Experimental Evolution of a Plant Pathogen into a Legume Symbiont

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Abstract

Rhizobia are phylogenetically disparate α- and β-proteobacteria that have achieved the environmentally essential function of fixing atmospheric nitrogen in symbiosis with legumes. Ample evidence indicates that horizontal transfer of symbiotic plasmids/islands has played a crucial role in rhizobia evolution. However, adaptive mechanisms that allow the recipient genomes to express symbiotic traits are unknown. Here, we report on the experimental evolution of a pathogenic *Ralstonia solanacearum* chimera carrying the symbiotic plasmid of the rhizobium *Cupriavidus taiwanensis* into *Mimosa* nodulating and infecting symbionts. Two types of adaptive mutations in the *hrpG*-controlled virulence pathway of *R. solanacearum* were identified that are crucial for the transition from pathogenicity towards mutualism. Inactivation of the *hrCV* structural gene of the type III secretion system allowed nodulation and early infection to take place, whereas inactivation of the master virulence regulator *hrpG* allowed intracellular infection of nodule cells. Our findings predict that natural selection of adaptive changes in the legume environment following horizontal transfer has been a major driving force in rhizobia evolution and diversification and show the potential of experimental evolution to decipher the mechanisms leading to symbiosis.

Introduction

Bacteria known as rhizobia have evolved a mutualistic endosymbiosis of major ecological importance with legumes that contributes ca. 25% of global nitrogen cycling. Rhizobia induce the formation on legumes of root nodules that they colonize intracellularly [1] and in which they fix nitrogen to the benefit of the plant. Rhizobia are taxonomically, metabolically, and genetically diverse soil bacteria [2,3]. They are currently distributed in 12 genera of α- and β-proteobacteria intermixed with saprophytes and pathogens. The occurrence of rhizobia in several distant genera is thought to have originated from repeated and independent events of horizontal transfer of key symbiotic functions in non symbiotic bacterial genomes [2,4]. Symbiotic plasmid/island transfer has been proven both in the field and in the lab [5,6]. However, horizontal gene transfer cannot solely account for the worldwide diversity of rhizobia, since only a few recipient bacteria—phylogenetically close to existing rhizobia [5–8]—turned into nitrogen-fixing legume symbionts. Which phylogenetic, genetic, or ecological barriers restrict evolution of symbiotic properties and how these barriers are overcome have not been investigated so far.

Experimental evolution [9] coupled with genome resequencing [10] is a powerful approach to address the evolution of rhizobia. *Ralstonia solanacearum* and *Cupriavidus taiwanensis* are plant-associated β-proteobacteria with drastically different lifestyles. *R. solanacearum* is a typical root-infecting pathogen of over 200 host plant species. It intercellularly invades root tissues and heavily colonizes the vascular system, where excessive production of extracellular polysaccharides blocks water traffic, causing wilting [11,12]. *Cupriavidus taiwanensis* is the major nitrogen-fixing symbiont of *Mimosa* spp. in Asia [13,14] (see Figure 1A). Due to their phylogenetic and genomic distance (Figure S1), *C. taiwanensis* and *R. solanacearum* are ideally suited to act as symbiotic gene provider and recipient, respectively, in experimental evolution.

Here, we report on the experimental evolution of *R. solanacearum* carrying the symbiotic plasmid of *C. taiwanensis* into *Mimosa*-nodulating and -infecting symbionts. Two types of key adaptive mutations are described that are crucial for the transition from pathogenicity to mutualism. One allows nodulation to occur,
Author Summary

Most leguminous plants can form a symbiosis with members of a group of soil bacteria known as rhizobia. On the roots of their hosts, some rhizobia elicit the formation of specialized organs, called nodules, that they colonize intracellularly and within which they fix nitrogen to the benefit of the plant. Rhizobia do not form a homogenous taxon but are phylogenetically dispersed bacteria. How such diversity has emerged is a fascinating, but only partly documented, question. Although horizontal transfer of symbiotic plasmids or groups of genes has played a major role in the spreading of symbiosis, such gene transfer alone is usually unproductive because genetic or ecological barriers restrict evolution of symbiosis. Here, we experimentally evolved the usually phytopathogenic bacterium *Ralstonia solanacearum*, which was carrying a rhizobial symbiotic plasmid into legume-nodulating and -infecting symbionts. From resequencing the bacterial genomes, we showed that inactivation of a single regulatory gene allowed the transition from pathogenesis to legume symbiosis. Our findings indicate that following the initial transfer of symbiotic genes, subsequent genome adaptation under selection in the plant has been crucial for the evolution and diversification of rhizobia.

Results/Discussion

Evolution of Symbiotically Proficient *R. solanacearum*

To generate our starting material, we transferred the 0.55-Mb symbiotic plasmid pRalts of *C. taiwanensis* LMG19424 into *R. solanacearum* strain GMI10000, generating the *Ralstonia* chimeric strain CBM124. pRalts carries nitrogen-fixation genes and a full complement of nodulation genes required for the synthesis of lipochitooligosaccharide Nod factors (NFs) [15] that trigger the plant developmental program of nodule organogenesis [16]. Nevertheless, CBM124 was unable to nodulate the *C. taiwanensis* legume host *Mimosa pudica* and retained the pathogenic properties of *R. solanacearum*, i.e., pathogenicity on *Arabidopsis thaliana* and hypersensitive response (HR) induction on tobacco (Figure S2). Note that *M. pudica* is not a host plant for *R. solanacearum*. Several lines of evidence indicated that CBM124 had a symbiotic potential that, for an unknown reason, could not be expressed. First, a nodB-lacZ transcriptional fusion was induced by the nod-inducer luteolin in a similar way in CBM124 and in *C. taiwanensis* (Table S1). Second, mass spectrometry analysis demonstrated that CBM124 produced NFs structurally identical to those of *C. taiwanensis* [15] (Figure S3). Third, CBM124 induced root hair proliferation and deformations on *M. pudica*, typical of those induced by NFs (see below), indicating that CBM124-produced NFs were active.

To isolate clones expressing symbiotic potential, we took advantage of specific traits of the rhizobium-legume symbiosis, (i) legume plants act as a trap by selecting rare, nodulation-proficient mutants in an otherwise non-nodulating population [17], (ii) a single bacterium enters and multiplies within the nodule [18], which implies that a rare nodulation-conferring mutation in a population is rapidly fixed, and (iii) nodulation, infection, and nitrogen fixation, are phenotypically clear-cut symbiotic stages. Both the original chimera CBM124 and a gentamicin-resistant derivative, CBM124GenR, were used to repeatedly inoculate sets of *ca.* 500 *M. pudica* seedlings grown in nitrogen-free conditions, as previously described [13]. Whereas no nodules were obtained using CBM124 as an inoculum, three nodules, which appeared 3–4 wk after inoculation, were recovered from three independent CBM124GenR inoculation experiments. One bacterial clone was isolated from each nodule, generating CBM212, CBM349, and CBM356. These three clones nodulated *M. pudica* with different kinetics and efficiencies (Figure 1). Their nodulation ability was, however, reduced relative to *C. taiwanensis* (Figure 1D and Figure S4), and all three clones were unable to fix nitrogen (Fix−).

Identification of Key Adaptive Mutations for Symbiosis

We re-sequenced the three experimentally evolved clones as well as their immediate ancestor, CBM124GenR, using paired-
end Illumina/Solexa sequencing technology (http://www.
illumina.com/). Sequence data were mapped to the reference
genome (6.37 Mb) based on the known genome sequences of *R.
solanacearum* GMI1000 [19] and *C. taiwanensis* LMG19424 [15],
and analyzed using the SNIPER software (S. Cruveiller and C.
Medigue, unpublished data). We identified indels, SNPs (single
nucleotide polymorphisms), and large deletions in the evolved
clones relative to the CBM124GenR ancestor (Table S2). Among
them, we focused on a large deletion as well as three SNPs that
affected the HrpG-controlled virulence pathway in all three clones
(Table 1). We confirmed the deletion and the SNPs by PCR
amplification and Sanger resequencing.

The ca. 33-kb deletion ([Rsp0128–Rsp0154) of the *R. solana-
cearum* chromosome 2 removed 27 genes, including the *pme*
gene coding for a pectin methyltransferase involved in virulence and genes
encoding a putative type II secretion system. This deletion was
reconstructed in the chimera CBM124 by using the cre-lox system
(see Material and Methods). The resulting strain did not nodulate
*M. pudica*, indicating that this deletion either was not adaptive or
alone could not account for nodulation. This region probably
 corresponds to an unstable region of the genome.

The regulatory protein HrpG controls the expression of many
virulence determinants in *R. solanacearum* [20]. These include a
type III secretion machinery (T3SS) and associate effector
proteins that are regulated via the intermediate regulator *hrpB*
[21] as well as a large ensemble of genes that are modulated by
*hrpG* in an unidentified circuitry [20]. A stop mutation in the *hrvG*
gen, which encodes a structural inner membrane protein at the
base of the T3SS apparatus [22], was observed in CBM356, whereas
both CBM124 and CBM349 harboured a stop mutation in the
master regulator *hrpB* gene itself (Table 1). Consistently, all
three clones exhibited a typical T3SS-defective phenotype, i.e.,
loss of HR induction on tobacco leaves (Figure S2). Although *C.
taiwanensis* also possesses a T3SS of unknown function, it is not
located on pRhaL, thus ruling out the possibility that the impact
on nodulation of the *R. solanacearum* virulence pathway was due to
a modulation of indigenous *C. taiwanensis* T3SS. To assess the
possible role of *hrvG* and *hrpG* gene inactivation in *M. pudica*
nodulation, we inactivated the *hrvG* gene in the CBM124
strain. Both CBM125 (hrvG) and
CBM664 (ΔhrpG) were indeed found to nodulate *M. pudica*.
Nonpolar disruption of *hrvG* and *hrpG* structural genes, as well as
independent *hrvG* inactivation by site-directed Tn5
mutation in the CBM124 background further confirmed the
role of the T3SS and the *hrpG* gene in nodulation of *M. pudica*.
The *hrvG* and *hrpG* mutants had symbiotic behaviours similar to
that of the *hrvG* and *hrpG* evolved clones, respectively (Figure 1C
and 1D).

**Table 1.** Validated SNPs affecting the HrpG-controlled virulence pathway and common deletion in all evolved clones.

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<td>148232–178934</td>
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<td>Q589*</td>
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*Stop codon.

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T3SS Inactivation Allows Chimeric *Ralstonia solanacearum* to Nodulate and to Enter Root Hairs via Infection Threads

Like most rhizobia, *C. taiwanensis* invades *Mimosa* roots by means of
transcellular infection threads (ITs), which are initiated from
microcolonies entrapped within the curled root hairs known as
shepherd’s crooks [14] (Figure S5). Later on, ITs elongate into
emerging nodules delivering bacteria into the plant cells. Each
infected cell houses thousands of symbiosomes composed of
internalized bacteria (called bacteroids) surrounded by plant-
derived peribacteroid membranes. Mature *Mimosa* nodules
induced by *C. taiwanensis* have the typical histology of indetemi-
dinate nodules, i.e., a single distal persistent meristem and peripheral
vascular bundles (Figure S5). The ancestral chimeras CBM124 and
CBM124GenR promoted root hair proliferation and deformations as well as shepherd’s crooks. However, these chimeras
showed a clear defect in IT initiation and elongation
(Figure 2A and Figure S6). In contrast, well-elongated ITs were
observed with the *hrvG* mutant CBM123 (Figure 2B). Nodules
formed, which displayed the typical nodule structure (Figure S7),
although often of irregular shape compared to those induced by *C.
taiwanensis*. However the *hrvG* mutant only partially and extracel-
larly invaded the nodule (Figure 2C and 2D). A two-step
inoculation experiment using differently labelled (*gfp* and *lacZ*)
strains confirmed that extracellular bacteria inside nodules
originated from ITs and did not result from intercellular
penetration of bacteria from the nodule surface. A necrotic dark
brown zone around which bacteria were distributed was often
observed in the distal part of the infected zone of the nodules
(Figure S7). Plant cell wall thickening next to extracellular bacteria
was also suggestive of a plant structural defence response. This
could act as a physical barrier to intracellular infection. In a similar
way, the evolved clone CBM356 (*hrvG*) was able to form elongated
ITs but did not permit invasion of nodule cells (Figure S7), strongly
indicating that the *hrvG* mutation indeed accounted for the
symbiotic phenotype of CBM356. We observed that a double
mutant of the PopF1 and PopF2 translocos, which do not inhibit
the formation of the T3SS apparatus in *R. solanacearum* but are
required for protein effector injection in plant cells [23], had a
similar phenotype (Figure S4), thus suggesting that a T3SS
effector(s) is involved in blocking nodulation and early infection.
Most interestingly, some rhizobia have been shown to use
specialized host-targeting type III or type IV secretion systems to
either extend or restrict legume host range (reviewed in [24]).
Expression of these secretion systems is coordinated to nodulation
gene expression. Effectors have been identified that can either be
rhizobium specific or pathogen related. They have been proposed to
modulate host (signalling) pathways, including plant-defence
reactions triggered by the presence of infecting rhizobia [24].
Because *R. solanacearum* has more than 70 effectors [21], identification of the effector(s) responsible for blocking nodulation requires further work. Either nodulation is inhibited by effector-triggered immunity [25] or a T3SS effector(s) specifically interferes with the NF-signalling pathway.

**hrpG Inactivation Allows Intracellular Invasion of Nodule Cells**

The *hrpG* mutant of CBM124 (CBM664), as well as the *hrpG* evolved clones CBM212 and CBM349, formed nodules on *M. pudica* that looked similar to those induced by *C. taiwanensis* (Figure S8). In young nodules, plant cells were massively intracellularly invaded (Figure 3A and 3B, and Figure S8), although the infected zone was restricted, compared to N\textsubscript{2}-fixing nodules formed by *C. taiwanensis*. Intracellular bacteria were surrounded by a peribacteroid membrane forming typical symbiosomes (Figure 3C). Nodules, however, showed early signs of degeneration generally 3 wk postinoculation, i.e., loss of cell-to-cell contact, cytoplasmic structure desegregation of nodule cells and degradation of the internalized bacteria (Figure S8). A few extracellular bacteria were found in nodules formed by the *hrpG* chimeric mutant and CBM212 and CBM349 clones (Figure 3B), which is never seen with *C. taiwanensis*. In these cases, no plant cell wall thickening could be observed in proximity to extracellular bacteria, suggesting that they did not induce plant defence reactions. To summarize, *hrpG* mutants and evolved clones were able to intracellularly...
invasive nodule cells, contrary to hrpV mutants, although bacteroids were impaired for long-term maintenance. The regulatory gene hpg thus controls one or several T3SS-independent functions interfering with plant cell entry. In plant-associated bacteria, massive intracellular infection is restricted to nodule bacteria. Hence, there is a paradox between the rarity of intracellular infection in plants and the ease with which this trait was acquired by a strictly extracellular pathogen. Mechanisms of plant cell entry in C. taiwanensis and in rhizobia in general are largely unknown, although it has been established that surface polysaccharides play a key role in host invasion [1]. Identification of the gene(s) downstream of hpg controlling intracellular infection should shed light to this key, but still obscure, step of the symbiotic interaction.

Conclusion
How rhizobia have emerged is a fascinating, but so far only partly documented, question. Although pioneering work 13 y ago established the role of lateral transfer in rhizobia evolution [5,6], we and others [26,27] have observed that in many instances, transfer of symbiotic loci did not increase symbiotic competence. Here, we show that a recipient genome—that is not immediately converted to a rhizobium upon transfer of a symbiotic plasmid—could rapidly evolve two specific symbiotic traits, i.e., nodulation and intracellular infection, under plant selection pressure. Although in our case, nitrogen fixation—and hence mutualism—was not achieved and evolved clones could be considered as cheaters [28], evolution of nodulation and infection capacities is the first step in the evolutionary process of reciprocal cooperation [29]. Extant rhizobial lineages diverged long before they acquired symbiotic properties [30], i.e., after legumes appeared on earth 60 million years ago. Our results show that adaptive genomic changes indeed affect the dissemination of symbiotic traits over large phylogenetic and ecological distances. The fact that a single gene played a major role in the shift from extracellular pathogenesis to endosymbiosis reinforces previous reports that global regulators preference targets for evolution [31] and supports fluid boundaries between parasitism and mutualism.

Our knowledge of the rhizobium-legume symbiosis mainly comes from gene inactivation studies. Although a gain-of-function approach was first initiated ca. 25 y ago on Agrobacterium [8,26,32] and used thereafter [7,33], the experimental evolution approach we describe here is novel, as it consists of the progressive and random mutagenesis of the regulatory gene downstream of hrpG controlling intracellular infection should shed light to this key, but still obscure, step of the symbiotic interaction.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions
Bacterial strains and plasmids used in this work are listed in Tables 2 and 3. C. taiwanensis strains were grown at 28°C on TY medium supplemented with 6 mM CaCl2 or quarter-strength minimal medium (MM) [34] supplemented with 10 mM disodium succinate and vitamin solution (1 mg/ml nicotinic acid, 1 mg/ml thiamine hydrochloride, 1 mg/ml pyridoxine hydrochloride, 100 μg/ml myo-inositol, 1 μg/ml calcium pantothenate, 1 μg/ml riboflavin, 1 μg/ml ascorbic acid, 1 μg/ml folic acid, 1 μg/ml cyanocobalamin, 1 μg/ml D-biotin). R. solanacearum strains were grown at 29°C on rich BG medium [35] or MM supplemented with 20 mM glucose. Antibiotics were used at the following concentrations (in micrograms per milliliter): streptomycin 600, spectinomycin 40, trimethoprim 100, tetracycline 10, gentamicin 25, chloramphenicol 50 for E. coli and 200 for C. taiwanensis, and kanamycin 50 for E. coli, and 30 for R. solanacearum.

Transfer of pRal from C. taiwanensis to R. solanacearum
Transfer of pRal to R. solanacearum was performed in three consecutive conjugation steps. Step 1. C. taiwanensis CBM32 was randomly transposon mutagenised using pMH1801 possessing the Tn5-B13S transposon which carries the mob site (oriT), an npt-sacB-sacR cassette and Tet-resistance. Step 2. Mutants were selected on TY supplemented with Tet and Str, and the helper plasmid, RP4-7, was individually introduced into each C. taiwanensis mutant. Step 3. C. taiwanensis::Tn5-B13S mutants carrying RP4-7 were then conjugated with R. solanacearum. Transconjugants were selected on MM supplemented with glucose and Tet. One Tn5-B13S mutagenised C. taiwanensis clone, CBM61, was successful in producing Tet-resistant R. solanacearum transconjugants. A selected transconjugant, CBM62, was verified as R. solanacearum containing pRal by 16SrDNA and nifH gene amplification, and a seemingly intact pRal was confirmed by a modified Eckhardt gel analysis [36]. The Tn5-B13S insertion in pRal of CBM62 was found located within a putative transposase (see DNA Manipulation), and thus had not disrupted any gene essential for symbiosis, as confirmed by nodulation tests and microscopic observation of the mutagenised C. taiwanensis strain CBM61 used as donor for pRal transfer. The Tn5-B13S, which contains sacRsacB genes that might interfere with plant tests, was exchanged in CBM62 with a trimethoprim (Tri) resistance cassette (see DNA Manipulation), giving rise to the Ralstonia chimeric strain GMI1000 (pRal::Tri), or CBM124.

The ancestral strain CBM124GenR was obtained by natural transformation [35] of CBM124 with genomic DNA from the R. solanacearum GRS412 strain (containing the GenK plasmid pCZ367 inserted in the Rsp1236 gene). Correct insertion of pCZ367 in CBM124GenR was verified by using a primer located upstream of the inactivated gene and a primer located in the lacZ gene of pCZ367. Transfer of pRal into CBM124 by conjugation. Transconjugants resistant to kanamycin and sensitive to tetracycline were screened. The replacement of the Rsp0128–Rsp0156 region by the kanamycin resistance cassette in strain CBM351 was verified by PCR.

Construction of Mutants
To construct CBM351, a CBM124 derivative deleted for the Rsp0128–Rsp0154 region, PCR fragments from the Rsp0125 and Rsp0157 genes (Rsp0126, Rsp0127, Rsp0155 and Rsp0156 are transposases) were amplified using oCBM494–oCBM495 and oCBM496–oCBM497 as primers and cloned into the EcoRI/SacI restriction sites of pGM184, respectively. The modified plasmid was introduced into CBM124 by conjugation. Transconjugants resistant to kanamycin and sensitive to tetracycline were screened. The replacement of the Rsp0128–Rsp0156 region by the kanamycin resistance cassette in strain CBM351 was verified by PCR.

To construct CBM125, a hrpV mutant of CBM124, pRal::Tn5-B13S, was transferred by conjugation from C. taiwanensis CBM61 to the R. solanacearum hrpV mutant GMI1694. The Tn5-B13S transposon was then replaced by the trimethoprim resistance cassette as described above.

To construct CBM142 and CBM145, the hrpV mutation and the popF1 popF2 double mutation were introduced into CBM124 by
### Table 2. Strains used in this study.

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**Chimeric Ralstonia**

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<td>pRala::Tri, Tri&lt;sup&gt;R&lt;/sup&gt;, Gen&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>CBM145</td>
<td>pRala::Tri, Tri&lt;sup&gt;R&lt;/sup&gt;, Gen&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>CBM212</td>
<td>pRala::Tri, Tri&lt;sup&gt;R&lt;/sup&gt;, Gen&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Table 3. Plasmids used in this study.**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant Characteristics</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>p34E-Tp</td>
<td>Cassette vector with trimethoprim resistance gene, Tri&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[50]</td>
</tr>
<tr>
<td>pCBM01</td>
<td>pCM184 containing 401 bp of the nod&lt;sub&gt;B&lt;/sub&gt; promoter, Gen&lt;sup&gt;R&lt;/sup&gt;, Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pCBM19</td>
<td>pCM184 containing Rsp0125 and Rsp0157 gene fragments, Amp&lt;sup&gt;R&lt;/sup&gt;, Kan&lt;sup&gt;R&lt;/sup&gt;, Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pCBM32</td>
<td>pCM184 containing hrg upstream and downstream fragments, Amp&lt;sup&gt;R&lt;/sup&gt;, Kan&lt;sup&gt;R&lt;/sup&gt;, Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pCM184</td>
<td>cre-lox allelic exchange vector, Amp&lt;sup&gt;R&lt;/sup&gt;, Kan&lt;sup&gt;R&lt;/sup&gt;, Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[51]</td>
</tr>
<tr>
<td>pCZ388</td>
<td>pLAFR6 derivative containing a promoterless lacZ gene, Gen&lt;sup&gt;R&lt;/sup&gt;, Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[521]</td>
</tr>
<tr>
<td>pMG02</td>
<td>pGEM-Teasy with a 2-kb fragment from pRala carrying a Tri&lt;sup&gt;R&lt;/sup&gt; cassette</td>
<td>This study</td>
</tr>
<tr>
<td>pMH1801</td>
<td>pQ18 derivative carrying a Tn5-B135, mob, sac&lt;sup&gt;δ&lt;/sup&gt;sac&lt;sup&gt;β&lt;/sup&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;, Chl&lt;sup&gt;R&lt;/sup&gt;, Tet&lt;sup&gt;R&lt;/sup&gt;, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[52]</td>
</tr>
<tr>
<td>RP4-7</td>
<td>Helper plasmid, Chl&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[54]</td>
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</table>
natural transformation [35] of CBM124 with genomic DNA from the R. solanacearum hrG3 mutant GMI1596 and the popF1 popF2 double-mutant GMI1667, respectively. The presence of an inserted cassette in hrG3, popF1, and popF2 was verified by PCR.

To construct the hpgG mutants, CBM663 and CBM664, two different methods were used. First the CBM124 strain was transformed with genomic DNA from R. solanacearum hpgG::Tn5-B20 mutant GMI1425. Transformants were selected on BG medium supplemented with trimethoprim and kanamycin. The Tn5-B20 insertion in hpgG was verified by PCR in strain CBM663. Second, PCR fragments upstream and downstream from hpgG were amplified using oCBM622-oCBM623 and oCBM624–oCBM625 as primers and cloned into the EcoR1/KpnI and SacII/HpaI restriction sites of pCM184, respectively. The resulting plasmid was introduced into CBM124 by conjugation. Transconjungants resistant to kanamycin and sensitive to tetracycline were screened. The replacement of hpgG by the kanamycin resistance cassette was verified by PCR in strain CBM664.

DNA Manipulation

Primers used for DNA amplification are listed in Table S3.

to determine the precise location of the Tn5-B13S insertion point in pRalta of CBM61 and CBM62, tail-PCR was performed with arbitrary primer AD1 or AD4 [37] in combination with three sequential Tn5-specific primers designed from the terminal arms of the Tn5 transposon, oCBM183, oCBM184, and oCBM185.

For Tn5-B13S insertion exchange by TriK cassette, a 2-kb PCR fragment, corresponding to approximately 1 kb each side of the Tn5-B13S insertion point, was amplified from LMGI19424 using primers oCBM196 and oCBM198 and cloned into pGEM-Teasy (Promega). The TriK cassette isolated from p34E-Tp digested by BamHI was then introduced in the BgIII site of the fragment, generating pMG02. This BgIII site was located only 6 bp from the Tn5-B13S insertion point in CBM62. ScaI linearized pMG02 DNA was used to transform naturally competent R. solanacearum chimeric strains containing pRalta::Tn5-B13S. The exchange of Tn5-B13S with the trimethoprim cassette was verified by establishing that the strain had lost resistance to tetracycline and could grow on 5% sucrose.

For the construction of pCBM01, the promoter region of nodB was amplified using oCBM203 and oCBM211 as primers and cloned into pGEM-Teasy (Promega), cleaved from pGEM-Teasy with HindIII and PstI, and then directionally cloned into the same sites of the lacZ transcriptional fusion in pCZ338. pCBM01 was introduced in C. taisiacensis and R. solanacearum strains by conjugation.

The lacZ- and gfp-derived strains were obtained by natural transformation with genomic DNA from strains GMI1485 and GMI1600, respectively.

Solexa Re-Sequencing and Mutation Analysis

Sequence data production was performed by the C.E.A/IGG-Genoscope (Evry). Paired-end libraries were prepared following the protocol recommended by Illumina Inc. (http://www.illumina.com). For each strain, more than 5 million paired-end reads (L = 72 bp = 2×36 bp) were generated with Genome Analyzer sequencing system, leading to a ca. 60× total coverage of the reference genome (Table S4). Taking advantage of the local production of raw sequencing data, a bioinformatic pipeline called SNIPer (S. Cruveiller and C. Medigue, unpublished data) and based on ssha2 alignment software (Sequence Search and Alignment by Hashing Algorithm [38] has been implemented. This pipeline allows the detection of small variations (SNPs and Indels) between a collection of short reads and a reference sequence, this latter being either a consensus produced by assemblers or a previously published one.

SNIPer is a shell script that automatically sets the alignments parameters depending on the kind of reads (ABI-3730/454-GSFLX/Solexa/SOLiD) being used, launches the various parts of the detection pipeline, and controls for all tasks having been completed without errors. The detection of SNPs and indels is achieved in four main steps: (1) The data preparation, which consists in (i) the conversion of sequencing raw data (i.e., reads files) into Sanger Institute FastQ formatted files; (ii) the removal of duplicated reads (quite common when using Solexa platform) so as to keep exactly one copy of each read; and (ii) the split of paired-ends reads into single-end reads when required. (2) Reads mapping onto a reference molecule using the ssha2 package [38]. This package combines the SSAHA searching algorithm (sequence information is encoded in a perfect hash function) aiming at identifying regions of high similarity, and the cross_match sequence alignment program (http://www.phrap.org/phredphrapconsed.html), which aligns these regions afterwards using a banded Smith-Waterman-Gotoh algorithm [39,40]. (3) Based on the characteristics of reads alignments onto the reference molecule, a file containing the lists of all possible events is generated. (4) Each event is then scored so as to keep only significant ones. This score takes into account the reference base coverage (i.e., the number of reads mapping a given location) and the quality of bases of reads displaying a change at that particular location as well.

The ca. 5 million paired-end reads were split into single reads and mapped on the reference genome (the two replicas of Ralstonia solanacearum GMI1000 [RefSeq acc. NC_003295.fna and NC_003296.fna for the chromosome and the megaplasmid respectively]+ the nodulation plasmid of Cupriavidus taiensis LMGI19424 [RefSeq acc. NC_010529.fna]) using SNIPer. Among the 10 million single reads, around 7 millions were successfully mapped, leading to an effective coverage of the three reference molecules higher than 30× (Table S4), hence warranting a reliable detection of changes. The remaining unmapped reads (3 million on average) correspond to reads that could neither be mapped unambiguously (i.e., repeat regions, insertion sequences, rDNA, etc.) nor be mapped at all (i.e., fragment of sequences not present in the references).

Plant Assays and Cytological studies

Pathogenicity assays with M. pudica and Arabidopsis thaliana ecotype Col-0 were performed according to Deslandes et al. [41]. Root inoculations used the method of cutting 2 cm from the bottom of Jiffy pot-grown plants, followed by immersion for 5 min in a suspension of bacteria grown overnight and diluted to an OD600 of 0.1 in water. R. solanacearum and derivatives were tested for the HR ability by infiltrating a bacterial culture adjusted to 108 cells/ml inlilite into tobacco (cultivar Bottom Special) leaf parenchyma as described previously [35].

For M. pudica nodulation assay and cytology, seeds were surface sterilised and planted under sterile conditions using the tube method of Gibson as previously described [13], (except tubes contained Fahraeus [42] slant agar and liquid water). For the selection of nodulating evolved clones, 106 bacteria par tube were used as inoculum. Otherwise, 108 bacteria were routinely inoculated per tube unless specified. Nitrogen fixation was estimated by visual observation of the vigour and foliage colour of 40/60-d-old plants on at least 20 plants. For resolution of nodule bacteria, nodules were surface sterilised 10 min with 2.6% sodium hypochlorite, rinsed five times, then crushed and dilutions plated on the appropriate solid medium. For each M. pudica tube,
ex planta number of bacterial generations is estimated at a maximum of 5, and in planta generation number is calculated using the formula log(number of bacteria/initial number of bacteria)/log2.

\[ \text{LacZ}-\text{tagged infecting bacteria were stained according to the standard procedure. Briefly, roots were fixed in glutaraldehyde 1.5% in K phosphate buffer for 30 min under vacuum condition followed by 1 h at room temperature. After washing, roots were incubated overnight with the staining solution at 28°C (0.1 M K phosphate [pH 7.4], 2 mM K ferricyanide, 2 mM K ferrocyanide, and 0.08% of X-gal in dimethylformamide). Roots were washed and used for microscopic analysis. To analyse infection of \text{gfp}-tagged bacteria, root and nodules were fixed in paraformaldehyde 3.7% in phosphate buffered saline (PBS) for 30 min under vacuum, then washed and used directly or cut for nodule sections 60-μm thick using a Leica VT1000S vibratome. Samples were observed by using a fluorescence (Zeiss Axioskop Fluorescence microscope) or confocal microscope (Leica SP2).}

For fine histological examination, nodules were fixed in glutaraldehyde 2.5% in phosphate buffer 0.1 M [pH 7.4], osmium treated, dehydrated in an alcohol series, and embedded in Epon 812. Semithin nodule sections were observed by brightfield microscopy after staining in 0.1% aqueous toluidine blue solution and observed under a Zeiss Axioskop light microscope. Ultrathin sections were stained with uranyl acetate and observed with a Hitachi EM600 electron microscope.

For the two-step infections, we proceeded as follows. \textit{M. pudica} plants, grown as described above, were first infected with the \text{lacZ}-tagged \textit{hcv} chimera strain. After 9 d of infection, once nodules were formed, a secondary infection was performed by using the \text{gfp}-tagged \textit{hcv} chimera strain. Two weeks after, nodules were fixed in paraformaldehyde 3.7% as previously described and used for cytological analysis.

\section*{β-Galactosidase Assays}

Strains were grown overnight at 28°C in MM supplemented with the appropriate carbon source, vitamins, and tetracycline. Overnight cultures were then diluted to an OD\text{600} of 0.005–0.01 in MM with tetracycline 0.15 mM final concentration of luteolin and grown a minimum of 16 h until an OD\text{600} of 0.7 was reached. The cultures were then assayed for β-galactosidase activity (Miller units) according to Miller, 1972 [43]. The β-galactosidase activities represent an average of quadruplicate samples from two separate experiments.

\section*{Nod Factor Purification and Characterization}

NFs were produced, purified, and characterized as previously described [15].

\section*{Supporting Information}

\textbf{Figure S1} Phylogenetic and genomic relationships between \textit{C. taiwanensis} and \textit{R. solanacearum}. (A) Rooted 16S rDNA tree of \textit{Cupriavidus} and \textit{Ralstonia} species. The scale bar represents 5% of sequence divergence. Adapted from [44]. (B) Genome organization of \textit{C. taiwanensis} LMG19424 and \textit{R. solanacearum} GMI1000. (C) Synteny plots between \textit{C. taiwanensis} LMG19424 and \textit{R. solanacearum} GMI1000 genomes. The line plots have been obtained using synteny results between chromosomes 1 as well as chromosomes 2 of both genomes. Synteny groups containing a minimum of three genes are drawn in green for colinear regions, and in red for inverted regions. The display has been obtained using the MaGe graphical interface of the CupriavidusScope project [https://www.genoscope.cns.fr/agc/mage].

\textbf{Figure S2} Hypersensitive response elicited on the nonhost plant \textit{Nicotiana tabacum}. The tobacco leaf was infiltrated with a 10\textsuperscript{8} colony-forming units/millilitre suspension of \textit{R. solanacearum} derivative strains. GMI1000, wild-type \textit{R. solanacearum}. CMB124GenR, ancestral chimeric \textit{Ralstonia}. CMB212, CMB349, CMB356, Mimosa-nodulating evolved clones. CMB125, \textit{hcv} chimera. The photograph was taken 48 h after infiltration. Found at: doi:10.1371/journal.pbio.1000280.s002 (2.49 MB TIF)

\textbf{Figure S3} Compared structures of Nod factors from \textit{C. taiwanensis} and chimeric CMB124. Electrospray ionisation-mass spectrometry (ESI-MS) spectrum in the negative ionisation mode of high-performance liquid chromatography fractions eluting at 36% AcCN in water obtained from LMG19424 (A), and CMB124 (B). Molecular ions [M-H]− at mass-to-charge ratio (m/z) 1391.8 correspond to an oligomer of five glucosamine units, substituted by a vaccenic acid (C\textsubscript{18,1}), a methyl, a carbamoyl, and a sulphate group. Species at m/z 1365.7 and at m/z 1348 correspond to the same basic structure with a palmitic acid (C\textsubscript{16,0}) instead of the vaccenic acid with or without the carbamoyl group, respectively. Found at: doi:10.1371/journal.pbio.1000280.s003 (0.66 MB TIF)

\textbf{Figure S4} Compared nodulation of \textit{M. pudica} by \textit{C. taiwanensis} LMG19424 and the evolved clone CMB124 (A), and by the \textit{hcv} and \textit{popFlpopF2} mutants of the chimeric \textit{Ralstonia} (B). Plants were grown in Gibson tubes containing Fařaæus slant agar and 0.25 × liquid Jensen. At least 20 plantlets were inoculated (10\textsuperscript{3} bacteria per tube) per strain. Found at: doi:10.1371/journal.pbio.1000280.s004 (0.76 MB TIF)

\textbf{Figure S5} Nodulation and infection of \textit{M. pudica} by \textit{C. taiwanensis}. (A) Root hair deformation following \textit{C. taiwanensis} inoculation. (B and C) Infection threads of green \text{gfp}-tagged bacteria growing from infection sites with especially pronounced examples of branched and multiple infection threads (C). (D) Young nodules. (E and F) Nodule sections showing cells infected with \text{gfp}-tagged (E) or bacteria stained with toluidine blue (F). (G and H) Intracellular invasion of vegetal cells. Note the absence of bacteria in intracellular spaces. (I) Intracellular bacteria (bacteroids) surrounded by a peribacteroid membrane (arrow) forming typical symbiosomes. Found at: doi:10.1371/journal.pbio.1000280.s005 (7.37 MB TIF)

\textbf{Figure S6} Infection of \textit{M. pudica} by ancestral chimeric \textit{Ralstonia} CMB124 (A, C, and D) and CMB124GenR (B). (A) Root hair deformation following inoculation. (B and C) Microcolony of green \text{gfp}-tagged bacteria in curled root hair structures, and abortive ITs ([C] white arrow). (D) Dead root hair completely filled with blue \text{lacZ}-tagged bacteria, occasionally observed. Found at: doi:10.1371/journal.pbio.1000280.s006 (2.78 MB TIF)

\textbf{Figure S7} Nodulation and extracellular infection of \textit{M. pudica} by the \textit{hcv} chimeric mutant CMB125 (A–F and H) and the evolved clone CMB356 (G and I–K). (A) Root hair deformation. (B and C) Formation of infection threads from infection sites with especially pronounced examples of branched and multiple infection threads (C). (D) Young nodules. (E and F) Nodule sections showing cells infected with \text{gfp}-tagged (E) or bacteria stained with toluidine blue (F). (G and H) Intracellular invasion of vegetal cells. Note the absence of bacteria in intracellular spaces. (I) Intracellular bacteria (bacteroids) surrounded by a peribacteroid membrane (arrow) forming typical symbiosomes. (J) Dead root hair completely filled with blue \text{lacZ}-tagged bacteria, occasionally observed. Found at: doi:10.1371/journal.pbio.1000280.s007 (2.2 MB TIF)
vation of intercellular bacteria and cell wall thickening (asterisks) (H, J, K), and ITs (J).

Found at: doi:10.1371/journal.pbio.1000280.s007 (7.51 MB TIF)

**Figure S8** Nodulation and intracellular infection of *M. pudica* by *hrg* chimeric mutant CBM664 (A and C) and evolved clones CBM212 (B, D, and F-K) and CBM349 (E, A and B) Young nodules. (C and D) Nodule sections showing the infected zone. (E-G) Massive intracellular invasion in nodules. (H) Note the presence of bacteria in intercellular spaces. (H) Intraacellular bacteria surrounded by a peribacteroid membrane forming typical symbiosomes (arrow). Osmophilic material containing vesicles (arrowhead), probably involved in premature symbiosome degradation, were often associated with symbiosomes. PHB (Polyhydroxybutyrate) storage granules were present in bacteria (asterisks). (I) Infection pocket within intercellular space. (J) PHB (Polyhydroxybutyrate) storage granules were present in symbiosome degradation, were often associated with symbiosomes. (K) Young nodules. (L) Nodule sections showing the infected zone. (M) Massive intracellular invasion in nodules. (N) Intraacellular bacteria surrounded by a peribacteroid membrane forming typical symbiosomes (arrow).

Table S1 Expression of a nodB::lacZ fusion in *C. taiwanensis* and chimeric *Ralstonia* in response to luteolin 15 𝜇M.

Found at: doi:10.1371/journal.pbio.1000280.s008 (9.12 MB TIF)

**Table S2** Number of mutations in evolved clones relative to the immediate ancestor CBM124GenR.

Found at: doi:10.1371/journal.pbio.1000280.s010 (0.03 MB DOC)

**Table S3** List of primers.

Found at: doi:10.1371/journal.pbio.1000280.s011 (0.06 MB DOC)

**Table S4** Characteristics of raw sequencing data output by the Illumina Genome Analyzer^a^ and SNiPer primary results^b^ for the strains under study.

Found at: doi:10.1371/journal.pbio.1000280.s012 (0.03 MB DOC)

**References**


**Acknowledgments**

We are grateful to J. Cullimore for careful reading of the manuscript, M. Hynes for advices in transferring pRalta in *R. solanacearum*, C. Boucher and S. Genin for fruitful discussions and for providing *R. solanacearum* strains, and F. de Billy for help with microscopic work.

**Author Contributions**

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: PH JB CMB. Performed the experiments: MM DC MG CG TT VP LBG. Analyzed the data: MM DC MG SC VP CM CMB. Contributed reagents/materials/analysis tools: SC BCWM CM. Wrote the paper: JB CMB.

**Table S2** Number of mutations in evolved clones relative to the immediate ancestor CBM124GenR.

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**Table S3** List of primers.

Found at: doi:10.1371/journal.pbio.1000280.s011 (0.06 MB DOC)

**Table S4** Characteristics of raw sequencing data output by the Illumina Genome Analyzer^a^ and SNiPer primary results^b^ for the strains under study.

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