gene (designated *sodB*) in *S. meliloti* strain Rm1021 has minimal effects on symbiosis in terms of plant growth and nitrogenase activity [67]. Because the *S. meliloti* strains Rm5000 and Rm1021 are derivatives of the same wild-type strain SU47 (synonym of Rm2011) that were selected for spontaneous rifampicin and streptomycin resistance, respectively [68, 69], the phenotypic difference is perplexing. However, SU47 has a slower response to NF than Rm1021 as analyzed by Ca^{2+} spiking, probably due to the

lower expression of *nod* genes [70]. We have disrupted the sole SOD gene (*sodA*) in *M. loti* MAFF303099, and the mutant displays markedly different symbiotic efficiencies depending on the *Lotus japonicus* cultivar used (Hanyu and Saeki, unpublished data). This suggests that the pairing of rhizobial strains and host cultivars significantly affects the outcome of symbiosis.

BacA protein

bacA was first described in *Sinorhizobium meliloti* as a gene essential for bacteroid formation after release into the cytoplasm of the host *Medicago sativa* [11]. The predicted product of *bacA* is an integral membrane peptide transporter that has 64% sequence similarity to *Escherichia coli* SbmA [11]. SbmA sensitizes *E. coli* to some peptide antibiotics including microcin B17, microcin J25 and bleomycin [71]. *S. meliloti bacA* is also involved in this sensitization and, most likely, in the uptake of peptide antibiotics [72, 73]. Homologues of *bacA* are found in *M. loti* MAFF303099 [58] and animal pathogens including *Brucella abortus* and *Mycobacterium tuberculosis* [74, 75]. In *B. abortus*, which induces abortion in chronically infected animals, the *bacA* homolog is required for effective survival in murine macrophages [74]. In *M. tuberculosis*, the causative agent of tuberculosis, the lack of the *bacA* homolog results in the compromised maintenance of persistent infection in mice [75]. Therefore, BacA homologues in symbiotic and pathogenic bacteria may support chronic intracellular survival in their eukaryotic hosts by counteracting host defense reactions [14, 19, 74, 75].

Although many rhizobia have *bacA* homologues, genetic studies were only recently performed in five rhizobial species other than *S. meliloti* [76–78]. Gene disruption studies revealed that the *bacA* homologs of *Mesorhizobium huakuii* and *Rhizobium leguminosarum* bv. *viciae* are essential for symbiosis with *Astragalus sinicus* (Chinese milk vetch) and *Pisum sativum* (pea) [76, 77], respectively. In contrast, similar gene disruption studies have revealed that the *bacA* homologs in *Rhizobium leguminosarum* bv. *phaseoli* and *Rhizobium etli* are dispensable for symbiosis with the host *Phaseolus vulgaris* (bean) [77] and that the *M. loti* MAFF303099 *bacA* homolog is dispensable for symbiosis with *L. japonicus* [78]. Legume hosts that do not require *bacA* in compatible rhizobia form determinate nodules, while those that require *bacA* form indeterminate nodules. The indeterminate hosts *Medicago*, *Pisum* and *Astragalus* belong to the inverted repeat-lacking clade (IRLC), whereas the two determinate hosts, *Lotus* and *Phaseolus*, belong to the robinoids and milletioids clades, respectively [79].

Despite their different contributions to the establishment of symbiosis, *bacA* and its homologues appear to perform similar functions under free-living conditions. BacA-lacking mutants of five rhizobial species have increased resistance to the glycopeptide antibiotic bleomycin and some aminoglycoside antibiotics and increased sensitivity to some membrane-disturbing reagents including detergents [72, 76–78, 80–82]. The *M. tuberculosis* BacA homologue is reported to be an ABC-transporter, but its ATP-binding cassette is located in an extended carboxyl-terminal portion that is not conserved with rhizobial BacA homologs [75]. This suggests that rhizobial BacA homologs may not function without other synergistic components. The *S. meliloti* and *M. huakuii bacA* mutants have abnormal LPS lacking the very-long-chain fatty acid (VLCFA) modification [76, 83, 84]. This is in accordance with the relatively weak but significant structural similarity with the human adrenoleukodystrophy protein (hALDP), and genetic disorders associated with this protein can result in the accumulation of VLCFAs in all body tissues [85]. These properties support a role of BacA homologs as transporters, although there is little direct biochemical evidence.

The rhizobial requirement for BacA is determined by its pairing to a host legume. Rhizobial species absolutely require BacA to establish symbiosis with the IRLC indeterminate legumes *Medicago*, *Pisum* and *Astragalus*, but it is not required for symbiosis with the non-IRLC determinate legumes *Phaseolus* and *Lotus*. In nodules of *M. truncatula* and *P. sativum*, bacteroids tend to undergo endoreproduction with fragmented DNA and have reduced reproductive viability [21]. A similar fragmentation of bacteroid DNA was also observed in *A. sinicus* [86]. In contrast, bacteroids in *L. japonicus* and *P. vulgaris* maintain quasi-normal sizes and reproductive viability [21]. The two cultivars of *R. leguminosarum* have essentially identical main chromosomes, which contain *bacA* and differ only in their symbiotic plasmids [87]; however, their *bacA* mutants display opposite symbiotic phenotypes. Similarly, although *M. loti* and *M. hukuii* are closely related and have similar BacA homologs that differ by only two amino acids, *bacA* mutants of these two non-IRLC legumes display opposite symbiotic phenotypes [76, 78]. *M. loti bacA* can partially complement the symbiotic defect of the *S. meliloti bacA* mutant [78], suggesting that the necessity of BacA to establish symbiosis is solely dependent on the host properties that determine the strength of the intermediate, and possibly maintenance, phase attacks.

IRLC legumes have various nodule-specific cysteine-rich (NCR) peptides that are similar to defensin-type antimicrobial peptides, which may be responsible for the strong attacks (Fig. 2c). The peptides are individually expressed in distinct nodule zones, but neither *Lotus* nor

Phaseolus possesses such peptides [88, 89]. Some NCR peptides have regulatory or bactericidal effects on rhizobial cells and attach to bacteroids in symbiosomes [43]. Notably, NCR peptides attached to bacteroids are delivered via a nodule-specific protein secretion pathway consisting of DNF1, a subunit of the signal peptidase complex [90].

Even if a specific NCR peptide degrades *bacA*-lacking mutants of *S. meliloti* and *R. leguminosarum* bv. *viciae*, it can be difficult to explain the mechanism underlying the BacA-mediated protection due to the pleiotropy of *bacA* deletions. All known BacA-lacking mutants have a compromised cell envelope, with the partial loss of VLCFA from LPS, and a decreased sensitivity to some antibiotics. In addition, *R. leguminosarum* species are deficient in the ability to utilize dicarboxylic acid as a growth substrate [77]. The lack of VLCFA modifications is not directly related to decreased bleomycin sensitivity [82]. To fully understand the protective mechanism of BacA, its biochemical properties, including physiological substrates and components with which it interacts, must be elucidated.

Although the requirement of BacA to establish symbiosis with five legume species has been investigated, it is unclear if the studies in these IRLC and non-IRLC legumes can be extrapolated to indeterminate and determinate legumes. Recently, Oono et al. [91] reported additional classification schemes of bacteroid morphology—swollen (longer than 4 μm or wider than 1.5 μm (for spherical bacteroids) or branched (regardless of size)) and non-swollen (smaller than 2.5 × 1.5 μm)—together with the conventional indeterminate and determinate nodule types. They observed at least four classes of nodules: indeterminate with non-swollen bacteroids, determinate with swollen bacteroids, indeterminate with swollen bacteroids and determinate with non-swollen bacteroids. Based on the distribution of the classes within a legume phylogenetic tree, they proposed multiple evolutionary origins for nodule types and bacterial deformations [91]. It will be of interest to investigate the response of legumes with determinate nodules and swollen bacteroids, as well as legumes with indeterminate nodules and non-swollen bacteroids, to rhizobial cells lacking BacA. Such analyses might help elucidation of factors used to evade host attacks during chronic infection.

Type III and type IV secretion systems and their effectors

The type III and type IV secretion systems (T3SS and T4SS) are important for the virulence of many animal and plant pathogenic bacteria. Bacteria use the T3/T4SS to transfer effector proteins from the bacterial cytoplasm into eukaryotic cells or the external milieu, where they can manipulate host cellular processes to facilitate pathogenicity [92, 93]. Phytopathogenic bacteria often use effector proteins to suppress the host immune response activated by pathogen-associated molecular patterns (PAMPs) [94]. To counteract bacterial effector proteins, host plants use resistance proteins (R proteins) that recognize effector proteins and trigger resistance responses, including the hypersensitive response (HR), which halts pathogen invasion and virulence [95, 96].

T3SS and T4SS are also found in many rhizobia that use effectors to modulate their host specificity and symbiotic efficiency [12, 97, 98]. Rhizobial T3SS genes are often designated *nop* (nodulation outer protein) [99]. Among the rhizobia of model legumes, the *Lotus* symbionts *M. loti* strains MAFF303099 and R7A possess T3SS and T4SS, respectively [58, 100]. The deletion of T3SS in MAFF303099 does not affect its symbiotic performance with its host *L. japonicus* but modulates its nodulation capacity with other species of the *Lotus* genus [101]. A negative effector (Mlr6361 protein) against *Lotus halophilus* possesses a conserved multidomain that is also found in the T3SS genes of several plant pathogens [101]. The absence of T4SS in R7A results in delayed nodulation with *L. corniculatus* but increases the capacity of productive symbiosis with the tree legume *Leucaena leucocephala* [102, 103]. Separate insertion mutations in two effectors (Msi059 and Msi061 proteins) have shown that these proteins are at least partially responsible for the positive and negative effects. Notably, it has been demonstrated using the bacteriophage P1 Cre/*lox*-based CRAft system [104] that these effectors can be transported via the *Agrobacterium tumefaciens* VirB/D4 system into *Arabidopsis thaliana* and *Saccharomyces cerevisiae* [102]. The Mlr6361, Msi059, and Msi061 effectors negatively affect symbiosis with certain hosts and could be recognized as PAMP or virulence factors by the host, whereas those with positive effects might successfully evade host defenses or reinforce symbiotic functions. The *Medicago* symbiont *S. meliloti* strain Rm1021 also encodes a T4SS [105], which does not affect its symbiotic capacity with *M. sativa* or *M. truncatula* [106, 107]. *Rhizobium* sp. NGR234, which has a broad host range, can establish symbiosis with *L. japonicus* [108] and has several T3SS and T4SS [109]; however, experimental studies have focused on one T3SS encoded by a symbiotic plasmid [99, 110–122].

Based on studies in which increased transcription of T3SS genes in *Rhizobium* sp. NGR234 was observed within 24 h of adding the flavonoid daidzein, T3SS have been assumed to counteract the initial and intermediate phase threats [121, 122]. This hypothesis was supported by the immunocytochemical observation that *Sinorhizobium fredii* NopX was detected in infection threads, but not within mature nodules, in *Glycine max* (soybean) and

Vigna unguiculata (cowpea) [123] as well as by other omics studies [124, 125]. However, recent experimental data have indicated that rhizobial T3SS function even in mature nodules. Gene-fusion analyses have shown that NopE1 of *B. japonicum* USDA110 is expressed in mature nodules (4 weeks after infection) and in infection threads [126]. This indicates that some rhizobial effectors are used to subvert the late-phase threats, thereby enabling chronic infection, as is the case in many T3SS effectors of animal pathogens [127, 128].

The presence of *B. japonicum* NopE1 or its closely related homolog NopE2 positively affects symbiosis with *G. max* and *Macroptilium atropurpureum* (Siratro or Purple Bush-Bean) but negatively affects symbiosis with *Vigna radiata* (mung bean or green gram) [129]. Both NopE1 and NopeE2 are transported into the *M. atropurpureum* (host) cytoplasm, as demonstrated using an in-frame fusion [129] to the *Bordetella pertussis* calmodulin-dependent adenylate cyclase (*cya* gene product), which becomes active only in the eukaryotic host cytoplasm where calmodulin is present [130, 131]. Both NopE1 and NopeE2 become physiologically active only after autonomous cleavage [129]. Modification is also required for the activation of NopL and NopP by phosphorylation by a plant cytosolic kinase in *Rhizobium* sp. NGR234 [114]. NopP is transported to the cytoplasm of host nodule cells, which was demonstrated using *Vigna unguiculata* as a host and the fusion of a closely related NopP from *S. fredii* to the *Bordetella* adenylate cyclase described above [132]. These studies indicate that some rhizobial T3SS effectors require post-translational modifications to function in their host.

S. fredii NopP is responsible for suppressing the expression of the host defense-related gene *PR1* in *G. max* [133]. It is plausible that rhizobial T3SS and T4SS effectors can suppress or induce PAMP-triggered host defense responses that might constitute the initial and intermediate phase threats. It is also possible that during the maintenance phase, in addition to defense measures that are common in other non-leguminous plants, legume hosts have nodule-specific attack mechanisms, as exemplified by the expression of NCRs by IRLC legumes. Some T3SS effectors can suppress nodule-specific plant defense measures while others can be activators that increase the expression of nodule-function host genes. Because at least some T3SS effectors are expressed in mature nodules and transported to the host cytoplasm, there can be molecular chaperones that control the timing and order of effector transport, as observed in pathogens [134, 135]. To decipher the function and mode of action for each T3SS or T4SS effector, it is necessary to identify the target molecules in the host plant and to investigate the biochemical properties of the effectors and targets, as well as their molecular chaperones.

Concluding remarks

Rhizobial measures to evade host-derived and endogenous threats do not function equally during the three threat phases (Fig. 2). Furthermore, the measures are not used in the same manner in the model rhizobial species *M. loti* and *S. meliloti*. To evade the initial and intermediate phase threats, ROS scavenging enzymes seem to be more critically are required in *S. meliloti* than in *M. loti*. It is possible that ROS scavenging subverts the maintenance phase threats in both rhizobial models. The BacA protein is essential to overcome intermediate threats in *S. meliloti*, but not *M. loti*. The T3SS and T4SS in *M. loti* are important for host-specific modulation of symbiotic efficiency, mostly by evading or triggering the host defense mechanisms during the initial and intermediate phases. However, it is possible that some effectors contribute to evade threats during the maintenance phase. The contribution of the T4SS in *S. meliloti* is currently not known.

Autoregulation of nodulation is necessary for legumes to balance energy expenditure and growth. Significant advances, including the identification of a receptor kinase and signaling peptide, have been made over the last decade (see reviews [18, 136]). However, it is currently not known how host plants arrest infecting rhizobial cells. Several specific questions remain: (1) Do hosts use ROS or other hazardous compounds to eliminate excess rhizobia? (2) Do hosts simply discontinue the organogenesis program as well as the elongation of infection threads? Combined cytological and biochemical analyses are necessary to address these questions.

Because the maintenance of active mature nodules for long periods would be directly beneficial to host legumes and ultimately to agronomy, it is important to investigate the nature of the late-phase threats. It is also important to determine whether they are involved in the plant sanction of inefficient rhizobial cells. This phenomenon has attracted attention because such a selection method for rhizobia would be beneficial for legumes and influence the co-evolution of legume–rhizobia [137]. It has been reported that the legume host *G. max* supplies less oxygen to nodule cells containing rhizobial *B. japonicum* cells unable to fix nitrogen than to those with nitrogen-fixing rhizobia [50]. However, whether this phenomenon exists is controversial because there is supporting [138] and opposing [139, 140] evidence. Although it is currently unclear whether the observed sanction is caused by simple metabolic imbalances or by complex surveillance machineries that detect commensal rhizobia, studies on late-phase threats will be useful to breed more efficient rhizobial species.

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It might be noted that cloning of soybean *R* gene probably to counteract rhizobial T3SS effectors has been reported by Zhu and colleagues [141] during revision process of this review.

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