

## Nickel Resistance Determinants in *Bradyrhizobium* Strains from Nodules of the Endemic New Caledonia Legume *Serianthes calycina*<sup>†</sup>

Clémence Chaintreuil,<sup>1\*</sup> Frédéric Rigault,<sup>2</sup> Lionel Moulin,<sup>1</sup> Tanguy Jaffré,<sup>2</sup> Joël Fardoux,<sup>1</sup>  
Eric Giraud,<sup>1</sup> Bernard Dreyfus,<sup>1</sup> and Xavier Bailly<sup>1</sup>

Laboratoire des Symbioses Tropicales et Méditerranéennes (UMR113), IRD/CIRAD/SUPAGRO/INRA/UM2, Montpellier, France,<sup>1</sup>  
and Laboratoire de Botanique et d'Ecologie Végétale, IRD, Nouméa, Nouvelle-Calédonie<sup>2</sup>

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***Bradyrhizobium* strains, isolated in New Caledonia from nodules of the endemic legume *Serianthes calycina* growing in nickel-rich soils, were able to grow in the presence of 15 mM NiCl<sub>2</sub>. The genomes of these strains harbored two Ni resistance determinants, the *cnr* and *nre* operons. By constructing a *cnrA* mutant, we demonstrated that the *cnr* operon determines the high nickel resistance in *Bradyrhizobium* strains.**

New Caledonia, an archipelago in the Pacific Ocean, is considered one of the main hotspots of biodiversity on earth (17). This is explained by both its geographical isolation and the presence of nickel-rich ultramafic soils that cover one-third of the main island. These soils contain 250-fold more nickel than is found in average soil. Moreover, they contain high concentrations of other metals and have strong deficiencies in nutritive elements (4, 22). Bacteria isolated from ultramafic soils have been shown to be adapted to these specific constraints (14). In addition, Stoppel and Schlegel (25) determined by DNA-DNA hybridization that nickel-resistant bacteria from New Caledonia soils harbor DNA fragments homologous to several nickel resistance determinants, including *nre* (nickel resistance), *cnr* (cobalt-nickel resistance), and *ncc* (nickel-cobalt-cadmium resistance) genes. Ultramafic ecosystems of New Caledonia are endangered due to nickel mining activities, which constitute the main economical resource of the islands. Therefore, restoration of current or old mining sites (i.e., bare slag heaps) is a priority for preservation of the unique diversity of New Caledonia's ecosystems. This first requires reestablishment of a vegetal cover to stabilize the soils and to control water flow. Native nitrogen-fixing trees belonging to the genus *Serianthes* appear to be among the best candidates for these purposes because of their capacity to fix nitrogen in symbiosis with rhizobia and to improve the fertility of mine spoils (3, 6). However, until now there has been no information available about the taxonomic and functional diversity of the symbiotic rhizobia associated with the genus *Serianthes* in New Caledonia. In order to obtain insight into the symbiotic partners associated with *Serianthes calycina*, we isolated bacteria from nodules obtained from two ultramafic nickel-rich soils (Mont Dore and Tiébaghi) and from one calcareous nickel-free soil (Nouméa Anse Vata) (Table 1). Ten strains were isolated and characterized by determining (i) their taxonomic positions, (ii)

their abilities to nodulate plants and fix nitrogen symbiotically, (iii) their capacity to grow on high nickel concentrations, and (iv) the presence of known nickel resistance determinants (*nre* and *cnr* operons). Furthermore, by constructing a *cnrA* mutant of strain STM2464, we confirmed that the *cnr* operon was responsible for the high nickel resistance of the most tolerant strains.

Soil analyses and isolation of rhizobia from nodules were carried out as described previously (2, 5). Total genomic DNA was extracted as previously described by Pitcher et al. (21). A nearly full-length 16S rRNA gene was amplified using primers FGPS-6 (GGACAGTTAGATCTTGGCTACG) and FGPS-1509 (AAGGAGGGGATCCAGCCGCA) as described by Normand et al. (19). The sequences of the PCR products were analyzed using the algorithm BLASTN (1) to identify the most related sequences. Multiple alignment was performed with ClustalX, version 1.63b (28). Phylogenetic analysis was performed using a distance approach (Kimura two-parameter method), and bootstrap values were obtained based on 1,000 replicates (9) using MEGA, version 3.0 (16).

All bacteria isolated from *S. calycina* clustered in a large group, which included the type strain of *Bradyrhizobium elkanii* (see Fig. S1 in the supplemental material). Within this group, three distinct clades of New Caledonia strains could be distinguished. Two strains (STM2464 and STM2465) belonged to the species *B. elkanii* as they shared 100% sequence similarity with each other and with two reference *B. elkanii* strains (LMG9520 and ORS133) and 99% sequence similarity with *B. elkanii* type strain USDA76. Three other strains (STM2457, STM2458, and STM2460) formed a second clade close to *B. elkanii*, showing 98% similarity to the *B. elkanii* type strain. Five strains (STM2456, STM2459, STM2461, STM2467, and STM2468) formed a third clade belonging to the genus *Bradyrhizobium*. This group, which also includes reference strain Ai1a-2 isolated from Costa Rica (20), is clearly distinct from the known species of *Bradyrhizobium*, suggesting that it could represent a new species of this genus. This analysis revealed that the main symbionts of *S. calycina* belong to the genus *Bradyrhizobium*.

All strains were first tested for in vitro nodulation on *Macropitium atropurpureum* (Siratro) as described previously (8).

\* Corresponding author. Mailing address: LSTM, Campus International de Baillarguet, TA A-82/J, 34398 Montpellier Cedex 5, France. Phone: (334) 67 59 38 51. Fax: (334) 67 59 38 02. E-mail: clemence.chaintreuil@mpl.ird.fr.

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TABLE 1. Symbiotic properties and nickel resistance genes in rhizobial strains isolated from different New Caledonia soils

| Strain <sup>a</sup> | Isolation site     | Total nickel soil content (mg/g of soil) | Nodulation efficiency <sup>b</sup> | Resistance to <sup>c</sup> : |              | <i>nreB</i> gene <sup>d</sup> | <i>cnrA</i> gene <sup>d</sup> |
|---------------------|--------------------|--|------------------------------------|------------------------------|--------------|-------------------------------|-------------------------------|
|                     |                    |  |                                    | 3 mM nickel                  | 15 mM nickel |                               |                               |
| STM2464             | Mont Dore          | 19.3 ± 1.0                               | E                                  | ++                           | ++           | (+)                           | (+)                           |
| STM2465             | Mont Dore          | 19.3 ± 1.0                               | E                                  | ++                           | ++           | (+)                           | (+)                           |
| STM2457             | Tiébaghi           | 10.4 ± 0.8                               | e                                  | ++                           | +            | (+)                           | (+)                           |
| STM2458             | Tiébaghi           | 10.4 ± 0.8                               | E                                  | ++                           | +            | (+)                           | (+)                           |
| STM2460             | Tiébaghi           | 10.4 ± 0.8                               | e                                  | ++                           | +            | (+)                           | (+)                           |
| STM2456             | Tiébaghi           | 10.4 ± 0.8                               | I                                  | +                            | 0            | (+)                           | —                             |
| STM2459             | Tiébaghi           | 10.4 ± 0.8                               | e                                  | +                            | 0            | (+)                           | —                             |
| STM2461             | Tiébaghi           | 10.4 ± 0.8                               | I                                  | +                            | 0            | (+)                           | —                             |
| STM2467             | Nouméa (Anse Vata) | 0  | e                                  | 0                            | 0            | —                             | —                             |
| STM2468             | Nouméa (Anse Vata) | 0  | e                                  | 0                            | 0            | —                             | —                             |

<sup>a</sup> STM, collection of the "Laboratoire des Symbioses Tropicales et Méditerranéennes," Montpellier, France. The bacterial strains listed are new isolates, and *S. calycina* was the original host plant for all of them.

<sup>b</sup> Nodulation efficiency on *M. atropurpureum*. I, ineffective root nodulation; e, partially effective root nodulation; E, effective root nodulation.

<sup>c</sup> Resistance based on bacterial growth. ++, strong growth; +, faint growth; 0, no growth.

<sup>d</sup> Signal determined by PCR amplification. (+), strong signal; —, no signal.

Siratro is a host legume known to form nodules with a very broad range of *Bradyrhizobium* strains. All 10 strains were able to form nodules on this host plant. They showed different levels of nitrogen fixation, as estimated by plant biomass and leaf color (Table 1). To study their resistance to nickel, the 10 strains were grown on solid or liquid YM (29) medium without Ni (control) or with NiCl<sub>2</sub> at concentrations of 3 and 15 mM. All cultures were incubated at 28°C on a rotary shaker. Bacterial growth rates were evaluated daily for up to 14 days by measuring the optical density at 620 nm. The effect of NiCl<sub>2</sub> on bacterial growth is shown in Table 1 and Fig. 1. The two strains isolated from *S. calycina* growing in the nickel-free soil

(STM2467 and STM2468) did not show any tolerance to 3 mM NiCl<sub>2</sub>. In contrast, the eight strains isolated from ultramafic nickel-rich soils were able to grow on solid or liquid YM medium supplemented with 3 mM NiCl<sub>2</sub>. However, the nickel tolerance varied significantly between the strains. While the growth rates of strains STM2456, STM2459, and STM2461 were strongly reduced in the presence of 3 mM NiCl<sub>2</sub>, the growth of strains STM2464, STM2465, STM2457, STM2458, and STM2460 was hardly affected (Fig. 1). In addition, strains STM2464, STM2465, STM2457, STM2458, and STM2460 were also able to grow in the presence of 15 mM NiCl<sub>2</sub> in either solid or liquid medium; strains STM2464 and STM2465

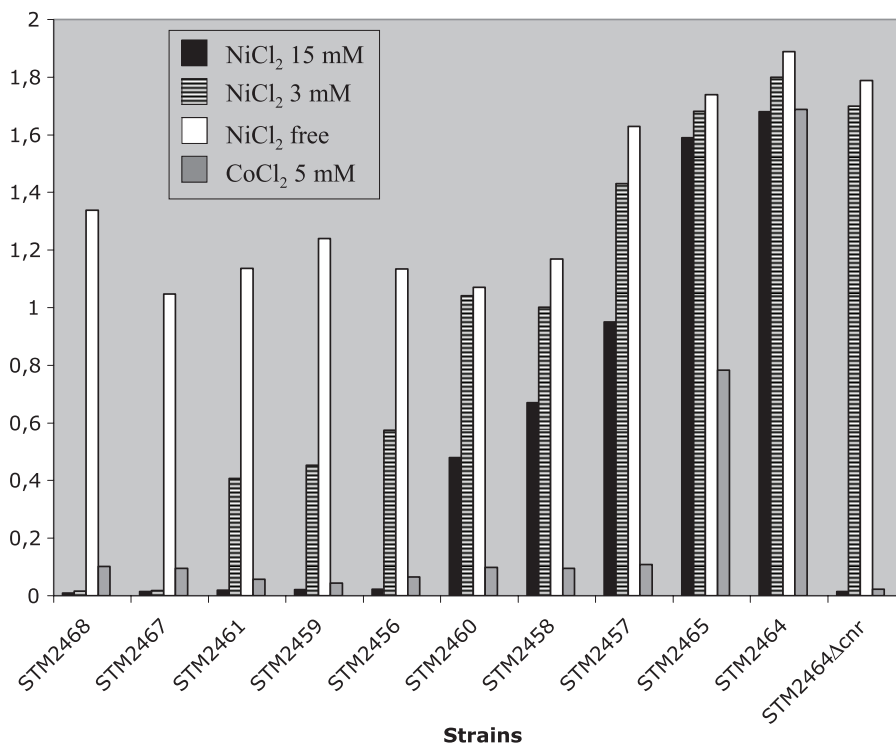


FIG. 1. Bacterial growth after addition of NiCl<sub>2</sub> or CoCl<sub>2</sub>. Optical density at 620 nm (indicated on the y axis) was measured 2 weeks after inoculation.

FIG. 2. Neighbor-joining phylogenies of metal resistance determinants based on amino acid alignments of (A) NreB (nickel resistance) and (B) NccA/CnrA (nickel-cobalt-cadmium/cobalt-nickel resistance) and the close homolog CzcA (cobalt-zinc-cadmium resistance). New strains in this study are indicated by bold type. Scale bars = number of substitution per site. The values at the nodes are bootstrap percentages based on an analysis of 1,000 replicates using distance criteria (only values of >50% are shown). The metal targeted by the resistance marker is indicated when a functional demonstration has been pub-

We studied the presence and sequence polymorphism of two resistance determinants, *nreB*, and *nccA/cnrA*, which have been found in New Caledonia soil bacteria (25). The presence of the *nreB* gene was analyzed by PCR using primers *nreB*-425F (CCTTCACGCCGACTTTCCAG) and *nreB*-1179R (CGGATAGGTAATCAGCCAGCA). Similarly, the presence of *nccA/cnrA*-like genes was analyzed by PCR amplification using primers *cnrA*-F (AACAAGCAGGTSCAGATCAAC) and *cnrA*-R (TGATCAGGCCGAAGTCSAGCG). These primers were designed based on conserved motifs present in *nreB* and *nccA/cnrA* sequences available in data banks, respectively. *Cupriavidus (Ralstonia) metallidurans* strain CH34 was used as a positive control. The different PCR products obtained were sequenced, aligned with *nreB* and *cnrA/nccA/czcA* sequences of reference strains, and translated into amino acids, and phylogenetic analyses were independently performed for each of these nickel resistance markers. The resulting phylogenetic trees, constructed by a distance approach (“Poisson p” distance; neighbor joining and bootstrap analysis based on 1,000 pseudoreplicates), are shown in Fig. 2A and B. Amplified *nreB* gene fragments (725 bp) were obtained from all eight strains growing on 3 mM NiCl<sub>2</sub> (Table 1). The NreB protein sequences of the eight New Caledonia *Bradyrhizobium* strains formed two main branches; the first branch was subdivided into two clades (99% homology) grouping strains STM2456 to STM2460 (100% identity) and strain STM2464, and the second branch contained strains STM2461 and STM2465 (100% sequence identity) (Fig. 2A). These two branches are closely

lished for the strain used in the phylogeny, as follows: Ni, nickel (11); Cd/Zn, cadmium/zinc (13); Co/Zn/Cd (7), Co/Ni: cobalt/nickel (12) and Ni/Co/Cd: nickel/cobalt/cadmium (24). The accession numbers for NreB homologs are as follows (clockwise from *Magnetospirillum magnetotacticum*): ZP\_00051068, ZP\_01018645, NP\_436142, AAR82963, AAK38164, EAM36311, ZP\_00654165, AAZ19430, YP\_145657, AAA72441, ZP\_01038231, BAD57616, ZP\_00413240, AAN05252, ZP\_01038464, ZP\_01037380, ZP\_00964062, ZP\_00955873, ZP\_00621039, AAV94683, and ZP\_010455481. The accession numbers for CzcA homologs (clockwise from *Methylobacillus flagellatus*) are as follows: ZP00564496, YP315085, NP841672, YP343437, AAM38995, YP245184, ZP00415898, ZP00242273, YP527427, ZP00803354, NP947401, ZP01199977, YP145656, CAB824253, Q44586, YP161705, YP497419, ZP00577412, ZP01039337, AAK24689, ZP00577310, ZP01048443, ZP00860443, ZP00625303, YP276927, ZP00899495, YP349350, NP251210, CAB56471, ZP00978841, YP373186, NP522054, and ZP00599347. Abbreviations: *Sil.*, *Silicibacter*; *Leg.*, *Legionella*; *C. met.*, *Cupriavidus metallidurans*; *Rh.*, *Rhodopseudomonas*; *X.*, *Xanthomonas*; *Xa.*, *Xanthobacter*; *P.*, *Pseudomonas*; *Sp.*, *Sphingopyxis*; *No.*, *Novosphingobium*; *Al.*, *Alcaligenes*; *B.*, *Burkholderia*; *Sac.*, *Saccharophagus*; *Ni.*, *Nitrobacter*.



related to *Nitrobacter* sp. strain Nb-311A (66% sequence identity) and *Magnetospirillum magnetotacticum* (62% similarity). They share 61% amino acid identity with the sequences from *C. metallidurans* and *Alcaligenes xylosoxidans*, two reference organisms for which the nickel resistance function of the *nre* operon has been demonstrated (11, 24, 26). Conversely, amplified *nccA/cnrA* gene fragments (1,066 bp) were obtained only from the five strains growing on 15 mM NiCl<sub>2</sub>, which also contained the *nreB* gene (Table 1). The CnrA protein sequences from the five strains resistant to 15 mM NiCl<sub>2</sub> were analyzed together with reference sequences encoded by both the NccA/CnrA genes and CzcA genes (i.e., a homolog involved in the resistance to cobalt, zinc, and cadmium). As shown in Fig. 2B, reference sequences of NccA/CnrA and CzcA determinants form two well-separated branches. The New Caledonia CnrA sequences form a single branch much closer to the NccA/CnrA clade than the CzcA clade. This New Caledonia branch shares 68, 63, and 62% amino acid identity with the closely related sequences from *Nitrobacter* sp. strain Nb-311A, *Nitrobacter hamburgensis* X14, and the photosynthetic organism *Bradyrhizobium* sp. strain BTai1, respectively. It also shares 51% amino acid identity with NccA from *A. xylosoxidans*, 49% amino acid identity with CnrA from *C. metallidurans*, and 47% amino acid identity with CzcA from *C. metallidurans*; all of the latter resistance determinants were functionally demonstrated in these organisms (7, 18, 27).

As we observed a clear correlation between high nickel resistance and the presence of the *cnrA* gene, but not the presence of the *nreB* gene, we performed a functional analysis of the former marker. To create the STM2464Δ*cnrA* mutant, the PCR fragment corresponding to the *cnrA* region from strain STM2464, obtained as previously described, was cloned into the pGEM-T vector (Promega Corporation, Madison, WI). The *lacZ*-Km<sup>r</sup> cassette of pKOK5 (15) was then inserted into the unique BamHI site present in this region. The construct liberated by ApaI/SpeI double digestion was then introduced into the pJQ200mp18 suicide vector (23) and finally delivered by conjugation into STM2464 as described previously (10). Double recombinants were selected on sucrose and confirmed by PCR. The effects of 3 and 15 mM NiCl<sub>2</sub> on the growth of strain STM2464 and the STM2464Δ*cnrA* mutant are shown in Fig. 1. Both the wild type and the mutant strain were able to grow on YM medium supplemented with 3 mM NiCl<sub>2</sub>. In contrast, no growth of mutant strain STM2464Δ*cnrA* was detected with 15 mM NiCl<sub>2</sub> (Fig. 1). In addition, mutant strain STM2464Δ*cnrA* also was not able to grow on 5 mM CoCl<sub>2</sub> (Fig. 1). These results clearly demonstrate that resistance to high levels of nickel and cobalt in *Bradyrhizobium* sp. strain STM2464 involves the *cnr* operon.

In order to determine if the *cnr* operon could confer to *Bradyrhizobium* sp. strain STM2464 a selective advantage for establishment of the symbiotic association with its host plant, *S. calycina*, the wild-type and mutant strains were inoculated onto young *S. calycina* plants growing in pots containing 0 or 15 mM NiCl<sub>2</sub> per kg of soil. Both strains formed effective nodules within 4 weeks. No difference in nodulation between the wild type and the mutant strain was observed in the nickel-free pots. In contrast, in the presence of 15 mM NiCl<sub>2</sub>, the number and size of nodules were doubled when the wild-type strain was used, showing that nodulation by mutant strain STM2464Δ

*cnrA* was affected by the presence of Ni. Taken together, our data suggest that the *cnr* operon found in some *Bradyrhizobium* strains gives these strains a competitive advantage for survival in metal-rich environments and therefore also for symbiotic efficiency that benefits the endemic leguminous tree. It remains to be determined whether the *cnr* operon was acquired by lateral gene transfer or whether it is an ancestral characteristic that is specifically conserved in the bacteria living in nickel-rich ultramafic soils.

**Nucleotide sequence accession numbers.** The *nreB* gene sequences of strains STM2456, STM2457, STM2458, STM2459, STM2460, STM2461, STM2464, and STM2465 have been deposited in the EMBL database under accession numbers AM236914, AM236915, AM179845, AM179846, AM179847, AM179848, AM179851, and AM236916, respectively. The *cnrA* gene sequences of strains STM2457, STM2458, STM2460, STM2464, and STM2465 have been deposited in the EMBL database under accession numbers AM260684, AM260685, AM260686, AM260687, and AM260688, respectively.

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