APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1983, p. 1865–1869
0099-2240/83/061865-05$02.00/0

Vol. 45, No. 6

Genetic Recombination in *Actinoplanes brasiliensis* by Protoplast Fusion

NORBERTO J. PALLERONI

Department of Microbiology, Hoffmann-La Roche Inc., Nutley, New Jersey 07110

Received 17 January 1983/Accepted 28 March 1983

Protoplast formation, fusion, and cell regeneration have been achieved with mutant strains of *Actinoplanes brasiliensis*. Three-, four-, and five-factor crosses have shown genetic recombination among the markers, and a five-factor cross is analyzed and discussed. Possibilities of using protoplast fusion for gene mapping and strain improvement are suggested.

A high frequency of genetic recombination can be achieved in streptomycetes by fusion of protoplasts (6), and methodologies for the genetic analysis of *Streptomyces* species are now available (1, 2, 5).

Protoplast fusion has also been demonstrated in *Streptosporangium viridogriseum*, a member of the family *Actinoplanaceae* (7). Organisms belonging to this family differ from streptomycetes in several important characters, including the cell wall composition and the type of sporulation. The most important genus of this family is *Actinoplanes*, which is characterized by the formation of spore vesicles enclosing globose spores that become actively motile at the time of their release (3). Many strains belonging to several species of the genus are available from various culture collections.

In the present communication, genetic recombination by protoplast fusion is demonstrated for one species of *Actinoplanes, A. brasiliensis*.

MATERIALS AND METHODS

Bacterial strains. *A. brasiliensis* ATCC 25844 was used in most of the experiments. Mutants of this strain were obtained by the methods described below. In some experiments, *Actinoplanes italicus* ATCC 27366 was used.

Media. Modified starch-casein agar medium (9) was supplemented with 0.1% vitamin-free casein hydrolysate (Nutritional Biochemicals Corp., Cleveland, Ohio). This medium will be referred to as FSC (fortified starch-casein) in the following discussion. It allows good growth and abundant sporulation of *A. brasiliensis* and its mutants.

Growth of the cultures for protoplast formation was carried out in Trypticase soy (TS) broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 0.6% glycine, which partially inhibits growth and increases the sensitivity of the cells to lysozyme (8).

Protoplast regeneration was obtained in medium R-3 described previously by Shirahama et al. (12), with 0.5 M sucrose instead of sodium succinate. This medium was supplemented with methionine, glutamate, tyrosine, and histidine (50 μg of each amino acid per ml), and was solidified with 1.8% Noble agar (Difco). The medium was poured into plates at about 42°C to prevent excess condensation of water on the agar surface after solidification, and the plates were dried half-open in an inverted position at 37°C for 40 to 60 min before use.

The chemically defined mineral medium previously described by Palleroni and Doudoroff (10) was used for the isolation and characterization of various auxotrophic and recombinant strains. Glucose was the main carbon source at a concentration of 0.2%, and the medium was solidified with purified agar (Difco Laboratories) at 1.8%. When required, amino acids were added to a final concentration of 50 μg/ml, and streptomycin to a final concentration of 10 μg/ml.

Mutagenesis experiments. *A. brasiliensis* strains were grown as lawns on FSC agar plates for 4 to 5 days at 28°C. Spores were released by flooding the plates with 5 mM phosphate buffer (pH 6.8) containing 0.01% Tween 80. Spore suspensions were exposed to low-wavelength (257 nm) UV light for lengths of time sufficient to kill 99.95% of the population. Samples of the treated suspensions were spread onto plates of FSC agar to obtain confluent growth, and after 4 to 5 days at 28°C, spores were released as indicated above. The spores were washed with 5 mM phosphate buffer at pH 6.8, and their concentration in the suspensions was measured by microscopic count. Samples of appropriate dilutions were spread over FSC agar plates to obtain 200 to 300 colonies per plate. When sporulation occurred (4 to 6 days at 28°C), the plates were replicated onto mineral agar plates without amino acid supplements for the identification of auxotrophic mutants, and onto plates with streptomycin for the screening of resistant mutants.

The inclusion of an enrichment step was found to be very convenient for the isolation of auxotrophic mutants (see below). Spores that had been subjected to mutagenesis were transferred to liquid mineral medium and incubated at 28°C for 4 to 5 days with rotatory shaking. A sample of the suspension was then filtered through cotton, and the filtrate was spread onto plates of FSC agar. The spores liberated from such lawns were screened for mutants.

Auxotrophic mutants were characterized on mineral agar medium supplemented with different amino acids.

Protoplast formation, fusion, and regeneration. Mutant strains of *A. brasiliensis* were grown in TS broth for 72 h at 28°C with rotatory shaking, and the culture was used as a 20% inoculum of a TS broth containing 0.6% glycine. Dispersion of the growing microbial mass into small clumps was achieved by including in each flask three pieces of nichrom wire bent into tight spirals approximately 8 mm in diameter and 25 mm in length. Incubation of the TS-glycine broth was continued for 24 to 36 h at 28°C with rotatory shaking at 250 rpm, after which each culture was transferred to a sterile cylinder, and the clear supernatant was poured off after sedimentation of the cell clumps. These were washed twice with 0.3 M sucrose and finally suspended in the P-3 medium of Shirahama et al. (12) containing 1 mg of lysozyme per ml. The suspensions were transferred to sterile screw-cap tubes, which were incubated with slow rotation (approximately 60 turns around the axis per minute, in an almost horizontal position) at 28°C. At 15-min intervals, each suspension was carefully pipetted in and out by using a pipette with a narrow tip, a step that facilitates the release of protoplasts from the cell clumps. After 1 h, the suspensions were filtered through sterile cotton, and the protoplasts were washed with PWP solution (12) and finally suspended in P-3 medium.

Protoplast fusion was done by mixing appropriate volumes of protoplast suspensions in the same Eppendorf centrifuge tube, centrifuging the mixture, suspending the pellet in 0.1 ml of P-3 medium, and adding 0.9 ml of 40% polyethylene glycol 6000 in P-3 medium. The addition of this solution was slow, with gentle stirring with the tip of the pipette. After 30 to 50 s, the suspension was centrifuged for 8 min in an Eppendorf centrifuge, the pellet was resuspended in 0.1 ml of P-3 medium, and the total volume was plated on a single plate of regeneration medium.

Intact or fused protoplasts were plated by placing the suspension on the surface of the regeneration agar medium, and 4 ml of the same medium with 0.4% low-melting-point agarose (gel electrophoresis grade; Bethesda Research Laboratories, Gaithersburg, Md.) at 39°C was poured onto a separate area of the agar plate. The two liquids were gently mixed, and the overlayer was left to solidify. The plates were incubated overnight in the same position, and the next day they were placed in an inverted position in plastic bags and incubated at 28°C. Growth was usually observed after 7 to 10 days.

The regeneration procedure just described is essentially the one recommended by Shirahama et al. (12), except that in our case the plates were dried before pouring the materials for the overlayer, and not after the overlayer had solidified.

Since *A. brasiliensis* does not sporulate in the regeneration medium, the overlayer with the colonies was scraped off with a sterile spatula and spread over FSC agar plates, where sporulation was invariably abundant in 4 to 5 days.

Recombinant screening. Spores from regenerated protoplast fusion cultures on FSC plates were released and washed, and appropriate dilutions were plated onto various differential agar media for recombinant screening. After 4 to 5 days at 28°C, when there was a clear size differential between the recombinants able to grow on a given medium and the rest of the auxotrophic population, larger colonies were picked up under a dissecting microscope and were immersed individually into 20 μl of 5 mM phosphate with 0.01% Tween 80 in wells of a multitest plate (Nunclon microtest plate, cat. no. 163118; Vangard International, Inc., Neptune, N.J.).

When the spores were released (about 1 h at room temperature), the liquid of each well was sucked up with a Gilson pipette and spread with the side of the pipette tip onto a defined area of a plate of mineral agar supplemented with all of the amino acids required by the parent strains. About 4 to 5 days later, material from each patch was cut and lifted with a sterile spatula, trying to carry as little agar as possible, and immersed into individual tubes with 2 ml of buffer-Tween 80 to release the spores. The suspensions obtained were printed onto special differential media with various amino acid supplements, and with or without streptomycin, using a multirod inoculator capable of simultaneously depositing 16 drops in a pattern of 4 by 4 on each plate. The plates were incubated at 28°C, and the results were scored after 1 week as growth or no growth for each recombinant in the various media.

RESULTS AND DISCUSSION

Colony variation in *A. brasiliensis*. Two main types of colonies of *A. brasiliensis* can be observed when the spores of this species are streaked onto mineral agar medium with glucose. One type is compact, with abundant substrate mycelium, and becomes intensely orange with age. Another type shows much lighter development of the immersed mycelium, but sporulation is more abundant than in the first type. Owing to lighter vegetative growth, the colonies of the second type appear slightly pigmented.

No efforts were made to isolate a single colony type of each of the mutants, even though the two types could be separated and seemed to maintain their respective characteristics on mineral agar at least for a few transfers. Both types of colonies have been scored as positive growth on special media, since they are easy to differentiate from the small colonies given by mutants or recombinants growing only marginally.

Among the products of mutagenesis experiments were observed white colony mutants and, with less frequency, mutants unable to form spore vesicles. At least some of these colony characters may in time become interesting markers for genetic recombination work in members of the genus.

Mutagenesis experiments. Even though *A. brasiliensis* sporulated very abundantly on starch-containing agar media and, in particular, in the FSC medium, the efficiency of replica plating was very low, and therefore, a simple comparison of a "minimal" printed plate with the original "complete" plate was not very helpful for the identification of auxotrophic mutants, since many of the missing colonies on minimal agar

represented failures of replication. Mutant detection was, however, very easy by examination of the minimal or mineral agar plates either directly or with the help of a dissecting microscope. Auxotrophic mutants were recognized by their scanty marginal growth and sporulation on the mineral agar.

The inclusion of an enrichment step in the mutagenesis experiments as explained above considerably improved the yield of auxotrophic mutants. In a typical mutagenesis experiment, a spore suspension of tyrosine-requiring strain 28, with 2.55×10^8 spores per ml, was exposed to UV light for 30 s, which killed 99.95% of the spores. A sample of the suspension was spread onto FSC agar plates to give confluent growth, and the spores were liberated and plated to give 200 to 300 colonies on each of 20 plates. Another sample of the mutagenized spore suspension was transferred to liquid mineral medium with 50 μg of tyrosine per ml and incubated with shaking at 28°C. The suspension was filtered through cotton, and a lawn was prepared on FSC agar plates, from which spores were liberated and plated onto another set of 20 plates. The direct isolation method only yielded three mutant colonies requiring methionine in addition to tyrosine, whereas the sample subjected to the enrichment step gave, in addition to the original tyrosine marker, the following numbers of mutants: histidine, 11; methionine, 7; lysine, 3; leucine, 2; phenylalanine, 4; threonine, 1; and arginine, 2. One of the arginine mutants was also able to grow with ornithine.

Regeneration of protoplasts. Regeneration of *A. brasiliensis* protoplasts was tested in various regeneration media mentioned in the literature, and also in some media whose formulation was designed in our laboratory. The most satisfactory of the media was found to be medium R-3 of Shirahama et al. (12), with sucrose and amino acid supplements (see above). However, it is not certain that this medium could be recommended for all *Actinoplanes* species. Preliminary observations showed that *A. italicus* protoplasts were formed very abundantly but regenerated very poorly in all known regeneration media, and only limited success was obtained with this species with the medium recommended by Oh et al. (7).

Quantitative estimations of regeneration of protoplasts to normal cells were performed only sporadically, but the efficiency of the process seemed to vary considerably from one experiment to another. Regeneration approaching 10% of the microscopic protoplast count could be reached only in a few experiments, whereas in most instances the regeneration only occurred in 0.1 to 1% of the protoplasts. No inhibitory effect on the regeneration of protoplasts in the proximity of other protoplasts regenerating early (autoinhibition) (1) was observed.

Microscopic protoplast counts may not be a very reliable reference to judge regeneration efficiency because, as mentioned by Hopwood et al. (6) for *Streptomyces* species, many of the *Actinoplanes* protoplasts are very small and probably do not contain a complete genome.

After 3 or 4 days of incubation, regenerating protoplasts of *A. brasiliensis* appeared on the agar as large cells 6 to 12 μm in diameter, but they never reached the gigantic sizes (up to 50 μm or more) observed in *Streptomyces* species (8).

Genetic recombination by protoplast fusion. Several conjugation attempts with mutant strains of *A. brasiliensis* in our laboratory gave negative results (J. Unowsky and N. J. Palleroni, unpublished observations). Preliminary experiments demonstrated that genetic recombination in *A. brasiliensis* was possible by protoplast fusion. These experiments involved strains 7-6 (*met glu*) and 28 (*tyr*), and were later repeated using strain 7-6 and a streptomycin-resistant (Str^r) mutant of strain 28. The protocol for this second experiment was as follows. Samples (0.5 ml) of each one of the two washed protoplast suspensions, containing 1×10^8 to 1.5×10^8 protoplasts per ml of each, were mixed in a centrifuge tube, and the pellet was subjected to fusion as described above. A total of 578 recombinant colonies were counted on mineral-methionine and mineral-glutamic acid plates (248 and 330 colonies, respectively) out of a total of 17,250 colonies. Of the recombinants isolated from these two selective media, 389 were characterized, and a summary of the results is presented in Table 1.

This experiment included two controls in which protoplasts of each of the parents were subjected individually to fusion, and a third control in which polyethylene glycol was excluded from the fusion mixture. As expected, the first two controls did not give any recombinants. On the other hand, by exclusion of poly-

TABLE 1. Analysis of four-factor protoplast fusion in *A. brasiliensis* (*met glu* × *tyr* Str^r)

Genotype/phenotype	No. of recombinants isolated from medium supplemented with:	
	Methionine	Glutamate
met$^+$ *glu*$^+$ *tyr*$^+$ Str^s	39	27
met$^+$ *glu*$^+$ *tyr*$^+$ Str^r	113	49
met glu$^+$ *tyr*$^+$ Str^s	29	
met glu$^+$ *tyr*$^+$ Str^r	16	
met$^+$ *glu tyr*$^+$ Str^s		4
met$^+$ *glu tyr*$^+$ Str^r		112

ethylene glycol from the fusion step, only three recombinants (all of genotype *met*$^+$ *glu tyr*$^+$ Str^s) could be isolated from a total of 10,900 colonies.

A new round of mutagenesis on strain 28 added new auxotrophic markers to the tyrosine and streptomycin mutations already present in this strain, as mentioned before. Some of these mutants were used in fusion experiments with strain 7-6, and in the following paragraphs one of these experiments will be discussed in some detail. The cross involved strain 7-6 (*met glu* Str^s) and strain 28-3417 (*tyr his* Str^r), and the protocol was essentially the same as for the previous experiment.

The total number of recombinants obtained on selective media in these experiments represented from 2.4 to 7% of the spores plated. Screening of the spores obtained in this last fusion experiment on four selective media gave the results presented in Table 2. The average frequencies from the last column of Table 2 were used for the estimation of the relative recombination frequencies for each pair of markers, and the data are presented in Table 3. Tables 2 and 3 have been constructed on the basis of the model used by Hopwood (4).

Recombination between the *tyr* and *his* markers was low (Table 3), which suggests that these two genes are closely linked, whereas the pairs *glu-str* and *met-tyr* seem to constitute less-tight linkage units.

Many different genotypes were identifiable by the use of selective media, but further analysis of the data does not support any conclusions on the arrangement of the genes in a circular or linear map. One of the main problems is the unequal frequencies of complementary genotypes. This is evident in Table 1, in which the discrepancy between the numbers of *met glu*$^+$ and *met*$^+$ *glu* recombinants is substantial. Inclusion of glutamate in all selective media seemed to have corrected this anomaly in the segregation of the above two genes in the second fusion experiment, but other abnormalities in reciprocal segregations are evident. Exclusion of data for the glutamic acid marker did not result in a significant improvement of the results of the analysis (D. Hopwood, personal communication).

For the moment, there is no satisfactory explanation for the anomalies, which may be an intrinsic property of the system, although additional experiments now in progress with other markers may justify the use of protoplast fusion recombination data for mapping purposes in *A. brasiliensis*.

The experiments reported here open possibili-

TABLE 2. Analysis of five-factor protoplast fusion in *A. brasiliensis* (*met glu tyr*$^+$ *his*$^+$ Str^s × *met*$^+$ *glu*$^+$ *tyr his* Str^r)

Genotype/phenotype	No. of recombinants in 10 plates of each medium supplemented with:[a]								Avg frequencies (10 plates)
	Glutamic acid, tyrosine		Glutamic acid, histidine		Glutamic acid, tyrosine, methionine, streptomycin		Glutamic acid, histidine, methionine		
	a	b	a	b	a	b	a	b	
met$^+$ *glu*$^+$ *tyr*$^+$ *his*$^+$ Str^s	21	67	28	58					63
met$^+$ *glu*$^+$ *tyr*$^+$ *his*$^+$ Str^r	21	67	41	85	8	85	30	86	81
met glu$^+$ *tyr*$^+$ *his*$^+$ Str^r					42	443	79	226	334
met$^+$ *glu tyr*$^+$ *his*$^+$ Str^s	92	294	105	218					256
met$^+$ *glu tyr*$^+$ *his*$^+$ Str^r	2	6	6	12	1	10	3	7	9
met$^+$ *glu*$^+$ *tyr his*$^+$ Str^s	1	3							3
met$^+$ *glu*$^+$ *tyr his*$^+$ Str^r	5	16			4	42			29
met$^+$ *glu*$^+$ *tyr*$^+$ *his* Str^r			2	4			2	6	5
met glu tyr$^+$ *his*$^+$ Str^r					31	327	36	103	215
met glu$^+$ *tyr his*$^+$ Str^r					4	42			42
met glu$^+$ *tyr*$^+$ *his* Str^r							4	11	11
met$^+$ *glu tyr his*$^+$ Str^r	27	86			15	158			122
met$^+$ *glu tyr*$^+$ *his* Str^r			1	2					2
No. of recombinants analyzed	169		183		105		154		
No. of recombinants per 10 plates		539		379		1,107		439	

[a] Column a, Sample of recombinants analyzed in each of the four selective media; column b, total count of recombinants per 10 plates of each medium.

TABLE 3. Relative recombination frequency for each pair of markers in the cross 7-6 × 28-3417

Expt no.	Avg frequencies[a]									
	met-glu	*met-tyr*	*met-his*	*met-str*	*glu-tyr*	*glu-his*	*glu-str*	*tyr-his*	*tyr-str*	*his-str*
1	334	63	63	63	63	63	63	3	81	81
2	256	81	81	334	81	81	9	29	334	334
3	9	256	256	256	334	334	3	5	9	9
4	42	9	9	3	5	3	215	42	3	29
5	11	5	3	215	11	29	122	16	5	215
6	122	42	29	42	122	42	2	122	215	42
7	2	2	11	11		2		2	11	122
8			122						2	

[a] From Table 2.

ties for genetic investigations in *Actinoplanes* species. Since members of this genus are producers of interesting antibiotics (11), strain improvement by protoplast fusion is also an open avenue for the immediate future.

ACKNOWLEDGMENTS

I am very grateful to David A. Hopwood for reading the manuscript and for his comments and suggestions. The kind collaboration of Joel Unowsky, who participated in the initial mutagenesis experiments on conjugation in our laboratory, is also acknowledged.

LITERATURE CITED

1. **Baltz, R. H.** 1978. Genetic recombination in *Streptomyces fradiae* by protoplast fusion and cell regeneration. J. Gen. Microbiol. **107**:93–102.
2. **Baltz, R. H.** 1980. Genetic recombination by protoplast fusion in *Streptomyces*. Dev. Ind. Microbiol. **21**:43–54.
3. **Bland, C. E., and J. N. Couch.** 1981. The family *Actinoplanaceae*, p. 2004–2010. *In* M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (ed.), The prokaryotes, vol. II. Springer-Verlag, New York.
4. **Hopwood, D. A.** 1972. Genetic analysis in microorganisms, p. 29–158. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 7B, Academic Press, Inc., New York.
5. **Hopwood, D. A., and H. M. Wright.** 1978. Bacterial protoplast fusion: recombination in fused protoplasts of *Streptomyces coelicolor*. Mol. Gen. Genet. **162**:307–317.
6. **Hopwood, D. A., H. M. Wright, M. J. Bibb, and S. N. Cohen.** 1977. Genetic recombination through protoplast fusion in *Streptomyces*. Nature (London) **268**:171–174.
7. **Oh, Y. K., J. L. Speth, and C. H. Nash.** 1980. Protoplast fusion with *Streptosporangium viridogriseum*. Dev. Ind. Microbiol. **21**:219–226.
8. **Okanishi, M., K. Suzuki, and H. Umezawa.** 1974. Formation and reversion of streptomycete protoplasts: cultural conditions and morphological study. J. Gen. Microbiol. **80**:389–400.
9. **Palleroni, N. J.** 1979. New species of the genus *Actinoplanes, Actinoplanes ferrugineus*. Int. J. Syst. Bacteriol. **29**:51–55.
10. **Palleroni, N. J., and M. Doudoroff.** 1972. Some properties and subdivisions of the genus *Pseudomonas*. Annu. Rev. Phytopathol. **10**:73–100.
11. **Parenti, F., and C. Coronelli.** 1979. Members of the genus *Actinoplanes* and their antibiotics. Annu. Rev. Microbiol. **33**:389–411.
12. **Shirahama, T., T. Furumai, and M. Okanishi.** 1981. A modified regeneration method for streptomycete protoplasts. Agric. Biol. Chem. **45**:1271–1273.