A selective bottleneck during host entry drives the evolution of new legume symbionts

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Abstract

During the emergence of new host-microbe symbioses, multiple selective pressures –acting at the different steps of the microbial life cycle– will shape the phenotypic traits that jointly determine microbial fitness. However, the relative contribution of these different selective pressures on the adaptive trajectories of microbial symbionts are still poorly known. Here we characterized the dynamics of phenotypic adaptation and the underlying genetic bases during the experimental evolution of a plant pathogenic bacterium into a legume symbiont. We show that fast adaptation was supported by the sequential fixation of mutational cohorts within populations, and dominated by selection for host entry competitiveness over within-host proliferation. Computer simulations predict that this effect emerges from the presence of a strong selective bottleneck at host entry before bacterial multiplication. However, identifying and characterizing adaptive mutations revealed recurrent couplings between the two phenotypic traits, further increasing the efficiency of host-mediated selection of new symbionts.

Main

Many bacterial lineages have evolved the capacity to establish symbiotic associations, either beneficial, neutral or parasitic, with eukaryotic hosts. These interactions form a dynamic continuum along which bacteria can move¹. Lifestyle changes may arise due to ecological (resource availability, host environment changes or host shifts) or genomic (mutations, acquisition of new genetic material) modifications. These new interactions are often initially sub-optimal for the bacterial partner²⁻⁴, which

may then adapt to the selective pressures associated with its new life-cycle. When the microbes are transmitted horizontally, they must alternate between at least two different habitats, the host and the environment, which will impose a variety of constraints and selective pressures^{5,6}. Biphase life cycles therefore require bacteria to be able to enter and exit their hosts, and replicate and persist within each habitat. Surviving and persisting in different niches requires adapting to abiotic stressors, host immunity, competitors, as well as being able to use specific nutrient sources. However, the relative influence of the different selective pressures on the dynamics of bacterial adaptation to a new interaction and on the evolution of phenotypic traits is poorly known.

Rhizobia are examples of facultative host-associated bacteria that can either live freely in soil or in symbiotic mutualistic associations with legume plants⁷. In most legumes, rhizobia penetrate the root tissue through the formation of so-called infection threads (ITs). Most of the time, only one bacterium attaches to the root hair and initiates the formation of ITs⁸, thus creating a strong population bottleneck between the rhizosphere and internal root tissues. Bacteria then divide clonally within ITs, while at the same time a nodule starts to develop at the basis of the infected root hair. Hundreds of millions of bacteria are then released from ITs inside the cells of the developing nodule and differentiate into nitrogen fixing bacteroids^{9,10}. After several months, in nature, nodule senescence leads to the release of a part of bacterial nodule population in the surrounding soil. To fulfil their life cycle, rhizobia rely on the activity of numerous bacterial genes, allowing signal exchanges with the host plant, proliferation and metabolic exchanges within nodule cells, while maintaining free-living proficiency⁷. Somewhat surprisingly given their complex and specialized lifestyle, rhizobia evolved several times independently. Horizontal transfer of key symbiotic genes is a necessary, though often not sufficient, condition for a new rhizobium to emerge¹¹⁻¹⁴. Additional steps of adaptation of the recipient bacterium, occurring during evolution under plant selection, are believed to be needed to actualize the symbiotic potential of emerging rhizobia¹⁵.

In a previous study, we used experimental evolution to convert the plant pathogen *Ralstonia solanacearum* into an intracellular legume symbiont¹⁶. Here, we analysed the dynamics of molecular evolution in 5 lineages evolved for 35 cycles of nodulation through whole-population genome sequencing and examined the selective and genetic bases of adaptation. Adaptation proceeded rapidly during the first cycles of evolution, and was underpinned by the fixation of successive cohorts of mutations within populations. We then identified adaptive mutations in two lineages and evaluated their effect on each symbiotic stage of the rhizobial life cycle. Our experimental data indicated that selection for host entry outweighs the selection for multiplication within host. Computer simulations further showed that the selective nodulation bottleneck and the chronology of symbiotic events are critical drivers of this evolutionary pattern.

Results

Fast adaptation of new legume symbionts during the first cycles of evolution

To investigate the genetic and evolutionary conditions that promote the evolution of new rhizobia, we previously transferred a symbiotic plasmid (pRalt) from the rhizobium *Cupriavidus taiwanensis* LMG19424 into the plant pathogen *Ralstonia solanacearum* GMI1000. We obtained three nodulating ancestors, two infecting nodules intracellularly (CBM212, CBM349) and one infecting nodules extracellularly (CBM356)¹⁴, that we evolved in 18 parallel bacterial lineages for 16 serial cycles of nodulation on *Mimosa pudica* plants¹⁶. Here we continued the evolution of five of these lineages (referred to as lineages B, F, G, K, and M) until cycle 35 (Fig. 1a, Extended Data Fig. 1, Supplementary Table 1). After 35 cycles, no nitrogen fixation sustaining plant growth was observed. Therefore, we focus exclusively on the analysis of changes in bacterial fitness in this manuscript. To analyse the dynamics of fitness changes over time, we compared the relative fitness of the nodulating ancestors and evolved clones from cycles 16 and 35 by replaying one nodulation cycle in competition with the natural symbiont *C. taiwanensis*. Fitness trajectories were similar in the five lineages. While the three nodulating ancestors were on average 10^6 times less fit than *C. taiwanensis*, fitness improved very quickly during the first 16 cycles, then slower until cycle 35 (Fig. 1b,c). On average, evolved clones isolated at cycle 16 and cycle 35 were 47 and 19 times less fit than *C. taiwanensis*, respectively (Fig. 1c, and Supplementary Table 2).

Because bacterial fitness in our system depends on both the capacity of strains to enter the host and induce nodule formation (nodulation competitiveness) and to multiply within these nodules (within-host proliferation), we analysed how each of these two fitness components changed during the experiment (Fig. 1b,c,d). In the five lineages, evolutionary trajectories of nodulation competitiveness resembled that of fitness with fast improvement during the first 16 cycles, and a slow-down during the last cycles. On average, nodulating ancestors were 5×10^4 times less competitive than *C. taiwanensis*, while cycle 16 and cycle 35 evolved clones were only 17 and 4 times less competitive than *C. taiwanensis*, respectively (Fig. 1c and Supplementary Table 2). Moreover, evolved clones B16, B35, G16, G35 and M35 were not statistically different from *C. taiwanensis* in terms of nodulation competitiveness. Within-host proliferation also improved mostly during the first 16 cycles in all lineages. The rate of improvement then slowed down in two lineages and continued to increase significantly in the three others. As previously published, the three nodulating ancestors display different capacities to infect nodules^{14,16}, the extracellularly infective ancestor CBM356 being the less infective (750 times less than *C. taiwanensis*) and the intracellularly infective CBM349 being the most

infective (45 times less than *C. taiwanensis*). On average, cycle 16 and cycle 35 evolved clones were 8 and 5 times less infective than *C. taiwanensis* (Fig. 1d and Supplementary Table 2). Altogether, the symbiotic properties of evolved clones improved rapidly during the first cycles of the evolution experiment and then adaptation slowed down. Both symbiotic traits, nodulation competitiveness (*i.e.* host entry) and within-host proliferation, improved greatly. However, gains in nodulation competitiveness (average factor of 11,500 between ancestors and cycle 35 clones) were *ca.* 150 times higher than gains in proliferation (average factor of 74 between ancestors and cycle 35 clones). Moreover, although the difference in nodulation competitiveness between *C. taiwanensis* and the nodulating ancestors was much more important ($>10^4$ fold) than the difference in proliferation ($<10^3$ fold), nodulation competitiveness of evolved clones reached the level of *C. taiwanensis* in three lineages (B, G and M) while within host proliferation remained lower than *C. taiwanensis* in all of them after 35 cycles.

a Experimental setup and phenotypic tests

b Relative in planta fitness

c Nodulation competitiveness

d Within-host proliferation

Fig. 1 | Evolution of the symbiotic properties of *Ralstonia* clones along evolution cycles.

a, Left: overview of the evolution cycles showing the symbiotic steps determining bacterial fitness (nodulation competitiveness and within-host proliferation) and human intervention (nodule harvest, grinding and re-inoculation). Nodule colors reflect bacterial genotypes: blue represents the ancestor, and red represents a mutant with increased fitness. Right: schematic representation of the main phenotypic measurements performed in this work, where the symbiotic phenotypes of one evolved strain can be compared to those of a reference strain (e.g. the reference symbiont *C. taiwanensis*). Competitive indexes for in planta fitness are calculated as the ratio of evolved vs. reference clones in nodule bacterial populations normalized by the ratio of strains in the inoculum. Competitive indexes for nodulation

competitiveness are calculated as the ratio of nodules formed by each strain normalized by the inoculum ratio. Within-host proliferation is measured in independent single inoculations of each strain. b-d, Relative in planta fitness (b), nodulation competitiveness (c) and within-host proliferation (d) of nodulating ancestors (cycle 0) and evolved clones isolated from cycles 16 and 35 were compared to *C. taiwanensis*. Values correspond to means $\pm$ standard deviations. Data were obtained from at least three independent experiments. For each experiment, nodules were harvested from 10 plants (b), 20 plants (c) and 6 plants (d). The sample size (n) is equal to n=3 (b,c) or comprised between n=15-18 (d). Raw data are available in Supplementary Table 2. cfu: colony-forming units.

The dynamics of molecular evolution is characterized by multiple selective sweeps of large mutational cohorts

The rapid adaptation in this experiment was striking given that strong nodulation bottlenecks (limiting effective population size at each cycle (Supplementary Table 1)) are generally known to limit the rate of adaptive evolution¹⁷. To describe the dynamics of evolution at the molecular level, we performed whole-population sequencing of evolved lineages using the Illumina sequencing technology. Populations of the five lineages were sequenced every other cycle until cycle 35 with a minimum sequencing coverage of 100x and a median of 347x (Supplementary Table 3). We detected a very large number of mutations in all sequenced populations (a total of 4114 mutations, between 382 and 1204 mutations per lineage with 23.6 new mutations per cycle on average) (Extended Data Table 1 and Supplementary Tables 4-8). This high number of mutations is a result of transient hypermutagenesis occurring in the rhizosphere¹⁸. Mutations accumulated in the populations throughout the experiment, showing no sign of slowing down until the end of the experiment, which suggests that no anti-mutator mutations established in these populations as we might have expected¹⁹. We evaluated genetic parallelism among mutations detected above a frequency of 5% in the populations by computing G scores²⁰, a statistics used to point out genes that could be mutated more often than expected by chance (Extended Data Table 2). In spite of high mutation rates that may obscure signals of genetic convergence, we detected signatures of parallelism at the gene level among our list of mutations (observed sum of G scores of 7,540.5, compared to a mean sum of 5,401.01 after 1,000 randomized simulations, $Z = 31.23$, $P < 10^{-200}$). The randomized simulations identified 171 genes with a higher number of mutations than expected by chance (Extended Data Table 2).

To simplify the analysis of mutational trajectories, we then focused on the 819 mutations (out of a total of 4414 detected mutations) that rose above a frequency of 30%. We observed that groups of mutations showed correlated temporal trajectories and clustered them in cohorts^{21,22} (Fig. 2 and Extended Data Table 3). Cohorts increased in frequency with variable speed, some being fixed within 1 to 3 cycles (representing *ca.* 25-75 bacterial generations) while others reached fixation in up to 21 cycles (representing more than 500 bacterial generations). Fixed cohorts can be very large (up to 30 mutations) (Extended Data Table 3), which suggests hitchhiking of neutral or slightly deleterious

mutations with one or several beneficial mutations acting as driver (or co-drivers) of the cohort^{21,23}. Among the mutations that rose above 30% frequency at some stage of the experiment, 35% later declined until extinction. This is indicative of clonal interference, *i.e.* the co-occurrence of multiple subpopulations competing against each other within populations²⁴, which can readily be observed on Muller plots for lineages B and G (Extended Data Fig. 2). Overall, in these 5 lineages, the pattern of molecular evolution was characterized by a steady accumulation of mutations along the successive cycles and the formation of mutational cohorts, some of which containing a large number of mutations. Despite strong selective population bottlenecks at the root entry, mutation supply was not limiting as evidenced by the co-occurrence of competing adaptive mutations. Yet, multiple (and sometimes rapid) selective sweeps occurred throughout the 35 cycles in all lineages, indicating that strong selection is acting on these populations.

Fig. 2 | Dynamics of molecular evolution

Allele frequency trajectories of mutations that attained a frequency of 30% in at least one population of the B, F, G, K and M lineages (a,b,c,d,e). Mutations with similar trajectories were clustered in cohorts and represented by different colors. For simplicity, mutations travelling alone were also called cohorts.

Large fixed cohorts contain multiple adaptive mutations

Next, we searched for driver mutations in fixed (or nearly fixed, >90% frequency) cohorts from the two lineages that have the highest symbiotic fitness after 35 cycles: B and G. To do so, we identified, for each cohort of interest, a clone carrying all (or, when not available, almost all) previously fixed cohorts of mutations and used it to reconstruct individual point mutations from this cohort. This allowed us to test the fitness effect associated with each mutation in a relevant genetic background, taking into account possible epistatic effects arising from mutations that were previously acquired in this clone. Competitive index (ratio of strains carrying the mutant vs. the wild-type alleles in nodule populations normalized by the inoculum ratio) were measured for 44 mutations belonging to 14 cohorts from the two lineages (Fig. 3a,d and Supplementary Table 7). Twenty-eight mutations significantly increased the fitness of evolved clones and were thus beneficial for symbiosis while 11 mutations were neutral and 5 were slightly deleterious for symbiosis (Fig. 3a,d, Table 1 and Extended Data Table 4). Among the 44 genes targeted by the reconstructed mutations, 8 have a higher number of mutations than expected by chance (Extended Data Table 2) and adaptive mutations were identified in 6 of them.

Several interesting results emerge from this data set. First, some mutations show very strong adaptive effects, improving more than 100 times the fitness of bacteria, in particular during the first cycles of evolution. Second, many cohorts carry more than 1 adaptive mutation: six cohorts contain between 2 and 7 adaptive mutations. Third, 35% of adaptive mutations were found to be synonymous mutations, confirming that these mutations can play a significant role in adaptive evolution²⁵. All adaptive synonymous mutations except one converted frequently used codons in *R. solanacearum* into unusual ones (Extended Data Table 5), which may explain their functional effects. Moreover, when inspecting gene annotations, we found that the 28 beneficial mutations target various biological functions (Table 1). Strikingly, nearly 40% affect regulatory functions such as global transcription regulators (*efpR* and Rsc0965)²⁶, putative quorum quenching (RSp0595), signal transduction (RSc1598), unknown transcription regulator (RSc0243), protein dephosphorylation (Rsp1469), protein folding (*ppiB*), degradosome (*rhIE1*), and DNA methylation (RSc1982). Another significant proportion of mutations (32%) affect metabolism and transport genes (*gloA*, *phbB*, RSc0194, RSc2277, Rsp1116, RSp1417, RSp1422, RSp1590 and pRALTA_0398) and 3 mutations are located in genes belonging to the type VI secretion system (T6SS) operon (*tssJ*, RSp0759, Rsp0769). The observed genetic architecture (*i.e.* the large number of genes and molecular functions targeted by adaptive mutations) indicates that symbiotic traits are complex and constitute a large mutational target for adaptive evolution.

Fig. 3 | Symbiotic phenotypes of reconstructed mutants and evolved clones from lineages B and G.

a,d, In planta relative fitness of evolved clones carrying reconstructed mutations from fixed mutational cohorts. Evolved clones in which mutations were reconstructed are indicated on the left of the graph. Horizontal lines separate the different mutational cohorts. Cohort number and the number of tested mutations from the cohort on the total number of mutations present in the cohort are indicated in brackets. Competitive indexes (CI) were calculated as the ratio of the mutant strain on the isogenic parental strain in bacterial nodule populations normalized by the inoculum ratio. GFP/mCherry correspond to control co-inoculation experiments of strains derived from the same evolved clone labelled with different fluorophore either GFP or mCherry. b,e, Nodulation competitiveness effect of adaptive mutants. CI were calculated as the ratio of the number

of nodules formed by the mutant strain on the number of nodules formed by the isogenic parental strain normalized by the inoculum ratio. a,b,d,e, vertical segments correspond to mean values of CI. Red segments indicate significantly beneficial mutations, gray segments indicate neutral mutations and blue segments indicate significantly deleterious mutations ($P < 0.05$, t-test). c,f, Distribution of within-host proliferation values (number of bacteria per nodule) of adaptive mutants and the corresponding isogenic parental clones. Rectangles span the first quartiles to the third quartiles, bold segments inside the rectangle show the median, unfilled circles represent outliers, whiskers above and below each box show the minimum and the maximum in the absence of suspected outlying data. Red stars indicate the mutations that significantly improved infectivity compared to the parental evolved clone ($P < 0.05$, Wilcoxon test). a,b,c,d,e,f, Data were obtained from 3 to 5 independent experiments. For each experiment, nodules were harvested from 10 plants (a,d), 20 plants (b,e) and 6 plants (c,f). The sample size (n) is comprised between $n=3-5$ (a,d), $n=3-4$ (b,e) and $n=15-24$ (c,f). Raw data are available in Supplementary Table 9.

Adaptive mutations predominantly improve nodulation competitiveness

Next, we measured the effect of each adaptive mutation on nodulation competitiveness (reflecting host entry) and within-host proliferation in order to uncover which of these two symbiotic traits can explain the observed fitness gain. All mutations increasing fitness improved nodulation competitiveness of evolved clones (Fig. 3b,e and Supplementary Table 9). Gains in nodulation competitiveness ranged from 1.6 to 58-fold, with three mutations providing the highest gains: RSc2277-V321G (58-fold), *efpR*-E66K (35-fold) and *rhIE1*-L368L (28-fold). By contrast, within-host proliferation was improved by only a subset of adaptive mutations (Fig. 3c,f and Supplementary Table 9). Only 3 mutations from lineage B (RSc1598-P455L, *up*¹¹⁵-RSc0965 and *rhIE1*-L368L) and 7 mutations from lineage G (*efpR*-E66K, RSc2277-V321G, *phbB*-S38F, pRALTA_0398-W719*, RSp0574-L15L, RSp0769-F416S and RSp1422-G1298E) significantly improved this fitness component. Moreover, gains in *in planta* proliferation were generally lower than gains in nodulation competitiveness and ranged from 1.6 to 6.4-fold. The highest proliferation gains were produced by the five mutations RSc1598-P455L (6.4-fold), *efpR*-E66K (3.7-fold), *up*¹¹⁵-RSc0965 (2.6-fold), RSc2277-V321G (2.5 fold) and *rhIE1*-L368L (2.4-fold), three of which also produced the highest nodulation gains.

To investigate whether improvements in nodulation competitiveness could be due to a better colonization of the plant culture medium or rhizosphere, we measured the survival of the 28 mutants in competition with their isogenic parental strain in these two compartments. Results from these assays showed that none of the adaptive mutations improved bacterial ability to colonize the culture medium and only 4 of them, including *up*¹¹⁵-RSc0965, *efpR*-E66K and *rhIE1*-L368L, improved the colonization of the rhizosphere (Extended Data Fig. 3 and Supplementary Table 10) although by smaller factors than improvements in nodulation competitiveness. These results showed that improvements in nodulation competitiveness are generally not associated with a better colonization of the culture medium or of the rhizosphere, indicating that host entry is mainly controlled directly by the plant and

is the dominant selective force driving the adaptation of legume symbionts in this evolution experiment.

Evolutionary modelling predicts that selection for host entry drives adaptation

The preferential selection of nodulation competitiveness in our experiment prompted us to investigate the origin of this phenomenon. In particular, we wondered if stronger selection for host entry over within-host proliferation could be a general feature of symbiotic life cycles, or, instead, if it is more likely to be a specific property of our experimental system that may arise due to genetic constraints on symbiotic traits and/or specific features of the selective regime. We used computer simulations to model the evolution of bacterial populations that cycle between two compartments: the rhizosphere, where bacteria accumulate mutations, and root nodules, where bacteria reproduce clonally (see Supplementary Information for full details on the model and Supplementary Fig. 1). In this setting (and similar to our experiment), bacterial fitness is mediated by two phenotypic components: competitiveness to enter the host (resulting in nodulation) and within-host proliferation. The nodulation step imposes a very stringent selective bottleneck during host entry, since (i) in most cases, each nodule is founded by one single bacterium^{9,27}, (ii) there were usually between 100 and 300 nodules per lineage collected at each cycle (Supplementary Table 1) and (iii) bacterial genotypes differ in their competitive ability to form nodules (Fig. 3b,e). Once inside the host, bacteria multiply to reach a carrying capacity that is directly proportional to their proliferation fitness (no competition assumed), before returning to the rhizosphere.

When following the evolution of bacterial phenotypes in populations founded by an ancestor with low initial fitness (10^{-4} fold compared to the theoretical optimum, for each phenotypic component), we observed, in most cases, a faster increase in nodulation competitiveness relative to within-host proliferation (Fig. 4a). Accordingly, the distribution of mutations that were selected at the early steps of the adaptive process was biased towards stronger improvement of nodulation over proliferation (Fig. 4b, Low fitness domain). This dominance decreased, and was sometimes reverted, as populations progressed towards high fitness values. Decreasing the strength of the nodulation bottleneck also reduced the dominance of early selection for nodulation competitiveness, until abolishing it for the largest bottleneck size tested (3000 nodules).

In our experiment, the ancestral nodulating strains had a much higher potential for improvement in nodulation competitiveness relative to proliferation (5×10^4 vs. 10^2 - 10^3 fold, respectively, compared to the natural symbiont *C. taiwanensis*) which could explain the faster evolution of the former trait. We performed additional simulations to test how evolutionary trajectories are affected by the genotype of the ancestor. As expected, starting from a higher proliferation fitness in the ancestor further increased the overall fold effect of early selected mutations (Fig. 4c,d). In contrast, starting with a

higher initial nodulation competitiveness value of 10^{-3} or 10^{-2} in the ancestor was not sufficient to lead to a stronger selection for proliferation, further confirming the asymmetry between selective pressures acting on the two phenotypes. These findings were qualitatively robust to our assumptions regarding the genetic architecture of the two symbiotic phenotypes (Supplementary Information, Supplementary Fig. 2-11).

Fig. 4 | Relative strength of selection for nodulation competitiveness and proliferation

a,c,e, Median fitness trajectories of 100 simulated populations evolving under different sizes of nodulation bottleneck (10 to 3000 nodules). Points indicate fitness values at cycles 0, 10, 20, 30, 40 and 50. Dotted grey lines represent iso-fitness lines (defined as the product of nodulation competitiveness and proliferation) used to delineate 3 fitness domains: Low (L; fitness $< 10^{-5}$), Mid (M; $10^{-5} < \text{fitness} < 10^{-3}$), and High (H; fitness $> 10^{-3}$). The black dotted line represents the diagonal, along which population would improve both phenotypic traits equally well. b,d,f, Fold effect (nodulation competitiveness effect divided by proliferation effect) of mutations that reached a frequency of at least 30% in simulated populations. Color shading indicate

the fitness domain (Low, Mid or High) in which each group of mutations arose. Note that fold effects of mutations are indicated even when median fitness trajectories do not reach the High fitness domain (e.g. 10 and 30 nodule lines in a) because some individual replicate simulations (shown in Supplementary Figures) do reach this fitness domain. The effect of different parameters on adaptive trajectories was tested: the size of nodulation bottleneck (a, b), the initial fitness values of the ancestor (c, d), and the size of nodulation bottleneck under a scenario where the nodulation bottleneck occurs after bacterial clonal proliferation (e, f).

Finally, we wondered if the chronology of symbiotic steps impacts on selective pressures and evolutionary trajectories. We modified the simulations so that clonal multiplication of bacteria (according to their proliferation fitness) occurs before the nodulation bottleneck. Although this model is not relevant in the context of the legume-rhizobium symbiosis, it may apply to other symbiotic or pathogenic life cycles, for example when a selective bottleneck occurs during dissemination in the environment or within the host^{28,29} or in the case of vertically transmitted symbioses³⁰. In this context, the dominance of nodulation competitiveness was strongly reduced, bringing the two selective forces close to equilibrium (Fig. 4e,f and Supplementary Fig. 12), in agreement with the expectation that each fitness component should have a similar influence on adaptation when they have a multiplicative effect on global fitness. Therefore, the dominance of nodulation competitiveness in the previous simulation runs is likely a consequence of the selective bottleneck occurring just after the transient hypermutagenesis phase in the rhizosphere.

Altogether, these simulations show that the selective pressures that are experienced by symbiotic bacteria are asymmetric, with a dominance of selection for nodulation competitiveness over within-host proliferation. This asymmetry occurs when a highly diverse bacterial population from the rhizosphere is exposed to the strong, selective nodulation bottleneck, allowing the efficient selection of the most competitive clones by the host plant. In our evolution experiment, the fact that the ancestor has a lower nodulation competitiveness value compared to proliferation (relative to that of the wild-type rhizobium *C. taiwanensis*) is an additional factor expected to have contributed to the stronger selection on nodulation competitiveness.

Discussion

Horizontally transmitted symbiotic bacteria have complex lifecycles during which they have to face multiple environmental constraints, both outside and inside the host. Assessing the relative influence of each of these selective pressures on the adaptive trajectories of emerging symbiotic bacteria is critically needed to better understand the eco-evolutionary dynamics of microbial populations⁵.

Here we show that selection on competitiveness for host entry is the dominant selective pressure shaping early adaptation of new rhizobia. This trait, mainly mediated by the plant, improved very fast during our evolution experiment, to a level comparable to that of the *Mimosa* rhizobium

C. taiwanensis, and all adaptive mutations identified in lineages B and G improved nodulation competitiveness. Evolutionary simulations led us to propose that the dominance of the selection on host entry over the selection on within-host proliferation that we observed in our system is strongly dependent on the strength of the selective nodulation bottleneck. These results shed light on the importance of selective bottlenecks in shaping the evolutionary trajectories of emerging microbial symbionts. Most theoretical^{31,32} and experimental³³⁻³⁸ works on the influence of bottlenecks on microbial adaptation have focused on non-selective bottlenecks that randomly purge genetic diversity and reduce the efficiency of natural selection. Yet, another aspect of bottlenecks emerges when considering that transmission and host colonization can be, at least partially, dependent on microbial genotype, prompting us to consider infection bottlenecks as selective events³⁹. Selective bottlenecks have already been described during virus infections^{28,40-43} but received less attention in bacteria, except for their role in the evolution of phenotypic heterogeneity^{17,44-46}. However selective bottlenecks are likely common both in mutualistic and pathogenic interactions. Indeed, potentially all symbiotic microorganisms (whether commensal, pathogenic or mutualists) experience bottlenecks of various intensities (but sometimes as strong as only a few cells⁴⁷) during their life cycles^{48,49}. Given that microbes rely on complex genetic networks to disseminate and infect their hosts^{7,50-53}, one can expect these traits to display extensive genetic and phenotypic variation within populations and evolve through competition with other members of the microbiome. Interestingly, strong selection for host entry in experimentally evolved symbionts was recently observed in several studies⁵⁴⁻⁵⁶, reinforcing the idea that selective bottlenecks for host entry may play a crucial role in the evolution of diverse symbiotic interactions.

The molecular dissection of bacterial adaptation confirmed that the genetic architecture of rhizobial symbiotic traits is complex, as it involves many different genes and physiological functions^{57,58}. Our study also unveiled a new characteristic of the symbiotic genotype-to-phenotype map: the recurrent genetic coupling between nodulation competitiveness and within-host proliferation. Indeed, all the 10 identified mutations that improved within-host proliferation also improved nodulation competitiveness, while the reverse was not true. This result corroborates our previous data showing that mutations promoting nodule cell infection always improved nodule formation^{14,59}. It is conceivable that the intracellular release and proliferation of rhizobia in nodule cells is dependent on the efficiency of the earliest symbiotic events, *i. e.* the entry and progression of bacteria in infection threads and the concomitant divisions of nodule cells preparing for the accommodation of bacteria. Consistently, delayed progression of infection threads across the root cell layers was shown to impair the release of bacteria in nodule cells⁶⁰. Moreover plant receptors (LYK3, NFR1, Sym37, SYMRK) and transcription factors (NIN, ERN1, ERN2) involved in both nodule organogenesis and rhizobial intracellular infection were identified⁶¹⁻⁶⁴, suggesting that common mechanisms controlling bacterial invasion are exerted by

the plant at the two entry points, the root hairs (host entry) and the nodule cells (within-host proliferation). Yet, this coupling is not strict, since some mutations increase nodulation competitiveness without increasing proliferation. These mutations may target bacterial functions specifically required for nodulation. Alternatively, the fact that most mutations with an effect on proliferation are the ones with the highest contribution to nodulation competitiveness could be explained by a threshold effect, *i.e.* the existence of a minimal level above which mutations that increase nodulation competitiveness also increase proliferation. Surprisingly, mutations only improving proliferation were not found in our study. At this stage, we cannot distinguish between two hypotheses, namely if these mutations do not exist (proliferation is always linked to nodulation and host entry) or if they were not yet selected in this experiment due to the dominance of nodulation competitiveness in the first steps of adaptation. Future functional analyses of the adaptive mutations identified in this study will expand our understanding of the molecular bases of nodulation competitiveness in rhizobia⁶⁵⁻⁶⁷, and its relationship to within-host proliferation. Interestingly, these two traits primarily selected by the plant, although necessary for the symbiotic interaction, are not, in themselves, beneficial for the host. Including nitrogen fixation and host growth promotion in the analysis of genetic couplings between the different symbiotic traits, will be fundamental to better understand the evolution of mutualism⁶⁷.

In all lineages of this experiment, the rate of adaptation was very high during the first cycles of evolution and then tended to decrease over time. This classical pattern of evolution, due to diminishing return epistasis among beneficial mutations^{24,68,69}, was likely favored by the low initial fitness values of nodulating ancestors compared to the natural symbiont *C. taiwanensis* allowing the rapid acquisition of highly beneficial mutations during the first cycles (Table 1). Elevated mutation rate in the rhizosphere, prior to the entry of bacteria in roots¹⁸, has also played an important role in the dynamic of adaptation by fueling the extensive genetic diversification of bacteria and exposing these populations to plant-mediated selection. A probable consequence of high mutation rate combined to strong selection is the presence of large cohorts of mutations that carry multiple beneficial mutations along with some neutral or slightly deleterious ones. Mutational cohorts were already described in previous evolution experiments^{21,70}, with cases of cohorts carrying 2 co-driver mutations²². Here we identified up to 7 adaptive mutations in one of our cohorts. The occurrence of multi-driver mutations per cohort might be explained by the nested, sequential fixation of multiple adaptive mutations in one lineage before it reaches detectable frequency (5% in our case)²². This phenomenon, creating a ‘travelling wave’ of adaptation⁷¹, was recently observed with high-resolution sequencing²³ and is possibly amplified by the strong selection/high mutation regime of our experiment. Alternatively, it was proposed that, in the case of hypermutagenesis, the likelihood of multiple adaptive mutations

arising simultaneously in a given genome becomes non-negligible and might promote saltational evolution⁷².

In conclusion, our work shows that the relative strengths of the selective forces imposed by hosts strongly influence the evolutionary trajectory of microbial populations. Selection for host entry is likely the predominant life-history trait selected during the evolution of new horizontally-transmitted symbiotic interactions, such as the rhizobium-legume symbiosis. This effect emerges from the presence of a selective bottleneck at host entry, although its predominance can be modulated by other genetic and ecological factors. Investigating the role of selective bottlenecks in other symbiotic interactions will improve our ability to manage and predict the eco-evolutionary dynamics of host-associated microbial populations.

Methods

Bacterial strains and growth conditions

Strains and plasmids used in this study are listed in Supplementary Table 11. *Cupriavidus taiwanensis* strains were grown at 28°C on tryptone-yeast (TY) medium⁷³. *Ralstonia solanacearum* strains were grown at 28°C either on BG medium⁷⁴ or on minimal MP medium⁷⁵ supplemented with 2% glycerol. *Escherichia coli* strains were grown at 37°C on Luria-Bertani medium⁷⁶. Antibiotics were used at the following concentrations: trimethoprim at 100 µg ml⁻¹, spectinomycin at 40 µg ml⁻¹, streptomycin at 200 µg ml⁻¹ and kanamycin at 25 µg ml⁻¹ (for *E. coli*) or 50 µg ml⁻¹ (for *R. solanacearum*).

Experimental evolution

Symbiotically evolved clones and populations were generated as previously described¹⁶. Five lineages, two (B and F) derived from the CBM212 ancestor, two (G and K) derived from the CBM349 ancestor and one (M) derived from the CBM356 ancestor, previously evolved for 16 cycles were further evolved until cycle 35. For each lineage, at each cycle, 30 plants were inoculated with nodule bacterial populations from the previous cycle. Twenty-one days after inoculation, all nodules from the 30 plants were pooled, surface-sterilized with 2.6% sodium hypochlorite for 15 min, rinsed with sterile water and crushed. Then, 10% of the nodule crush was used to inoculate a new set of 30 plants the same day. Serial dilutions of each nodule crush were plated and one clone was randomly selected from the highest dilution and purified. The selected clones, the rest of the nodule crushes, and a 24-h culture of bacteria from an aliquot of nodule crushes were stored at -80°C at each cycle.

Sequencing bacterial populations and clones

Aliquots of frozen nodule bacterial populations or single colonies from purified clones were grown overnight in BG medium supplemented with trimethoprim. Bacterial DNA was extracted from 1 ml of culture using the Wizard genomic DNA purification kit (Promega). Evolved population DNAs were sequenced at the GeT-PlaGe core facility (<https://get.genotoul.fr/>), INRAE Toulouse. DNA-seq libraries have been prepared according to Illumina's protocols using the Illumina TruSeq Nano DNA HT Library Prep Kit. Briefly, DNA was fragmented by sonication, size selection was performed using SPB beads (kit beads) and adaptators were ligated to be sequenced. Library quality was assessed using an Advanced Analytical Fragment Analyzer and libraries were quantified by qPCR using the Kapa Library Quantification Kit (Roche). Sequencing has been performed on a NovaSeq6000 S4 lane (Illumina, California, USA) using a paired-end read length of 2x150 pb with the Illumina NovaSeq Reagent Kits. Evolved clones were re-sequenced either by C.E.A/IG/Genoscope using the Illumina GA2X technology (clones B8, B16, F16, G8, G16, K16 and M16), or by the GeT_PlaGe core facility using the Illumina technology HiSeq2000 (clones B1, B2, B3, B4, B5, B6, B7, B9, B10, B11, B12, B13, B14, B15, G1, G2, G3, G4, G5, G6, G7, G9, G10, G11, G12, G13, G14, and G15), or HiSeq3000 (clones B20, B25, B30, G25 and G30), or NovaSeq6000 (clones B35, F35, G35, K35 and M35).

Detection of mutations and molecular analyses

Sequencing reads from NovaSeq6000 runs (whole-populations and clones from cycle 35) were mapped on the chimeric reference genome of the ancestral strain, comprising *R. solanacearum* GMI1000 chromosome (GenBank accession number: NC_003295.1) and megaplasmid (NC_003296.1) together with *C. taiwanensis* symbiotic plasmid pRaltA (CU633751). Mutations were detected using breseq v0.33.1(Ref. ⁷⁷) with default parameters, either using the polymorphism mode (for whole-population sequences) or the consensus mode (for individual clones). Mutation lists were curated manually in order to remove mutations present in the ancestral strains as well as false positive hits arising from reads misalignments in low complexity regions. Moreover, alleles showing aberrant trajectories in the time-course whole-population sequencing data were checked manually, by inspecting either breseq output files and/or read alignments with IGV⁷⁸, and corrected as needed. In this work, we focused our attention on SNPs and indels detected above 5% frequency in the populations. Recombinations, rearrangements and IS movements were not analyzed exhaustively. Mutations detected in populations are listed in Supplementary Tables 4-8.

Sequencing data of the remaining clones were analyzed as described previously²⁶ with the PALOMA bioinformatics pipeline implemented in the Microscope platform⁷⁹. The complete list of mutational events generated for these clones are available on the Microscope platform (<https://mage.genoscope.cns.fr/microscope/expdata/NGSProjectEvo.php>, SYMPA tag). Mutations detected in clones are listed in Supplementary Tables 12-16.

In order to identify mutations forming temporal clusters (cohorts) through populations, we selected mutations having a frequency of 30% in at least one cycle and clustered their frequency using the *hclust* function in R v3.6.1⁸⁰. Then, we separated cohorts using a cutoff distance of 0.3.

Sub-population genealogies in lineages B and G, shown as Muller plots in Extended Data Figure 2, were reconstructed by comparing mutations found in cohorts and in individually sequenced clones. Cohorts for which ancestry cannot be ascertained (mutations not found in any clone) were not included in these plots. Relative frequencies of genotypes were calculated manually and plotted with ggMuller⁸¹. G scores were calculated as described²⁰. Since we found that synonymous mutations can be adaptive, we used all types of mutations in coding regions to calculate this statistics. Moreover, we included all mutations beyond 5% frequency in this analysis since we assumed that strong clonal interference may prevent adaptive mutations to rise to high frequency. To evaluate statistical significance of G scores, we ran 1,000 simulations where the total number of mutations used for the calculation of the observed G scores (3330) were randomly attributed in the coding genes according to their respective length to compute simulated G scores for each bacterial gene. The sum of simulated G scores was compared to the observed sum and we computed a Z score and *P*-value from these simulated G statistics. These simulations were also used to compute mean G scores for each gene, and to calculate the associated Z scores and *P*-values (adjusted using a Bonferroni correction).

Reconstruction of mutations in evolved clones

Mutant alleles and constitutively expressed reporter genes (GFPuv, mCherry) were introduced into *Ralstonia* evolved clones by co-transformation using the MuGent technique^{26,82}. Briefly, two DNA fragments, the first one carrying an antibiotic (kanamycin) resistance gene prepared from the pRCK-Pps-GFP or pRCK-Pps-mCherry linearized plasmids allowing the integration of the resistance gene in the intergenic region downstream the *glmS* gene, and the second one carrying the mutation to be introduced prepared by PCR amplification of a 6 kb region using genomic DNA of evolved clones as template and high fidelity Phusion polymerase (ThermoFisher Scientific), were co-transformed into naturally competent cells of *Ralstonia* evolved clones. Co-transformants resistant to kanamycin were screened by PCR using primers specifically amplifying mutant or wild-type alleles and verified by Sanger sequencing. Primers used in mutation reconstructions are listed in Supplementary Table 17.

Analyses of symbiotic phenotypes on *M. pudica*

Mimosa pudica seeds from LIPME production (2019 batch) were sterilized as described⁸³ by immersion in 95% H₂SO₄ during 15 minutes and 2.4% sodium hypochlorite solution during 10 minutes and rinsed in sterile distilled water 5 times. Seeds were soaked in sterile water at 28°C under agitation for 24 hours and then deposited on soft agar (9.375 g/L) and incubated at 28°C during 24 more hours in

darkness. Then, seedlings were cultivated in glass tubes (2 seedlings/tube) under N-free conditions, each tube containing 20 mL of solid Fahraeus medium⁸⁴ and 40 mL of liquid Jensen medium⁸⁵ diluted 1:4 with sterile water. Plants were incubated in a culture chamber at 28°C under a photoperiod day/night of 16 h/8 h.

For *in planta* relative fitness and nodulation competitiveness assays, two strains of *Ralstonia solanacearum* expressing differential constitutive fluorophores (GFPuv or mCherry) or one strain of *R. solanacearum* and one strain of *C. taiwanensis*, both expressing differential antibiotic resistance (streptomycin and trimethoprim), were co-inoculated onto *M. pudica* plantlets grown for 3 to 4 days in the culture chamber. Both strains were inoculated in equivalent proportion (*ca.* 5×10^5 bacteria of each strain per plant) except for the comparisons of the *Ralstonia* nodulating ancestors with *C. taiwanensis* where strains were mixed in a 1000:1 ratio (*ca.* 5×10^3 bacteria of *C. taiwanensis* and *ca.* 5×10^5 bacteria of nodulating ancestors per plant). Nodules were harvested 21 days after inoculation, surface sterilized by immersion in 2.4% sodium hypochlorite solution for 15 minutes and rinsed with sterile water. For *in planta* relative fitness measurements, sterilized nodules from 20 plants (see the exact number of harvested nodules in Supplementary Table 1) were pooled and crushed in 1 mL of sterile water, diluted and spread on selective solid medium using an easySpiral automatic plater (Interscience). After 2-day incubation at 28°C, colonies were screened either by plating on selective medium in case of co-inoculations of *Ralstonia* evolved clones with *C. taiwanensis* or based on fluorescence using a stereo zoom microscope (AxioZoom V16, Zeiss, Oberkochen, Germany) for co-inoculations of *Ralstonia* evolved clones with *Ralstonia* reconstructed mutants. For nodulation competitiveness assays, *ca.* 96 individual nodules per experiment were crushed separately in 96-well microtiter plates and droplets were deposited on selective medium. Nodule occupancy was determined by screening bacteria grown in the droplets either on selective medium or based on their fluorescence as described for fitness measurements. Both nodulation competitiveness and relative fitness assays were measured in at least three independent experiments. Competitive indexes (CI) were calculated by dividing the ratio of the number of test strain (evolved strains or reconstructed mutants) vs. reference strain (*C. taiwanensis* or evolved parental strains, respectively) in nodules normalized by the inoculum ratio. CI values above 1 were transformed by their inverse and compared to the value 1 using a one-sided Student *t*-test ($P < 0.05$).

For within-host proliferation assays, plantlets were inoculated with a single strain (5×10^5 bacteria per plant). In each experiment, nodules from 6 individual plants were collected separately 21 days after inoculation, surface sterilized for 15 minutes in a 2.4% sodium hypochlorite solution, rinsed with sterile water and crushed in 1 mL of sterile water. Dilutions of nodule crushes were plated on selective solid medium using an easySpiral automatic plater (Interscience). Two days after incubation at 28°C, the number of colonies was counted. Within-host proliferation was estimated as the number of bacteria

per nodule. For each strain, 15 to 24 measurements from three independent experiments were performed. Pairwise comparisons of proliferation values were compared using a two-sided Wilcoxon rank sum test.

For Jensen culture medium and rhizosphere colonization assays, plantlets were co-inoculated with pair of strains expressing differential constitutive fluorophores (GFPuv or mCherry) in equivalent proportion (5×10^6 bacteria of each strain per plant). Seven days after inoculation, bacteria present in the culture medium were diluted and plated on selective solid medium using an easySpiral automatic plater (Interscience). Bacteria attached to the roots were resuspended in 4 ml of sterile water by strongly vortexing for 1 minute, diluting and plating on selective medium using an easySpiral automatic plater (Interscience). Two days after incubation at 28°C, colonies were screened for fluorescence using a stereo zoom microscope (AxioZoom V16, Zeiss, Oberkochen, Germany). Competitive indexes (CI) for Jensen medium or rhizosphere colonization were calculated as described above. CI values were compared to the value 1 using a two-sided Wilcoxon rank sum test with Benjamini-Hochberg correction.

Modelling/simulations

Full details on evolutionary simulations are available as Supplementary Information.

Data availability

Sequencing data are available under NCBI SRA BioProject ID PRJNA788708 and SRP353965. Raw experimental data are available as Supplementary Information. Raw data generated from computer simulations are deposited on the Data INRAE dataverse (<https://doi.org/10.15454/QYB2S9>).

Code availability

Computer code used to analyze genomic data and to perform computer simulations are deposited on the Data INRAE dataverse (<https://doi.org/10.15454/QYB2S9>).

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Figure legend

Fig. 1 | Evolution of the symbiotic properties of *Ralstonia* clones along evolution cycles.

a, Left: overview of the evolution cycles showing the symbiotic steps determining bacterial fitness (nodulation competitiveness and within-host proliferation) and human intervention (nodule harvest, grinding and re-inoculation). Nodule colors reflect bacterial genotypes: blue represents the ancestor, and red represents a mutant with increased fitness. Right: schematic representation of the main phenotypic measurements performed in this work, where the symbiotic phenotypes of one evolved strain can be compared to those of a reference strain (*e.g.* the reference symbiont *C. taiwanensis*). Competitive indexes for *in planta* fitness are calculated as the ratio of evolved vs. reference clones in nodule bacterial populations normalized by the ratio of strains in the inoculum. Competitive indexes for nodulation competitiveness are calculated as the ratio of nodules formed by each strain normalized by the inoculum ratio. Within-host proliferation is measured in independent single inoculations of each strain. **b-d**, Relative *in planta* fitness (**b**), nodulation competitiveness (**c**) and within-host proliferation (**d**) of nodulating ancestors (cycle 0) and evolved clones isolated from cycles 16 and 35 were compared to *C. taiwanensis*. Values correspond to means $\pm$ standard deviations. Data were obtained from at least three independent experiments. For each experiment, nodules were harvested from 10 plants (**b**), 20 plants (**c**) and 6 plants (**d**). The sample size (*n*) is equal to *n*=3 (**b,c**) or comprised between *n*=15-18 (**d**). Raw data are available in Supplementary Table 2. cfu: colony-forming units.

Figure 2. Dynamics of molecular evolution

Allele frequency trajectories of mutations that attained a frequency of 30% in at least one population of the B, F, G, K and M lineages (**a,b,c,d,e**). Mutations with similar trajectories were clustered in cohorts and represented by different colors. For simplicity, mutations travelling alone were also called cohorts.

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mutations from the cohort on the total number of mutations present in the cohort are indicated in brackets. Competitive indexes (CI) were calculated as the ratio of the mutant strain on the isogenic parental strain in bacterial nodule populations normalized by the inoculum ratio. GFP/mCherry correspond to control co-inoculation experiments of strains derived from the same evolved clone labelled with different fluorophore either GFP or mCherry. **b,e**, Nodulation competitiveness effect of adaptive mutants. CI were calculated as the ratio of the number of nodules formed by the mutant strain on the number of nodules formed by the isogenic parental strain normalized by the inoculum ratio. **a,b,d,e**, vertical segments correspond to mean values of CI. Red segments indicate significantly beneficial mutations, gray segments indicate neutral mutations and blue segments indicate significantly deleterious mutations ($P < 0.05$, t -test). **c,f**, Within-host proliferation (number of bacteria per nodule) of adaptive mutants and the corresponding isogenic parental clones. Rectangles span the first quartiles to the third quartiles, bold segments inside the rectangle show the median, unfilled circles represent outliers, whiskers above and below each box show the minimum and the maximum in the absence of suspected outlying data. Red fillings indicate the mutations that significantly improved within-host proliferation compared to the parental evolved clone ($P < 0.05$, Wilcoxon test). **a,b,c,d,e,f**, Data were obtained from 3 to 5 independent experiments. For each experiment, nodules were harvested from 10 plants (**a,d**), 20 plants (**b,e**) and 6 plants (**c,f**). The sample size (n) is comprised between $n=3-5$ (**a,d**), $n=3-4$ (**b,e**) and $n=15-24$ (**c,f**). Raw data are available in Supplementary Table 9.

Figure 4. Relative strength of selection for nodulation competitiveness and proliferation

a, Median fitness trajectories of 100 simulated populations evolving under different sizes of nodulation bottleneck (10 to 3000 nodules). Points indicate fitness values at cycles 0, 10, 20, 30, 40 and 50. Dotted grey lines represent iso-fitness lines (defined as the product of nodulation competitiveness and proliferation) used to delineate 3 fitness domains: Low (L; fitness $< 10^{-5}$), Mid (M; $10^{-5} < \text{fitness} < 10^{-3}$), and High (H; fitness $> 10^{-3}$). The black dotted line represents the diagonal, along which population would improve both phenotypic traits equally well. **b**, Fold effect (nodulation competitiveness effect divided by proliferation effect) of mutations that reached a frequency of at least 30% in simulated populations. Color shading indicate the fitness domain (Low, Mid or High; shown in panel **a**) in which each group of mutations arose. Note that fold effects of mutations are indicated even when median fitness trajectories do not reach the High fitness domain (e.g. 10 and 30 nodule lines in **a**) because some individual replicate simulations (shown in Supplementary Figures) do reach this fitness domain. Figures **c** and **d** are built following the same principles than **a** and **b**, but explore the effect of differences in initial fitnesses. Note that the dashed diagonal (in **b**) or horizontal (in **d**) lines represent the expected evolutionary trajectories when initial fitnesses are unequal, and upon the null hypothesis that adaptation would proceed proportionally to the differences between the ancestral and the theoretical

optimum values for each phenotypic component. Figures **e** and **f** are as **a** and **b** but explore a situation where the nodulation bottleneck occurs after bacterial clonal proliferation.

Tables

Table 1. Symbiotic phenotypes of beneficial mutations improving *in planta* fitness.

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Authors contributions

C.M.-B, D.C. and P.R. designed the project. G.G.D.M. performed most experiments and analyze the data. S.M., N.G., A.-C.C., D.C. and P.R. performed experiments. J.-B.F. and P.R. performed computer simulations. M.M., T.B. and S.V. performed NGS sequencing at GeT-PlaGe. D.R. analyzed mutations in clones and deposit data in the microScope platform. D.C. and P.R. supervised experimental work and data analysis. C.B.-M., J.-B.F and P.R. acquired funding for the project. G.G.D.M., J.B-F., D.C. and P.R. wrote the manuscript, which was approved by all authors.

Competing interests

The authors declare no competing interests.

Extended data

Extended Data Fig. 1. Experimental evolution of *Ralstonia solanacearum* GMI1000 pRalta through serial cycles of plant (*Mimosa pudica*) inoculation-isolation of nodule bacteria.

The 5 bacterial lineages, B, F, G, K, and M lines were independently derived from the spontaneous nodulating mutants either CBM212, or CBM349, or CBM356. At each evolution cycle, bacterial nodule populations were isolated either 21 days (black lines) or 42 days (blue lines) after plant inoculation and partly directly re-inoculated to new plants to initiate the next cycle. At each cycle, one individual clone representative of the bacterial population was isolated and stored. Sequenced populations and clones are indicated. The number of nodules harvested at each evolution cycle is reported in Supplementary Table 1.

Extended Data Fig. 2. Evolution of population composition over time in lineages B and G.

Muller plots representing the relative frequency of each genotype in lineages B (a) and G (b) for each sequenced population (uneven cycles). Each color represent a different genotype, and its relative frequency is shown as the vertical area at each sequenced time point. The genotypes containing cohorts for which adaptive mutations were identified experimentally are indicated. For each cohort, non-adaptive or deleterious mutations are shown in black, adaptive mutations are in red.

Extended Data Fig. 3. Survival of reconstructed mutants from lineages B and G in the Jensen medium and rhizosphere.

Survival of evolved clones carrying reconstructed adaptive mutations from fixed mutational cohorts in the Jensen medium (a) and in rhizosphere (b). Competitive indexes (CI) were calculated as the ratio of the mutant strain on the isogenic parental strain in Jensen medium populations normalized by the inoculum ratio. Red points correspond to mean values of CI. Rectangles span the first quartiles to the third quartiles, bold segments inside the rectangle show the median. Red stars beside strain names indicate the mutations that significantly improved survival in the rhizosphere compared to the parental evolved clone ($P < 0.05$, Wilcoxon test with Benjamini-Hochberg correction). Data were obtained from at least 3-5 independent experiments for each comparison and the sample size (n) is comprised between n=9-14. Raw data are available in Supplementary Table 10.

Extended Data Table 1. Summary of detected mutations in the five lineages

Extended Data Table 2. Analysis of genetic parallelism in the five lineages.

Extended Data Table 3. Global analysis of mutational cohorts

Extended Data Table 4. Neutral and deleterious reconstructed mutations

Extended Data Table 5. Frequencies of usage of codons modified by adaptive synonymous mutations

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Supplementary information

Supplementary Table 1. Number of nodules harvested at each evolution cycle.

Supplementary Table 2. Raw data obtained for the phenotypic characterization of *Ralstonia* evolved clones compared to *C. taiwanensis*.

Supplementary Table 3. Coverage depth for whole-population sequencing

Supplementary Table 4. List of mutations detected in evolved populations of the B lineage.

Supplementary Table 5. List of mutations detected in evolved populations of the F lineage.

Supplementary Table 6. List of mutations detected in evolved populations of the G lineage.

Supplementary Table 7. List of mutations detected in evolved populations of the K lineage.

Supplementary Table 8. List of mutations detected in evolved populations of the M lineage.

Supplementary Table 9. Raw data obtained for the *in planta* phenotypic characterization of reconstructed mutants compared to their respective isogenic parental strain.

Supplementary Table 10. Raw data obtained for the *ex planta* phenotypic characterization of reconstructed mutants compared to their respective isogenic parental strain.

Supplementary Table 11. Strains and plasmids used in this study.

Supplementary Table 12. List of mutations detected in evolved clones of the B lineage.

Supplementary Table 13. List of mutations detected in evolved clones of the F lineage.

Supplementary Table 14. List of mutations detected in evolved clones of the G lineage.

Supplementary Table 15. List of mutations detected in evolved clones of the K lineage.

Supplementary Table 16. List of mutations detected in evolved clones of the M lineage.

Supplementary Table 17. Oligonucleotides used in this study.

Supplementary Table 18. Parameter values used in computer simulations

Supplementary Information. Model framework and supplementary simulation results

Correspondence and requests for materials should be addressed to P.R. or D.C.

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Table 1. Symbiotic phenotypes of beneficial mutations improving *in planta* fitness

Lineage	Gene ID ^a	Gene name	Mutation	Type of mutation ^b	Description	Cohort	Mean CI for	Mean CI for	Mean CI for	Mean gain	Symbiotic phenotypes ^f	G score ^g	Adjusted P value ^h	
							Mean CI for <i>in planta</i> fitness ^c	plant culture medium colonization ^d	rhizosphere colonization ^d	Mean CI for nodulation competitiveness ^e				factor in within-host proliferation ^e
B	RSp0595		P132P	Syn	Hypothetical protein	CoB1	77	1	1	5.2	1.6	Nod+	2.80	1
	RSc1598		P455L	Nonsyn	Transmembrane sensor histidine kinase	CoB2	24.2	1	1.4	4.7	6.4	Nod+ Pro+	1.04	1
	RSp0788		E225K	Nonsyn	Carbamoyl-transferase	CoB3	1.9	1	1.1	2	1.2	Nod+	-0.09	1
	RSp1417		S417L	Nonsyn	Multidrug efflux transmembrane protein	CoB3	1.6	0.8	1.4	3.9	0.9	Nod+	12.47	2.58E-04
	RSp1590		S153L	Nonsyn	FAD-dependent oxidoreductase	CoB3	6.3	1	0.9	2.4	0.6	Nod+	4.10	1
	up ¹¹⁵ -RSc0965		G/A	Intergenic	Unknown function, negatively regulates <i>efpR</i> expression	CoB6	31.1	0.9	2.9	8.2	2.6	Rhizo+ Nod+ Pro+	nd	nd
	Rsc0539	<i>rhlE1</i>	L368L	Syn	ATP-dependent RNA helicase protein	CoB7	46.2	1.1	4	28.2	2.4	Rhizo+ Nod+ Pro+	2.88	1
	RSc0243		L213L	Syn	LysR-type transcription regulator protein	CoB10	3	1.3	1.4	3.9	1.1	Rhizo+ Nod+	1.16	1
	Rsp1116		I32I L33F	Syn Nonsyn	Putative low specificity L-threonine aldolase	CoB10	2.1	1.2	1.2	2.1	0.9	Nod+	0.84	1
	up ⁸ -RSp1469		C/T	Intergenic	Putative tyrosine specific protein phosphatase	CoB10	2.1	0.9	1	2	1.0	Nod+	nd	nd
	pRALTA_0230		G242E	Nonsyn	Putative ATP-dependent DNA helicase	CoB10	1.5	1.5	1.5	2.1	0.9	Nod+	6.34	1
	pRALTA_0552		I100I	Syn	Pseudogene, putative membrane bound hydrogenase	CoB10	2.9	1.2	1.4	2.3	0.8	Nod+	nd	nd
	G	RSc1097	<i>efpR</i>	E66K	Nonsyn	Transcriptional regulator	CoG1	212.1	1.3	4	35	3.7	Rhizo+ Nod+ Pro+	3.17
RSc1982			I100I	Syn	DNA methyltransferase	CoG2	3.3	3.1	1.3	4.0	0.7	Nod+	1.44	1
Rsc1164		<i>ppiB</i>	Q112*	Nonsense	Peptyl-prolyl-cis/trans isomerase	CoG4	24.5	1	1.0	5.0	1.4	Nod+	2.26	1
RSc2277			V321G	Nonsyn	Putative transporter protein	CoG4	133.9	9.3	10.1	58	2.5	Nod+ Pro+	36.95	4.68E-50
up ⁸⁵ -RSc0520		<i>gloA</i>	T/C	Intergenic	Lactoylglutathione lyase	CoG7	2.8	1.2	1.5	2.1	1.1	Nod+	nd	nd
up ¹⁸⁰ -RSp0637			G/C	Intergenic	Hypothetical protein	CoG7	1.8	1	1.2	2.6	0.9	Nod+	nd	nd
RSc0194			S102F	Nonsyn	Zinc-dependent alcohol dehydrogenase	CoG13	5.5	1.1	1.6	3.1	1.4	Nod+	4.67	1
RSc1633		<i>phbB</i>	S38F	Nonsyn	Acetoacetyl-CoA reductase	CoG13	7.9	2	2.1	3.3	1.7	Rhizo+ Nod+ Pro+	1.62	1
RSp0741		<i>tssJ</i>	I157N	Nonsyn	Type VI secretion system, lipoprotein TssJ	CoG13	6.7	1	1.1	2.9	1.7	Nod+	6.99	1
RSp0574			L15L	Syn	Hypothetical protein	CoG15	3.2	1.1	1.3	2.1	1.6	Nod+ Pro+	4.36	1
RSp0587			P84P	Syn	Putative signal peptide hypothetical protein	CoG15	2	1.1	0.8	2.6	1.3	Nod+	28.74	1.04E-46
RSp0759			F91F P92S	Syn Nonsyn	Putative type VI secretion-associated protein	CoG15	5.4	0.7	0.9	2.5	1.4	Nod+	1.00	1
RSp0769			F416S	Nonsyn	Putative type VI secretion-associated protein	CoG15	6.3	1.1	1.3	1.6	1.8	Nod+ Pro+	17.20	3.98E-10
up ¹¹⁶ -RSp0780			C/T	Intergenic	Hypothetical protein	CoG15	2.5	1.1	1.1	3	1.1	Nod+	nd	nd
RSp1422			G1298E	Nonsyn	Non-ribosomal peptide synthase	CoG15	2	1.2	1.2	2.4	1.6	Nod+	18.28	2.79E-03
pRALTA_0398			W719*	Nonsense	Phosphoenol pyruvate synthase	CoG15	2.2	0.8	0.8	1.6	1.6	Nod+ Pro+	151.77	0

^aup*, intergenic mutations located x nucleotides upstream the gene indicated. ^bSyn, synonymous mutations. Nonsyn, non synonymous mutations. ^cMean values of competitive indexes (CI) obtained from 3 to 4 independent experiments. Figures in bold are statistically different from 1 (Student t-test $P < 0.05$). ^dMean values obtained from at least 15 measurements from 3 to 4 independent experiments. Figures in bold are statistically significant (Wilcoxon test, $P < 0.05$). ^eMean values obtained from at least 15 measurements from 3 to 4 independent experiments. Figures in bold are statistically different from 1 (Wilcoxon test, $P < 0.05$ with the Benjamini-Hochberg correction). ^fRhizo+, improvement in rhizosphere colonization. Nod+, improvement in nodulation competitiveness. Pro+, improvement in within-host proliferation. ^gG scores were calculated as proposed by Tenailon et al. (2016). nd, G scores were not determined for mutations in pseudogenes or intergenic regions. ^hP-values associated with G scores were adjusted using a Bonferroni correction to evaluate genetic parallelism.