Transfer of Nitrate Reductase Genes of the Cyanobacterium *Nostoc muscorum* into *Rhizobium japonicum*

By RAMA K. SINGH,* RAMESH K. SINGH AND BRAHMA D. SINGH

Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi-221 005, India

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Transformation of *Rhizobium japonicum* CB1809 was studied using DNA from the cyanobacterium *Nostoc muscorum* ATCC 27893. A spontaneous nitrate reductase deficient (Nar-) mutant (NR-6) of *R. japonicum* CB1809 was isolated with a frequency of $8.4 \times 10^{-7}$. Streptomycin (Sm) and Neomycin (Neo) resistance markers were introduced into strain NR-6, and the resulting strain was designated NR-6 Sm$^R$ Neo$^R$. Experiments with cyanobacterial DNA and live cells of strain NR-6 Sm$^R$ Neo$^R$ indicated transformation of nitrate reductase (nar) genes of *N. muscorum* into this strain. This conclusion was supported by the reversion frequency of strain NR-6 Sm$^R$ Neo$^R$ to Nar$^+$ and the transformation frequency when recipient cells were exposed to *N. muscorum* DNA (with heat-treated DNA as control). Comparisons of growth, nitrate uptake, assimilatory nitrate reductase activity and nodulation of parent CB1809, NR-6 Sm$^R$ Neo$^R$ and five transformant clones (Nar$^+$) suggest that there may be considerable homology between the nar genes of *R. japonicum* CB1809 and *N. muscorum*.

INTRODUCTION

Devilly & Houghton (1977) reported the intergeneric transformation of drug resistance in cyanobacteria, from *Gloeocapsa alpicola* to *Anacystis nidulans*. Intra- as well as inter-generic transformation of drug resistance, nitrogen fixation, infectivity and effectiveness in *Rhizobium* has also been accomplished (Schwinghamer, 1976; Brill, 1980). However, information on genetic transformation among phylogenetically distant prokaryotes is scarce. A glutamine synthetase mutant of *Escherichia coli* was transformed by a cloned glutamine synthetase gene of the cyanobacterium *Anabaena* 7120 to yield a functional glutamine synthetase (Fisher et al., 1981).

Here, we report genetic transformation, using DNA from the cyanobacterium *Nostoc muscorum* (a filamentous nitrogen-fixing photoautotroph) in a nitrate reductase deficient (Nar$^-$), streptomycin and neomycin resistant mutant of *Rhizobium japonicum* CB1809 (a symbiotic nitrogen-fixing heterotrophic bacterium).

METHODS

Organisms. The donor strain *N. muscorum* ATCC 27893 (ISU) was grown in modified Chu No. 10 medium (Gerloff et al., 1950) supplemented with 5 mM-KNO$_3$ as described by Singh & Singh (1981). DNA was isolated from exponential phase cultures by the method of Marmur (1961).

*Rhizobium japonicum* CB1809, a gift from Dr A. H. Gibson, CSIRO, Canberra, Australia, was maintained on yeast extract/mannitol agar slants (Vincent, 1970) and grown routinely in a minimal medium (MM) supplemented with sodium glutamate (1 mg ml$^{-1}$) or KNO$_3$ (10 mM) (Ludwig & Signer, 1977). Nitrate reductase deficient (Nar$^-$) mutants of parent CB1809 were isolated by the method of Pagan et al. (1977), by plating approximately $10^9$ cells per plate from exponential phase cultures on to plates of MM plus glutamate containing 5 mg KClO$_3$ ml$^{-1}$, a medium which is extremely toxic to the parental strain. A mutant clone thus isolated (NR-6) showed no growth in

Abbreviations: Neo, neomycin; Sm, streptomycin.
MM plus nitrate and it did not revert to the parental type (Nar+). Therefore, after several serial subcultures in medium lacking KClO₃, Streptomyces Sm (10 µg ml⁻¹) and neomycin (Neo; 100 µg ml⁻¹) resistance markers were introduced into strain NR-6 by plating exponentially growing cells (approximately 10⁹ per plate) on to plates of MM containing glutamate plus 10 µg Sm ml⁻¹ or 100 µg Neo ml⁻¹. Concentrations of 5 µg Sm ml⁻¹ and 50 µg Neo ml⁻¹ were extremely toxic to both the parent CB1809 and strain NR-6. A mutant thus obtained was unable to grow in MM plus nitrate, was resistant to Sm and Neo, and did not show loss of Sm or Neo resistance or reversion to Nar+ type even after ten subsequent passages through nodules. This clone, designated NR-6 SmR NeoR, was used as recipient in the present study.

**Assays.** Growth of cultures was measured on the fourth day as optical density (OD) changes at 420 nm in a Bausch and Lomb spectrophotometer. Nitrate uptake in NR-6, NR-6 SmR NeoR and parent CB1809 was measured according to Herbert et al. (1971). Exponential phase cells grown on MM plus glutamate were transferred to MM plus 100 µM nitrate; 2 h after the transfer, samples of the cell suspension were removed and nitrate was determined in the supernatant after centrifugation. Assimilatory nitrate reductase activity in whole-cell suspensions was assayed according to Snell & Snell (1949). Protein was estimated by the Lowry method. Infectivity (nodulation) of parent CB1809, NR-6, NR-6 SmR NeoR and five transformants from the tenth serial subculture was tested by inoculating 25 seedlings of soybean plants (Glycine max L., cv. Brag) with each strain as described by Vincent (1970).

**Transformation experiments.** These were done according to Stevens & Porter (1980). Nostoc muscorum DNA (in 0.15 M-NaCl/0.015 M-trisodium citrate) was mixed with exponentially growing cells of NR-6 SmR NeoR (10⁹ ml⁻¹) to give a final concentration of 10 µg DNA ml⁻¹. After incubation with gentle shaking for 3 h at 25 °C, DNAase I (Sigma) was added to a final concentration of 10 µg ml⁻¹ and approximately 10⁹ cells per plate were plated on to MM plus nitrate plates, to screen out transformant (Nar+) clones. In a parallel set of experiments, NR-SmrR NeoR cells exposed to heat-treated (100 °C for 10 min) N. muscorum DNA were plated as above to determine whether the cyanobacterial DNA was solely responsible for complementing the nitrate reductase defect in the NR-6 SmR NeoR strain. Samples of control NR-6 SmR NeoR cultures, not exposed to cyanobacterial DNA, were plated on to MM plus glutamate and MM plus nitrate plates, for viable colony counts and for the determination of reversion frequency to Nar+ type, respectively. Transformants (Nar+) were purified by restreaking twice on the selective medium and their infectivity was tested on soybean plants.

**RESULTS AND DISCUSSION**

Parent *R. japonicum* CB1809 mutated with a frequency of 8.4 × 10⁻⁷ to the Nar- phenotype, and mutants of the Nar- strain NR-6 resistant to Sm and Neo were obtained with a frequency of 8.3 × 10⁻⁷ and 5.3 × 10⁻⁷, respectively. Drug resistance in strain NR-6 SmR NeoR seemed to be stable as it was not lost during several serial subcultures in antibiotic-free medium. Neither strain NR-6 nor strain NR-6 SmR NeoR reverted spontaneously to the parent type (Nar+) when approximately 10⁹ exponentially growing cells per plate were plated on to MM plus nitrate plates. Transformant (Nar+) clones were recovered with a frequency of 1.2 × 10⁻⁵ from NR-6 SmR NeoR cultures exposed to *N. muscorum* DNA without prior heat-treatment, whereas no Nar+ clones could be isolated from cells exposed to heat-treated *N. muscorum* DNA. All the transformants (Nar+) were resistant to Sm (10 µg ml⁻¹) and Neo (100 µg ml⁻¹) (Table 1). Five transformant clones (out of 185 clones isolated) were serially subcultured ten times and cells from the tenth subculture were used in soybean plant infectivity (nodulation) tests. These clones retained the Nar+ phenotype, and Sm and Neo resistance, throughout subculture and passage through nodules, indicating that the reversion of Nar- to Nar+ was stable. It is suggested that these Nar+ clones are transformants. This conclusion is supported by the observation that all the transformants (Nar+) selected for nar genes were also resistant to Sm and Neo.

The inability of strains NR-6 and NR-6 SmR NeoR to grow in MM plus nitrate could be due to a defect in nitrate uptake, a mutation in structural/regulatory genes of the nitrate reductase system, or a mutation in a gene controlling post-translational modification or assembly of the functional enzyme complex (Guerrero et al., 1981). Although strains NR-6 and NR-6 SmR NeoR showed normal nitrate uptake [142 nmol h⁻¹ (µg protein)⁻¹] comparable to that of the parent CB1809 [148 nmol h⁻¹ (µg protein)⁻¹], they neither grew in MM plus nitrate, nor showed detectable nitrate reduction, whereas parent CB1809 grew well and reduced nitrate at a rate of 820 nmol nitrite formed h⁻¹ (mg protein)⁻¹ under similar conditions (Table 2). Furthermore, both strain NR-6 and strain NR-6 SmR NeoR nodulated soybean plants as effectively as the
Table 1. Frequency of mutation to Nar\(^{-}\), Sm\(^{R}\) and Neo\(^{R}\), and reversion to the wild type (Nar\(^{+}\)) in Rhizobium japonicum, and transformation of Nar\(^{-}\) mutants to Nar\(^{+}\) by Nostoc muscorum DNA

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation to:</th>
<th>Reversion to Nar(^{+})</th>
<th>Transformation to Nar(^{+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent CB1809</td>
<td>Nar(^{-}): 8.4 (\times) 10(^{-7})</td>
<td>0.0</td>
<td>1.2 (\times) 10(^{-5})</td>
</tr>
<tr>
<td>NR-6</td>
<td>Sm(^{R}): 8.3 (\times) 10(^{-7}) Neo(^{R})</td>
<td>5.3 (\times) 10(^{-7})</td>
<td>(0.0)*</td>
</tr>
<tr>
<td>NR-6 Sm(^{R}) Neo(^{R})</td>
<td>0.0</td>
<td>5.3 (\times) 10(^{-7})</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Transformation frequency when heat-treated N. muscorum DNA was used. For experimental details see Methods.

Table 2. Comparison of growth in MM plus nitrate, assimilatory nitrate reductase activity and infectivity (nodulation) of parent CB1809, NR-6, NR-6 Sm\(^{R}\) Neo\(^{R}\) and transformants (Nar\(^{+}\) Sm\(^{R}\) Neo\(^{R}\)) of Rhizobium japonicum

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth(^{*}) ((\Delta)OD(_{420}))</th>
<th>Nitrate reductase activity(†) [nmol h(^{-1}) (mg protein(^{-1}))]</th>
<th>Infectivity(‡) (no. of nodules per soybean plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent CB1809</td>
<td>0.68</td>
<td>820</td>
<td>14</td>
</tr>
<tr>
<td>NR-6</td>
<td>0.05</td>
<td>ND</td>
<td>10</td>
</tr>
<tr>
<td>NR-6 Sm(^{R}) Neo(^{R})</td>
<td>0.05</td>
<td>ND</td>
<td>11</td>
</tr>
<tr>
<td>Transformants(§)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT-1</td>
<td>0.65</td>
<td>768</td>
<td>11</td>
</tr>
<tr>
<td>RT-2</td>
<td>0.64</td>
<td>786</td>
<td>12</td>
</tr>
<tr>
<td>RT-3</td>
<td>0.69</td>
<td>804</td>
<td>14</td>
</tr>
<tr>
<td>RT-4</td>
<td>0.65</td>
<td>832</td>
<td>12</td>
</tr>
<tr>
<td>RT-5</td>
<td>0.67</td>
<td>794</td>
<td>10</td>
</tr>
</tbody>
</table>

ND, Not detectable in assays containing up to 0.42 mg protein.

* Growth was measured after 4 d, as changes in OD\(_{420}\).

† Measured, as nitrite formation, after 12 h incubation of cells in MM plus nitrate.

‡ Nodules were counted after 4 weeks growth of the plants; the values are means of 20 random countings.

§ Transformants are representative types from five independent experiments.

parent CB1809 (Table 2). These results, along with the frequency of mutation to Nar\(^{-}\) phenotype, clearly suggest that strain NR-6 Sm\(^{R}\) Neo\(^{R}\) is, due to mutation, defective in nar genes, involved either in the regulation/synthesis of the enzyme protein or in the post-translational modification of precursor proteins into active enzyme complex. The five transformants (NR-6 Sm\(^{R}\) Neo\(^{R}\)) showed nodulation of soybean plants comparable to that of parent CB1809, indicating that they are strains of R. japonicum. Moreover, all transformants showed rates of growth and assimilatory nitrate reductase activity in MM plus nitrate comparable to those of parent CB1809, while strains NR-6 and NR-6 Sm\(^{R}\) Neo\(^{R}\) failed to grow or to show nitrate reductase activity under identical conditions (Table 2). These observations suggest a considerable degree of homology between the nar genes, involved in the reduction of nitrate, of R. japonicum and N. muscorum.

It is thus evident that all the transformants (NR-6 Sm\(^{R}\) Neo\(^{R}\)) are strains of R. japonicum CB1809 and that they resulted from transfer of nar genes, concerned with the synthesis, regulation or post-translational maturation of the nitrate reductase enzyme complex, from N. muscorum. These data further advance the prospects for cloning genes of nitrogen metabolism by complementation of deficient mutants among phylogenetically distant prokaryotes.

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REFERENCES


