IMPROVEMENT OF MACROLIDE ANTIBIOTIC-PRODUCING STREPTOMYCETE STRAINS BY THE REGENERATION OF PROTOPLASTS

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Three macrolide antibiotic-producing strains, the spiramycin producer *Streptomyces ambofaciens*, the tylosin producer *Streptomyces fradiae*, and the cirramycin producer *Streptomyces cirratus* easily formed protoplasts when treated with lysozyme in hypertonic medium. Each type of protoplast was regenerated to a mycelial form at a frequency of 90 to 100% in regeneration medium supplemented with a plasma expander.

In the spiramycin producer *S. ambofaciens* and the tylosin producer *S. fradiae*, antibiotic productivities of the regenerated progeny were drastically changed. Some regenerated progeny from the three strains gave higher production. One from *S. ambofaciens* showed about twice the productivity of the original strain and one obtained after two rounds of protoplast regeneration from *S. fradiae* produced about three times as much as the original strain.

Conditions suitable for the preparation and regeneration of streptomycete protoplasts were developed by OKANISHI *et al.*[1] These procedures have been modified and improved by SHIRAHAMA *et al.*[2] and BALTZ and MATSUSHIMA.[3] Subsequently, two very important techniques, protoplast fusion[4] and transformation[5]/transfection,[6] useful for genetic manipulation, have been developed and refined.

Increases in antibiotic productivity have generally been achieved by mutation through the use of chemical mutagens and/or ultraviolet irradiation and selection of high producing strains. ICHIKAWA *et al.*[7] obtained a genetic recombinant with higher kasugamycin productivity by crossing auxotrophic mutants of *Streptomyces kasugaensis*. This method needs two complementary types of auxotrophic mutants for selection among recombinants. Not all of the recombinant progeny show increased antibiotic productivity.

The authors[8] and OKANISHI[9] have observed morphological changes and restoration of antibiotic productivity in regenerated protoplasts. Genetic variation of this type was relatively frequent with streptomycetes. In this paper, we report on the efficiency of regeneration obtained by using a regeneration medium supplemented with plasma expander. We also demonstrate the possibility of improving macrolide antibiotic-production by selection among strains regenerated from protoplasts.

Material and Methods

Organisms

Three 16-membered macrolide antibiotic-producing strains, the spiramycin producer *Streptomyces ambofaciens* KA-1028 (ISP 5053), the tylosin producer *Streptomyces fradiae* KA-427 (C-373), and the cirramycin producer *Streptomyces cirratus* KA-412 (JTB-3) were used throughout the experiments.

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Media

Strains were maintained on slants of 4 g of yeast extract (Difco), 10 g of malt extract (Difco), 4 g of soluble starch (Difco), 20 g of Bacto-agar (Difco), and distilled water to one liter (adjusted to pH 7.5 with 2 N KOH prior to autoclaving). The basal regeneration medium (basal medium) contained 171 g of sucrose, 0.25 g of K_2SO_4, 10.12 g of $MgCl_2 \cdot 6H_2O$, 10 g of glucose, 1.0 g of yeast extract (Difco), 2 ml of trace element solution, 22 g of Bacto-agar (Difco), and distilled water to 770 ml. After autoclaving, the following solutions were added to each 77 ml: 8 ml of $CaCl_2 \cdot 2H_2O$ (3.68%), 1 ml of KH_2PO_4 (0.5%), 3 ml of L-asparagine·H_2O (6.0%), 10 ml of *N*-2-hydroxyethylpiperazine-*N'*-2-ethansulfonic acid (HEPES buffer) (0.25 M, pH 7.2), and 0.5 ml of 1 N NaOH. Hypertonic soft agar overlays (3 ml) contained sucrose, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, and HEPES buffer at the concentrations used above, plus 0.65% Bacto-agar (Difco). Hypertonic soft agar was kept at 42°C before use. Spiramycin[8]- and tylosin[10]-production media were described previously. Cirramycin-production medium consisted of 30 g of glycerol, 5 g of glucose, 5 g of yeast extract, 5 g of peptone, 3 g of NaCl, 1 g of $NaNO_3$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 100 ml of 0.25 M HEPES buffer (pH 7.2), and distilled water to one liter.

Preparation and Regeneration of Protoplasts

Conditions for the cultivation and preparation of protoplasts were as described previously[8] except that mycelia were grown at 27°C instead of 32°C. Protoplasts were sedimented by centrifugation at 500 to 600 × *g* for 10 minutes. Protoplasts were regenerated as described by BALTZ and MATSUSHIMA.[8] The conditions used for protoplast fusion were those reported by BALTZ[11] except that polyethylene glycol #4,000 was substituted for polyethylene glycol #6,000.

Antibiotic Production

The antibiotic productivity of each colony was examined by the agar-piece method using *Micrococcus luteus* PCI 1001 as the test organism. Antibiotic titers in the culture broths were measured by a paper disc method using standard curves obtained with authentic antibiotic samples. In addition, the products were detected by silica-gel thin-layer chromatography.

Chemicals

Lysozyme (five times crystallized) was purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan), bovine serum albumin (Fraction V, fatty acid-free) was from Sigma Chemical Co. (Saint Louis, U.S.A.), and polyvinyl pyrrolidone (K-90) was from Wako Chemical Industries, Ltd. (Osaka, Japan).

Results and Discussion

Effect of Plasma Expanders on Regeneration Frequency

The protoplasts of some macrolide antibiotic-producing streptomycete strains regenerated the mycelial form at a relatively high frequency. However, the regeneration frequencies of others were often low and protoplasts of some auxotrophic and idiotrophic mutants regenerated at quite low frequencies. The plasma expanders, horse serum, bovine serum albumin, and polyvinyl pyrrolidone are often used in regeneration media, and such substances stimulate regeneration in *Bacillus subtilis*.[12,13] Initially, the effects of plasma expanders on streptomycete regeneration frequency were examined by supplementing the basal regeneration medium with 0.02% bovine serum albumin (Fraction V) or 0.03 to 0.3% polyvinyl pyrrolidone (K-90). As shown in Table 1, the regeneration frequencies of *S. ambofaciens* and *S. fradiae* on basal medium supplemented with 0.03% of polyvinyl pyrrolidone were about twice as high as on basal medium. However, the frequencies with 0.3% polyvinyl pyrrolidone showed smaller increases. There was little effect of polyvinyl pyrrolidone on *S. cirratus*, but no decrease was observed. Regeneration frequencies of all three strains on basal medium supplemented with 0.02% of bovine serum albumin were higher than on any other media tested. Moreover, the concentration of bovine serum albumin was not as critical as it was for polyvinyl pyrrolidone (data not shown).

Table 1. Effect of plasma expanders on regeneration frequency of protoplasts from three macrolide antibiotic-producing streptomycetes.

Agar medium	Regeneration frequency (%)		
	S. ambofaciens	*S. fradiae*	*S. cirratus*
Basal medium*	41	35	75
〃 +PVP** (0.03%)	84	81	79
〃 +PVP (0.3%)	63	55	81
〃 +BSA*** (0.02%)	100	92	102

* Composition was described in "Material and Methods".
** Polyvinyl pyrrolidone (K-90, MW. 360,000).
*** Bovine serum albumin (Fraction V).

These media containing plasma expanders can be adopted to regenerate many streptomycete protoplasts. A medium with the same components as the basal regeneration medium except that yeast extract is omitted and polyvinyl pyrrolidone (0.03%) is added is useful for selecting prototrophs after protoplast fusion (data not shown).

Variation in Macrolide-antibiotic Productivity Caused by the Regeneration of Protoplasts

In the three macrolide antibiotic-producing strains used in these experiments, some progeny after regeneration of protoplasts were different in morphology from the original strains. Most of them produced more aerial mycelium. Because of this evidence of genetic variation, we examined the antibiotic productivities of regenerated protoplasts. Progeny regenerated on basal medium supplemented with 0.02% of bovine serum albumin were used in these experiments.

Histograms of antibiotic productivity showed that the distribution of this property was changed drastically in regenerated protoplasts of *S. ambofaciens* and *S. fradiae* (Figs. 1 and 2). Moreover, to examine whether the productivity of regenerated protoplasts is stable, they were replicated to yeast-malt extract agar plates and each progeny (second generation) that appeared was assayed for antibiotic productivity. Some progeny showed instability in the second generation (Figs. 1 and 2). In the case of *S. cirratus* (Fig. 3), the increased antibiotic productivity of regenerated protoplasts was lower than in the other two strains. It is also more difficult to isolate high producing progeny by mutation from *S. cirratus* than from *S. ambofaciens* and *S. fradiae* (unpublished observation).

We examined whether the high producing progeny were formed during regeneration of protoplasts *per se* or from genetic recombination induced by fusion of protoplasts. As shown in Figs. 1, 2, and 3, the frequencies with which high producing strains appeared after simple regeneration of protoplasts were very similar to those found when protoplast fusion was promoted by adding polyethylene glycol. We also observed that intraspecific recombinants appeared at a frequency of about 10^{-1} in fusion between protoplasts from complementary types of auxotrophic mutants in the presence of polyethylene glycol, but were not detected without polyethylene glycol (data not shown). In *S. cirratus*, high antibiotic producers were more frequent after simple regeneration than when the fusion step was included. Consequently, we suspected that the appearance of high producing progeny was due to genetic variation associated with regeneration of the protoplasts rather than by the result of genetic recombination following protoplast fusion.

To determine whether regenerated protoplasts produced the same antibiotics as the original strains,

Fig. 1. Distribution of spiramycin productivity in the original strain, regenerated protoplasts, their second generation progeny, and strains obtained after protoplast fusion.

Those strains indicated by shaded areas were used to measure spiramycin production in liquid medium (Table 2).

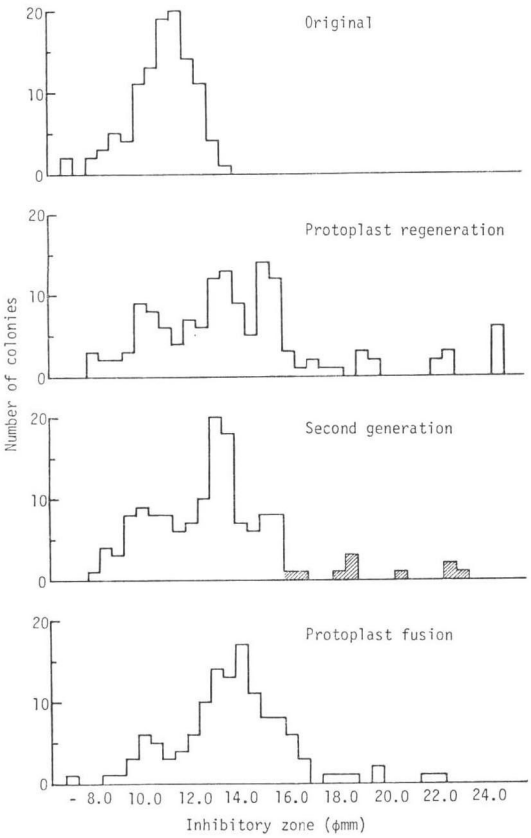
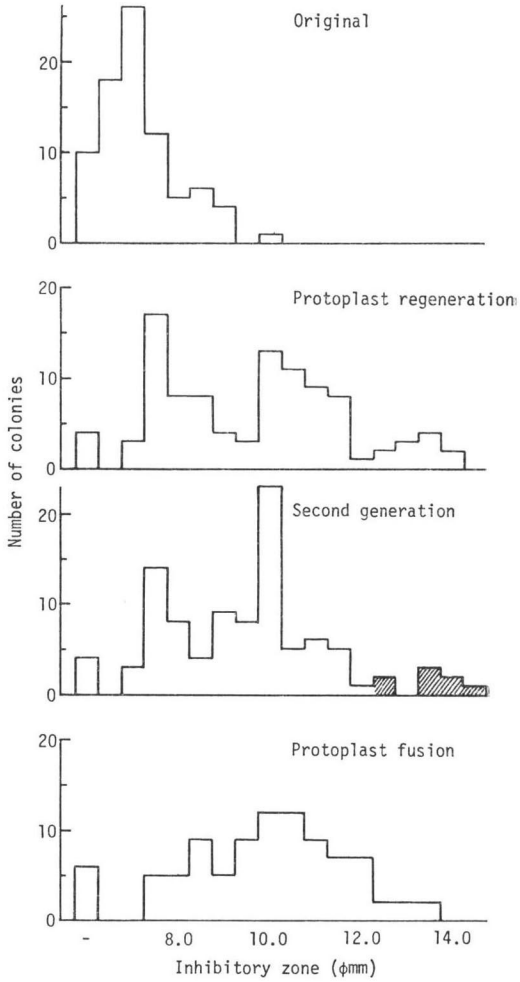


Fig. 2. Distribution of tylosin productivity in the original strain, regenerated protoplasts, their second generation progeny, and strains obtained after protoplast fusion.

Those strains indicated by shaded areas were used to measure tylosin production in liquid medium (Table 3).



we examined the products of *S. ambofaciens* and *S. fradiae* strains in which antibiotic productivity was changed drastically by regeneration of protoplasts. As shown in Fig. 4, PR-7-2 and PR-4, which were the highest yielding progeny, produced mainly tylosin (TYL) and spiramycins (SPM), respectively. The pattern of products in each case was the same as in the original strain. Similar results were obtained with other regenerated protoplast strains—*i.e.* each gave the same pattern of products as the parent (data not shown).

To determine quantitatively the productivity of regenerated protoplasts, we measured antibiotic production by relatively high producing progeny (those indicated by shaded areas in Figs. 1 and 2) grown in liquid media. Except for strain PR-3, spiramycin production by regenerated protoplasts was higher than that of the original strain (Table 2). In particular, strain PR-4 yielded about twice as much. Tylosin

Fig. 3. Distribution of cirramycin productivity in the original strain, regenerated protoplasts, their second generation progeny, and strains obtained after protoplast fusion.

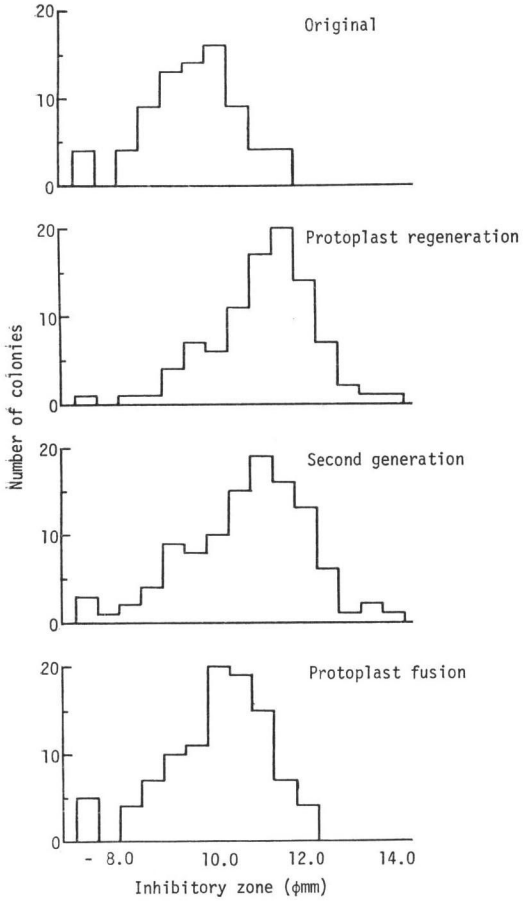


Fig. 4. Silica-gel thin-layer chromatograms of products from *S. fradiae* (A) and *S. ambofaciens* (B).

Chromatograms were developed with ethyl acetate - diethylamine (95: 5, A) or chloroform - methanol - 1.5 N NH_4OH (2: 1: 1, bottom layer, B).

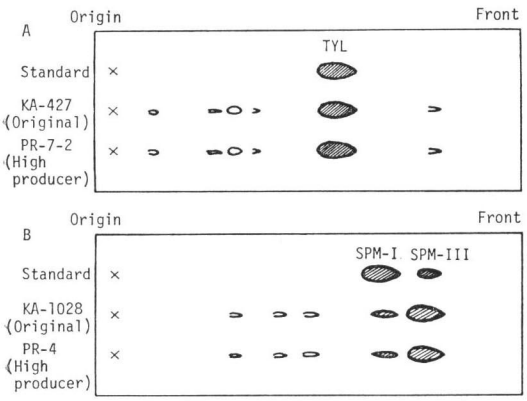


Table 2. Spiramycin production in liquid medium by the original strain of *S. ambofaciens* and by regenerated protoplasts.

Strain	Spiramycin production* (μg/ml)	Strain	Spiramycin production* (μg/ml)
KA-1028 (Original)	52	PR-5	98
		PR-6	68
PR-1	74	PR-7	101
PR-2	87	PR-8	57
PR-3	46	PR-9	77
PR-4	113	PR-10	89

* This value represents maximum antibiotic titer.

Table 3. Tylosin production in liquid medium by the original strain of *S. fradiae* and by regenerated protoplasts.

Strain	Tylosin production** (μg/ml)	Strain	Tylosin production** (μg/ml)
KA-427 (Original)	70	PR-6	180
		PR-7	184
PR-1	72	PR-8	58
PR-2	54	PR-7-1*	198
PR-3	115	PR-7-2*	253
PR-4	96	PR-7-3*	221
PR-5	140	PR-7-4*	227

* These strains were obtained by regenerating protoplasts of strain PR-7.

** This value represents maximum antibiotic titer.

production by regenerated protoplasts, except for strains PR-2 and 8, was also higher than that of the original strain (Table 3). Moreover, the productivity of regenerated protoplasts derived from strain PR-7, which gave the highest titer after the first round of protoplast regeneration was extremely high. Strain PR-7-2 made about 3.6 times as much as antibiotic as the original strain (Table 3). We assume that the increased productivity of regenerated protoplasts originates from genetic variation, as also do the changes in morphology,[8,9] the increases in antibiotic resistance,[14] the restoration of antibiotic productivity[8,9] and the elimination of extrachromosomal elements[15] all of which have been observed after regeneration of streptomycete protoplasts.

BALTZ[16] reported that several percent of the colonies obtaind by regenerating protoplasts of *S. fradiae* produced very low to undetectable levels of tylosin. However, our results show that high antibiotic-producing strains can be obtained by regeneration of protoplasts.

The overall results indicate that regeneration of protoplasts is a useful method for improving antibiotic production in streptomycetes.

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