Interspecific Electrofusion between Protoplasts of *Streptomyces antibioticus* and *Streptomyces fradiae*

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Received December 14, 1987

The interspecific electrofusion between protoplasts of *Streptomyces antibioticus* a20 (Ala−) and *S. fradiae* f2 (His+) was examined. The pulse intensities required for the breakdown of half of the protoplasts were 7 kV/cm for *S. antibioticus* a20 and 5 kV/cm for *S. fradiae* f2. The optimum pulse intensity for the fusion was found to be 6 kV/cm. When the density of a protoplast suspension was $1 \times 10^8$ protoplasts/ml in a 0.3 M sucrose solution containing 2.5 mM MgCl$_2$ and CaCl$_2$, the maximum fusion frequency of 2.7% was obtained on the application of one square field pulse of 6 kV/cm for 20 usec after dielectrophoresis in an alternating field of 800 V/cm (1 MHz) for 60 sec. The fusion frequency was much higher than that obtained on polyethylene glycol-induced fusion (0.15%). This is the first case of electrofusion of *Streptomyces* protoplasts, which are as small as those of some bacteria.

Many streptomycete strains synthesize a variety of important products such as antibiotics, enzymes and antitumor agents. Recently, protoplast fusion has become a valuable technique for strain improvement, i.e., improvement in the yields$^1$ and species$^2$ of products. Polyethylene glycol (PEG) has been used almost exclusively as the fusogenic agent for the fusion of *Streptomyces* protoplasts.$^6$ This method, however, has a number of disadvantages in comparison with the newly developed electrical fusion method.$^{10}$ Therefore, the electrical fusion method is becoming a routine method for the fusion of yeast, plant and animal cells.$^{11}$ Although the use of this method is expanding in various directions, there has been little application yet of this method to the fusion of such small protoplasts as those of *Streptomyces* spp.

In this paper, we describe the electrofusion of protoplasts prepared from *Streptomyces antibioticus* a20 and *S. fradiae* f2. A fusion microchamber, constructed from a slide glass for microscopy and aluminum foil, was used to monitor the process of fusion of the *Streptomyces* protoplasts.

**MATERIALS AND METHODS**

Organisms. *S. antibioticus*, kindly provided by Kumiai Chemical Co., Tokyo, Japan, and *S. fradiae* IFO 12773 (ISP 5063) were used for the present study. These strains synthesize antimicrobial agents, i.e., *S. antibioticus* and *S. fradiae* synthesize multiomycin$^{14}$ and neomycin,$^{15}$ respectively.

From *S. antibioticus* and *S. fradiae*, amino acid auxotrophs were isolated by the method of Coats and Roeser$^{16}$ with a slight modification. A suspension of spores of each of the strains was treated with 1 mg/ml of N-methyl-N'-nitro-N-nitrosoguanidine (Sigma Chemical Co., St. Louis, U.S.A.) in 50 mM Tris-maleic acid buffer (pH 9.0) at 30°C for 30 min. After cultivation on a complete agar plate at 30°C for 1 week, the colonies appeared were examined for amino acid requirement by the auxanographic method. The strains requiring L-alanine (*S. antibioticus* a20) and L-histidine (*S. fradiae* f2) were selected as test organisms for

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Abbreviations: AC, alternating current; DC, direct current.
the following experiment. These auxotrophs were comparatively stable, and when their spores were inoculated on the minimal medium, neither of them grew at a frequency higher than $10^{-8}$.

**Media.** The media described by Okanishi et al.,\(^{17}\) Baltz,\(^{6}\) and Shirahama et al.,\(^{18}\) were used. TS broth (pH 7.2) contained 3.0% tryptoyc soy broth (Difco Laboratories, Detroit, U.S.A.) in deionized water. The glycine–TS broth, for the cultivation of cells to obtain protoplasts, comprised 0.2% (for *S. antibioticus* a20) or 0.8% (for *S. fradiae* f2) glycine in TS broth. The complete medium consisted of 1.0% maltose, 0.2% NZ amine type A (Difco Laboratories), 0.1% yeast extract, 0.1% beef extract and 2.0% agar (pH 7.3) in tap water. The minimum medium contained 1.0% glucose, 0.1% l-asparagine, 0.5% NaCl, 0.05% MgSO\(_4\), 7H\(_2\)O, 0.05% K\(_2\)HPO\(_4\) and 1.8% agar (pH 7.2) in deionized water. The minimum medium was supplemented with 50 \(\mu\)g/ml L-alanine or 70 \(\mu\)g/ml L-histidine for auxotrophic mutants (the minimal supplemented medium). Medium R3 for the regeneration of protoplasts consisted of 10.3% sucrose (0.3M), 1.0% glucose, 0.4% polypeptone, 0.4% yeast extract, 0.05% KCl, 0.81% MgCl\(_2\)-6H\(_2\)O, 0.22% CaCl\(_2\)-2H\(_2\)O, 0.02% K\(_2\)HPO\(_4\) and TES buffer (pH 7.2) in deionized water. Medium P, recommended by Okanishi *et al.*,\(^{17}\) was used as a protoplasting buffer.

**Preparation of protoplasts.** From a stock culture, one loopful of spores was transferred to a test tube containing 5 ml of TS broth and the same glass beads (approximate 4 mm), and then incubated at 30°C with shaking for the preculture. After the precultivation (2 days for *S. fradiae* f2 and 3 days for *S. antibioticus* a20), 1 ml of the culture was transferred to glycine-TS broth (5 ml) and incubated further for 24 hr on a shaker. Protoplasts were prepared according to Okanishi’s procedure\(^{17}\) with a slight modification. The mycelia harvested by centrifugation were ground in a teflon pestle tissue grinder, washed twice with medium P and then suspended in an appropriate volume of medium P. To the mycelial suspension, lysozyme (Sigma Chemical Co.) and achromopeptidase (Wako Pure Chemical Industries, Osaka, Japan) were each added at a concentration of 1 mg/ml, followed by incubation at 30°C for 50 min in a microtube. The protoplasts formed were separated from the remaining mycelia with a sterilized membrane filter (5 \(\mu\)m pore size; Gelman Science, Ann Arbor, U.S.A.), harvested by centrifugation (3,000 rpm, 10 min), washed and then suspended in a 0.3 M sucrose solution. The concentration of protoplasts was regulated by direct counting with a Thoma haemacytometer under a phase contrast microscope.

**Protoplast regeneration.** The regeneration of protoplasts was carried out according to the method of Shirahama *et al.*,\(^{18}\) except that a 0.3 M sucrose solution was substituted for the 0.555 M disodium succinate solution as the osmotic stabilizer. Serial dilutions of the protoplast suspension in medium P (1.0 ml) were placed on agar plates (φ, 90 mm) of medium R3 hardened beforehand. Then, soft agar medium R3 (0.4% low melting point agarose; Wako Pure Chemical Industries) was overlaid and mixed well, followed by incubation at 30°C for 2 weeks. The frequency of protoplast reversion to filamentous cells was 5% for *S. antibioticus* a20 and 50% for *S. fradiae* f2.

**Fusion equipment.** A Shimadzu Somatic Hybridizer SSH-1 was used as a fusion apparatus and the fusion process was observed under an Olympus phase contrast microscope, BHS-323, equipped with an Olympus camera, OM-1. In order to observe the process of fusion of small *Streptomyces* protoplasts, a fusion microchamber, which was constructed from a slide glass with two parallel (0.5 mm apart) aluminum foil (15 \(\mu\)m thick) electrodes, was used. After electrofusion in the microfusion chamber or Shimadzu SSH-C11 fusion chamber (in the case of a large scale fusion), the protoplasts were regenerated on medium R3 as described above.

**Selection of fusants.** The plates containing the colonies of regenerants were replica-plated onto agar plates of the complete medium (or the minimum supplemented medium) and ones of the minimum medium to select the hybirs, and the replica-plates were incubated at 30°C until colonies appeared. The ratio of hybrids to regenerants was defined as the fusion frequency. Since no reversion of auxotrophs could be observed after self-fusion (Table I), this method seemed to be reliable for the detection of fusants.

<table>
<thead>
<tr>
<th>Strains used for fusion treatment</th>
<th>Number of colonies appeared</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None + Alanine + Histidine</td>
</tr>
<tr>
<td><em>S. antibioticus</em> a20</td>
<td>0</td>
</tr>
<tr>
<td><em>S. fradiae</em> f2</td>
<td>0</td>
</tr>
<tr>
<td>+ <em>S. antibioticus</em> a20</td>
<td>140</td>
</tr>
</tbody>
</table>

After the application of a field pulse, the protoplasts of each strain were regenerated on R3 medium. *S. fradiae* f2 grows faster than *S. antibioticus* a20 on R3 medium. When both strains are co-cultured, the growth of *S. antibioticus* a20 is repressed by *S. fradiae* f2. The colonies appeared on R3 medium were cultured on the minimum medium and that supplemented with l-alanine (50 \(\mu\)g/ml) or l-histidine (70 \(\mu\)g/ml). The data represent the total numbers of colonies obtained in experiments with different pulse-intensities.
RESULTS AND DISCUSSION

Dielectrophoresis conditions for pearl chain formation

The process of electrofusion can be divided into 3 stages, namely: close contact of protoplasts due to dielectrophoresis in an alternating electric field; temporary breakdown of plasma membranes at the contact zone due to a unidirectional DC pulse of high intensity; and rearrangement of the lipid bilayer leading to the fusion of apposed protoplasts. The dielectrophoresis induces the formation of a chain of cells (pearl chain) of variable length, because of the attractive forces between the dipoles of adjacent cells. Preliminary experiments showed that an alternating field of 800 V/cm strength, which is much stronger than that applied to plant protoplasts by Togawa et al.,19) is required for the proper drift of small Streptomyces protoplasts. The dielectrophoretic behaviors of the protoplasts prepared from S. fradiae f2 and S. antibioticus a20 were very similar to each other, regardless of their difference in size.

As it was thought that the fusion of the two types of protoplasts was desirable for hybrid formation, conditions preferable for the formation of a two-celled pearl chain (single pair) were sought. The experimental results obtained can be summarized as follows: (1) The protoplast density favorable for pearl chain formation was about $10^8$ protoplasts/ml, and no essential differences in the pearl chain formation were observed in the concentration range of $0.7 \times 10^8$ to $3 \times 10^8$ protoplasts/ml (Fig. 1A). (2) The yield of single pairs was maximum (20%) after 60 sec dielectrophoresis at 800 V/cm and 1 MHz, and then decreased gradually as more multi-celled pearl chains formed (Fig. 1B). (3) The addition of 2.5 mM MgCl$_2$ and 2.5 mM CaCl$_2$ did not cause a significant difference in the yield of single pairs (Fig. 1C).

Lopetz et al.20) reported that the addition of cations to a yeast cell suspension in a fusion chamber equipped with pin-pin type electrodes led to a considerable decrease in the yield of single pairs. Such an effect, however, was not observed in the present experiment, as described above.

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**Fig. 1.** Effects of Protoplast Concentration, Duration of Dielectrophoresis and Inorganic Salts on the Pearl Chain Formation.

*S. fradiae f2* protoplasts were suspended in a 0.3 M sucrose solution and then subjected to dielectrophoresis in a field of 800 V/cm (1 MHz). Then the pearl chains formed were counted photomicrographically.

**A.** Effect of protoplast concentration: $-\circ-$, $0.7 \times 10^8$; $-\bullet-$, $1 \times 10^8$; and $-\triangle-$, $3 \times 10^8$ protoplasts/ml. The duration of dielectrophoresis was 30 sec.

**B.** Effects of duration: a protoplast suspension ($1 \times 10^8$ protoplasts/ml) was subjected to dielectrophoresis for 15, $-\circ-$; 30, $-\bullet-$; 60, $-\triangle-$; and 90 sec, $-\Delta-$.

**C.** Effect of inorganic salts: a protoplast suspension ($1 \times 10^8$ protoplasts/ml) was subjected to dielectrophoresis for 60 sec with $-\circ-$ or without $-\bullet-$ 2.5 mM MgCl$_2$ and CaCl$_2$. 
Effect of pulse field strength on protoplast breakdown

For protoplast fusion, the breakdown of the plasma membrane through electric stimulation at the contact zone with an apposed protoplast is essential. Therefore, the electrical breakdown of *Streptomyces* protoplasts by field pulses of various intensities was measured by counting both the remaining protoplasts and the regenerated colonies. Figure 2 shows that the amount of protoplasts remaining unbroken decreased with increasing pulse intensity and that the addition of MgCl₂ and CaCl₂ accelerated the electrical breakdown. The survival rate of protoplasts determined by direct counting (Fig. 2A) under a microscope and that determined by counting the regenerated colonies (Fig. 2B) agreed with each other. The field intensity required to decompose half of the *S. fradiae f2* protoplasts was 7 kV/cm in a 0.3 M sucrose solution and 5 kV/cm in a sucrose solution containing 2.5 mM MgCl₂ and CaCl₂, whereas, in the case of *S. antibioticus a20*, it was 9 kV/cm and 7 kV/cm, respectively. Thus, the addition of inorganic ions to the fusion medium accelerated the protoplast destruction. This salt effect may be explained by the increase in electric power caused by the increased conductivity.

Interspecific protoplast fusion between *S. fradiae* and *S. antibioticus*

Interspecific protoplast fusion was attempted between *S. fradiae f2* and *S. antibioticus a20*. On the basis of the experimental results described above, the concentration of protoplasts, the intensity of the alternating electric field and the time for dielectrophoresis were fixed at 1 x 10⁸ protoplasts/ml, 800 V/cm strength and 1 MHz frequency, and 60 sec, respectively.

Since the field pulse intensity required to break 50% of the protoplasts was different for the two organisms, as described above, the effect of the field pulse intensity on the fusion frequency (production rate of hybrids) was measured. The intensity of the field pulse was changed in the range of 4 to 10 kV/cm, and

![Graph A](image1)

**Fig. 2. Effects of Pulse Strength and Inorganic Salts on the Breakdown of Protoplasts.**

A protoplast suspension (1 x 10⁸ protoplasts/ml) was exposed to DC fields of various strengths for 20 μsec. Then, the number of remaining protoplasts were determined by (A) direct counting under a microscope and (B) counting of regenerated colonies. —O—, 0.3 M sucrose solution; —#—, 0.3 M sucrose solution containing 2.5 mM MgCl₂ and CaCl₂.

![Graph B](image2)

**Fig. 3. Effects of Inorganic Salts and the Mixing Ratio of Parent Protoplasts on the Fusion Frequency.**

A mixture (1 x 10⁸ protoplasts/ml) of *S. antibioticus a20* (Ala⁻) and *S. fradiae f2* (His⁺) protoplasts was subjected to dielectrophoresis (800 V/cm, 1 MHz, 60 sec), and then one square field pulses of various intensities were applied. The fusion products were regenerated as described in the text.

A. Effects of inorganic salts: the mixing ratio of the protoplasts was 1:1. —O—, 0.3 M sucrose solution; —#—, 0.3 M sucrose solution containing 2.5 mM MgCl₂ and CaCl₂.

B. Effect of mixing ratio: the mixing ratios of the protoplasts (*S. antibioticus a20: S. fradiae f2*) were 1:1 (—O—), 3:1 (—#—) and 5:1 (—△—).
TABLE II. DISTRIBUTION OF ANTIBIOTIC PRODUCERS AND ANTIBIOTIC RESISTANT COLONIES AMONG REGENERANTS

<table>
<thead>
<tr>
<th>Strains used for fusion treatment</th>
<th>Resistant No./Total</th>
<th>Antibiotic producer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>N</td>
</tr>
<tr>
<td>S. antibioticus a20</td>
<td>4961/4961</td>
<td>0</td>
</tr>
<tr>
<td>S. fradiae f2</td>
<td>4490/4490</td>
<td>0</td>
</tr>
<tr>
<td>S. antibioticus a20 + S. fradiae f2</td>
<td>8'/140</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3/140</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>129/140</td>
<td>0</td>
</tr>
</tbody>
</table>

The regenerants obtained in the experiment shown in Table I were used. Antibiotic producers were detected by bioautography according to the procedure of Coats and Roeser.16) Antibiotic resistant colonies were detected on the complete medium containing 100 μg/ml of antibiotic. UI, unidentified antibiotic; ND, not detected; M, multithiomyein; N, neomycin; * denotes resistant.

* Two of these produced both N and UI.

MgCl₂ and CaCl₂ were each added to the isotonic medium at a concentration of 2.5 mM. After the application of a high DC voltage for electrofusion, a low AC voltage was applied (400 V/cm, 1 MHz) for 60 sec to the protoplast mixture as follow-up dielectrophoresis to promote sufficient fusion. As shown in Fig. 3A, the addition of 2.5 mM MgCl₂ and CaCl₂ caused an increase in fusion frequency. The maximum fusion frequency of 1.8% was obtained at a field strength of 8 kV/cm in the plain isotonic medium, whereas in the salt-containing isotonic medium, it was about 2.7% at 6 kV/cm. This difference may be attributed to the increase in electric energy and the stabilizing effect of salt ions on the plasma membrane.17) Most of the fusants exhibited phenotypes similar to that of S. fradiae f2, but differed individually in antibiotic productivity and antibiotic resistance, as shown in Table II. Electrofusion seemed to be a very effective means of cell fusion on comparison of the fusion frequency with that of polyethylene-glycol-induced fusion (0.15%*).

Since there was a considerable difference in the size of protoplasts between S. fradiae f2 (ϕ, 3 μm) and S. antibioticus a20 (ϕ, 1 μm), the effect of the mixing ratio of the protoplasts on the frequency of fusion was investigated. As shown in Fig. 3B, the mixing ratio of 1:1 gave the most effective interspecific fusion.

This is the first case of the electrofusion of Streptomyces protoplasts. Recently, a genetic engineering method was applied by Hopwood et al.21) to the production of hybrid antibiotics. Since the electric cell-fusion technique, however, is a simple and reliable method, it will still attract much attention. At present, detailed studies on the fusants are in progress in our laboratory.

Acknowledgments. We thank Dr. R. Kobayashi of Kumiai Chemical Co. for providing Streptomyces strain and Mr. Y. Togawa of Shimadzu Co. for his interest.

REFERENCES


* Unpublished data obtained using the method described by Shirahama et al.18