Introduction

Whereas intracellular infection by bacteria is widespread in the animal kingdom, most often as part of a pathogenic interaction, it is more sporadic in the plant kingdom. One noticeable instance is the intracellular colonization of legume root nodules by phylogenetically diverse bacteria collectively called rhizobia [1]. Rhizobia enter a mutualistic symbiosis with legumes resulting in a chronic infection of legume cells by endosymbiotic bacteria that fix nitrogen to the benefit of the plant. The plant, in turn, provides host bacteria with carbon resources and a privileged niche.

Rhizobia vary in their mode of initial penetration of root tissues [2]. Some enter root tissues by crack entry at the emergence of host bacteria with carbon resources and a privileged niche.

In a search for a suitable experimental assay, we reasoned that symbiosome formation by rhizobia was reminiscent of the chronic extinction of infection threads inside the differentiating nodule cells and become enclosed in a plant-derived peribacteroid membrane to form symbiosomes, reminiscent of phagosomes found in animal systems. One single nodule cell typically contains thousands of symbiosomes.

Key molecules for nodulation and infection thread formation are the well-described lipo-chitooligosaccharides called Nod factors that are specifically recognized by plant receptor-like kinases [3,4]. Bacterial surface lipo- and exopolysaccharides, such as low molecular weight succinoglycan, are also often required for successful infection thread formation although their actual role has not been completely elucidated yet [3]. The process of intracellular infection that was recently shown to involve both exocytic and endocytic cellular pathways [5], remains poorly understood because whole plant assays do not permit specific insight into this late symbiotic infection step.

In a search for a suitable experimental assay, we reasoned that symbiosome formation by rhizobia was reminiscent of the chronic...
invasion of animal cells by intracellular pathogens such as *Brucella*, *Salmonella* or *Legionella* (reviewed in [6]). Some of these animal pathogens are phylogenetically interspersed with rhizobia (e.g. *Brucella* or *Bartonella* and *Sinorhizobium*) and common genetic determinants of symbiosis and pathogenicity have been reported [6–8]. This prompted us to assay and co-culture *in vitro* the model rhizobium *Sinorhizobium meliloti* [9] with human HeLa cells, a widely-used experimental infection model for animal pathogens for which many molecular and cellular tools are available.

Here we report that *S. meliloti*, and other phylogenetically distant rhizobia as well, were able to induce major actin cytoskeleton rearrangements on HeLa cells. We isolated mutants of the *S. meliloti* wild-type strain that were defective for actin cytoskeleton modifications on HeLa cells. We found that these mutants were affected in queuosine biosynthesis, a modified nucleoside that affects gene expression post-transcriptionally. We showed that an intact queuosine biosynthetic pathway was also required for efficient symbiosis with *Medicago truncatula*. Possible links between actin cytoskeleton modification, queuosine biosynthesis and symbiotic proficiency are discussed.

**Results**

1. **Rhizobia induce drastic actin cytoskeleton modifications on HeLa cells**

Subconfluent HeLa cells were incubated with *S. meliloti* bacteria at a multiplicity of infection of 100 (i.e. 100 bacteria per eukaryotic cell). The morphology of HeLa cells was observed at different time points after bacterial inoculation and fluorescent phalloidin staining of actin. Epithelial cells infected with the wild type strain 1021 of *S. meliloti* displayed an extended, slender and elongated morphology that was not observed in non-infected cells (Fig. 1AB and Fig. S1). Cell deformations could be observed ca 30 hours after inoculation (hpi) and increased with time so that 80% of Hela cells presented drastic morphological changes at 48 hpi. *S. meliloti* also induced a loss of stress fibers in HeLa cells (Fig. 1AB) as well as a block in cell cycle (Fig. S2).

Rhizobia are phylogenetically disparate bacteria among the α- and β-subclasses of proteobacteria that share the ability to form an endocellular symbiosis with plants [1]. We thus tested rhizobia belonging to different genera for their impact on HeLa cell morphology. *Rhizobium leguminosarum*, *Azorhizobium caulinodans* and the β-rhizobia *Capriovicis taiwanensis* and *Burkholderia tuberum* induced cytoskeleton changes on HeLa cells similar to those induced by *S. meliloti* (Fig. 1). Instead, no cytoskeleton modifications were observed upon inoculation of HeLa cells with the aquatic bacterium *Caulobacter crescentus* -an α-proteobacterium closely related to *S. meliloti*- and with the γ-proteobacterium *E. coli* (Fig. 1, Fig. S3). This set of observations indicated that different rhizobia share the ability to promote actin cytoskeletal rearrangements on eukaryotic cells, which could be symbiotically relevant [10,11].

This result prompted us to explore in more detail the molecular mechanisms underlying the cellular changes induced by *S. meliloti* strain 1021 on HeLa cells. No cellular modification was observed when HeLa cells were separated from *S. meliloti* cells by a 0.2 μm anapore membrane or incubated with a bacteria-free culture supernatant (data not shown). This suggested that cytoskeleton modifications were not provoked by a diffusible bacterial molecule and, instead, required a physical contact between bacteria and HeLa cells. Heat-killed *S. meliloti* cells did not trigger morphological changes thus indicating that live bacteria were needed for HeLa cell deformation (data not shown).

**Figure 1. Bacteria-induced cytoskeleton modifications of HeLa cells.** HeLa cells untreated (A), inoculated with *S. meliloti* (B), *R. leguminosarum* (C), *A. caulinodans* (D), *C. taiwanensis* (E), *B. tuberum* (F), *C. crescentus* (G) and *E. coli* (H). HeLa cells were stained with phalloidin-Texas red and observed by fluorescence microscopy 48 hours after bacterial inoculation. Arrow: stress fiber. doi:10.1371/journal.pone.0056043.g001

HeLa cells were inoculated with a GFP-tagged *S. meliloti* strain in order to monitor bacterial entry and survival. Confocal analyses showed rare (2–3/cell) live, GFP-expressing, bacteria in ca 50% of HeLa cells, between 18 and 48 hpi (Fig. S4). Instead, electron microscopy analyses showed a high number of intracellular bacteria within vacuoles, most often in a degraded state thus indicating that bacteria were efficiently internalized but did not maintain (Fig. 2).

As small GTPases Cdc42, Rac1 and RhoA are known to coordinate signaling cascades that produce both morphological
and nuclear responses to a variety of extracellular signals [12,13] we examined whether these GTPases were involved in the S. meliloti-induced cellular responses of HeLa cells. The activation state of Cdc42, Rac1 and RhoA of HeLa cells challenged with S. meliloti was measured by pull down assays (Fig. 3). At different time points following HeLa cell infection with S. meliloti, lysates were prepared and the amount of active, GTP-bound, Cdc42, Rac1 and RhoA precipitated with the GST-CRIB fusion protein was determined by western blotting [14]. A decrease in the level of the active form of the three small RhoGTPases was detected at 48 hpi with live S. meliloti (Fig. 3AB) that was not detected after heat-killing of bacteria (Fig. 3C). A kinetic analysis showed that Cdc42 inhibition was already detectable at 24 hpi (Fig. 3D).

2. HeLa cell invasion, cytoskeleton modifications and modulation of RhoGTPases activity by S. meliloti required an intact queuosine biosynthetic pathway

With the aim to identify bacterial genes involved in HeLa cell infection and cytoskeleton modifications, we screened a library of S. meliloti mutants for their ability to induce HeLa cell elongation. Mutants in genes known to be essential for symbiosis such as genes involved in Nod Factor production (nodA, nodD1nodD2nodD3), exopolysaccharide (exoITWY) or lipopolysaccharide (lpsB) synthesis, bacteroid differentiation and survival (bacA), nitrogen fixation and microoxic respiration (fixJ), stress adaptation (tp5A) or the crp-like regulator Clr (Table S1) all triggered HeLa cells deformations as wild-type thus indicating that cytoskeleton modifications were independent from known symbiotic genes. Upon testing S. meliloti strains carrying large deletions on the pSymB megaplasmid [15], we found that a 120 Kb-large deletion (Rm541) prevented HeLa cell elongation. Nested site-directed deletions in the same region (GMI11660, GMI11661, GMI11662; Table S1) indicated that the locus responsible for HeLa cell deformations mapped into the exs gene cluster required for succinoglycan biosynthesis [16]. Systematic individual inactivation of genes located in this region showed that the exsBCD gene cluster was responsible for actin cytoskeleton modifications on HeLa cells. Expert sequence analysis of the exsBCD genes indicated that they are the likely orthologues of the queCDE genes from E. coli and B. subtilis involved in the biosynthesis of queuosine (Fig. 4), a modified nucleoside that replaces guanosine at position 54 of tRNAsGUN [17]. We thus renamed the exsBCD genes of S. meliloti queCDE.

Looking for additional genes belonging to this pathway (Fig. 4) we identified queF (SMc02723), tgt (SMc01206) and queA (SMc01207) genes on the S. meliloti main chromosome. These genes were individually inactivated and corresponding mutants triggered reduced HeLa cell deformations 48 hpi in regular HeLa culture medium (DMEM) containing 10% Foetal Calf Serum (FCS). As FCS is known to contain queuine [18], the base moiety of queuosine, we cultured HeLa cells in a medium containing only 0.5% FCS. We then observed that the queC, queF and tgt mutants were completely unable to induce actin cytoskeletal changes on HeLa cells in this medium (Fig. 5). This was not due to an altered growth of que mutants as compared to wild-type under the assay conditions (Fig. S5). Supplementation of the 0.5% FCS culture medium with 300 nM of the queuosine precursor preQ1 completely restored an elongated HeLa phenotype for the queC and queF mutants, but not for the tgt or queA mutant consistently
with the proposed queuosine biosynthetic pathway (Fig. 4). Genetic complementation of the queF null mutant by a plasmid-encoded queF gene (GMI11686, Table 1) restored HeLa cell deformation, as expected (Fig. 5J). Pull down experiments performed on HeLa cells inoculated with the queF mutant at 48 hpi revealed high activity of the Rho-GTPases, as in non-inoculated cells or cells inoculated with live and heat-killed wild-type bacteria 48 hpi. (D) Kinetics of Cdc42 activation. Actin, total Cdc42, Rac1 and RhoA or active GTP-bound forms of Cdc42, Rac1 or RhoA were detected by immuno-blotting of SDS-PAGE gels.

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Figure 3. Determination of GTPases activation state in bacteria-treated HeLa cells. (A) Representative pull down assays of active Cdc42, Rac1 and RhoA GTPases at 48 hpi in non-inoculated (control), S. meliloti 1021- and queF-inoculated HeLa cells. (B) Quantification of pull down assays using ImageJ software. Means ± S.D. were calculated from three independent experiments for Cdc42-GTP and mean from two independent experiments for Rac1-GTP and RhoA-GTP. Results were normalized to the corresponding total protein. Statistical significance (P<0.001) is shown (*) with respect to the control. (C) Immunoprecipitation of active and total Cdc42 from non-inoculated (control) HeLa cells or cells inoculated with live and heat-killed wild-type bacteria 48 hpi. (D) Kinetics of Cdc42 activation. Actin, total Cdc42, Rac1 and RhoA or active GTP-bound forms of Cdc42, Rac1 or RhoA were detected by immuno-blotting of SDS-PAGE gels.

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Figure 4. The S. meliloti queuosine biosynthetic pathway. preQ0: 7-cyano-7-deazaguanine, preQ1: 7-(aminomethyl)-7-deazaguanine, AdoMet: S-adenosyl-L-methionine, EpoxyQ: epoxyqueuosine, Q: queuosine. Adapted from [49,50].

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with the proposed queuosine biosynthetic pathway (Fig. 4). Genetic complementation of the queF null mutant by a plasmid-encoded queF gene (GMI11686, Table 1) restored HeLa cell deformation, as expected (Fig. 5J). Pull down experiments performed on HeLa cells inoculated with the queF mutant at 48 hpi revealed high activity of the Rho-GTPases, as in non-inoculated cells or cells inoculated with heat-killed bacteria (Fig. 3AB), thus reinforcing the link between Rho-GTPases inhibition and actin cytoskeleton modifications.

Electron microscopy indicated that HeLa cells were not, or very poorly, invaded by the queF mutant (Fig. 2E). We thus wondered whether the defect of this mutant in promoting actin cytoskeleton reorganization originated from its defect in invading HeLa cells. This was ruled out by treating HeLa cells with the E. coli CNF-1 toxin, that is known to promote internalization of bacteria in cultured cells [19]. CNF-1 restored entry of the queF mutant without restoring actin cytoskeleton modifications (Fig. S4). Furthermore, the CNF-1 toxin promoted an increase in wild-type bacteria internalization, as compared to untreated cells, without exacerbating or accelerating the extent of cytoskeleton modifications (data not shown). This suggests that bacterial entry might not be required for cytoskeleton modifications.
3. A *S. meliloti* 1021 *queF* mutant is severely affected in late symbiotic stages

We assessed the symbiotic performances of the various *S. meliloti* queuosine deficient mutants on the model legume *Medicago truncatula*. At 40 dpi, aerial parts of *Medicago* plants grown in nitrogen-free medium and inoculated with the mutants were small and yellowish suggesting a defect in nitrogen fixation. Dry-weight measurements of aerial parts confirmed a profound defect of the *queF*, *queC* and *tgt* mutants in nitrogen fixation ability (Fig. 6A).

Surprisingly, no or little symbiotic phenotype was observed for the *queA* mutant. Genetic complementation of the *queF* null mutant by a wild type allele restored symbiotic proficiency (Fig. 6).

A more detailed phenotypic characterization of plants inoculated with the *queF* mutant was performed. The kinetics of nodulation by the *queF* mutant was indistinguishable from wild-type (Fig. S6), thus pointing to a late symbiotic defect. The indeterminate nodules formed by *S. meliloti* on *M. truncatula* display a longitudinal gradient of bacteroids at different stages of differentiation, the most differentiated forms (type 4/5 bacteroids) in the central zone of the nodule being responsible for nitrogen fixation [20]. Optical and electron microscopy analyses of *queF*-induced nodules showed a sharp decrease in the number of successfully infected cells (Fig. 6C). Bacteroids formed by the *queF* mutant differentiated into type 4/5 bacteroids (Fig. 6E) that were, however, randomly organized within the infected cells, in striking contrast to the normal radial organization of wild-type *S. meliloti* bacteroids (Fig. 6D). This indicates a defect in symbiosome

**Table 1. Bacterial strains and plasmids.**

| Strains Description | Reference |
|---------------------|-----------|
| **S. meliloti strains** | |
| 1021 Str<sup>e</sup> derivative of *S. meliloti* strain SU47 | [51] |
| Rm5416 ΔΩ5007-5011-Tn5-233, Gen<sup>e</sup> Str<sup>e</sup> Spe<sup>e</sup> | [52] |
| GMI11655 1021 queA:pVO155 (221 bp), Str<sup>e</sup>, Neo<sup>e</sup> | This work |
| GMI11656 1021 queC:pVO155 (63 bp), Str<sup>e</sup>, Neo<sup>e</sup> | This work |
| GMI11657 1021 tgt:pVO155 (128 bp), Str<sup>e</sup>, Neo<sup>e</sup> | This work |
| GMI1546 1021 queF:pVO155 (18 bp), Str<sup>e</sup>, Neo<sup>e</sup> | This work |
| GMI11658 1021 SMc02721:pVO155 (654 bp), Str<sup>e</sup>, Neo<sup>e</sup> | This work |
| GMI11659 1021 SMc02722:pVO155 (881 bp), Str<sup>e</sup>, Neo<sup>e</sup> | This work |
| GMI11669 1021 pHC60 Str<sup>e</sup> Tet<sup>e</sup> | This work |
| GMI11686 GMI11546 pGMI(queF), Str<sup>e</sup>, Neo<sup>e</sup>, Gen<sup>e</sup> | This work |
| **Other bacterial strains** | |
| 248 *Rhizobium leguminosarum* bv *viciae* | [43] |
| ORS571 *Azorhizobium caulinodans* | [44] |
| LMG19424 *Cupriavidus taiwanensis* | [45] |
| STM678 *Burkholderia tuberculosis* | [46] |
| NA1000 *Caulobacter crescentus* | [47] |
| DH5 alpha *Escherichia coli* | [42] |
| **Plasmids** | |
| pH650 pHC41 containing GFP-S65T, Tet<sup>e</sup> | [53] |
| pRK600 Helper plasmid, Chi<sup>e</sup> | [52] |
| pVO155 Suicide plasmid, Kan<sup>e</sup>, Amp<sup>e</sup> | [41] |

The location of plasmid insertions (number of nucleotides after the start codon) is indicated between brackets.

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A more detailed phenotypic characterization of plants inoculated with the *queF* mutant was performed. The kinetics of nodulation by the *queF* mutant was indistinguishable from wild-type (Fig. S6), thus pointing to a late symbiotic defect. The indeterminate nodules formed by *S. meliloti* on *M. truncatula* display a longitudinal gradient of bacteroids at different stages of differentiation, the most differentiated forms (type 4/5 bacteroids) in the central zone of the nodule being responsible for nitrogen fixation [20]. Optical and electron microscopy analyses of *queF*-induced nodules showed a sharp decrease in the number of successfully infected cells (Fig. 6C). Bacteroids formed by the *queF* mutant differentiated into type 4/5 bacteroids (Fig. 6E) that were, however, randomly organized within the infected cells, in striking contrast to the normal radial organization of wild-type *S. meliloti* bacteroids (Fig. 6D). This indicates a defect in symbiosome
organization in queF-infected nodules. Numerous starch granules were observed consistent with the carbon imbalance resulting from the defect in nitrogen fixation. Finally, an early degeneration of infected nodule cells was observed as soon as 21 dpi. At 40 dpi, no more live queF bacteria were observed in nodule cells indicating a strong defect in bacteroid maintenance (data not shown).

Discussion

Despite obvious intrinsic limitations, in vitro assays and surrogate hosts are useful simplified experimental systems to help decipher complex biological processes taking place during host-microbe interactions [21]. Our understanding of rhizobial intracellular infection, a late step in the symbiotic interaction, has been so far impaired by the lack of a dedicated assay. In this study, we report on the ability of S. meliloti to trigger a dramatic rearrangement of actin cytoskeleton on HeLa cells, resulting in severely elongated cells, reminiscent of the “hummingbird” phenotype induced by Helicobacter pylori [22]. As with H. pylori, a long (48 hpi) incubation time was needed to observe a full cytopathic effect on HeLa cells [23]. Interestingly, other phylogenetically distant rhizobia induced similar modifications on HeLa cells, whereas C. crescentus and E. coli did not.

Actin cytoskeleton rearrangements are known to occur at different stages of the symbiotic interaction between rhizobia and their host plants, including root hair growth and curling, infection thread formation, bacterial internalization and positioning in plant nodule cells, cell trafficking and possibly defence reactions [24,25]. It is thus tempting to speculate that the actin cytoskeleton deformations elicited by rhizobia on HeLa cells may reflect the ability of these bacteria to manipulate legume plant actin cytoskeleton. In animal systems, actin-based cytoskeleton rearrangements are regulated by small GTPases of the Rho family, which are often hijacked by animal pathogens [26,27]. We took advantage of the molecular tools available on human Rho-GTPases to demonstrate that the actin cytoskeleton modifications induced by S. meliloti on HeLa cells were associated with an inhibition of the activity of the three major Rho-GTPases, RhoA, Rac and Cdc42. This suggests that S. meliloti and possibly other rhizobia may manipulate plant Rho-GTPases in a symbiotic context.

Further experiments are now required to assess this possibility in a homologous context. In contrast to animal and fungi, plants possess a single family of closely related members known as ROPs (Rho-GTPases of plants), similar to animal Rac-GTPases [28]. Medicago and Lotus Rho-GTPases-encoding genes have been identified recently that express at different stages of the symbiotic interaction [28–32]. It would be very interesting to characterize both the expression and activity of these Medicago Rho-GTPases in response to infection by wt S. meliloti and que mutants.

A novel finding reported here is that actin cytoskeletal modifications as well as Rho-GTPases inhibition on HeLa cells required an intact pathway for queuosine biosynthesis. Queuosine is a hyper-modified guanosine whose function is not completely understood. Previous studies have shown that the queuosine modification of rRNAsGUN, specifying aspartic acid, asparagine, tyrosine and histidine, may affect the translation efficiency of target proteins by either modulating the interaction of tRNAs with the different degenerate codons [33] or by enhancing the binding efficiency of tRNAs to the ribosomes [34]. Interestingly, it was shown recently that some mRNAs could be directly queuosine-modified in vitro [35] suggesting that queuosine may modulate protein translation by targeting either tRNAs or mRNAs. Contrary to eukaryotes that obtain queuosine or queuine from their diet, bacteria can synthesize queuosine de novo [36]. We have identified the corresponding biosynthetic pathway in S. meliloti whose genes are split on three loci on two different chromosomes. Although the queuine base was previously shown to affect cell proliferation and intracellular signaling in HeLa cells [37], no cytological modifications similar to those described here have been reported, to our knowledge, upon queuine addition. Furthermore, we observed no cytoskeleton modification of HeLa cells upon addition of (wild-type) bacterial culture supernatants thus making it unlikely that free queuine or queuosine potentially secreted by wild-type rhizobia could be responsible for the cell deformations.

Figure 6. Symbiotic phenotype of S. meliloti queuosine mutants. Dry weight of M. truncatula seedlings inoculated with S. meliloti 1021, different queuosine-deficient mutants and the queF complemented (GMI11186) strain at 40 dpi. Statistical significance (P<0.01) is shown with respect to strain 1021(*) and the queF mutant (**), respectively. (B, C) Sections of M. truncatula 21-day old nodules induced by 1021 (B) and the queF isogenic mutant (C). (D, E) Electron micrographs of nodule cells infected with 1021 (D) or the queF mutant (E). queF mutant bacteroids are randomly organized within the infected cell whereas 1021 bacteroids show a radial organization. (Insert panel in E): arrows point to symbiosome membranes detached from queF bacteria (*). Arrowhead, type 4/5 bacteroid. *, starch granules.

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described here. Instead, a physical contact between live bacteria and HeLa cells was required for HeLa cell deformation to occur. Bacterial entry however might not be required for cytokskeleton reorganization, based on E. coli CNF1 toxin experiments. Altogether this suggests that a S. meliloti protein(s) whose synthesis is under queuosine control, or its product, might be involved in actin cytoskeletal modifications. In S. meliloti, as in S. fredii, the queCDExsCDOE gene cluster is physically linked to genes involved in exopolysaccharide synthesis. Furthermore, a queC (exsC) mutant of S. meliloti was shown to overproduce succinoglycan, a symbiotically important exopolysaccharide, thus indicating a functional link between the two pathways [16]. Succinoglycan itself is not responsible for eliciting HeLa cell deformation since all exo mutants tested, including exoT that is completely defective in succinoglycan production, induced HeLa cell deformation as wild-type. A queA mutant of S. meliloti also has modified lipopolysaccharides [38]. However a lpsB mutant affected in a mannosyltransferase required for LPS core biosynthesis and symbiosis with Medicago sp. [39], triggered HeLa cell deformation as wild-type (Table S1). Hence the que-dependent functions responsible for triggering actin cytoskeleton modifications remain to be identified.

The queF, queC and tgt mutants of S. meliloti 1021 had a severe defect in symbiotic phenotype on M. truncatula. Surprisingly, no conspicuous symbiotic defect was observed with the queA mutant. A possible reason could be the complementation of the queA mutant by a plant-derived metabolite or, alternatively, an alternate bacterial gene/pathway specifically expressed mutant. In planta, we observed a parallel between the intensity of the symbiotic type. A que (Table S1). Hence the Medicago sp. transferase required for LPS core biosynthesis and symbiosis with lpsB and infection thread formation and progression into the cortex. In early symbiotic stages, including nodulation, root hair infection precisely the queuosine-dependent functions in S. meliloti is now required to challenge this possibility.

1. Experimental procedures

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Tables 1 and S1. For genetic purposes, S. meliloti strains were grown at 28°C in TY (Tryptone-Yeast) medium supplemented with 6 mM of CaCl2. Antibiotics were used at the following concentrations: streptomycin 200 μg/ml, neomycin 100 μg/ml, tetracyclin 10 μg/ml. The 1021 queA, queC, queF and tgt mutants were obtained by site-specific insertion of the pVO155 plasmid as previously described [41]. Primers used for mutagenesis are listed in Table S2. pVO155 plasmids for mutants generation and the pHCG6 plasmid for the construction of GFP expressing bacteria were introduced in S. meliloti by triparental mating using the pRK600 as helper plasmid. Mutants were checked by PCR. Rhizobium leguminosarum, Agrobacterium tumefaciens, Burkholderia tuberum, Caulobacter crescentus, and E. coli were grown as previously described [42–47].

2. HeLa cell culture and infections

HeLa cells were routinely cultured in DMEM (Invitrogen) medium supplemented with 2 mM L-glutamine and 10% heat inactivated FCS (Invitrogen) at 37°C in a 5% CO2 incubator. For immunoprecipitation and electron microscopy experiments, cells were cultured in 10 cm petri dishes. For immunofluorescence experiments and confocal microscopic observations, 106 cells were grown with either 10% or 0.5% FCS on glass coverslips in 24-well plates. All bacterial species were grown to mid-log phase and added to HeLa cells 24 hours after eukaryotic cell replication at a multiplicity of infection of 100. After infection, HeLa cells were grown as usual at 37°C in a 5% CO2 incubator. For long incubation times (>24 hours) the culture medium was replaced by fresh medium at 24 hpi. Cell-bacterium separation assays were performed using 0.2 μm Anopore™ membrane (Nunc). For supplementation experiments, the queuosine precursor preQ1 (300 nM final) was added just before bacterial inoculation. The E. coli CNF1 toxin was used at 10−8 M and added to the culture medium just before bacterial infection.

3. Immunoprecipitation assays

Activation of endogenous Cdc42, Rac1 and Rho GTase was measured by p21-activated kinase (PAK)-Cdc42-Rac1 (CRIB) and Rhoetkin-Rho interaction binding pull-down assays as previously described [14]. In brief, at different time points after bacterial inoculation, HeLa cells were washed twice with cold PBS and lysed by scraping on ice in cold radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM MgCl2). Lysates were cleared by centrifugation at 13,000 rpm for 15 min at 4°C, an aliquot was saved to assess total protein level and the remaining lysates were used to assess level of active GTases. Lysates were incubated for 1 h at 4°C with ca. 30 μg of gluthathione sepharose beads (GST) fused to the CRIB domain of PAK, WASP or Roketkin. GST beads (Amersham) were added (50% slurry) for 1 h at 4°C to precipitate GTases. Beads were subsequently washed twice in cold washing buffer (50 mM Tris pH 7.5, 137 mM NaCl, 10 mM MgCl2, 1% Triton X-100). Equal amount of beads and total cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted using anti-Cdc42, Rac1 or Rho antibodies (Santa Cruz). Blots were visualized using chemiluminescence reagents (Amer sham).

4. HeLa cells microscopic and flux cytometry examination

For fluorescence microscopy analysis, cells were washed 3 times with PBS and fixed with 3.7% Paraformaldehyde (PFA) for 30 min, free aldehyde groups were blocked with 50 mM NH4Cl for 15 minutes, then cells were permeabilized with 0.3% Triton X-100 for 10 min and stained with Texas red-labelled phalloidin (Molecular Probes) to visualize F-actin cytoskeleton. Cells were mounted with mounting medium (DakoCytomation) and observed with an inverted LEICA microscope (Axiohot I) or a confocal LEICA SP2 AOBS microscope.

For electron microscopy analysis, untreated or bacteria-inoculated HeLa cells were recovered by scraping of 9 cm Petri dishes and fixed in 2.5% glutaraldehyde in phosphate buffer. The fixed material was postfixed in 1% osmium tetroxide, dehydrated in an ethanol series and then embedded in Epon 812 for electron microscopy analysis. Semithin (1 μm) and ultrathin (0.1 μm) sections were taken using a Reichert Ultracut ultramicrotome. The semithin sections were collected on glass slides and stained with 1% toluidine-0.1% methylene blue in borax. The ultrathin sections were stained with uranyl acetate and lead citrate before...
being viewed under a Hitachi EM600 transmission electron microscope. For scanning electron microscopy, control or infected cells (48 hpi) were grown on 0.5 mm slides, fixed in glutaraldehyde (4%) in cacodylate buffer, washed, dehydrated, and metallized (1.2 V, 10 mA) before observation under a MAB Hitachi S4500 microscope.

For cell cycle analyses, untreated and bacteria-inoculated HeLa cells were grown on 9 cm Petri dishes in DME medium with 0.5% FCS and proceeded as described [48]. Briefly, cells were recovered by scraping 48 hpi into PBS-EDTA 0.02% at 4°C. After centrifugation the cell pellet was washed in cold Propidium Iodide/Triton X-100 staining solution (0. 1% (v/v) Triton X-100 (Sigma) in PBS, 2 mg DNase-free RNase A (Sigma), 0.40 ml of 500 µg/ml PI) and analysed on a cyto Facsalibur (Becton Dickinson).

5. Plant assays

Seeds of Medicago truncatula cv. Jemalong A17 were surface sterilized, germinated and grown in test tubes containing slanting nitrogen-free Fahraeus agar medium for three days at 22°C with day and night cycles of 16 and 8 hours, respectively. 20 plants nitrogen-free Fahraeus agar medium for three days at 22°C in phase G0/G1 with cells untreated (A) or inoculated with S. meliloti 1021 (B).

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