Fusion of Protoplasts and Genetic Recombination of 
Brevibacterium flavum

Harumi KANEKOK and Kenji SAKAGUCHI

Laboratory of Microbiological Chemistry, Mitsubishi-Kasei Institute of Life Sciences,
11 Minamiooya, Machida-shi, Tokyo, Japan
Received October 31, 1978

Protoplasts of Brevibacterium flavum were obtained by treatment with 0.3 units of penicillin per ml during exponential growth, and followed by lysozyme treatment in a hypertonic medium. Protoplasts of antibiotic-resistant and auxotrophic strains were fused by a modified polyethyleneglycol technique, and allowed to revert to bacillary form on selective media. The clones were analyzed and recombinants were found not to segregate.

Artificially induced somatic cell fusion is a useful technique in studying genetics of eukaryotic and prokaryotic organisms of which no technique to exchange the genetic principles has been discovered. Recently, the polyethyleneglycol 6000 (PEG 6000) method has been developed and used because of its high fusion inducing ability and low specificity among various organisms.1,2) PEG 6000 has been applied to plant protoplast fusion to construct hybrid plants.3) The technique also allows genetic information exchange between protoplasts of sexually inert cells such as fungal cells of the same mating type.4) Protoplast fusion of bacterial cells was recently reported with respect to Bacillus subtilis5) and Bacillus megaterium.6) However, no transformation, transduction or conjugation mechanism has ever been found in Brevibacterium flavum.

We report here the procedure for protoplast preparation, protoplast fusion, cell wall regeneration and genetic recombination of fused cells of B. flavum to which no accessible lytic enzymes have been found.

MATERIALS AND METHODS

Bacterial strains. Brevibacterium flavum ATCC14067
Trp"Str" and Thr" strains were kindly supplied by Dr. H. Momose, Central Research Laboratories, Ajinomoto Co. Other mutants were derived from these two strains by N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis or UV irradiation. Str" strains were resistant to 200 μg/ml of streptomycin and sensitive to 5 μg/ml of rifampicin. Rif" strains were resistant to 10 μg/ml of rifampicin and sensitive to 3 μg/ml of streptomycin. Tryptophan and threonine auxotrophs were slightly leaky, and the strains carrying these markers formed colonies on minimal agar in 2 weeks of incubation.

Chemicals. Penicillin G was purchased from Meiji Seika Co., Ltd. (Tokyo). Lysozyme was the product of Seikagaku-Kogyo Co., Ltd. (Tokyo).

Media. Minimal medium (MM) had the following composition (per liter): (NH4)2SO4, 10 g: urea, 3 g; KH2PO4, 1 g; NaCl, 50 mg; MgSO4·7H2O, 0.4 g; FeSO4·4·H2O, 2 mg; MnSO4·4·H2O, 2 mg; glucose, 20 g; biotin, 50 μg, and thiamine, 200 μg. MMYE, MM supplemented with 0.1% yeast extract (Difco), was used as the ordinary culture medium. Regeneration medium was a nutrient broth containing 0.5 M sodium succinate (RNB), or MM containing 0.5 M sodium succinate (RMM). Solid and soft agar media contained 1.5 and 0.8% agar, respectively. The dilution fluid (DF), used for protoplast dilution, was DF of Landman and Halle,7) except that pH was adjusted to 6.5. Fusion fluid (FF) was DF supplemented with 5 mM EDTA.

Protoplast formation. An overnight culture of bacteria, 0.2 ml, was inoculated into fresh MMYE 20 ml, and the culture was incubated at 30°C with shaking. In the mid-exponential phase (about 1 x 10^9 cells/ml), penicillin G was added at a rate of 0.3 unit/ml. At this concentration, no growth inhibition was observed. After 1.5 more hours of shaking, cells were harvested and suspended into 10 ml of lysis fluid (LF) in a 100-ml conical flask. The flask was incubated
1008 H. KANEKO and K. SAKAGUCHI

at 30°C without shaking for 16 hr. LF was twice
diluted MMYE supplemented with 0.41 M sucrose,
0.01 M MgSO₄, and 300 µg/ml lysozyme.

Reversion of protoplasts (viable protoplast count).
Protoplasts were diluted with DF, and plated with
3 ml of RNB soft agar onto an RNB solid agar layer.
Colonies were counted after 7 days' incubation at 30°C.

Microscopic observation of reverting protoplasts. Re
verting protoplasts were taken from the soft agar layer
for microscopic observation at intervals. Protoplasts
were put on a thin RNB agar (1.5%) film on a slide
glass, covered with a cover slip, sealed with paraffin and
observed under a microscope at 30°C from time to
time. Otherwise, protoplasts, mixed with RNB contain-
ting 30% gelatin instead of agar, were put on a slide
glass, covered, sealed and observed at intervals.

Normal viable cell count. Cells were diluted with
TM buffer containing Trizma base 50 mM, maleic acid
50 mM, MgSO₄(7H₂O) 0.4 mM, (NH₄)₂SO₄ 7.5 mM, and
sodium citrate 1.7 mM, pH 6.5, and spread onto the
nutrient broth agar. Colonies were counted after 2
days' incubation at 30°C.

Protoplast fusion. Protoplasts of two strains, of
which one was streptomycin-resistant and the other
was rifampicin-resistant, were used throughout these
experiments. They were mixed and centrifuged
(3000 rpm 15 min at 4°C). The pellet was resuspended
in a one-tenth volume of FF. This dense suspension
was then diluted 10-fold with 33% solution of poly-
ethylene glycol 6000 (Iwai Kagaku) in distilled water.
After incubation at 30°C for 15 min, the suspension
was twice diluted with FF. Samples (0.1 ml) were
then plated with 3 ml of RNB soft agar on RNB solid
agar containing rifampicin (5 µg/ml) and streptomycin
(100 µg/ml). After 14 days incubation at 30°C, colonies
in the selection medium were isolated, and their auxo-
trophic properties were checked.

RESULTS

Protoplast formation

The conditions suitable for protoplast forma-
tion by lysozyme treatment were studied. The appearance of spherical cells and the
rupture of these cells at low osmotic pressure
under a microscope were used as protoplast
formation indices.

Mild penicillin treatment of the cells in their
mid-exponential growth phase, which gave no
growth inhibition, had a remarkable effect on
protoplast formation. Also the sucrose con-
centration of LF, 0.41 M was important. Su-
crose (0.5 M) reduced the number of spherical
cells. The existence of growth medium,
MMYE, during the lysozyme treatment en-
hanced the formation of protoplasts. Gentle
shaking during the lysozyme treatment acce-
lerated the appearance of round cells, but partial
cell lysis occurred simultaneously.

Cells suspended in LF became sensitive to
low osmotic pressure after 2-hr incubation.
After 4-hr incubation spherical cells were
observed amongst rod-shaped cells, which were
also sensitive to low osmolarity. After 16-hr
incubation, almost all cells turned into a spheri-
cal shape and sensitive to low osmotic pressure.

Figure 1 shows the time course of the lysozyme

![Fig. 1. Occurrence of Osmotically Fragile Cells
upon Lysozyme Treatment.](image)

After penicillin treatment, cells were transferred into
a hypertonic medium containing lysozyme (LF), and
sampled at intervals. They were appropriately
diluted with DF, and the ability to form colonies was
measured by spreading on a hypotonic NB solid
agar (○), or by plating with a hypertonic RNB soft
agar onto RNB solid agar (□). The colony numbers
were counted after 2 days' and 7 days' incubation,
respectively.

Regeneration and colony formation of proto-
plasts

When the protoplasts were overlaid with
TABLE I. COLONY FORMATION FROM PROTOPLASTS OF B. flavum Trp-Str<sup>a</sup>

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Overlay medium</th>
<th>Basal agar medium</th>
<th>Viable count/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>_ &lt;sup&gt;b&lt;/sup&gt;</td>
<td>NB 1.5% agar</td>
<td>8 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>RNB 0.8% agar</td>
<td>RNB 1.5% agar</td>
<td>2 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>RNB 0.5% agar</td>
<td></td>
<td>1.9 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>RNB 0.2% agar</td>
<td></td>
<td>1.5 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>RNB 30% gelatin</td>
<td></td>
<td>2.1 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>RNB 25% gelatin</td>
<td></td>
<td>6 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>RNB 20% gelatin</td>
<td></td>
<td>6 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>RMM&lt;sup&gt;c&lt;/sup&gt; 0.8% agar</td>
<td>RMM&lt;sup&gt;c&lt;/sup&gt; 1.5% agar</td>
<td>3 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>RNB 0.8% agar</td>
<td>RNB 1.5% agar</td>
<td>2.4 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>RMM&lt;sup&gt;c&lt;/sup&gt; 0.8% agar</td>
<td>RMM&lt;sup&gt;c&lt;/sup&gt; 1.5% agar</td>
<td>1.6 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Protoplast suspension (about 2 × 10<sup>8</sup> protoplasts/ml) was diluted with DF, plated and incubated at 30°C for one week.

<sup>b</sup> The cells were spread onto basal agar without overlay agar.

<sup>c</sup> RMM supplemented with 50 µg of tryptophan per ml was used.

RNB soft agar onto the RNB solid agar, colonies were formed at an efficiency of ca. 30%. RMM instead of RNB slightly reduced the colony numbers. The agar concentration in the soft agar most intensely affected the frequency of colony formation with the best results at 0.5%. Colonies appeared after three days incubation at 30°C, but their number increased during the following 4 days. When gelatin was used instead of soft agar, a gelatin concentration between 20 and 30% gave a colony forming efficiency corresponding to 3% of that given by soft agar. The protoplasts spread on the surface of RNB solid agar also formed colonies at an efficiency corresponding to 5% of the standard method. Table I shows several conditions and the numbers of colonies which appeared.

Microscopic observation of reverting protoplasts

Samples taken from soft agar layer were observed under a microscope with phase contrast illumination. Figure 2 shows normal cells and protoplasts of B. flavum. Protoplasts were slightly enlarged and divided, forming clusters. Cell division occurred in protoplast and bacillary forms (Fig. 3A, B, C). In the course of cell division, some or all protoplasts in the cluster turned into a bacillary form, forming a colony of bacillary cells (Fig. 3A). In some cases dividing protoplasts formed colonies consisting of a mixture of spheric cells and rod shaped cells (Fig. 3B). Among these cells complete bacillary form appeared. In some clusters protoplasts continued to divide without turning into a bacillary form.

Fig. 2. (A) Normal Cells of B. flavum ATCC14067 Trp<sup>-</sup>Str<sup>+</sup>. (B) Protoplasts Appeared after 18 hr of Lysozyme Treatment. The bar represents 10 nm.
form (Fig. 3C). When cultured on a slide glass with a thin film of RNB agar, (as described in MATERIALS AND METHODS), the dividing protoplasts gave different morphology from those on dish culture. They enormously enlarged and divided to form huge mosaics. Some of them divided into small spheres (not shown), and then turned into bacillary forms (Fig. 3D, E). In the case of gelatin slide culture, protoplast enlarged two or three times in size, and divided to form protoplast clusters. Bacillary cells budded directly from round protoplasts, forming clusters of bacillary cells (Fig. 3F, G).

Conditions for protoplast fusion

The procedures of protoplast fusion on this microorganism were studied with reference to (i) the concentration of polyethylene glycol (PEG), (ii) the length of incubation period with PEG, (iii) pH and additives to PEG solution, and (iv) other conditions.

Mutants with auxotrophic markers and drug-resistance, namely rifampicin-resistance (5 μg/ml) or streptomycin-resistance (100 μg/ml), were used as parent strains. They were protoplasted, mixed, suspended in PEG solution and plated on a selection medium containing both rifampicin (5 μg/ml) and streptomycin (100 μg/ml). For the control, parental strains were treated by the same procedure without mixing the two strains. Fusion frequency was calculated in terms of colony number per total protoplast number plated. (i) PEG concentrations between 30~50% gave good results. For the sake of handling ease, 30% PEG was used throughout these experiments. (ii) The viable counts of protoplast did not change during 30-min incubation with PEG solution, and the fusion frequency did not differ between 1 min and 15 min. The treatment was continued for 15 min. (iii) The pH of the fusion mixture did not much affect the fusion frequency. Therefore pH 6.5 was chosen with a view to the stability of protoplast. The effect of additives were tested as follows:
mixed protoplasts were centrifuged and the pellet was suspended in a one-tenth volume of the basal medium containing additives. The basal medium consisted of 0.25 M sodium-succinate, 0.25 M sucrose and 0.01 M MgSO₄. PEG solution in water was added to give a final concentration of 30%. The addition of 5 mM EDTA and 0.1 M K₂HPO₄ stimulated the fusion (Table II). Therefore, this medium was further used as the fusion fluid (FF) (iv) Protoplast suspension in PEG solution (0.1 ml) was twice diluted with FF and plated with RNB soft agar onto the selection RNB solid agar medium. The centrifugation (3000 rpm 15 min) of protoplast mixture before PEG treatment was essential to obtain a higher fusion frequency. Under the best condition, the fusion frequency was 5 x 10⁻⁶ of plated protoplasts.

Table II. Effect of Phosphate and EDTA on the Fusion Frequency

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Fusion frequency (x 10⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal buffer⁵</td>
<td>1</td>
</tr>
<tr>
<td>+ EDTA 1 mM</td>
<td>16</td>
</tr>
<tr>
<td>5 mM</td>
<td>21</td>
</tr>
<tr>
<td>+ Phosphate 0.1 M</td>
<td>20</td>
</tr>
<tr>
<td>0.2 M</td>
<td>8.8</td>
</tr>
<tr>
<td>0.3 M</td>
<td>3.3</td>
</tr>
</tbody>
</table>

⁵ Protoplasts were suspended in the basal medium (0.25 M Na₂ succinate, 0.25 M sucrose and 0.01 M MgSO₄) or basal buffer plus additives before the addition of PEG solution. Viable numbers were counted on the selection medium containing both 100 μg/ml of streptomycin and 10 μg/ml of rifampicin. In this experiment PEG-treated samples were not diluted.

⁶ K₂HPO₄ solution adjusted to pH 6.5 with 1 N HCl was added to the basal medium.

Characteristics of fusants

The genetic characteristics of clones which appeared on the selective medium was analyzed on the combination of various parental strains of different genetical markers. Table III clearly shows that the genetic recombination occurred through this fusion technique. Many of the isolated and purified clones showed one of the parental phenotypes except drug-resistance, but some showed mixed phenotypes of both parents at a high frequency amongst the isolated clones. Spontaneous development of drug-resistant mutants occurred at a frequency of 10⁻⁷ of plated protoplasts, which was one order lower than our fusion rates.

To examine whether the complemented clones such as Thr⁺Trp⁺ Rif⁺Str⁺ had diploid or haploid characteristics, 4 clones of these phenotypes were allowed to grow for 10 generations in a broth culture. Neither Thr⁻ nor Trp⁻ colonies could be detected amongst 2,000 progeny of each of the four clones.

The presence of 5 μg of deoxyribonuclease I per ml during the fusion process had no effect on fusion frequency, indicating that the occasional transformation by free DNA derived from lysed cells did not occur (data not shown).

The microscopical observation revealed that the protoplast suspension aggregated immediately upon addition of PEG. Among these aggregates apparently fused amorphous protoplasts were often observed (Fig. 4a). Sometimes free protoplasts like a dumbbell were also observed. Upon dilution with FF, some of the “fused” protoplasts formed a large sphere (Fig. 4b).

DISCUSSION

This paper describes the formation and reversion of B. flavum protoplasts, and protoplast fusion induced by PEG to construct genetic recombinant.

In the genus Brevibacterium or related genera, no genetic transfer system has ever been known. The discovery of the genetic system to raise recombinants described in this report is valuable in connection with the industrial importance of this group of bacteria as potent amino acid producers. In Bacillus subtilis⁵ and Bacillus megaterium,⁶ they were easily protoplasted and reverted to a bacillary form, both necessary conditions for their fusion and cloning. In Brevibacterium and in its related genera, the formation of protoplast-like body from a short rod form was reported only in Microbacterium ammoniaphilum,⁸ which is...
one of the L-glutamic acid producers. The present report describes an efficient method of protoplast formation on this genus of bacteria and also probably on the bacteria which had not been accessible by simple treatment of various cell wall lytic enzymes.

Reversion of protoplasts to a bacillary form was reported in several bacteria. In *B. subtilis* and *Streptococcus faecium*, hyper-tonic gelatin medium could revert protoplasts. In *B. megaterium*, hypertonic soft agar overlay onto a solid agar layer enhanced the reversion of protoplasts to a bacillary form. In *B. flavum*, soft agar was more effective than gelatin as the reversion medium. Under these conditions almost 30% of the protoplasts were

![Table III. Recombinant Formation by Protoplast Fusion](image)

<table>
<thead>
<tr>
<th></th>
<th>Thr</th>
<th>His</th>
<th>Arg</th>
<th>Trp</th>
<th>Met</th>
<th>Rif</th>
<th>Str</th>
<th>Number of clones obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>S</td>
<td>-</td>
</tr>
<tr>
<td>Fusant</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>1</td>
</tr>
<tr>
<td>Exp. 2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>S</td>
<td>R</td>
<td>-</td>
</tr>
<tr>
<td>Fusant</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>1</td>
</tr>
<tr>
<td>Exp. 3</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>R</td>
<td>S</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>S</td>
<td>R</td>
<td>-</td>
</tr>
<tr>
<td>Fusant</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>R</td>
<td>R</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>R</td>
<td>R</td>
<td>1</td>
</tr>
</tbody>
</table>

Fig. 4. (A) Protoplast Aggregation Formed in the 30% Polyethylene glycol Solution (PEG). (B) A Large Protoplast was formed after Dilution of (A) with FF.

The bar represents 10 nm.
reverted to form colonies that consisted of normal bacillary cells. Protoplasts in agar slide culture or in gelatin slide culture took different morphological processes in reversion. Aeration and surrounding materials probably affected the reverting process.

The efficiency of protoplast fusion was rather low although an improvement was observed upon supplementation of EDTA and phosphate. However, the definite occurrence of recombinant clones by the described procedure gives a method for genetical analysis and breeding of this group of bacteria including *Corynebacterium* and *Arthrobacter*, and further of bacteria which may be protoplasted only by combined treatment with penicillin and lytic enzymes. The genetical stability of the obtained clones is especially noteworthy, suggesting the formation of recombinants, not heterocaryons.

**Acknowledgment.** The authors are grateful to Dr. H. Momose of Ajinomoto Co. for the supply of mutants he isolated.

**REFERENCES**