INTRODUCTION OF COSMID pHU 52 INTO HUP⁻ *AZOSPIRILLUM* SP. BSs 8 BY TRANSCONJUGATION AND TRANSFORMATION

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ABSTRACT

Cosmid pHU 52 carrying hydrogen uptake genes from hup⁺ *Rhizobium japonicum* and harbored in *Escherichia coli* was introduced into *Azospirillum* sp. BSs 8 by triparental mating and transformation.

The frequency of transconjugation was 1.4×10^{-4} . The transfer was facilitated by the help of the suicidal helper plasmid pRK 2013.

The frequency of transformation per μg DNA was 3.5×10^3 .

The method of Hanahan was modified for the transformation of BSs 8 wherein CaCl_2 concentration was increased from 10 mM to 75 mM. Rb (I) and Co (III) salts were not added to the competence buffer. The modified procedure increased transformation frequencies.

Hydrogen uptake (hup) activity was assayed by a modified methylene blue method. Hup activity was expressed by the transconjugants and most of the transformants.

INTRODUCTION

Biological nitrogen fixation is always linked to proton reduction and hydrogen evolution. Hydrogen evolution by the nitrogen fixing enzyme complex nitrogenase represents a loss of energy and reduction equivalent. Some strains of rhizobium possess an uptake hydrogenase which recycles the hydrogen evolved by hydrogenase regenerating ATP as well as protecting nitrogenase from oxidative damage¹. The possession of an uptake hydrogenase system increases the efficiency of nitrogen fixation as reported by several researchers^{2,3}. Soybean plants inoculated

with *Rhizobium japonicum* with an active *hup⁺* system were reported to contain higher nitrogen content in their tissues and seeds than soybean plants inoculated with *hup⁻* strains^{4,5}. *Hup⁻ Rhizobium* were converted to *hup⁺* strains by the introduction of *hup⁺* genes⁶. In this study, we attempted to introduce pHU 52, a cosmid carrying genes responsible for *hup* activity into *hup⁻ Azospirillum sp. BSs 8*, an associative diazotroph isolated from the roots of *Saccharum spontaneum*.

MATERIALS AND METHODS

Bacterial Strains and Culture

Azospirillum sp. BSs 8, *E. coli* HB 101 (pHU 52) and *E. coli* HB 101 (pRK 2013) were the bacterial strains used. The *E. coli* strains were cultured in Luria Broth (LB) and BSs 8 in Nutrient Broth (NB). To assay for hydrogen uptake activity based on hydrogen-dependent methylene blue reduction, the donor and recipient strains were grown in NB and after incubation for a specified period were grown in Repaske's medium (RM)⁷.

Plasmid/Cosmid DNA Isolation

Plasmid/Cosmid DNA was isolated by the modified alkaline method of Birnboim and Doly⁸. Antibiotics, tetracycline (Tc), kanamycine (Km) and nalidixic acid (Na) were added to the culture when appropriate. Modification of the method consists of increasing cell sample, increasing period of lysing with lysozyme from 1/2 hr to 1 hr and enzymic and chemical lysis were all done at room temperature (25°C).

The plasmid/cosmid DNA was banded by cesium chloride/ethidium bromide density gradient and the DNA band collected. Ethidium bromide was extracted from the DNA preparation by isoamyl alcohol until the last traces of color was removed from the DNA solution.

Transconjugation/Bacterial Matings

The triparental mating system of Ditta *et al.*⁹ was used for conjugative transfer of pHU 52 from *E. coli* HB 101 (donor cells) to BSs 8 (recipient cells) by the help of pRK 2013 in *E. coli* HB 101 (helper cells). One ml of an exponentially growing culture of *E. coli* HB 101 (pH 52, Tc^r) and *E. coli* HB 101 (pRK 2013, Km^r) and 4 ml of BSs 8 (Na^r, > 10⁶ cells) were mixed and centrifuged at 5,000 rpm for 5 min. The pellet was suspended in 1.0 ml of NB and incubated overnight (16–18 hrs) at 35°C. Appropriate dilutions were made with 1% peptone of the cell suspension and plated on selective plates of NB medium containing the appropriate antibiotics (15 µg/ml Tc, 50 µg/ml Km and 50 µg/ml NaI). Incubation was at 35°C for 3–4 days.

The BSs 8 cell culture was cultured for 4 days while those for *E. coli* cells were grown for 5 hrs at 37°C before mating with BSs 8 cells.

Transformation

Competent cells of BSs 8 were prepared using a modified method of Hanahan¹⁰. The concentration of CaCl₂ on FSB competence buffer was increased from 10 mM to 75 mM. Rubidium (I) salts and cobalt (III) salts were not added to FSB.

200 µl of competent cells were used and incubated on ice for 60 min. 30 µl of plasmid preparation (equivalent to 1 µg DNA) was added to the cells. The mixture was heat pulsed without agitation at 42°C for 1½ min and then placed on ice for another 1½ min. Then 1.8 ml of NB was added and the mixture was incubated at 35°C for 4 hrs with gentle agitation. The appropriate dilutions of the transformation mixture were made and planted on nutrient agar plates containing 15 µg Tc/ml and incubated at 35°C for 3 days. A set of control sample cells without addition of pHU 52 were also plated.

Uptake Hydrogenase Assay

Single colony strains of *E. coli* HB 101 (pHU 52), *E. coli* HB 101 (pRK 2013), *Azospirillum* sp. BSs 8 (wild type), BSs 8 (transformant) and BSs 8 (transconjugant) were separately cultured in 0.5 ml NB medium contained in Eppendorf tubes provided with silicone stopper for 4 days at 35°C. After this period of incubation, the cell culture was centrifuged and cell pellet washed with RM, then centrifuged and washings decanted and 0.5 ml RM added and the cells cultured in this medium. The hup activity was derepressed by incubating the cells in an atmosphere of 5% H₂, 5% CO₂, 0.5% O₂ and 89.5% N₂. Replenishment of the gas atmosphere was made at 8:00 A.M. and at 7:00 P.M. during the derepression period of 4–6 days.

After the derepression period, the culture was centrifuged and the RM was decanted off. 0.5 ml of 0.10 mM methylene blue reagent (MB) was added to the cells. Another set of assays were made on serological plates wherein 200 µl of the cell suspension in MB were transferred to the wells. The third set of hup assays used filter paper discs following the method developed by Haugland and co-workers¹¹ for rapid colony screen for hup⁺ strains.

The tubes, serological plates and filter paper discs with the cells and treated with MB were placed inside a plexiglass chamber fitted with inlet and outlet ports for the entry and exit of gases. The air in the chamber was first flushed with N₂. Then a continuous stream of H₂ was passed through the chamber for 6 hours. Hup⁺ strains will decolorize MB while Hup⁻ will not during this exposure time to H₂. Strains with strong hup⁺ are decolorized rapidly while the weak ones decolorize the dye slowly.

RESULTS AND DISCUSSION

Azospirillum sp. (Nal^r) are slow growers and form small pink colonies on (NA) plates (Figure 1a). *E. coli* (pHU 52, Tc^r) and *E. coli* HB 101 (pRK 2013, Km^r) are fast growers and form large whitish colonies (Figures 1 b and 1c). BSs 8 transformants (Figure 1d) show morphological characteristics intermediate between *E. coli* HB 101 and BSs 8.

Plasmid/Cosmid Isolation

Using the modified method, BSs 8 did not show any plasmid band (Figure 2a) while a distinct plasmid band was exhibited by *E. coli* HB 101 (pHU 52, Tc^r) (Figure 2b) and BSs 8 transformant (Figure 2c).

Transconjugation

Table 1 shows the confirmation of the isolated conjugants using antibiotic resistance as selective markers by the triparental mating system.

TABLE 1
CONFIRMATION OF THE ISOLATED TRANSCONJUGANTS OF
AZOSPIRILLUM SP. BSs 8 USING ANTIBIOTIC RESISTANCE MARKERS

Bacterial Strains	Growth in the presence of*			
	Km	Tc	Nal	Km + Tc + Nal
Parental strains (Donor) <i>E. coli</i> HB 101 (pHU 52, Tc ^r)	-	+	-	-
(helper) <i>E. coli</i> HB 101 (pRK 2013, Km ^r)	+	-	-	-
Recipient <i>Azospirillum</i> sp. BSs 8	-	-	+	-
Transconjugant	+	+	+	+

*20 µg /ml Km, 15 µg /ml Tc, 50 µg /ml Nal

Fig. 1 Photographs of cell colonies of (a) *Azospirillum* sp. BSs 8 (Nal^r) (b) *Escherichia coli* HB 101 (pHU 52, Tc^r) (c) *E. coli* HB 101 (pRK 2013) cultured on nutrient broth agar and (d) BSs 8 transformants (pHU 52, Tc^r, Nal^r).

Fig. 2 Cesium chloride-ethidium bromide density gradient centrifugation profile of extracted DNA by the modified alkaline method from (a) *Azospirillum* sp. BSs 8 (wild type) (b) *E. coli* HB 101 (pHU 52, Tc^r) (c) BSs 8 transformant (pHU 52, Tc, Nal^r).

The conjugal transfer frequency of pHU 52 into *Azospirillum* sp. BSs 8 has an average value of 1.4×10^{-4} . The transfer was facilitated by the suicidal helper plasmid pRK 2013. pRK 2013 does not seem to replicate in many gram negative bacteria. pRK 2013 is a recombinant of the plasmid Col EI and *tra* genes of RK 2. The conjugants with the exception of enterobacteria often lose pRK 2013 and therefore this plasmid is called suicidal.

Transformations of BSs 8 with pHU 52

Increasing the concentration of CaCl₂ from 10 mM to 75 mM increased transformation frequencies. The transformation efficiency of BSs 8 with pHU 52 is 3.5×10^3 transformants per μg of DNA when the concentration of CaCl₂ in FSB was increased from 10 mM to 75 mM. Previous results showed 1.2×10^3 transformants per μg DNA using 10 mM CaCl₂ in FSB. These results show that transformation efficiency was improved using 75 mM CaCl₂.

Uptake Hydrogenase Assay

The results for the hup assay for *E. coli* HB 101 (pHU 52), *Azospirillum* sp. BSs 8 (wild type) and BSs 8 (pHU 52) transconjugants and transformants (pHU 52) are given in Table 2 and the qualitative results are shown in Figure 3.

TABLE 2
CONJUGAL TRANSFER, TRANSFORMATION AND EXPRESSION OF
HYDROGEN UPTAKE ACTIVITY (METHYLENE BLUE METHOD) OF
***AZOSPIRILLUM* SP. BSs 8**

Transconjugation				
Donor	Recipient	Frequency of Tc ^r colonies per recipient 1.4×10^{-4}	Phenotype Hup Activity	
<i>E. coli</i> HB 101 (pHU 52)	BSs 8		Recipient -	Transconjugant +
Transformation pHU (μ g DNA)	BSs 8	Transformants per μ g 35×10^3	DNA -	Transformants +

Fig. 3 Photograph of the results of the hydrogen uptake activity assay by the modified methylene blue method of transformants (Tf) and control (c).

The results of the filter paper disc method were not quite conclusive after a period of hours. The intense dark blue background of the paper impregnated with 10mM MB made it difficult to discern the tiny white spots that show up hup⁺ strains, transformants and transconjugants. The decolorization of the diluted MB solution (0.10 mM) was faster and easily detected during this period (6 hrs) of observation by the hup⁺ strains. BSs 8 (wild type) did not show hup⁺ activity during this period of observation while *E. coli* HB 101 (pHU 52) and BSs 8 transconjugants and transformants with pHU 52 showed decolorization of MB under the conditions of the assay. Gas chromatographic analysis of uptake hydrogenase activity of BSs 8 (wild type) showed negligible values.

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