INTERSPECIFIC PROTOPLAST FUSION AMONG MACROLIDE-PRODUCING STREPTOMYCETES

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(Received for publication March 29, 1984)

From auxotrophic and idiotrophic mutants of *Streptomyces fradiae* (tylosin producer) and *Streptomyces* sp. AM 4900 (pikromycin producer) or *Streptomyces narbonensis* (narbomycin producer), prototrophic fusants were obtained at a low frequency by the protoplast fusion technique.

In the cross of *S. fradiae* 261-27E (mycaminose-idiotroph, *ilv*) and *Streptomyces* sp. AM 4900 N3-4, (pikronolide-idiotroph, *arg*), an unstable prototrophic fusant, strain No. 14, produced a macrolide antibiotic which was not produced by the wild type, parent strains, and the productivity was lost within a few times transfer. It was concluded that the fusant was not a recombinant, but a heterokaryon. On the other hand, relatively stable fusants were obtained from the cross of *S. fradiae* TBM (mycaminose-idiotroph, *met*) and *S. narbonensis* NA12US3 (narbonolide-idiotroph, *his*, *str*) at a frequency of 3.2×10^{-5}. One of the prototrophic fusants produced narbomycin, which is believed to be due to a result of interspecific recombination.

Several studies on interspecific recombination in antibiotic-producing *Streptomyces* species have been reported[1~4]. It is considered to be particularly suitable for streptomycete strain improvement. Recently, interspecific recombinants were efficiently obtained by protoplast fusion techniques[4]. To examine the possibility that hybrid antibiotic molecules are obtained by the co-operation of segments of the genes of strains producing different antibiotics, we have tried interspecific protoplast fusion between 14- and 16-membered macrolide antibiotics-producing streptomycete strains.

We have reported that 5-*O*-desosaminylprotylonolide (M-4365 G_1), composed of the 16-membered lactone ring protylonolide and desosamine which, in general, is a component of 14-membered macrolide antibiotics, was obtained by the "hybrid biosynthesis" technique using cerulenin, an inhibitor of fatty acid and polyketide biosyntheses[5]. The antibiotic M-4365 G_1 is produced by *Micromonospora capillata*[6]. Since the organism grows slowly and the antibiotic productivity is low, the aim of our investigation is to provide a *Streptomyces* strain producing M-4365 G_1. After we obtained the complementary idiotrophic mutants of macrolide producers, mycaminose-idiotroph of the tylosin producer *Streptomyces fradiae* and pikronolide-idiotroph of the pikromycin producer *Streptomyces* sp. AM 4900 or narbonolide-idiotroph of the narbomycin producer *Streptomyces narbonensis*, interspecific fusion was performed and the properties of the fusants derived from the above idiotrophic mutants were examined.

In this paper, we describe the properties of prototrophic fusants isolated from protoplast fusion between *S. fradiae* and *Streptomyces* sp. AM 4900 or *S. narbonensis*.

Materials and Methods

Strains

Strains used in interspecific crosses and DNA-DNA hybridization are listed in Tables 1 and 2. Auxotrophic markers, *ilv*, *arg*, *met* and *his*, indicate requirements of isoleucine/valine, arginine, methio-

Table 1. Mutants used in genetic crosses.

Strain	Original strain	Treatment	Genetic marker
Streptomyces fradiae N29	C373	NTG*	*met*
261-27	C373	NTG*	Mycaminose-idiotroph, prototroph
261-27E	261-27	NTG*	Mycaminose-idiotroph, *ilv*
TBM	N29×261-27	Protoplast fusion	Mycaminose-idiotroph, *met*
S. narbonensis NA8	KCC S-0147	NTG*	Narbonolide-idiotroph, *arg*
NA12US3	KCC S-0147	NTG* and UV**	Narbonolide-idiotroph, *his*, *str*
Streptomyces sp. AM-4900 N3-4	AM-4900	NTG*	Pikronolide-idiotroph, *arg*

* NTG: *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine.
** UV: Ultraviolet.

Table 2. DNA homology between *S. fradiae* and producers of desosamine-containing macrolides.

Strain	Homology index	Antibiotic produced
Streptomyces fradiae 261-27E (derived from *S. fradiae* C-373*)	(100)	Protylonolide
S. felleus KCC S-0031**	82	Pikromycin
S. narbonensis KCC S-0147**	93	Narbomycin
Streptomyces sp. AM-4900***	63	Pikromycin
Streptomyces sp. AM-7650***	83	Methymycin, neomethymycin, narbomycin, pikromycin
Streptomyces sp. AM-7762***	55	Neomethymycin, YC-17, narbomycin, pikromycin

* This strain was obtained from Dr. C. W. PETTINGA of Eli Lilly & Co.
** These strains were obtained from Dr. A. SEINO of Kaken Chemical Co. Ltd.
*** These strains were isolated from a soil sample in our laboratory.

nine and histidine for growth, respectively. The marker *str* indicates resistance to 5 μg/ml of streptomycin.

Media

All strains were maintained on the slants of yeast extract - malt extract - soluble starch agar. Minimal medium was used for selection of prototrophs. Antibiotic production medium consisted of 15 g of glycerol, 1 g of yeast extract, 5 g of Casamino Acids, 2 g of L-asparagine·monohydrate, 3 g of NaCl, and 1 liter of distilled water (adjusted to pH 7.4 with 2 N KOH before autoclaving). Antibiotic production agar medium for the agar-piece method was solidified by adding 20 g of Bacto-agar (Difco) per liter to the above medium. Minimal regeneration medium for protoplast regeneration was described by IKEDA *et al.*[7]. The medium for the preparation of labelled total DNA was described by IKEDA *et al.*[8].

Isolation of Prototrophic Progeny by Interspecific Crosses

The preparation of protoplasts was carried out as described previously[7] except that the cultivation of mycelia was performed at 27°C instead of 32°C. The conditions for protoplast fusion were as described previously[7] except that polyethylene glycol #1,000 was substituted for #4,000. When only minute colonies difficult to be picked up grew on the regeneration medium, the hypertonic soft agar layer in which prototrophic progeny grew was scraped off and then homogenized with an Ultra Turrax homogenizer (Ika-Werk, Lank & Kunkel KG, West Germany). The homogenate was passed through cotton wool and the mycelium was washed with sterile distilled water. An aliquot (0.1 ml) of the suspension of homogenized mycelia was spread onto a minimal medium plate and the plate was incubated at 27°C for 5 days.

Detection of Antibiotics

The antibiotic productivity of prototrophic progeny grown on a minimal medium was assayed by the conventional agar-piece method using a macrolide-sensitive mutant of *Escherichia coli* as the test organism. An antibiotic-producing progeny was cultivated in a 500-ml Sakaguchi flask containing 100 ml of the production medium for 4 days at 27°C. After the cultured broth was adjusted to pH 8 to 9 with aqueous ammonia, fermentation products were extracted with 100 ml of benzene and the extract was evaporated to dryness. The crude residue from the extract was analyzed by silica gel TLC. Fermentation products were detected by bioautography using a macrolide-sensitive mutant of *E. coli*.

Preparation of Labelled DNA and DNA-DNA Hybridization

For the preparation of labelled DNA, *S. fradiae* 261-27E (mycaminose-idiotroph, *ilv*) was inoculated into a medium[8] supplemented with 50 μg of isoleucine and valine and 10 μCi of [*methyl*-^{3}H]thymidine per ml, and then incubated for 24 hours at 27°C. The isolation of total DNA was performed as described previously[8]. The DNA was sheared by sonication to fragments of 0.5 to 1.0 kbp and DNA-DNA hybridization was performed by the S1 nuclease method[9].

Co-fermentation

S. fradiae 261-27E (mycaminose-idiotroph, *ilv*) and *Streptomyces* sp. AM 4900 N3-4 (pikronolide-idiotroph, *arg*) were grown separately in Trypticase Soy broth (BBL) for 2 days at 27°C. After the cultures were homogenized and passed through cotton wool, each culture was diluted to an absorbance of 0.3 at 600 nm with sterile distilled water. One milliliter of the homogenized culture of *S. fradiae* 261-27E and 0.01 ml of *Streptomyces* sp. AM 4900 N3-4, were inoculated into a 100 ml of production medium in a 500-ml Sakaguchi flask, and incubated for 3 days at 27°C.

Chemicals

Egg white lysozyme was purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan), polyethylene glycol #1,000 was from Nakarai Chemical Co., (Kyoto, Japan), polyvinyl pyrrolidone (K-90) was from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan), [*methyl*-^{3}H]thymidine (42 Ci/mmol) was from The Radiochemical Centre Amersham (England), and authentic samples of tylosin and M-4365 G_1 were generous gifts from Eli Lilly & Co. and Dr. T. OKUDA of Tanabe Seiyaku Co., Ltd., respectively. Protylonolide, pikromycin and narbomycin were isolated from the cultured broths of mycaminose-idiotroph of *S. fradiae*, *Streptomyces* sp. AM 4900, and *S. narbonensis*, respectively.

Results

Protoplast Fusion between *S. fradiae* 261-27E (Mycaminose-idiotroph, *ilv*) and *Streptomyces* sp. AM 4900 N3-4 (Pikronolide-idiotroph, *arg*)

The suspension (0.32 ml) containing about 4.7×10^{10} protoplasts of each of the above strains was carefully added to 1.68 ml of 50% (w/v) polyethylene glycol #1,000 using a Pasteur pipette and allowed to stand at room temperature for 1 minute. Then the suspension was diluted with P medium 2[8]. For regeneration to mycelial form, an aliquot (0.1 ml) of a suspension of washed protoplasts (total volume 1 ml) was put onto the minimal regeneration medium which does not permit the regeneration of the parent strains to mycelial form but does that of nutritionally complementing fusants, and then 3 ml of molten hypertonic soft agar (42°C) was poured onto the basal layer of the minimal regeneration medium. Innumerable minute colonies, the diameters of which were less than 0.2 mm, appeared after incubation at 27°C for 14 days, however, these minute colonies did not grow to larger size after additional incubation for 14 days. After 28 days the hypertonic soft agar layer was scraped off and spread onto a minimal medium. From the hypertonic soft agar layer of one plate, 4.2×10^3 prototrophic colonies appeared. When the colonies were transplanted onto minimal and complete media and incubated at 27°C, they did not grow on minimal medium and the colonies grown on a complete medium were of two types having the characteristics of the parental auxotrophic strains, respectively. These results showed that the prototrophic fusants easily segregated to individual parental auxotrophic strains.

Fig. 1. Distribution of antibiotic productivity of colonies obtained by protoplast fusion of *S. fradiae* 261-27E and *Streptomyces* sp. AM 4900 N3-4.

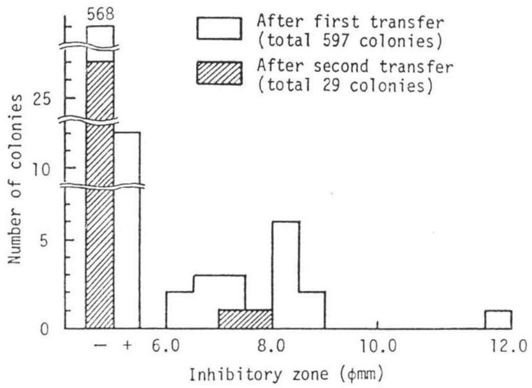
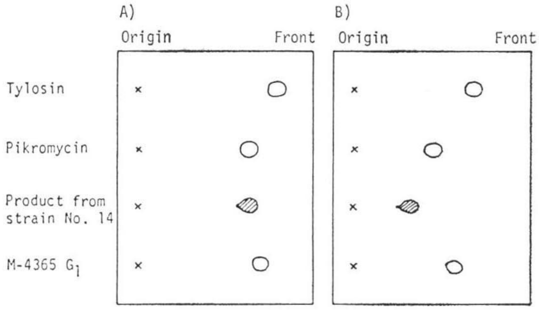


Fig. 2. Bioautograms of the product from strain No. 14.

Silica gel TLC was developed with A) EtOAc - EtOH - 15% NH_4OAc (pH 9.6) (9:4:8, upper layer), B) $CHCl_3$ - EtOH - 15% NH_4OAc (pH 7.0) (85:15:1).



Characterization of Fusant Strain No. 14 and Its Fermentation Products

We examined whether the prototrophic fusants produced antibiotics. Protylonolide which is the fermentation product of *S. fradiae* 261-27E (mycaminose-idiotroph, *ilv*), possesses no antimicrobial activity and also *Streptomyces* sp. AM 4900 N3-4 produces no antibiotics active against a macrolide-sensitive mutant of *E. coli*. As shown in Fig. 1, about 4.9% of primary prototrophic fusants produced antimicrobial products after the first transfer; however, after the second transfer the colonies did not produce any antimicrobial products except for two fusants. These two antibiotic-producing fusants, No. 12 and 14, were examined for antibiotic productivity in a liquid production medium, and antibiotic activity was detected in the cultured broth of fusant No. 14, but not No. 12. The extract of the cultured broth of the fusant No. 14 showed an inhibitory zone on an agar plate containing a macrolide-sensitive mutant of *E. coli*, but did not with an erythromycin-resistant *Staphylococcus aureus* strain (data not shown). The fermentation product of the fusant No. 14 had a different mobility from that of an authentic sample of M-4365 G_1 on silica gel TLC (Fig. 2). The antibiotic productivity was rather low and easily lost after further transfer. The progeny, which then were unable to produce antibiotic, on transfer segregated into two types of colonies, showing the same morphology and nutritional requirements as the original mutant strains, respectively. The above data indicate that the fusant No. 14 is not a recombinant, but a heterokaryon. The possibility was also considered that the antibiotic detected in the culture broth of the fusant was brought by co-fermentation between two types of segregated organisms. However, this possibility was rejected since in the co-fermented broth of the parent strains, *S. fradiae* 261-27E and *Streptomyces* sp. AM 4900 N3-4, M-4365 G_1 was detected but the antibiotic produced by fusant No. 14 could not be detected.

Thus, we believe that the fusant No. 14 was a heterokaryon but not a recombinant, and that genetic recombination between *S. fradiae* and *Streptomyces* sp. AM 4900 did not occur at a significant frequency. Consequently, we examined the DNA homology between *S. fradiae* and streptomycete strains maintained in our laboratory which produce 14-membered macrolide antibiotics containing desosamine in their molecules (Table 2). Among the strains tested, *S. narbonensis* (narbomycin producer) possessed the highest homology with *S. fradiae*. Therefore, we attempted the construction of recombinants between *S. fradiae* and *S. narbonensis*.

Table 3. Frequency of recombination after protoplast fusion.

Cross	Selective marker	Frequency of occurrence of prototroph
S. fradiae × *S. narbonensis*		
261-27E (*ilv*) × NA8 (*arg*)	Prototroph	(Many minute colonies)*
261-27E (*ilv*) × NA12US3 (*his*)	Prototroph	7.6×10^{-7}
TBM (*met*) × NA8 (*arg*)	Prototroph	(Many minute colonies)*
TBM (*met*) × NA12US3 (*his*)	Prototroph	3.2×10^{-5}

* These colonies seem to be heterokaryons.

Fig. 3. Colonies which appeared after the treatment of protoplasts of *S. fradiae* TBM (mycaminose-idiotroph, *met*) and *S. narbonensis* NA12US3 (narbonolide-idiotroph, *his*, *str*) with (right) and without (left) polyethylene glycol #1,000 on minimal regeneration medium.

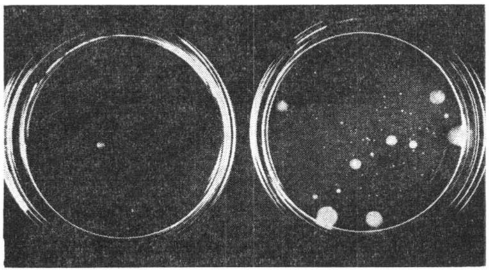
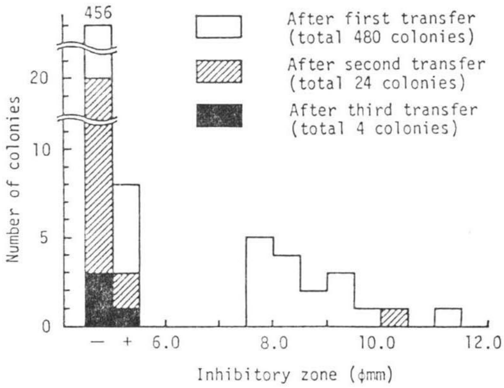


Fig. 4. Distribution of antibiotic productivity of colonies obtained by protoplast fusion of *S. fradiae* TBM and *S. narbonensis* NA12US3.



S. fradiae TBM (Mycaminose-idiotroph, *met*) × *S. narbonensis* NA12US3 (Narbonolide-idiotroph, *his*, *str*)

The results of crosses when 2.0×10^9 protoplasts of each strain were fused are summarized in Table 3. In 2 of 4 crosses, only minute colonies grew as in the cross of *S. fradiae* and *Streptomyces* sp. AM 4900. However, in the cross between *S. fradiae* TBM (mycaminose-idiotroph, *met*) and *S. narbonensis* NA12US3 (narbonolide-idiotroph, *his*, *str*), colonies as large as the usual regenerated colonies from protoplasts of both parent strains grew at the frequency of 3.2×10^{-5} on the minimal regeneration medium (Fig. 3). Under the same conditions, the spontaneous prototrophic reversion rates of *S. fradiae* and *S. narbonensis* were 1.6×10^{-7} and 1.0×10^{-7}, respectively, and no streptomycin-sensitive revertants were detected among 1.2×10^3 prototrophic revertants from *S. narbonensis* (*his*, *str*). Moreover, when the mixture of protoplasts of both parent strains was plated without prior polyethylene glycol #1,000 treatment and incubated at 27°C, only few colonies grew on the regeneration medium. Thus almost all the colonies which appeared on the minimal regeneration medium after polyethylene glycol treatment are obviously fusants. Eighty-seven percent of the primary fusants retained their prototrophy even after the third transfer. Moreover, 477 fusants among 480 prototrophic ones were sensitive to streptomycin.

Characterization of the Fermentation Product of Fusant No. 4-7

The antibiotic productivity of the fusants was examined as described above. As shown in Fig. 4, about 5.0% of the primary prototrophic fusants produced antibiotic activity, however, in this case also the productivity was lost during several times transfer of subculture. The antibiotic-producing fusant No. 4-7 resembled morphologically *S. narbonensis*, and was sensitive to streptomycin and prototrophic even after several times transfer. This fusant also produced an antibiotic in a liquid medium. The antibiotic was identified as narbomycin, which was produced by a wild type strain of *S. narbonensis*. The narbomycin productivity of the fusant was lost after two times transfer, whereas, the narbomycin productivity of antibiotic-producing revertants obtained at a frequency of 1.7×10^{-3} by the treatment of *S. narbonensis* NA12US3 (narbonolide-idiotroph, *his*, *str*) with UV was not lost even after four times transfer. The antibiotic productivity of prototrophic revertants obtained from protoplast regeneration of *S. narbonensis* NA12US3 was tested, and no antibiotic producers could be detected.

Discussion

Attempts to produce new antibiotics by the interspecific crosses among streptomycetes have been reported. However, the production of hybrid antibiotic molecules by the interspecific crosses has been observed only by MAZIERES *et al.*[3] and SCHLEGEL & FLECK[2]. We attempted interspecific protoplast fusion between macrolide-producing streptomycetes to produce hybrid macrolide antibiotics. In the cross of *S. fradiae* and *Streptomyces* sp. AM 4900, we isolated the fusant No. 14 producing an antibiotic of the macrolide family, which was not detected in the cultured broths of either of its parents and which was not produced by co-fermentation of these two strains. However, its productivity was extremely low and the fusant segregated to two types of colonies after a few times transfer. Each segregated colony had the same morphology and nutritional requirements as those of the original mutant parental strain. This result means that the prototrophic fusant derived from *S. fradiae* and *Streptomyces* sp. AM 4900 was a fully diploid heterokaryon capable of producing the antibiotic.

In the cross between *S. fradiae* and *S. narbonensis*, one of prototrophic fusants continued to produce narbomycin after two times transfer. This antibiotic was identical with that produced by wild type *S. narbonensis*. The strain of *S. narbonensis* used in this cross was a streptomycin-resistant mutant, while the prototrophic fusant producing narbomycin, which resembled morphologically *S. narbonensis*, was sensitive to streptomycin, and did not segregate to individual auxotrophic colonies. These results indicate that the fusant was not a heterokaryon like the fusant from the cross between *S. fradiae* and *Streptomyces* sp. AM 4900. We speculate that the production of narbomycin by the fusant No. 4-7 occurred as the result of recombination. If all of the gene(s) of *S. fradiae* for protylonolide production were exchanged into the chromosome of *S. narbonensis* as a recipient, the genes for M-4365 G_1 production could be constructed. However, if only a part of the former was exchanged to the latter and complemented the defective part of the gene(s) for narbonolide production in *S. narbonensis* NA12US3 (narbonolide-idiotroph), the fusant No. 4-7 could produce narbomycin. Since the productivity of fusant No. 4-7 which might be heteroclone was unstable, this is consistent with the instability of heterogeneous recombination[10] between antibiotic production genes of limited base sequence homology.

On the other hand, prototrophy of fusants, obtained from between *S. fradiae* TBM (*met*) and *S. narbonensis* NA12US3 (*his*), was stable after several times transfer. Also prototrophy of fusants between *S. fradiae* 261-27E (*ilv*) and *S. narbonensis* NA12US3 (*his*) was stable. These prototrophic fusants are obvious recombinants. These results indicate that *met*, *ilv*, and *his* genes of two strains are closely related on alleles of these markers and on DNA sequence homology, respectively.

No prototrophic recombinants were obtained between *met* or *ilv* of *S. fradiae*, and *arg* of *Streptomyces* sp. AM 4900 or *S. narbonensis*. However, prototrophic recombinant was obtained by intraspecific fusion between *S. narbonensis* NA8 (*arg*) and NA12US3 (*his*) at a frequency of 5.2×10^{-2} (data not shown). Alleles of *arg* between *S. fradiae* and *S. narbonensis* might not be fitted each other, consequently no prototrophic recombinants could occur but heterokaryons appeared temporarily by interspecific crosses (*met* $\times$ *arg* and *ilv* $\times$ *arg*).

Acknowledgments

We wish to thank Dr. C. W. PETTINGA of Eli Lilly & Co., for providing *S. fradiae* C373, Dr. A. SEINO of Kaken Chemical Co. Ltd., for providing macrolide-producing streptomycete strains, and Dr. T. OKUDA of Tanabe Seiyaku Co. Ltd., for a gift of M-4365 G_1. We also thank Mrs. Y. TAKAHASHI and Miss M. HIROKAWA for isolation of some auxotrophic and idiotrophic mutants and Miss K. KAWAUCHI for her technical assistance.

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