Enhanced Nitrogen Fixation Capabilities of Soybean Rhizobia by Inter- and Intra-specific Cell Fusion

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Abstract: A procedure for protoplast isolation and regeneration in rhizobia has been established. The use of 1% N-laurylsarcosine in pre-washing of the cells facilitated the cellular lysis by incubation for 1 hr in a reaction solution composed of Tris-HCl buffer (pH 7.5) containing 0.6 M MgSO4 and 5 mg/ml lysozyme. Exchanging of the solution at the half-way point of the reaction led to sufficient protoplast formation. The prepared protoplasts could regenerate at rates ranging from $3 \times 10^{-3}$ to $6.4 \times 10^{-3}$. The polyethylene glycol treatment was adopted for inter- and intra-specific fusion of protoplasts. The fused protoplasts between rhizobia with two different auxotrophic markers were regenerated by plating in a soft agar layer of a minimum medium containing 0.6 M mannitol at the regeneration rate of $10^{-4}$ levels. After the several dozens of repeated subcultures the intra-specific fusion products between Bradyrhizobium japonicum and the inter-specific fusion products of B. japonicum with Sinorhizobium fredii were found capable of forming nodules against the host plant in the pot experiment. The number of nodules produced by some of the intra-specific fusion products, after repeated subcultures, was 1.5 times higher than in the parental stocks. Besides, more than twice the nitrogenase activity was detected in the nodules of some of the intra- and inter-specific fusion products. These results suggested that production of highly effective nitrogen fixing strains by cell-fusion technique is possible.

Key words: (Brady) Rhizobium, Cell fusion, Nitrogenase, Nodulation, Protoplast, Soybean.

Rhizobium and Bradyrhizobium are Gram negative bacteria that have a unique capability to infect the roots of leguminous plants and establish a nitrogen-fixing symbiosis10. As this symbiosis acts the most ecologically sound and cheapest source of nitrogen13, it has been targeted by many investigators14, and the rapid advances in molecular genetics may allow the development of genetically engineered rhizobial strains with enhanced nitrogen fixation12. Most of the genetic operations of rhizobia are carried out either by transduction or conjugation11. Besides, the recent advanced cell fusion technology makes it possible to improve the genetic traits of bacteria, as reported in many species of Gram positive bacteria4,7,14 and some of the Gram negative bacteria, such as E. coli8,20 and Providence alcalifaciens11. However, extremely slow growth of rhizobia and their poor cell wall degradation against the usual lytic treatments make them difficult to handle13, and these factors remain as barriers to genetic interactions. In spite of the progressive use of cell-fusion techniques as described above, no information regarding rhizobia has been reported.
Since many of the genes concerned with nodulation and nitrogen fixation (nif and nod) are carried and closely arranged on chromosomes and megaplasmid\(^8\), we attempted to transfer these genes intra- and interspecifically, and allow them to recombine and segregate to form new cells capable of fixing an enhanced amount of nitrogen. The process of the transfer has been carried out by cell fusion techniques. In this article, we describe a highly efficient protoplast preparation and fusion of *Bradyrhizobium japonicum* with the same or other rhizobial species. Furthermore, we describe the symbiotic properties of the fusion products, such as nodulation and nitrogen-fixing capabilities with soybean (*Glycine max* L. Merr).

### Materials and Methods

1. **Strains**

*Bradyrhizobium japonicum* strains TAL 377, TAL 379 and TAL 1904, along with *Sino-

rhizobium fredii* TAL 1783, were obtained from NiTAL Project and Mircen, Faculty of A-

griculture, University of Hawaii. *Rhizobium leguminosarum* bv. *viciae* IFO 14778 was obtained from the Institute for Fermentation in Osaka. *Bradyrhizobium japonicum* TAL 377 was used in the major parts of the present experiments. Unless otherwise mentioned, the reference to *B. japonicum* will refer to this strain. Auxotrophic mutants used for the experimentation were derived by UV treatment\(^9\).

2. **Media**

Yeast Extract Mannitol Broth (YEMB) medium\(^10\) was used as the complete medium (CM). The medium contained 0.5% mannitol, 0.3% yeast extract, 0.05% MgSO\(_4\), 0.07% K\(_2\)HPO\(_4\), 0.01% KH\(_2\)PO\(_4\), and 0.004% FeCl\(_3\). The pH was adjusted to 7.0. For preparing the solid and soft media 1.5% and 0.7% agar were added, respectively. The minimal medium (MM) contained HEPES (N-2-hydroxyethyl-piperazene-N-ethansulfonic acid) 0.65%, MES [2-(N-morpholino) ethansulfonic acid] 0.55%, FeCl\(_3\) 0.0067%, MgSO\(_4\)·7H\(_2\)O 0.18%, CaCl\(_2\)·2H\(_2\)O 0.013%, Na\(_2\)SO\(_4\) 0.25%, NH\(_4\)Cl 0.32%, Na\(_3\)HPO\(_4\) 0.125% and L- (+) arabinose 0.10%\(^9\). The pH was adjusted to 6.6. Arabinose was dissolved in water, sterilized by filtration with Millipore filter, and then added to the autoclaved MM without sugar.

3. **Growth conditions**

Cultivation of the rhizobial cells was carried out in 500 ml shaking flasks containing 70 ml of YEHB. Flasks were inoculated with 1 ml of the seed culture of rhizobial cells and incubated for 48 h at 30°C in a reciprocal shaker at a speed of 120 stroke per min.

4. **Protoplast preparation**

Cells were harvested at the mid-point of the log phase by centrifugation at 12,000 × g for 10 min, and washed once with 1% N-laurylsarcosine, followed by washing three times with 30 mM Tris [tris (hydroxymethyl) aminomethane]-HCl buffer (pH 7.5) containing 0.6 M MgSO\(_4\) as an osmotic stabilizer. The bacterial cells were then pelleted by centrifugation. Lysozyme was dissolved in the same Tris-HCl buffer in ratios to make two different final concentrations (1.0 and 5.0 mg/ml), followed by Millipore filter-sterilization, added to the cell pellets and mixed thoroughly to make the suspension. The resulting mixture was incubated at 37°C and the lysis of the bacterial cell wall was monitored at intervals by measuring the decrease in the turbidity at 660 nm. To decrease the concentration of the viscous compound produced by the degradation of the cell wall components, the reaction was first left to continue for 30 min and the mixture was then centrifuged at 2,000 × g for 10 min. After discarding the supernatant solution, the enzyme solution (prepared as described above) was then added and the reaction continued for a further 30 min. Viscosity, turbidity and the rates of protoplast formation were determined at these time intervals. Protoplasts were observed by phase contrast microscopy. Counting of protoplasts was determined in 10 grid areas of a haemocytometer. Viscosity of the reaction mixture was determined by using a rotary viscometer (Type visconic EHD, Tokyo Keiki Co., Japan).

5. **Regeneration of protoplasts**

The protoplasts in the reaction mixture were collected by centrifugation at 2,000 × g for 10 min. The precipitate was washed with the same Tris-HCl buffer with an osmotic stabilizer and the resulting precipitate was resuspended in the same buffer. Protoplast suspension (0.1 ml) was then added to the soft agar supplemented with the osmotic stabilizer and overlaid on the stabilized MM agar in...
Petri dishes. The colonies were counted after incubation for 7 days due to slow growth of the regenerated cells. Osmotic resistant cells that escaped the lytic reaction, were estimated by suspension first in water. The rate of regeneration was calculated on the bases of the percentage input of the osmotic sensitive protoplasts which gave rise to colonies in the regeneration media.

6. Fusion of protoplasts

To establish an effective and reliable method of gene transfer by protoplast fusion in soybean microsymbionts, auxotrophic mutants of *B. japonicum* TAL 377 (Ade- and Pro-) and *B. japonicum* TAL 1904 (Ade-) were selected for intra-specific fusion. The later was also used for inter-specific fusion experiment with *S. fredii* TAL 1783 (Leu-). Protoplasts of the auxotrophic strains were obtained as described above. Aliquots (1.0 ml each) of the parental protoplasts were mixed (1×10^10 protoplasts/ml for each strain) and sedimented by centrifugation to 2000×g to form pellets. Fusion was induced by adding 1.0 ml of Millipore-filtered PEG solution which consisted of 50 mM glycine-NaOH buffer (pH 7.5) containing 50% polyethylene glycol (PEG) 4,000 and 100 mM CaCl_2_. Mixtures were immediately homogenized at 30°C with gentle shaking. After incubation for 30 min, 5 ml of Tris-HCl buffer (pH 7.5) contained 0.6 M mannitol was added and the suspension was centrifuged. Washing was done three times with the same buffer as previously described, and finally resuspended in 3 ml of the same buffer. After appropriate dilution, the samples were then pipetted into 1.2 ml of soft agar MM with 0.6 M mannitol (maintained at 45°C) and immediately poured onto a surface of MM agar plates with the same stabilizer. Parental protoplasts were pipetted separately into stabilized soft agar CM and over layered onto a surface of MM agar plates. The over layered plates were then incubated at 30°C for 7 to 10 days. The appearance of the colonies on MM plates could therefore be attributed to protoplast fusion and complementation.

7. Bacteriological characterization of the fusion products

Morphological measurement was carried under light microscope after Gram staining technique. The acid reaction was performed in shaking tubes containing 8 ml YEMB includ-
ed 25 ppm bromthymol blue as pH indicator and the initial pH was adjusted to 6.8. Absorption of Congo red was made in YEM agar plates containing 25 ppm Congo red. For assessment of sensitivity to high pH, YEM agar plates with the pH adjusted to 9.0 were used and the addition of 2% NaCl to the medium was used to test resistance to NaCl.

8. Nodulation capability of the fusion products

Strains and the fusion products used in this experiment, are shown in Tables 4 and 5. The experiments were performed in plastic pots. Pots of 2.5 kg capacity were filled with sterilized vermiculite saturated with nitrogen-free nutrient solution. The sterilization was carried out by autoclaving the vermiculite in polypropylene bags at 121°C for 1 hr. Seeds of soybean (*Glycine max* L. Merr. cv Tamaharame) were obtained from the local market. Seeds were surface-sterilized with 5% NaClO solution, followed by washing 5 times with sterile distilled water and were germinated on absorbent paper. Three-day old seedlings were transferred to the pots (three seedlings per pot), and then inoculated with 1.0 ml (10^9/ml) of the required rhizobial inoculum. Treatments were replicated by three pots and the non-inoculated plants were used as the controls. Plants were then maintained to grow under normal environmental conditions. Pots were checked daily for water requirements. After 30 days, plants were examined for nodulation and nitrogenase activity.

9. Nitrogenase assay

Nitrogenase activity was determined in excised root parts. The excised parts from the root system with twenty nodules were placed in 2 ml vacuum vials. Air/acetylene mixture was added at a ratio of 4:1 (v/v) into vials under normal atmospheric pressure, and then incubated at 25°C for 1 hr. A Hitachi 165 gas chromatograph, fitted with a hydrogen flame ionized detector and a 2-meter column of Porapak T, was implemented for the measurement of the evolved ethylene.

10. Chemicals

The lytic enzyme used for the study was lysozyme chloride (Nacalai Tesque Inc., Kyoto, Japan). N-Laurylsarcosine (Nacalai Tesque Inc., Kyoto, Japan) was used as a washing agent. All the other chemicals were obtained from commercial sources.
Results

1. Effect of pre-washing with N-laurylsarcosine on the preparation of bacterial protoplasts

In preliminary tests we observed that there was no effect of lysozyme on the lysis of bacterial cells, and indeed, a slight increase in turbidity over protracted incubation periods was sometimes observed. Upon introduction of 1.0% (v/w) of the ionic detergent, N-laurylsarcosine, for the pre-washing of bacterial cells, a decrease in turbidity of cell suspension by lysozyme reaction was observed (Fig. 1). During 60 min of the reaction, the reaction mixture became highly viscous through the accumulation of the lysed products. Turbidity after 1 hr of the reaction was decreased to 68% of the initial value before the lytic reaction.

2. Conditions for protoplast preparation

The optimal conditions for preparation of rhizobial protoplasts by using lysozyme as the lytic enzyme were determined as follows:

1) pH of the reaction mixture

The maximum productivity for protoplasting as estimated by a decrease in turbidity of the reaction mixture was observed at pH 7.5.

2) Osmotic stabilizer

The use of mannitol (0.6 M) as osmotic stabilizer for the lytic reaction gave higher protoplast productivity (82%) than that of MgSO₄ (78%), but an extremely high viscous fluid was produced after 30 min of incubation, and there was no sedimentation by gravity. The yield of protoplasts was somewhat lower in the use of sucrose (65%).

3) Enzyme concentration

In a preliminary experiment when we had carried out the lytic reaction at an enzyme concentration of 5.0 mg/ml, the highest productivity of protoplasts over 70% and the maximum decrease in the turbidity were observed after 1 hr of incubation, but the viscosity of the reaction mixture was greatly increased (the data not shown). To decrease the viscosity of the reaction mixture, two methods for the enzyme treatments were tried. In the first method, the reaction, at 1 or 5 mg/ml of enzyme, was left to continue for 15 min followed by the exchange of the new reaction solution and incubated for a further 45 min. In the second method used the reaction time was partitioned into 30+30 min. Table 1 shows that higher productivity of protoplasts (82.1%) was obtained when the second method was applied at a lysozyme concentration of 5.0 mg/ml, and the concentration of the viscous compound was decreased to a minimum level.

![Graph](image_url)

Fig. 1. Effect of addition of N-Laurylsarcosine on the cell wall lytic reaction in *B. japonicum.* ○: no addition, ●: 0.1%, ■: 1.0%

Table 1. Effect of lysozyme concentrations on the cellular lytic reaction and protoplast formation in *B. japonicum.*

<table>
<thead>
<tr>
<th>Time in min*</th>
<th>Viscosity (ηP)</th>
<th>Reduction of the turbidity at 660 nm치(%)</th>
<th>Protoplast formation(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 mg/ml</td>
<td>5 mg/ml</td>
<td>1 mg/ml</td>
</tr>
<tr>
<td>15+45</td>
<td>5.08</td>
<td>5.54</td>
<td>56.0</td>
</tr>
<tr>
<td>30+30</td>
<td>5.81</td>
<td>5.18</td>
<td>63.1</td>
</tr>
</tbody>
</table>

*Intervals of the reaction times at which solutions were exchanged.
†Decrease in the visual optical density of cell lysozyme mixture.
3. Regeneration of protoplasts

Regeneration of *B. japonicum* TAL 377 protoplasts to intact cells was examined using different concentrations of various osmotic stabilizers.

(1) Effect of different osmotic stabilizers

Mannitol and sucrose were tested for their capacity to support protoplast regeneration. The highest rate of regeneration and the consequent colony formation were obtained with 0.6 M mannitol \(1.8 \times 10^{-3}\), and the lowest rate with sucrose \(1.6 \times 10^{-4}\).

(2) Effect of mannitol concentration

Optimal concentration of mannitol for protoplast regeneration was found to be 0.6 M (Fig. 2).

4. Protoplast formation from other rhizobial species

From the cells of other soybean symbionts, over 75% of the rate of protoplasting was obtained by using the procedures described above. It was also observed that different species of rhizobia carried different regeneration rates (Table 2).

5. Efficiency of prototrophic colony formation by fusion of parental auxotrophic strains

When a high density \(2 \times 10^{10}\) of the protoplasts of either of two auxotrophic strains as shown in Table 3 was plated separately or when they were mixed without the PEG treatment, very few colonies were emerged on MM, and these colonies might be due to reversion to prototrophs. The rates of reversion to the prototrophy in both of the auxotrophic strains were less than \(3.0 \times 10^{-11}\) as measured by plating on MM and CM. Besides, the regeneration rates of protoplasts from these strains were ranged between \(10^{-4}\) and \(10^{-5}\) levels. When two different auxotrophic strains were mixed, PEG-treated, and plated on a soft layer of MM medium, the colonies emerged on the plate at the rate of about \(4 \times 10^{-7}\) of the original number of protoplasts. Therefore, it seems that most of the prototrophic colonies from the PEG-treated protoplast mixtures were the fusion products by complemented the different nutritional requirements of the parental strains.

6. Some bacteriological observations on the fusion products

All of the isolated fusion products were found to be Gram-negative, non-spore forming and rod-shaped. The fusant colonies on the plates were large (reaching 3 to 5 mm in diameter in 3 days of culture), circular, convex, glistening, with entire margins, and showed no absorption of Congo red.

Some cultural characteristics of the fusion products were shown in Table 4 and 5. All of the fusant cultures showed acidic reaction against BTB, although the parental strains showed alkaline reactions to the same reagent.

![Graph](image)

**Fig. 2.** Effect of mannitol concentrations of the regeneration medium on the regeneration of protoplasts in *B. japonicum*.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Viscosity (cP)</th>
<th>Protoplast formation</th>
<th>Rate of regeneration of protoplasts↑</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. japonicum</em> TAL 377</td>
<td>5.18</td>
<td>80.0%</td>
<td>1.8 \times 10^{-3}</td>
</tr>
<tr>
<td><em>R. leguminosarum</em> IFO 14778</td>
<td>4.74</td>
<td>76.7%</td>
<td>3.0 \times 10^{-2}</td>
</tr>
<tr>
<td><em>S. fredii</em> TAL 1783</td>
<td>5.48</td>
<td>77.7%</td>
<td>6.4 \times 10^{-3}</td>
</tr>
</tbody>
</table>

↑Rate of protoplast regeneration to intact cells was calculated on the percentage ratio of the number of regenerated colonies to the number of the originally plated cells.
Table 3. Fusion frequency between protoplasts of auxotrophic mutants of B. japonicum after the PEG treatment.

<table>
<thead>
<tr>
<th>Parental strains</th>
<th>Frequency of reversion* (less than)</th>
<th>Rate of regeneration of protoplasts</th>
<th>Rate of protoplast fusion†</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. japonicum TAL 377(Pro⁺)</td>
<td>3.0 × 10⁻¹¹</td>
<td>1.9 × 10⁻⁴</td>
<td>3.9 × 10⁻⁷</td>
</tr>
<tr>
<td>B. japonicum TAL TAL 377(Met⁻)</td>
<td>2.0 × 10⁻¹³</td>
<td>2.8 × 10⁻⁵</td>
<td>—</td>
</tr>
</tbody>
</table>

*The frequency of protoplast reversion to prototroph was calculated on the bases of percentage ratio of the number of colony formed on MM to the number of the originally plated cells.

†Rate of protoplast fusion as calculated on the bases of the percentage number of colonies regenerated on MM to that on CM.

Table 4. Some characteristics of the intra-specific fusion products of B. japonicum and their nodulation and nitrogenase activity in symbiosis with soybean as compared to the parental strains.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Reaction in BTB medium</th>
<th>Growth at 2% NaCl</th>
<th>Growth at pH 9</th>
<th>Number of nodules/plant</th>
<th>Acetylene reduction (µmole)/g fresh nodules/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. japonicum TAL 377*</td>
<td>Alkaline</td>
<td>—</td>
<td>—</td>
<td>62³</td>
<td>1.59³</td>
</tr>
<tr>
<td>B. japonicum TAL 1904*</td>
<td>Alkaline</td>
<td>—</td>
<td>—</td>
<td>46³</td>
<td>3.95³</td>
</tr>
<tr>
<td>BB-1*</td>
<td>Acidic</td>
<td>—</td>
<td>±</td>
<td>46³</td>
<td>1.43³</td>
</tr>
<tr>
<td>BB-2</td>
<td>Acidic</td>
<td>—</td>
<td>—</td>
<td>30³</td>
<td>1.92³</td>
</tr>
<tr>
<td>BB-3</td>
<td>Acidic</td>
<td>—</td>
<td>±</td>
<td>40³</td>
<td>2.47³</td>
</tr>
<tr>
<td>BB-4</td>
<td>Acidic</td>
<td>—</td>
<td>±</td>
<td>34³</td>
<td>8.85³</td>
</tr>
<tr>
<td>BB-5</td>
<td>Acidic</td>
<td>—</td>
<td>±</td>
<td>94³</td>
<td>8.82³</td>
</tr>
</tbody>
</table>

*Parental wild type strains.

*Fusion products of the cross of B. japonicum TAL 377 Pro⁺ with B. japonicum TAL 1904 Ade⁻.

Numbers in the same column flanked by the same letter are not significantly different at P = 0.05 as determined by Duncan’s multiple range test.

Some of the intra-specific fusion products (BS-1 and BS-2) could grow in the nutrient broth containing 2% NaCl. Further, several of the intra- and inter-specific products showed a high tolerance against alkaline pH condition.

7. Nodulation and nitrogenase efficiency of the fusion products

The intra- and inter-specific fusion products were tested for their nodulation capability and nitrogenase activity after the several dozens of repeated subcultures.

(1) Intra-specific fusion products

The symbiotic attributes of the fusion products resulted from the intra-specific fusion between B. japonicum TAL 377 Pro⁺ and B. japonicum TAL 1904 Ade⁻ were listed in Table 4. It seems that the nodules by the fusion products were generally scattered around the lateral roots and few nodules were formed along the primary root. Nodules induced by these fusion products were smaller than those by their parental wild types. Analysis of the variance showed that the average number of nodules induced by the fusion products BB-1, BB-2, BB-3 and BB-4 did not significantly exceed those by the parental strains TAL 1904 and TAL 377. Besides, the fusion product BB-5 induced the highest number of nodules among the fusion products and the parental strains tested. The highest nitrogenase activities of nodules were produced by the fusion products BB-4 and BB-5 (P = 0.05), and their activities exceeded significantly over those of the parental strains.
Table 5. Some characteristics of the inter-specific fusion products of *B. japonicum* and *S. fredii* and their nodulation and nitrogenase activity in symbiosis with soybean as compared to their parental strains.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Reaction in BTB medium</th>
<th>Growth at 2% NaCl</th>
<th>Growth at pH 9.0</th>
<th>Number of nodules/plant</th>
<th>Acetylene reduction (μmole)/g fresh nodules/h</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. japonicum</em></td>
<td>alkaline</td>
<td>−</td>
<td>−</td>
<td>46a</td>
<td>3.95b</td>
</tr>
<tr>
<td>TAL 1904*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. fredii</em></td>
<td>Acidic</td>
<td>−</td>
<td>−</td>
<td>26a</td>
<td>.81d</td>
</tr>
<tr>
<td>TAL 1783*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BS-1**</td>
<td>Acidic</td>
<td>+</td>
<td>±</td>
<td>51a</td>
<td>4.91b</td>
</tr>
<tr>
<td>BS-2</td>
<td>Acidic</td>
<td>+</td>
<td>±</td>
<td>40a</td>
<td>8.83b</td>
</tr>
<tr>
<td>BS-3</td>
<td>Acidic</td>
<td>−</td>
<td>−</td>
<td>42a</td>
<td>1.50b</td>
</tr>
<tr>
<td>BS-4</td>
<td>Acidic</td>
<td>−</td>
<td>−</td>
<td>40a</td>
<td>2.90b</td>
</tr>
<tr>
<td>BS-5</td>
<td>Acidic</td>
<td>−</td>
<td>−</td>
<td>19b</td>
<td>1.30b</td>
</tr>
</tbody>
</table>

*Parental wild type strains.
**Fusion products of the cross of *B. japonicum* TAL 1904 Ade− with *S. fredii* TAL 1783 His−.
Numbers in the same column flanked by the same letter are not significantly different at *P*=0.05 as determined by Duncan’s multiple range test.

(2) **Inter-specific fusion products**

Nodulation of soybean was also induced by all of the inter-specific fusion stocks crossed between *B. japonicum* and *S. fredii* (Table 5). Nodules formed by this group of fusion products were large, crowned and mainly found on the tap roots. The number of nodules per plant was greater with four of the fusion products than that with the parental wild-type TAL 1783 and not significantly different from that induced by TAL 1904 (*P*=0.05). The fusion product BS-5 showed poorly induced nodulation as compared to the parental strains and other fusion products. When the nitrogen fixation capability of nodules was measured by acetylene reduction assay, the fusion products BS-1 and BS-2 gave the highest nitrogenase activities and they differed significantly over both of the parental wild-types (*P*=0.05).

**Discussion**

It has been reported that gram-negative bacteria produce a rigid layer of high molecular-weight polysaccharides. These polysaccharides are apparently crucial to commensal bacteria. However, their elimination is probably an essential step if efficient fusion to be achieved. Introduction of a detergent (1% *N*-laurylsarcosine) in our experiment disrupted the protective layer of bacterial cells and the treated cells became susceptible to the lytic enzyme. A similar result was reported by Metcalf when he used NaCl and sodium lauryl sulfite for the cellular lysis of *Streptococcus faecium*.

In our experiment, viscous compounds in the reaction mixture accumulated as lytic products. These viscous compounds cause poor handling in protoplast preparation. Therefore, the washing process to decrease viscosity was tested by exchaining the reaction solution at the half-way points of the reaction time, and it was proved to be very effective for sufficient production of protoplasts.

By applying the optimized procedure, we obtained almost same number of protoplasts from three tested rhizobial strains. However, the regeneration rate of protoplasts was very low as compared with other bacteria (see Table 2).

In addition to improve the procedures for protoplast preparation and regeneration, an efficient genetic recombination is contingent as means to fuse protoplasts and thus introduction of genetic materials from two different strains into common protoplast could be accomplished. We obtained the fusion products between *B. japonicum* auxotrophs with PEG treatment at the frequency of 1.2×10−7. Since the prototrophs did not appear after plating the parental auxotrophic strains on the MM, this is believed to be a result of protoplast fusion.

The intra-specific fusion products resulted from the slow grower strains of *B. japonicum*...
(TAL 377 and TAL 1904) showed fast growing characters. These fusion products showed high nodulation with soybean and produced more than 200% nitrogen activity over the parental strains. In the case of the interspecific fusion products between B. japonicum TAL 1904 and S. fredii, some of them showed higher nitrogen activity than those of the parental strains. The nodulation experiments using fusion products have been confirmed that successful transfer of symbiotic characters between nitrogen fixing rhizobia has been achieved, and the prototrophic segregation obtained via protoplast fusion are agronomically sound.

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References