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Characterization of a Mannitol-Utilizing, Nitrogen-Fixing *Bradyrhizobium japonicum* USDA 110 Derivative†

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We have isolated a colonial derivative of *Bradyrhizobium japonicum* USDA 110 (designated MN-110) that is both mannitol utilizing and N_2 fixing. Derivative MN-110 showed growth on mannitol and glucose similar to that of non-N_2-fixing, mannitol-utilizing L2-110. Derivative MN-110 showed high constitutive and induced D-mannitol dehydrogenase activity (similar to L2-110) relative to N_2-fixing, non-mannitol-utilizing I-110. Hybridization to *Eco*RI and *Hin*dIII total DNA digests with cloned USDA 110 *nif* DK and *nif* H genes revealed similar patterns for non-N_2-fixing mannitol-utilizing derivative L1-110 and derivative MN-110. Symbiotic tests with soybean cultivars Ransom and Lee indicate MN-110 to be a superior N_2-fixing derivative compared with derivative I-110 and the parent strain USDA 110. However, these differences were not revealed when comparing 28-day-old soybean-*B. japonicum* associations but were apparent in 49-day-old associations. It was apparent from this work that mannitol utilization was not necessarily correlated to symbiotic effectiveness in *B. japonicum* and that gene rearrangements were not responsible for differences in N_2 fixation between L1-110 or L2-110 and MN-110.

Colony morphology variants of *Bradyrhizobium japonicum* USDA 110 were first demonstrated by Kuykendall and Elkan (9). These derivatives were isolated on the basis of colonial morphology on the yeast extract mannitol medium developed by Cole and Elkan (2). Mannitol-utilizing (slimy) derivatives L1-110 and L2-110 have been shown to lack symbiotic N_2 fixation ability (15), whereas non-mannitol-utilizing (nonslimy) derivatives I-110 and S-110 have symbiotic N_2 fixation ability. Upchurch and Elkan (20) furthered the correlation of mannitol utilization and N_2 fixation ability for *B. japonicum* derivatives by showing that derivatives of strains 61A76, USDA 76, and USDA 140 also show this pattern. Vincent (21, 22) and Herridge and Roughley (8) previously reported the correlation of nonslimy, N_2-fixing and slimy, non-N_2-fixing colonies for other species of rhizobia. Recently, we have isolated a derivative from strain USDA 110 that is both slimy and N_2 fixing (14). This derivative has been shown to share greater than 90% DNA-DNA homology with other USDA 110 derivatives and to have restriction enzyme digest patterns similar to those of other USDA 110 derivatives (14). Additional serological data also indicate this derivative to be in the 110 serogroup (A. G. Wollum, personal communication). We have called this derivative MN-110, standing for mannitol-utilizing, nitrogen-fixing USDA 110 derivative. The culture of USDA 110 from which MN-110 was isolated was obtained from the U.S. Department of Agriculture culture collection, Beltsville, Md., in December 1984. In this report, MN-110 is characterized with respect to *nif* structural gene organization, growth on glucose and mannitol, mannitol dehyrogenase activity, and symbiotic effectiveness.

MATERIALS AND METHODS

Bacterial strains. Derivatives I-110, L1-110, and L2-110 were isolated by Kuykendall and Elkan (9) from *B. japonicum* USDA 110, which was obtained from D. F. Weber, U.S. Department of Agriculture (USDA). Derivatives MN-110 and NI-110 were isolated by Mathis et al. (14) from strain USDA 110 which was recently obtained from L. D. Kuykendall, USDA. To determine mean N_2 fixation values for symbiotically competent mannitol-utilizing and non-mannitol-utilizing derivatives, 10 individual colonial derivatives of similar colony morphology were combined to form substrains NI-110 and MN-110. In the case of MN-110, these 10 individual colonial derivatives were the 10 which gave intermediate total N and acetylene reduction values from the 20 individual colonial derivatives screened by Mathis et al. (14). Cloned *B. japonicum nifDK* (from pRj4034) (4) and *nifH* (from pRj7001) (5) genes were the kind gift of H. Hennecke, Mikrobiologisches Institut, Zurich, Switzerland.

Growth of derivatives in glucose and mannitol. Bacterial growth for derivatives I-110, NI-110, L2-110, and MN-110 were determined in cultures containing 1% (wt/vol) glucose or mannitol, 0.025% (wt/vol) yeast extract, 0.05% (wt/vol) K_2HPO_4, 0.08% (wt/vol) $MgSO_4 \cdot 7H_2O$, 0.02% (wt/vol) NaCl, and 0.001% (wt/vol) $FeCl_3 \cdot 6H_2O$ (pH 6.7) (23). Cultures were incubated at 150 rpm at 26°C on a gyratory shaker. Optical densities were measured at 650 nm, at 12-h intervals for a period of 200 h. Optimal generation times were determined during early log phase when measurements were taken every 30 to 60 min for a period of 7 to 12 h. Cultures (100 ml) used for growth determinations were

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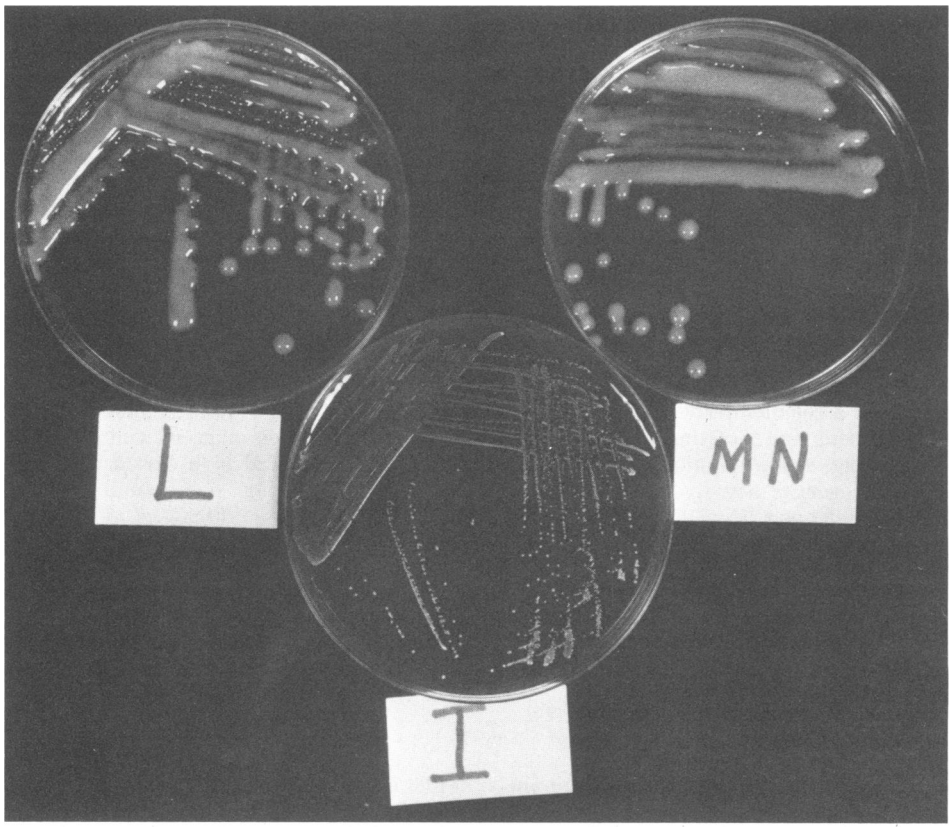

FIG. 1. Colony morphology of *B. japonicum* USDA 110 derivatives L(2)-110 (L), MN-110 (MN), and I-110 (I) on yeast extract-mannitol agar plates.

started from 1-ml cultures grown to log phase in yeast extract-gluconate (1% [wt/vol] gluconate, 0.1% [wt/vol] yeast extract, 0.05% [wt/vol] K_2HPO_4, 0.08% [wt/vol] $MgSO_4 \cdot 7H_2O$, 0.02% [wt/vol] NaCl, and 0.001% [wt/vol] $FeCl_3 \cdot 6H_2O$ [pH 6.7]).

Mannitol dehydrogenase activity. D-Mannitol dehydrogenase activities were determined as described previously by Kuykendall and Elkan (10). Constitutive levels were measured for derivatives I-110, NI-110, L2-110, and MN-110 by using yeast extract-gluconate-grown cells (as described above). Inducible levels of mannitol dehydrogenase were measured with cells grown in 1% (wt/vol) mannitol containing 0.1% (wt/vol) yeast extract and the salt concentrations described above. Formation of NADH was measured by monitoring changes in optical density at 340 nm to determine enzymatic activities. Protein concentrations of extracts were determined by the method of Layne (11). Between 100 and 500 μg of protein per ml was used for each assay.

Southern hybridization. Total DNAs from USDA 110 derivatives were isolated on CsCl-ethidium bromide step gradients by the procedure of Glisin et al. (6). Derivatives L1-110 and MN-110 were cultured in tryptone yeast extract (TY medium) (1) for the purpose of total DNA isolation. TY medium was chosen because the production of extracellular slime is limited in this medium. *Eco*RI and *Hin*dIII restriction enzyme digestion of total DNA from MN-110 and L1-110 was carried out by using the digestion protocols of the vendor (Bethesda Research Laboratories, Gaithersburg, Md.). Digestions were conducted overnight with a 5- to 10-fold excess of enzyme to ensure complete digestion. DNA was then separated on 1.5% agarose gels at 150 V for 3 h for *nifH* probing or 4 h for *nifDK* probing with 90 mM Tris borate–2 mM EDTA (pH 8.2) as a buffer. Total DNA digests were transferred to nitrocellulose by the procedure of Southern (18) as modified by Wahl et al. (24) with a Bethesda Research Laboratories blot transfer system.

Recombinant plasmids pRj4034 (4) and pRj7001 (5) were isolated by the procedure of Norgard (17). Purified *nifH* fragments were prepared by digesting pRj7001 with *Hin*dIII before electrophoresis on a 5% acrylamide gel at 250 V for 75 min by the methods of Maniatis et al. (12). To make *nifH* probe, an 800-base-pair fragment was excised and electroeluted from the acrylamide gel by the technique of Yang et al. (25). Plasmid pRj4034 containing *nifDK* was digested with *Eco*RI and also used as probe DNA. Probes were nick-translated with [α-^{32}P]dCTP by the procedure of Davis et al. (3). DNA hybridizations of labeled probe to Southern transfers were also done by the procedure of Davis et al. (3). Hybridization was carried out for 16 h with 50% formamide at 37°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Autoradiography was conducted for 12 to 72 h at −70°C with Kodak XAR-5 film.

Symbiotic tests. Symbiotic tests were carried out by the methods of McClure and Israel (16) as modified by Mathis et al. (13). Strains I-110, NI-110, MN-110, and USDA 110 (obtained recently from L. D. Kuykendall) were grown in yeast extract-gluconate medium (as described above). Stationary-phase cultures (>10^9 CFU/ml) were used to inoculate Lee and Ransom soybean seed (obtained from J. Burton, USDA Agricultural Research Service and Department of Crop Science, N.C. State University). Plants were harvested at 28 and 49 days after inoculation and transplant-

ing. At 28 days, nodule number, mass, acetylene reduced per plant and per gram of nodule tissue, plant dry weight, percent nitrogen, and total nitrogen were determined (13, 16). At 49 days, plant dry weight, percent nitrogen, and total nitrogen were determined. Since plants were grown in a nitrogen-free culture system, accumulation of nitrogen represented N_2 fixation (13, 16). To ensure that the proper strain had infected the various cultivars, 2 nodules per plant were purified (19, 23), and the colonial morphologies of the nodule occupants were checked. Data obtained from the symbiotic tests were subjected to analysis of variance by using computer programs developed by the SAS Institute (7).

RESULTS

Colony morphology and carbohydrate utilization. *B. japonicum* USDA 110 derivative MN-110 was morphologically indistinguishable from derivative L2-110 on yeast extract-mannitol agar plates (Fig. 1). Derivatives MN-110 and L2-110 both appeared morphologically larger, shinier, and smoother relative to derivative I-110. Derivative I-110 produced a more basic reaction on yeast extract-mannitol plates to which bromthymol blue had been added as an indicator.

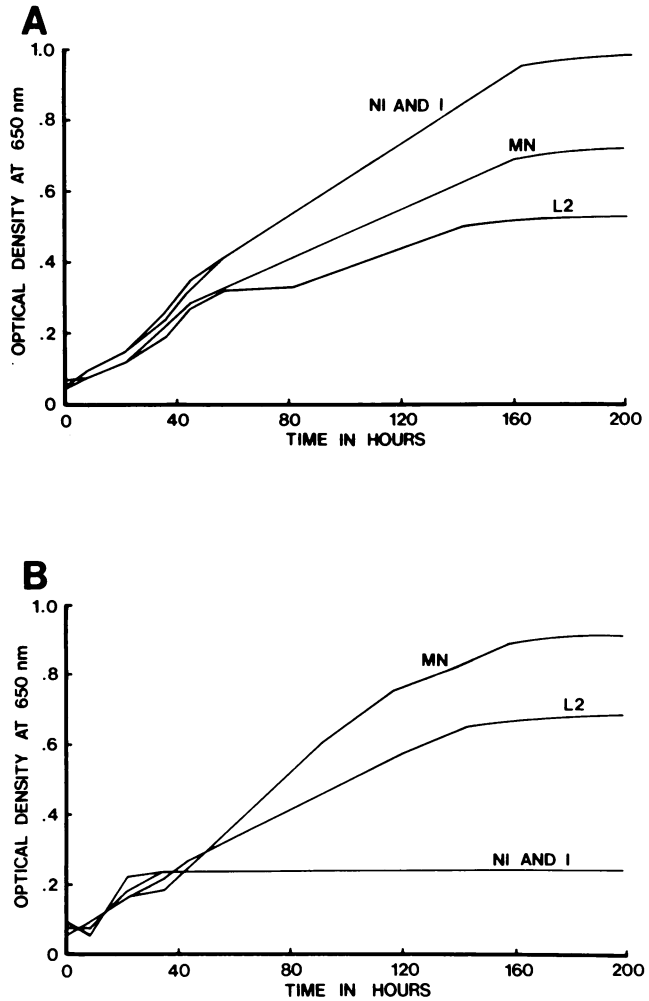

FIG. 2. Growth (expressed as optical density at 650 nm) of *B. japonicum* USDA 110 derivatives L2-110 (L2), I-110 (I), NI-110 (NI), and MN-110 (MN) with 1% glucose (A) or 1% mannitol (B) as the sole carbon source.

TABLE 1. Constitutive and induced D-mannitol dehydrogenase activity in USDA 110 derivatives

Derivative	Sp act (nmol of NADH formed per min per mg of protein)[a]	
	Constitutive (gluconate-grown cells)	Induced (mannitol-grown cells)
MN-110	10.2	49.1
L2-110	6.4	80.4
NI-110	1.6	ND[b]
I-110	0	ND

[a] Each number is the mean of three independent replicates.
[b] ND, Not determined.

With respect to optical density, derivatives NI-110 and I-110 produced indistinguishable growth curves on glucose and mannitol (Fig. 2). Neither of these derivatives grew with D-mannitol as a sole carbon source. Initial intrinsic growth for these derivatives was due to utilization of the gluconate carried over in the starter culture and the 0.025% (wt/vol) yeast extract. NI-110 and I-110 showed superior growth to MN-110 and L2-110 on D-glucose. Kuykendall and Elkan (9) have previously shown that I-110 utilizes D-glucose better than does L2-110. MN-110 appeared to exhibit slightly better growth than L2-110 on both D-mannitol and D-glucose. However, these differences may not be significant, since they did not become apparent until 60 h and may be due to extracellular slime rather than cell number. Both MN-110 and L2-110 in the early log phase of these cultures had similar optimal generation times of approximately 21 h in D-mannitol and 29 h in D-glucose. NI-110 and I-110 had an optimal generation time of 23 h in D-glucose. Optimal generation times were determined for a period of 7 to 12 h during early log phase, during which time measurements were taken every 30 to 60 min. During this phase of growth, production of extracellular slime is very limited and optical density accurately reflects cell number.

Constitutive and induced D-mannitol dehydrogenase. Derivative MN-110 demonstrated sixfold-higher levels of constitutive D-mannitol dehydrogenase than did NI-110, whereas I-110 altogether lacked D-mannitol dehydrogenase activity (Table 1). Kuykendall and Elkan (10) have previously shown a low level of constitutive activity for I-110 similar to that obtained for NI-110. Derivative L2-110 showed 63% of the constitutive D-mannitol dehydrogenase activity displayed by MN-110. However, L2-110 was induced to a 164% higher level of D-mannitol dehydrogenase activity than was derivative MN-110. Induced levels of D-mannitol dehydrogenase were not measured for I-110 or NI-110 since growth data indicate that these derivatives did not utilize D-mannitol. Previously, Kuykendall and Elkan (10) demonstrated a lack of inducible activity for enzyme extracts from glycerol- and glucose-grown derivative I-110 to which D-mannitol was added.

Southern hybridization. Hybridization of *B. japonicum nifDK* DNA (4) to *Hin*dIII restriction enzyme digests of L1-110 and MN-110 total DNA revealed a single major band of homology at 8.5 kilobases (kb) (Fig. 3). An additional minor band at approximately 6 kb was also detected, but this band probably represents homology to another *nif* promoter sequence, since this band was not previously demonstrated for either L1-110 (15) or USDA 110 (4). Homologous fragments of 6, 1.2, and 1.0 kb were detected when *Eco*RI total DNA digests of L1-110 and MN-110 were probed with *nifDK*.

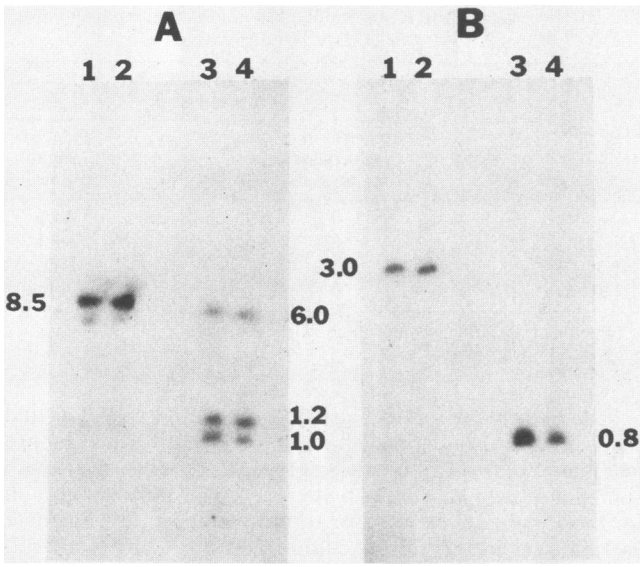

FIG. 3. Hybridization of *nifDK* and *nifH* probes to Southern blots of restriction enzyme-digested L-110 and MN-110 total DNA. (A) Hybridization of *Eco*RI-digested pRj4034. Lanes: 1, L1-110 digested by *Hin*dIII; 2, MN-110 digested by *Hin*dIII; 3, L1-110 digested by *Eco*RI; 4, MN-110 digested by *Eco*RI. Samples were separated in a 1.5% agarose gel in 90 mM Tris borate–2 mM EDTA for 4 h at 150 V. (B) Hybridization of purified *nifH*. Lanes: 1, L1-110 digested by *Eco*RI; 2, MN-110 digested by *Eco*RI; 3, L1-110 digested by *Hin*dIII; 4, MN-110 digested by *Hin*dIII. Samples were separated in a 1.5% agarose gel in 90 mM Tris borate–2 mM EDTA for 3 h at 150 V.

A single homologous fragment of 3 kb was detected in *Eco*RI digests of L1-110 and MN-110 total DNA (Fig. 3) when they were probed with *B. japonicum nifH* DNA (5). *Hin*dIII total DNA digests of MN-110 and L1-110 revealed a single *nifH* homologous fragment of 0.8 kb.

TABLE 2. Nodulation and nitrogenase activity by *B. japonicum* derivatives on Lee and Ransom soybeans 28 days after inoculation

Cultivar	Derivative or strain	No. of nodules per plant	Nodule fresh wt (g/plant)	Ethylene produced (μmol/h):	
				Per nodulated plant	Per g of nodule (fresh wt)
Ransom	I-110	94	1.00	18.1	17.8
	NI-110	88	0.97	16.3	16.8
	MN-110	82	0.90	18.3	20.2
	USDA-110	86	1.00	18.4	18.8
Lee	I-110	82	1.00	19.3	19.2
	NI-110	98	1.12	22.0	19.7
	MN-110	120	1.40	30.1	21.7
	USDA-110	90	1.10	22.0	19.6
Pooled	I-110	88	1.00	18.7	18.5
	NI-110	93	1.05	19.2	18.2
	MN-110	101	1.15	24.2	21.0
	USDA-110	88	1.05	20.2	19.2
Ransom	Pooled	88	0.97[a]	17.8[a]	18.4
Lee	Pooled	98	1.16	23.4	20.1

[a] Results for cultivar Ransom inoculated with the pooled strains were significantly different ($P < 0.05$) from those obtained for cultivar Lee inoculated with the pooled strains.

Symbiotic tests. Symbiotic tests revealed that derivative MN-110 was superior symbiotically to derivative I-110, derivative NI-110, or the parent strain USDA 110 (Tables 2 and 3). At 28 days after inoculation, higher, although not statistically significant, values were obtained for nodule number, nodule mass, nitrogenase activity per plant, nitrogenase specific activity, plant dry weight, and nitrogen accumulation for derivative MN-110. On pooled cultivars Lee and Ransom and on cultivar Lee alone, MN-110 demonstrated statistically higher dry weight and total nitrogen per plant at 49 days after inoculation. Cultivar Lee was found to be superior to cultivar Ransom with respect to nodule fresh weight and nitrogenase activity per plant at 28 days after inoculation (Table 2). Both dry weight and total nitrogen per plant were higher for cultivar Lee than for cultivar Ransom at 49 days, and these differences approached statistical significance ($0.05 < P < 0.10$). These data are in close agreement with those previously obtained by Mathis et al. (15), in which cultivar Lee was found to be superior to cultivar Ransom for N accumulation when in symbiosis with derivative I-110 and strain USDA 110.

DISCUSSION

Nitrogen-fixing *B. japonicum* USDA 110 derivative MN-110 was found to have similar mannitol utilization to non-nitrogen-fixing derivative L2-110. Derivatives MN-110 and L1-110 were found to have similar *nif* gene organization. Previously, Mathis et al. (14) have shown derivatives I-110 and L1-110 to have a *nif* gene organization similar to that of their parent strain USDA 110. These data, in conjunction with results of DNA-DNA hybridization and restriction enzyme analyses of *B. japonicum* derivatives (15) and the similar serologies observed for the derivatives (A. G. Wollum, personal communication), indicate that derivatives I-110, S-110, L1-110, L2-110, and MN-110 originated from a common ancestor.

Derivative MN-110, despite its shared characteristics with derivatives L1-110 and L2-110, has been found to be symbiotically superior to derivatives I-110 and NI-110 and strain USDA 110. This difference, however, was not statistically significant in data collected 28 days after inoculation but was

TABLE 3. Dry weight and nitrogen accumulation by *B. japonicum* derivatives on Lee and Ransom soybeans at 28 and 49 days after inoculation

Cultivar	Derivative or strain	Whole plant dry wt (g)		Whole plant nitrogen (g)	
		28 days	49 days	28 days	49 days
Ransom	I-110	2.5	17.1	0.10	0.60
	NI-110	2.5	17.4	0.10	0.62
	MN-110	2.4	24.8	0.08	0.86
	USDA-110	2.5	20.2	0.09	0.69
Lee	I-110	2.4	22.7	0.09	0.81
	NI-110	2.8	19.0	0.11	0.67
	MN-110	3.1	31.5[a]	0.12	1.13[a]
	USDA-110	2.7	22.5	0.11	0.78
Pooled cultivars	I-110	2.5	19.9	0.10	0.69
	NI-110	2.6	18.2	0.10	0.64
	MN-110	2.8	28.2[a]	0.10	1.00[a]
	USDA-110	2.6	21.4	0.10	0.74

[a] Significantly different ($P < 0.05$) result compared with those obtained for the other strains in the same experimental group.

significant in dry weight and N accumulation data collected 49 days after inoculation. These data reveal the shortcomings of basing N_2 fixation comparisons on a single measurement period at 28 to 30 days. Derivatives I-110 and NI-110 had similar symbiotic performance at both 28 and 49 days after inoculation. Their similar growth on glucose and mannitol, D-mannitol dehydrogenase activities, and symbiotic performances indicate that I-110 and NI-110 may be identical.

From these data it must be concluded that mannitol utilization in *B. japonicum* is not necessarily correlated to symbiotic effectiveness. It can also be concluded that *nif* gene rearrangements are not responsible for the loss of N_2 fixation competence in L1-110 and L2-110, since symbiotically effective derivatives I-110 and MN-110 have the same pattern of homology. Instead, another gene involved in N_2 fixation competence or a point mutation must be responsible for the loss of N_2 fixation competence in L1-110 and L2-110. Since strain USDA 110 derivatives with various levels of N_2 fixation have been discovered, the importance of screening strains for stable colonial morphology before genetic studies on *Rhizobium*, and *Bradyrhizobium* spp. is apparent. Since MN-110 and L2-110 were morphologically indistinguishable from one another, screening several individual colonies of similar morphology for N_2-fixing ability before genetic analysis of *nif* genes may also be important. The availability of naturally occurring strain derivatives or strains which are superior at N_2 fixation, such as MN-110, is also illustrated by this work.

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