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Differing Courses of Genetic Evolution of *Bradyrhizobium* Inoculants as Revealed by Long-Term Molecular Tracing in *Acacia mangium* Plantations

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Introducing nitrogen-fixing bacteria as an inoculum in association with legume crops is a common practice in agriculture. However, the question of the evolution of these introduced microorganisms remains crucial, both in terms of microbial ecology and agronomy. We explored this question by analyzing the genetic and symbiotic evolution of two *Bradyrhizobium* strains inoculated on *Acacia mangium* in Malaysia and Senegal 15 and 5 years, respectively, after their introduction. Based on typing of several loci, we showed that these two strains, although closely related and originally sampled in Australia, evolved differently. One strain was recovered in soil with the same five loci as the original isolate, whereas the symbiotic cluster of the other strain was detected with no trace of the three housekeeping genes of the original inoculum. Moreover, the nitrogen fixation efficiency was variable among these isolates (either recombinant or not), with significantly high, low, or similar efficiencies compared to the two original strains and no significant difference between recombinant and nonrecombinant isolates. These data suggested that 15 years after their introduction, nitrogen-fixing bacteria remain in the soil but that closely related inoculant strains may not evolve in the same way, either genetically or symbiotically. In a context of increasing agronomical use of microbial inoculants (for biological control, nitrogen fixation, or plant growth promotion), this result feeds the debate on the consequences associated with such practices.

Biological N_2 fixation by legume-rhizobium symbioses, and as a consequence the nitrogen input in the soil agroecosystem, has been exploited for centuries by farmers, first empirically in legume-cereal crop rotation and more recently by using artificial bacterial inoculation. Indeed, inoculation of either seeds at sowing or seedlings at a nursery stage is often recommended to maximize nodulation and N_2 fixation (1). Inoculation can be required when the specific symbiotic bacteria of the plant is absent in soil. Soybean (*Glycine max*) is, for instance, unable to associate efficiently with bacteria outside its natural geographical area (eastern Asia). Therefore, artificial inoculation of this species is required in America and Europe to achieve satisfying nodulation and nitrogen fixation levels. One recurrent question raised by this inoculation is the fate of the introduced strains. This question is essential in both agronomical and ecological frames of reference. For instance, does the strain persist in the soil between two crop cycles, which means that it does not need to be inoculated every year? If the inoculant strain is still present, does its nitrogen fixation efficiency remain at the same level? The genomic stability of an inoculated strain is also a major question. Indeed, an introduced strain may remain genetically isolated in soil, or gene exchanges might occur between this strain and indigenous bacteria. This can have a huge impact on indigenous rhizobial genetic diversity and also on competition for efficient nodulation. Can we thus prevent such recombination events or at least predict the fate of an introduced strain and its ability to recombine with local bacteria?

We explored these questions in the *Acacia mangium*-*Bradyrhizobium* symbiotic pair. *A. mangium* is a tree species native to Australia, Papua New Guinea, and Indonesia that has been widely used since the 1980s in forestry (2). The nitrogen fixation capacity of *A. mangium*, due to its symbiotic association with root nodule bacteria, includes several genera among which *Bradyrhizobium* is the most dominant genus (3, 4). Few field trials have been set up to test the effects of controlled inoculation with selected bradyrhizobia versus natural colonization with indigenous strains. For example, Galiana et al. (5) showed that inoculation with Australian strains had a positive effect on *A. mangium* growth, whatever the soil type and country, and improvement was still detectable after 2 to 3 years. More recently, in two Brazilian experimental *A. mangium* fields, Perrineau et al. (4) showed the erratic persistence of inoculum strains, probably due to competition with local rhizobia coupled with environmental conditions.

Recombination between inoculated and indigenous rhizobium strains nodulating herbaceous legumes has been documented several times. Sullivan et al. (6) and Sullivan and Ronson (7), using *Lotus corniculatus*, showed that a *Mesorhizobium loti* strain could not be recovered 7 years after inoculation but that its 500-kb chromosomal symbiosis island (SI) had been transferred to local nonsymbiotic rhizobia. Similarly, symbiotic gene transfer from *Bradyrhizobium japonicum* to *Bradyrhizobium elkanii* and *Ensifer meliloti* local isolates was shown by Batista et al. (8). Nandasena et al. (9, 10) also showed that 5 to 6 years after inoculation with a *Mesorhizobium ciceri* strain, some endemic Australian *Me-*

Received 17 June 2014 **Accepted** 1 July 2014
Published ahead of print 7 July 2014
Editor: C. R. Lovell
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.02007-14.

doi:10.1128/AEM.02007-14

sorhizobium sp. isolates acquired symbiotic genes carried on a mobile symbiotic island from the inoculant strain. Finally, in a nice study analyzing the persistence of strains, Tang et al. (11) showed that in a soybean field inoculated in each of 5 years, with corn cultivated in intervening years, only 20% of putative inoculant strains were recovered a few months after the last inoculation. Moreover, the ability of local strains to nodulate and fix nitrogen with soybean was not due to the acquisition of symbiotic genes from the inoculant strain; their data indicated that the inheritance of symbiotic gene variants was predominantly by vertical transmission. Parker (12) also suggested, based on three loci in the symbiotic island region and four nonsymbiotic loci, that lateral transfer of the symbiosis island region has been common throughout the evolution of *Bradyrhizobium* strains and that acquisition of a novel SI variant confers a strong selective advantage for recipient cells.

All of these studies of artificial inoculation, however, concern only annual leguminous species from which rhizobia are periodically released to the soil. Reinfection during the successive rotations of different individual plants may enhance recombination with the soil bacterial pool and apply a higher selective pressure on symbiotic gene evolution. The long-term nitrogen fixation efficiency, genetic evolution, and stability of nitrogen-fixing bacteria nodulating perennial species (shrub or tree) over a period of years are, conversely, poorly documented. These perennial species offer inoculants, at minimum, a constant rhizospheric habitat and potentially a long-term (perhaps pluri-annual) nodular niche even though the duration of indeterminate nodule life in legume trees in the humid tropics is not known. Truly, no study has ever coupled a fine molecular typing of strains isolated several years after the initial inoculation with symbiotic efficiency tests of both original and recovered isolates.

In this context, the goals of our study were (i) to estimate the long-term (i.e., after 5 and 15 years) persistence of selected *Bradyrhizobium* strains in *A. mangium* inoculation trials, (ii) to analyze their genomic stability and possible recombination events with local bacteria, and (iii) to compare the nodulation and nitrogen fixation efficiencies of potential recovered isolates with the original introduced strains.

MATERIALS AND METHODS

Study sites, sampling, and rhizobium isolation. We sampled nitrogen fixation bacteria nodulating *A. mangium* trees in Malaysia and Senegal inoculation field trials. In Malaysia, the site was provided by the Yayasan Sabah Group, in the Luasong Forestry Center (Sabah, East Malaysia). It was initially occupied by a very degraded forest that had been logged in the early 1970s and subsequently destroyed by fires, which damaged an important part of the Sabah forest in 1983 to 1984. Soil characteristics and a description of the field trial and inoculant strains used are given elsewhere (13, 14). In 1993, after 4 months in nursery, inoculated *A. mangium* seedlings were transferred to the field. In 2008, we collected root nodules and soil samples from one noninoculated control plot and from plots inoculated with four *Bradyrhizobium* sp. strains (Aust11c, Aust13c, Lu4, and Was3). The first two strains were originally isolated from *A. mangium* nodules harvested in a natural stand in Queensland (Australia) (15), whereas Lu4 and Was3 were isolated from *A. mangium* nodules in Taliwas (Malaysia) (14). The latter were no longer available in any collection or laboratory. We were thus not able to characterize them.

In this sampling in Malaysia, few nodules could be harvested directly from 15-year-old trees, and only two isolates were obtained after isolation and *in vitro* renodulation tests (see reference 4 for protocols), and none were obtained from the noninoculated plot. We supplemented our sampling by trapping, using *A. mangium* seedlings, some rhizobia contained in the soil collected around the roots of each tree in the Malaysian field. For this trapping, we used the same confined system as used for renodulation tests. One week after germination of surface-sterilized seeds, 5 g of soil was introduced in each tube and mixed with the liquid medium covering the root. The first nodules appeared 7 days postinoculation (p.i.) and were collected 30 days p.i. For each soil sample, three plants were grown, and three nodules per plant were used for subsequent characterizations. In Senegal, the *A. mangium* inoculation trial was set up in 2003, close to the city of Richard Toll on the Senegal River. In this trial, the only inoculant strain tested was *Bradyrhizobium* sp. strain Aust13c. In 2008, root nodules were collected from four *A. mangium* trees from the inoculated plot and from two noninoculated control trees.

Amplification and sequencing. We sequenced three housekeeping genes, *recA* (DNA recombination protein), *dnaK* (heat shock chaperone protein), and *glnII* (glutamine synthetase II), and two symbiotic genes, *nodA* (acyltransferase) and *nifH* (nitrogenase subunit), both located within the symbiotic cluster (16), for the two original inoculant strains (Aust11c and Aust13c) and for all the isolates recovered from field nodules or trapped from soils in Malaysia. Due to the very low divergence detected among Senegalese isolates for the *recA* sequence, we sequenced the five loci for only 17 of the isolates and two to four loci for the remaining isolates. The three housekeeping genes, evenly distributed along the *Bradyrhizobium* chromosome, were selected for their phylogenetic congruence in the *Bradyrhizobium* genus (17).

Total genomic DNA of each isolate was extracted as described in Chen and Kuo (18). DNA amplification and sequencing were performed according to Perrineau et al. (4). The partial genes *recA*, *dnaK*, and *glnII* were amplified by primers from Stepkowski et al. (19), with some modifications (Table 1). The *nodA* and *nifH* gene fragments were amplified with different pairs of primers depending on the strains.

Nucleotide sequence analyses. All sequences obtained were checked and manually corrected using Chromas Pro, version 1.34, software (Technolysium, Ltd.). Inferences about strain survival and recombination with local strains were based on comparisons of these sequences with those obtained from isolates trapped locally. Only nucleotide sequences strictly identical to those from Aust11c and Aust13c were considered to have resulted from either "identity by descent" or recombination (i.e., vertical or horizontal gene transfer). The various haplotypes were detected using Mothur, version 1.33 (20), and a multilocus haplotype data matrix was obtained using Concatenator, version 1.1 (21). We reconstructed phylogenic trees for each locus separately and concatenated in two matrices the three housekeeping genes and the two symbiotic genes; in either of these groups we did not detect any evidence of recombination using RDP4 software (22). Maximum-likelihood phylogenies were reconstructed using MEGA6 (23) with a GTR+I+G model (general time-reversible [GTR] model of nucleotide substitution with a proportion of invariant sites [I] and gamma-distributed rate heterogeneity [G]). We included several external reference sequences obtained from the NCBI GenBank database and the Joint Genome Institute (JGI)/integrated microbial genome platform.

We tested whether the local strains that acquired the symbiotic cluster from the inoculant strain were genetically closer to the inoculant than the nonrecombinant local strains. Genetic distances (Kimura two-parameter model), based on the three housekeeping loci were measured among all of these strains with MEGA6. Due to the small number of values, we used a nonparametric Kolmogorov-Smirnov test implemented in XLSTAT software (Addinsoft), with two classes of isolates (recombinant and nonrecombinant) to compare the two groups.

Nodulation and nitrogen fixation efficiency tests. We compared the symbiotic efficiency of the sampled strains that shared genetic similarity (either partially or totally) with the Australian strains originally inoculated. The strains tested fall within two classes: (i) one group of isolates identical for the five loci to the strain Aust11c and (ii) one group of isolates identical for both *nodA* and *nifH* loci to the strain Aust13c but with three

TABLE 1 Primers used to amplify the gene fragments

Gene	Organism(s)	Name	Sequence	Reference[a]	T_m (°C)[b]
recA	*Bradyrhizobium*	TS2recAf	GCCCTGCGTATCGTCGAAGG	19*	59
		TS2recAr	CGGATCTGGTTGATGAAGATCACC	19*	
	Mesorhizobium	recA(6)f_bis	GTAGAGGAYAAATCGGTGGA	37*	56
		TS2recAr	CGGATCTGGTTGATGAAGATCACC	19*	
dnaK	*Bradyrhizobium* and *Mesorhizobium*	TSdnaK2	GTACATGGCCTCGCCGAGCTTCA	19	58
		TSdnaK4	GGCAAGGAGCCGCAYAAGG	38	
glnII	*Bradyrhizobium* and *Mesorhizobium*	TSglnIIf	AAGCTCGAGTACATCTGGCTCGACGG	19	58
		TSglnIIr	SGAGCCGTTCCAGTCGGTGTCG	19	
nodA	*Bradyrhizobium*	nodAf.brad	GTYCAGTGGAGSSTKCGCTGGG	39	60–50 (20)
		nodAr.brad	TCACARCTCKGGCCCGTTCCG	39	
	Mesorhizobium	TSnodB1	AGGATAYCCGTCGTGCAGGAGCA	40	60–50 (20)
		TSnodD1-1a	CAGATCNAGDCCBTTGAARCGCA	40	
nifH	*Bradyrhizobium* and *Mesorhizobium*	nifHF	TACGGNAARGGSGGNATCGGCAA	41	57
		nifHI	AGCATGTCYTCSAGYTCNTCCA	41	

[a] Primers from references marked with an asterisk were modified.
[b] T_m, annealing temperature. Values in parentheses are numbers of cycles for the decrease from the first temperature to the second temperature listed in the touchdown amplification protocol.

different housekeeping genes. In the first group, we selected five isolates from three different trees in Malaysia, and in the second group were four isolates trapped from Malaysia and three isolates from Senegal. We also included in the test one noninoculated control, one strain sampled in Malaysia belonging to the *Mesorhizobium* genus, and the two original inoculant strains (Aust11c and Aust13c) that were stored at −80°C in the Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM) bacterial collection.

For the nitrogen fixation efficiency test, we used the same confined system that was used for *in vitro* renodulation tests. Three days after surface-sterilized seeds broke dormancy, the germinated *A. mangium* seeds were immersed in 10 ml of bacterial liquid culture (exponential growth phase, 10^7 bacteria/ml) for 10 to 15 min and then introduced in each tube. Twelve replicates were used per strain and for the noninoculated control, and 13 weeks after, the number of nodules and the dry weight of the nodules, roots, and aerial shoots on all plants were measured.

Overall significant strain parameter effects and significant differences among each strain for dry weights and nodule numbers were tested by an analysis of variance (ANOVA) test coupled with a *post hoc* Newman-Keuls test implemented in XLSTAT (2014 version; Addinsoft, Ltd.). In a second step, to confirm the significant difference with respect to the original inoculant stains, each treatment (i.e., strain) was compared to Aust11c and Aust13c as a single control, using a two-tailed Dunnett's test.

Nucleotide sequence accession numbers. Newly determined sequence data were deposited in GenBank under the accession numbers listed in Table 2.

RESULTS

After isolation and renodulation tests on *A. mangium*, 29 and 52 renodulating isolates were obtained from Malaysia and Senegal, respectively. All isolates from Senegal were obtained from field nodules. Conversely, only two isolates were recovered from field nodules in Malaysia, with all others obtained by trapping. The different isolates and the two inocula, their geographic origins, genotypes, and GenBank accession numbers are listed in Table 2. Names and genotypes of all isolates are given in Table S1 in the supplemental material.

Of the Malaysian isolates, 27.5% of the isolates sampled do not share any allele with the two Australian strains used for inoculation. Two of them even belonged to the *Mesorhizobium* genus and are different one from the other, whereas the six other *Bradyrhizobium* isolates resulted in three different multilocus genotypes. The two *Mesorhizobium* isolates share the same *nodA-nifH* haplotype but with different genetic backgrounds. All of these sequences were related, however, to *Mesorhizobium* haplotypes retrieved from GenBank (data not shown). Fifteen years after the introduction of inoculated *A. mangium* plants in the Malaysian field, 48.2% of the isolates harbored exactly the same multilocus haplotype as one of the original inoculant strains (Aust11c). Finally, even though none of the three housekeeping genes of the second inoculant strain (Aust13c) was identified among all of the Malaysian and Senegalese isolates, 24.1% of the Malaysian and 100% of the Senegalese isolates harbored *nodA* and *nifH* sequences identical to those of Aust13c (525 bp and 720 bp, respectively). Five years after this introduction in the Senegalese field, we never recovered the original Aust13c housekeeping haplotypes.

The concatenated *recA-glnII-dnaK* phylogeny clusters all isolates but one in a main clade, together with *Bradyrhizobium* sp. genospecies XI and several *Bradyrhizobium elkanii* sequences (Fig. 1). Within this main clade, the two isolates from Senegal are closely related and fall in an isolated branch. Only isolate Sab13b2 appears related to *Bradyrhizobium yuanmingense*. The two Australian strains used for inoculation fall in two different clades, each close to different Malaysian isolates. In the *nodA-nifH* phylogeny, the two Australian haplotypes clustered together and are closely related to two Malaysian haplotypes. Sab13b2, the third Malaysian haplotype that was associated with the diverging genomic background, also harbors a diverging symbiotic cluster relative to the other Malaysian isolates. The two *Mesorhizobium* isolates fall in a typical *Mesorhizobium* clade for both housekeeping and symbiotic sequences (data not shown).

This lack of recombinant isolates involving Aust11c does not result from a higher genetic divergence between this inoculant and local strains ($P = 0.8$, Kolmogorov-Smirnov test). Similarly, the local strains for which we detected recombination with Aust13c are not genetically closer to Aust13c than other native strains ($P = 0.6$).

TABLE 2 Origin and genotypes of the different isolates recovered in the study

Sampling country	Inoculant strain[a]	Representative isolate[b]	No. of identical isolates[c]	Haplotype code by locus (GenBank accession no.)[d]				
				Housekeeping gene			Symbiotic gene	
				recA	*glnII*	*dnaK*	*nodA*	*nifH*
Australia	None	Aust11c	1	R1 (KJ649514)	G1 (KJ649496)	D1 (KJ649482)	N1 (KJ649504)	F1 (KJ649523)
Malaysia	Aust11c	Sab17.07b	14	R1	G1	D1	N1	F1
Malaysia	Lu4	Sab12b4	1	R1	G1	D1	N1	F1
Malaysia	Was3	Sab15b2	1	R1	G1	D1	N1	F1
Australia	None	Aust13c	1	R2 (KJ649515)	G2 (KJ649497)	D2 (KJ649483)	N2 (KJ649505)	F2 (KJ649524)
Malaysia	Aust13c	Sab14a′7	1	R3 (KJ649512)	G3 (KJ649494)	D3 (KJ649480)	N2	F2
Malaysia	Aust13c	Sab14.02c	2	R4 (KJ649511)	G4 (KJ649493)	D4 (KJ649479)	N2	F2
Malaysia	Aust13c	Sab14.05a	2	R5 (KJ653440)	G5 (KJ649490)	D5 (KJ649476)	N2	F2
Malaysia	Aust13c	Sab14.08a	1	R6 (KJ649506)	G6 (KJ649486)	D6 (KJ649472)	N2	F2
Malaysia	Aust13c	Sab14.09a3	1	R7 (KJ653441)	G7 (KJ649489)	D7 (KJ649475)	N2	F2
Senegal	Aust13c	Sene01a2	24	R8 (KJ649516)	G8 (KJ649498)	D8 (KJ649484)	N2	F2
Senegal	None	Sene02a2	20	R8	G8	D8	N2	F2
Senegal	Aust13c	Sene01c1	1	R9 (KJ649517)	G9 (KJ649499)	D9 (KJ649485)	N2	F2
Senegal	None	Sene01c1	4	R9	G9	D9	N2	F2
Senegal	Aust13c	Sene02b2	2	R10 (KJ649518)	G10	D10	N2	F2
Senegal	None	Sene02b2	1	R10	G10	D10	N2	F2
Malaysia	Aust11c	Sab13b2	1	R11 (KJ649508)	G11 (KJ649488)	D11 (KJ649474)	N11 (KJ649501)	F11 (KJ649520)
Malaysia	Aust11c	Sab17.03b	2	R12 (KJ649507)	G12 (KJ649487)	D12 (KJ649473)	N12 (KJ649500)	F12 (KJ649519)
Malaysia	Aust13c	Sab14e1	3	R13 (KJ649510)	G13 (KJ649492)	D13 (KJ649478)	N13 (KJ649503)	F13 (KJ649522)
Malaysia	Aust13c	**Sab14.01d**	1	R14 (KJ649509)	G14 (KJ649491)	D14 (KJ649477)	N14 (KJ649502)	F14 (KJ649521)
Malaysia	Aust13c	**Sab14.04a**	1	R15 (KJ649513)	G15 (KJ649495)	D15 (KJ649481)	N14	F14

[a] Aust11c, Aust13c, Lu4, and Was3 were used as inoculant strains in different plots. The two Aust11c and Aust13c strains were originally sampled from nodules in the native area of *A. mangium* in Australia. None, the plantation was settled without any inoculant strain.
[b] Boldface isolates belong to the *Mesorhizobium* genus; all others are bradyrhizobia. Sab and Sene indicate isolates from Sabah (Malaysia) and Senegal, respectively.
[c] The list of all isolates is given in Table S1 in the supplemental material.
[d] Haplotype codes shaded in gray are identical to haplotypes of Aust11c or Aust13c. Haplotype numbers for each locus were assigned arbitrarily to make distinctions among them and do not reflect any sequence similarity.

We compared several isolates sampled from the field with the original two Australian strains in terms of symbiotic efficiency. Among the 13 strains tested, none was significantly different from the two inocula in terms of nodule numbers (see Fig. S1 in the supplemental material), but six were significantly better in terms of shoot dry weight, and one was significantly worse (Fig. 2).

DISCUSSION

Indigenous versus inoculant descendant strains. Based on our multilocus sequence typing scheme, we could discriminate between inoculant, indigenous, and recombined strains. Such distinction is based on the hypothesis that local soil did not contain strains genetically identical to the two introduced Aust11c and Aust13c strains. Several arguments are in favor of this hypothesis. In 1999, Fremont et al. (14) sampled 33 isolates from *A. mangium* plantations in Sabah. Based on 16S and 16S/23S intergenic spacer (IGS) ribosomal DNA restriction fragment length polymorphism (RFLP) typing, they showed that the Sabah isolates clustered in several clades but that the three Australian strains, including the two inoculant strains Aust11c and Aust13c, fell in a single separate clade. We also sampled four other noninoculated plantations 25 to 100 km distant from the inoculated site (data not shown). None of the 33 isolates from these sites had sequences identical to the those of the two Aust11c and Aust13c strains for any locus. We assume that this is in agreement with the hypothesis that the 10 specific haplotypes (five loci per strain) of the two inoculant strains were not locally present before their introduction. We also rejected the hypothesis of an emergence of new haplotypes derived from the inoculant strains due to mutation accumulation. Indeed, the number of differences is too high to accept such a hypothesis as nucleotide divergence ranges from 2.3% to 9.04%, representing from 41 to 165 mutations along the 1,846 bp of housekeeping genes sequenced. For the *nodA* sequence, divergences range from 6.3% (33 mutations) to 29.1% (152 mutations). Based on these data, we believe that we can confidently consider isolates sharing all or some haplotypes identical to the inoculant strains to be either descendant or recombined isolates.

Contrasted genetic fates of closely related inoculant strains. The genetic/genomic fate of inoculant strains in soil after their introduction has been the subject of many studies since the 1980s, especially in the case of the soybean-*Bradyrhizobium* pair since soybean is the most widely inoculated crop in the world. The most frequent outcome is the emergence of recombining strains in the short or long term, i.e., local isolates acquiring the symbiotic cluster from inoculated strain(s) (7, 8, 10, 24). When a new plant species is introduced together with its bacterial symbiont, selective pressures favor both the acquisition of symbiotic ability by local isolates (in order to gain the ability to nodulate the new host plant) and usually a loss of the chromosomal background of the introduced strain (usually due to a maladaptation to local environmental conditions). Several studies have also suggested either the persistence of the inoculant strain (25–27) or its apparent disappearance (4, 11, 28). Persistence or disappearance of the introduced isolates seems to be related to and under the influence of

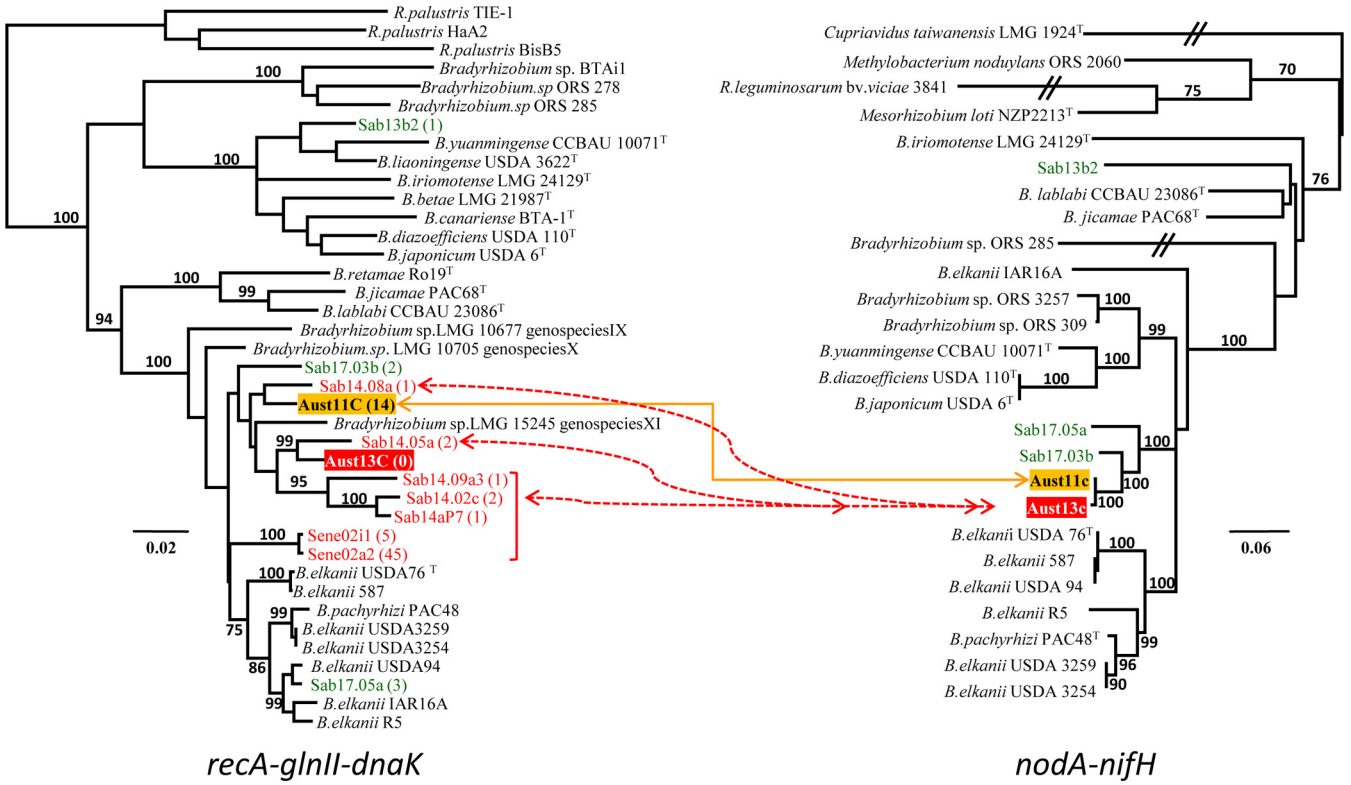

FIG 1 Concatenated housekeeping *recA-glnII-dnaK* and symbiotic *nodA-nifH* maximum-likelihood phylogenetic reconstructions. Each different combination of haplotypes was kept in the reconstruction. Numbers of isolates with the same multilocus haplotypes in our sampling are given in brackets following isolate names. A GTR+G+I molecular model of evolution was independently applied to each concatenated data set. Due to several partial sequences, in each data matrix, only sites that were present for at least 85% of the sequences were kept in the analysis. Double lines across branches indicate that branch length was artificially reduced in the figure. Inoculant strains Aust11c and Aust13c are shaded in orange and red, respectively. Haplotype isolates in red are those that harbored the Aust13c symbiotic cluster. Isolates shown in green are different for both core genome and symbiotic cluster compared to the two inoculant strains. A solid arrow indicates the persistence of inoculant strain Aust11c in soil, whereas the dashed arrow indicates symbiotic cluster acquisition from the inoculant strain Aust13c. Node bootstrap support was estimated with 100 resamplings. Only values higher than 70% are shown. The two *Mesorhizobium* isolates clustered with other *Mesorhizobium* sequences found in the GenBank (data not shown).

many parameters, including abiotic conditions, local flora, time after inoculation, and rhizobium characteristics (both introduced and native) and their competitiveness for nodulation (29).

Only one study analyzed the natural diversity of rhizobia nodulating *A. mangium* in Senegal (5). It suggested that local strains had a very low efficiency and nodulation ability. Moreover, soils in Saint Louis, located in the far north of Senegal, are irrigated but poor, depleted of nutrients. The mean annual rainfall in northern Queensland (where Australian strains were initially isolated) reaches 2,000 mm, whereas rainfall can reach up to 3,000 mm in Malaysia-Sabah but only 300 mm in northern Senegal. The introduction of *A. mangium* in this area in Senegal, where local rhizobia might not be able to nodulate this species efficiently, together with stress related to environmental conditions (such as high temperatures that often reach 40°C), must have resulted locally in a strong selection for the emergence of recombinant strains sharing the symbiotic cluster with a chromosomal background adapted to the arid conditions. This is not surprising since it has been shown that factors such as nutrient availability or bacterial density might positively influence horizontal gene transfer (HGT) rate and frequency (see reference 30 and references therein). In Malaysia, environmental conditions are similar to those observed in northern Queensland, with both environments considered wet tropics. Soils in Malaysia harbored local rhizobia that efficiently (but perhaps not with high competitiveness) nodulate *A. mangium*. With these rather good biotic and abiotic conditions, selection pressure for recombinants, although present, is lower than in Senegal, resulting in the coexistence of several strains, including native nodulating strains.

However, these results with respect to Aust13c evolution and recombination 5 and 15 years after inoculation are in contradiction with those of previous experiments. Galiana et al. (15) showed, in a field trial in the Ivory Coast, that 90 to 100% of nodules from trees inoculated with various *Bradyrhizobium* strains and from noninoculated trees exclusively contained the Aust13c strain 23 months after tree transplantation. This predominance was still observed 42 months after tree transplantation. Prin et al. (31) also showed, in a Madagascar field trial, that this strain spread to all plots 6 months after inoculation. Such an ability to propagate has been confirmed in Senegal and, to a lesser extent, in Malaysia. Of course, duration of the period between inoculation and resampling could then be a key parameter affecting the fate of introduced strains. A longer duration might explain the emergence of a recombinant in our study (5 and 15 years after its introduction), compared to 4 years in Ivory Coast or 19 months in Madagascar when the original strain survived as a whole.

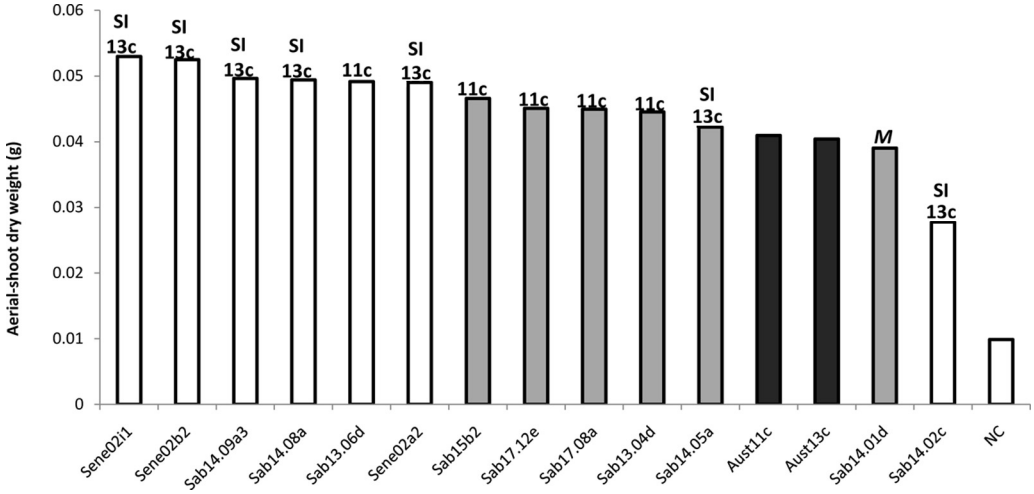

FIG 2 Mean aerial shoot dry weight of the *A. mangium* specimens inoculated with 15 different strains. The two black bars represent the original Aust13c and Aust11c inoculated strains. White bars indicate mean weights that are significantly different from the two original strains, either higher or lower as shown (see the text for a description of the statistical tests used). Gray bars indicate values that are not statistically different from Aust11c or Aust13c. SI 13c, the isolate tested has acquired the symbiotic cluster of the Aust13c strain; 11c, the isolate is identical to Aust11c on the five loci sequenced; M, the single *Mesorhizobium* isolate tested (all others are *Bradyrhizobium* isolates); NC, negative control (no inoculation). The value for each strain is the mean of 10 to 12 individual weights.

Finally, we observed a strict behavioral divergence between the two strains inoculated in Malaysia. Even though only the symbiotic Aust13c cluster was detected in the native genomic background, all Aust11c-derived strains shared the same multilocus genotype as the original inoculum. The two strains Aust11c and Aust13c were originally isolated in 1986 from Australia from two sites located in the Daintree Rainforest only 40 km from each other (32). They had been chosen as inocula based on their good infectivity and nitrogen fixation efficiencies. They are phylogenetically related and fall within the same main clade of the *Bradyrhizobium elkanii* species (Fig. 1). There was thus no prior indication that would have suggested a different ability to recombine. In contrast, in Malaysia, the indigenous rhizobia did not seem especially predisposed to recombination of either the housekeeping genes or those on the symbiotic island. In fact, we have no example of shared symbiotic polymorphism among various housekeeping loci. The small number of isolates obviously does not support the detection of rare recombinant strains, which is all the more reason that the local diversity appears rather high, with four symbiotic haplotypes among the six bradyrhizobium isolates. But this perfect linkage disequilibrium is in opposition to the five different genetic backgrounds hosting the Aust13c symbiotic cluster, suggesting several independent recombination/transfer events (Fig. 1). Furthermore, the same recombination pattern was observed in Senegal, where all isolates harbored Aust13c symbiotic loci but with various genomic backgrounds. Symbiotic cluster transfer from Aust13c to native isolates is thus frequent and recurrent, in contrast to Aust11c, for which no recombinant strains had been sampled in Malaysia. This difference between the two strains did not result from a higher genetic divergence of one inoculant from local strains. Minamisawa et al. (33) detected *nod* gene transfers among bradyrhizobia and other bacterial populations in soil and microcosms and showed that *B. japonicum* isolates harboring a high copy number of insertion sequences in their genome might make them potentially more efficient as donors. The different patterns detected between the two strains might be explained by such genomic particularity, probably coupled with a higher selection pressure promoting the stability of the local core genome diversity and the spread of the efficient symbiotic cluster.

We finally did not formally prove that the entire symbiotic cluster was transferred to indigenous strains. Several arguments, however, are in favor of transmission of the entire cluster, or at least a large part of it. The two loci we sequenced, *nodA* and *nifH*, are colocalized in this cluster, with variable lengths between them (255 kb within the 681-kb symbiosis island of *Bradyrhizobium diazoefficiens* USDA110 [34] and 232 kb in *B. japonicum* USDA6). We did not detect any case of an isolate with only one of the two inoculant symbiotic loci, a case that would have been theoretically possible if each locus had been transferred individually. We may then consider that the two loci have been cotransferred each time, reinforcing the hypothesis of a global symbiotic cluster transfer among isolates. Draft genomes of our recombined isolates should confirm our conclusion that recombination and symbiotic cluster transfer at a population level are also common.

Nitrogen fixation efficiency of descendant and recombinant inoculum strains. Beyond the question of the genomic evolution of introduced strains, we also explored the question of nitrogen efficiency of both evolved Aust11c (Aust11c strain recovered from the field) and Aust13c recombinant isolates. There was no clear tendency in our results; i.e., we cannot predict the efficiency of an isolate according to its genotype. Indeed, three Senegalese Aust13c recombinants, two Malaysian Aust13c recombinants, and one Malaysian evolved Aust11c isolate were among the best strains, and, conversely, one Malaysian Aust13c recombinant gave the worst result in terms of efficiency. One may just notice that four of the five Aust11c isolates are similar to the original inoculant, suggesting that there is no significant change in terms of nitrogen fixation efficiency even after more than 15 years in the field. This result may have a major impact on the inoculation frequency, the management of introduced strains, and their influence on local biodiversity. Very few studies have estimated the change in nitrogen fixation efficiency of strains after inoculation in soil. Gibson et

al. (35) detected little loss of symbiotic effectiveness of one strain 8 years after introduction in a field but did not clearly estimate its genetic evolution. Nandasena et al. (10) characterized recombinant *Mesorhizobium* isolates 5 years after inoculation and showed that they were less effective than the original inoculant strain or even ineffective in N_2 fixation. They concluded that there was rapid evolution of competitive, yet suboptimal strains for N_2 fixation following the lateral transfer of a symbiosis island. van Rensburg and Strijdom (36) showed that 4 to 8 years after inoculation, there was no difference in terms of effectiveness between the original strain and isolates recovered from nodules. At best, these studies showed a stability of the nitrogen fixation efficiencies of the introduced strains and, more frequently, the decrease of this efficiency in introduced and recombining strains. The large variation of efficiencies in our tests once again suggests that even locally, under the same conditions, both recombining Aust13c genotypes and evolved Aust11c isolates can result in strong diverging symbiotic efficiencies. Long-term evolution and coexistence (here, 15 years) is thus not sufficient to predict the evolution of the symbiotic properties of the isolates. It can result in, alternatively, selection for better adapted strains (in terms of nitrogen fixation efficiency) or a lower symbiotic efficiency.

It is always easier to explain afterwards the results obtained in an experiment. One may, in our case, logically suggest that abiotic (arid versus dry) and biotic conditions or genetic divergence between isolates can explain why recombination occurred here and not elsewhere, why strains evolved or not, or why they are persistent or not. However, our experiments suggest that it is complicated and hazardous today to predict the fate of inoculant strains in the soil. Many more investigations, especially on the genetic predisposition of a strain to recombine, including long-term experiments and sampling in natural areas of the origin of inocula, are urgently needed.

ACKNOWLEDGMENTS

This work was funded by the French Ministry of the Environment and Sustainable Development (Ecofor/Ecosystèmes Tropicaux Programme) and was supported by CIRAD (Ph.D. grant to M.M.P.).

We thank Moustapha Lo, the owner of the *Acacia mangium* plantation in Senegal, for his kind collaboration with Senegalese Research Institutes. We are very grateful to Naïma Rezkallah for roots and aerial-shoot weighing.

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