JOURNAL OF BACTERIOLOGY, Jan., 1966
Vol. 91, No. 1

Printed in U.S.A.

Genetic Recombination in Crosses Between *Streptomyces aureofaciens* and *Streptomyces rimosus*

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Received for publication 29 July 1965

ABSTRACT

POLSINELLI, M. (University of Pavia, Pavia, Italy), AND MARIA BERETTA. Genetic recombination in crosses between *Streptomyces aureofaciens* and *Streptomyces rimosus*. J. Bacteriol. **91**:63–68. 1966.—Biochemical mutants were obtained from *Streptomyces rimosus* and *S. aureofaciens* by ultraviolet irradiation. Crosses were performed between auxotrophic strains of *S. rimosus* and *S. aureofaciens* with positive results. Data are reported which indicate that the interaction observed in some crosses is due to gene recombination.

In 1955, Sermonti and Spada-Sermonti (17) first observed gene recombination in *Streptomyces coelicolor* [*S. violaceoruber* according to Kutzner and Waksman (11)]. Since then, recombination has been shown to occur in different species of *Streptomyces*, such as *S. fradiae* (6), *S. rimosus* (3), *S. griseoflavus* (13), *S. griseus* (7), and *S. aureofaciens* (2, 10). The most complete analysis of the process of recombination in *Streptomyces* was done, however, in *S. coelicolor* (9, 15).

Data have also been reported which suggest interspecific recombination between strains of *S. rimosus* and *S. coelicolor*, *S. aureofaciens* and *S. coelicolor*, and *S. rimosus* and *S. aureofaciens* (1). However, the evidence provided is not sufficient to discriminate between interspecific recombination and heterokaryosis. In some instances, even the possibility of back mutations has not been completely ruled out, especially when prototrophs were recovered from crosses between strains carrying only one biochemical mutation. Heterokaryosis has been shown to occur between *S. griseus* and *S. cyaneus* (5), and between *S. griseus* and *S. venezuelae* (4). In the present paper, we report results of interspecific crosses between *S. rimosus* and *S. aureofaciens*, which show the occurrence of true recombination.

MATERIALS AND METHODS

Culture media. Medium TSSA contained: oat flakes, 60 g; casein hydrolysate (Costantino, Favria, Turin, Italy), 1 g; nucleic acids (Eastman, Rochester, N.Y.), 10 mg; agar, 20 g; and distilled water, 1,000 ml; after sterilization at 120 C for 20 min, 1 ml of vitamin solution was added (100 ml of vitamin solution contained 2 mg each of thiamine, riboflavine, pyridoxine, nicotinic acid, *p*-aminobenzoic acid, and calcium pantothenate, 20 μg of biotin, and 100 mg of inositol). Medium BTT-lac contained: yeast extract (Costantino), 1 g; meat extract (Costantino), 1 g; casein hydrolysate (Costantino), 2 g; lactose, 20 g; agar, 25 g; and distilled water, 1,000 ml; sterilization was at 120 C for 20 min. Medium M40 contained: KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $(NH_4)_2SO_4$, 3.5 g; asparagine, 1.5 g; glucose, 10 g; solution A, 1 ml; solution B, 1 ml; solution C, 1 ml; agar, 20 g; and distilled water, 1,000 ml; sterilization was at 115 C for 20 min [solution A: NaCl, 10 g; distilled water, 100 ml; solution B: $CaCl_2 \cdot 6H_2O$, 10 g; distilled water, 100 ml; solution C: H_3BO_3, 50 mg; $CuSO_4 \cdot 5H_2O$, 4 mg; KI, 10 mg; $FeCl_3 \cdot 6H_2O$, 20 mg; $MnSo_4 \cdot H_2O$, 40 mg; $FeSO_4 \cdot 7H_2O$, 40 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 20 mg; distilled water 100 ml]. All three types of media were adjusted to *p*H 6.8 to 7 after sterilization.

Isolation of mutants. Biochemical mutants listed in Table 1 were induced by means of ultraviolet radiation in two strains of *S. aureofaciens* and one of *S. rimosus*. The prototrophic strains used were: *S. aureofaciens* Duggar ATCC 10762, *S. aureofaciens* 1361, *S. rimosus* R. Strains 1361 and R were two soil isolates, first identified by their capacity for producing chlortetracycline and oxytetracycline, respectively, and later classified as *S. aureofaciens* and *S. rimosus* by their morphological and physiological characteristics according to Waksman (18).

For antibiotic production, fermentation was carried out in V6 medium (for composition, see 12) for 72 hr at 28 C in an alternating shaker. At the end of the fermentation period, the mycelium was removed by

TABLE 1. *Genotypes and derivation of the strains of Streptomyces aureofaciens and S. rimosus used**

S. aureofaciens strain	Genotype	Derivation	*S. rimosus* strain	Genotype	Derivation
DR	Prototroph	Duggar strain ATCC 10762	R	Prototroph	Soil isolate
1361	Prototroph	Soil isolate	R 10	*his*$^-$	R
DR 1	*arg*$^-$	DR	R 13	*cys*$^-$	R
DR 12	*met*$^-$	DR	R 15	*cys*$^-$ *leu*$^-$	R 13
DR 11	*arg*$^-$ *met*$^-$	DR 1	R 16	*cys*$^-$ *ura*$^-$	R 13
DR 32	*arg*$^-$ *thr*$^-$	DR 1	R 17	*his*$^-$ (*isol* + *val*)$^-$	R 10
DR 321	*arg*$^-$ *thr*$^-$ *met*$^-$	DR 32	R 18	*his*$^-$ *arg*$^-$	R 10
DR 35	*arg*$^-$ *tyr*$^-$	DR 1	R 151	*cys*$^-$ *leu*$^-$ *his*$^-$	R 15
DR 352	*arg*$^-$ *tyr*$^-$ *ade*$^-$	DR 35	R 152	*cys*$^-$ *leu*$^-$ (*isol* + *val*)$^-$	R 15
1361/1	*met*$^-$	1361	R 161	*cys*$^-$ *ura*$^-$ (*isol* + *val*)$^-$	R 16
1361/1C	*met*$^-$ *bio*$^-$	1361/1	R 164	*cys*$^-$ *ura*$^-$ *rib*$^-$	R 16
			R 171	*his*$^-$ (*isol* + *val*)$^-$ *pan*$^-$	R 17
			R 182	*his*$^-$ *arg*$^-$ (*phe* + *tyr* + *try*)$^-$	R 18
			R 183	*his*$^-$ *arg*$^-$ (*isol* + *val*)$^-$	R 18
			R 187	*his*$^-$ *arg*$^-$ *ade*$^-$	R 18

* All mutants were induced by ultraviolet exposure. Symbols: *ade* = adenine; *arg* = arginine; *bio* = biotin; *cys* = cysteine; *his* = histidine; *isol* = isoleucine; *leu* = leucine; *met* = methionine; *pan* = pantothenate; *phe* = phenylalanine; *rib* = riboflavine; *thr* = threonine; *try* = tryptophan; *tyr* = tyrosine; *ura* = uracil; *val* = valine.

filtration, and the antibiotic was extracted from the liquid medium at *p*H 8 with 0.5 volume of *n*-butanol. The butanol extract was carefully neutralized with HCl and evaporated in vacuo in a rotary evaporator. The dry residue was taken up in a few milliliters of water and samples were used for paper chromatography. Chromatographic separation of the tetracyclines was achieved by spotting samples of the extract on Whatman no. 1 filter paper buffered at *p*H 3 with phosphate buffer. Two solvent systems were employed. One was composed of water-saturated ethyl acetate, and was used for ascending chromatography for 36 hr. The second solvent system was butanol saturated with phosphate buffer at *p*H 3, and was employed for descending chromatography for 12 hr. Chromatography was performed in the dark at 28 C. Tetracycline was localized under ultraviolet light in the presence of ammonia fumes and then was bioassayed on agar plates seeded with *Staphylococcus aureus*. Under such conditions, strain R showed a single area with antibiotic activity which was indistinguishable from oxytetracycline. Strain 1361 showed a single area with antibiotic activity which was indistinguishable from chlortetracycline. Therefore, strain R was identified as *S. rimosus* and strain 1361 as *S. aureofaciens*. It may be added that, under such conditions, the production of antibiotic ranged from 50 to 100 μg/ml.

A spore suspension of the prototrophic strains (0.5 ml containing approximately 10^8 spores) in a dish (diameter, 40 mm) was irradiated with a low-pressure mercury ultraviolet lamp to a survival of 10^{-2} to 10^{-3}. The irradiated spores were seeded on complete medium (TSSA for *S. aureofaciens* and BTT-lac for *S. rimosus*) and were incubated at 28 C. Generally, after 4 days, the colonies were replicated on minimal medium, and those which failed to grow were characterized for their growth requirements.

Some difficulty has been encountered in obtaining biochemical mutants from *S. aureofaciens*. In fact, in this species most of the auxotrophic mutants were arginine-dependent, as already reported by Alikhanian and Borisova (2). However, the filtration method described by Fries (8) for *Neurospora* has permitted mutants with different requirements to be obtained. Mutants of *S. aureofaciens* and *S. rimosus* were maintained on TSSA and BTT-lac, respectively.

Crossing procedure. Strains of *S. aureofaciens* and *S. rimosus* to be crossed were inoculated on slants of TSSA and BTT-lac medium, respectively, and were incubated at 28 C. When complete sporulation had taken place (8 to 15 days, according to the strain), spores were harvested, and the suspension was adjusted to about 10^8 spores per ml of distilled water.

Crosses were always performed on minimal medium M 40, fully or partially supplemented with all the nutrilites required by the parental strains. Full supplement consisted of the addition of 20 μg/ml of each amino acid or nitrogenous base and 0.1 μg/ml of vitamin. When partial supplement was used, only 1.3 μg/ml of amino acids or nitrogen bases and 0.01 μg/ml of vitamins were added to the minimal medium.

A simple technique was used to test the interaction between different strains. Interaction may result in true recombination, formation of heterokaryons, simple syntrophism, or a combination of these. Crosses were made according to the following procedure. Drops of spore suspension (about 5 mm in diameter) of one strain were placed on an agar plate of partially supplemented medium and were allowed to dry; then a

drop of suspension of the other strain was placed on the plate, and slight overlapping of the edges of the two drops was ensured. Plates were incubated at 28 C. When the cross was successful, an abundant growth of aerial mycelium was noticed within 2 to 3 days at the point of contact between the two drops, whereas the remaining part of the area occupied by the drop showed only poor growth. A few days later, the growth would invade either one or both of the parental colonies, with abundant production of spores. When crosses were unsuccessful, no extra growth was noticed at the contact edges of the two drops. When good sporulation had occurred, spores were collected and tested for recombination on selective media. These media, prepared with M 40 fully supplemented with some of the required growth factors, were used for selecting recombinants and for scoring their phenotype.

RESULTS

Growth interaction in crosses between auxotrophic mutants of S. aureofaciens and S. rimosus. By means of the procedure described above, with the use of partially supplemented media, the interaction between a number of strains of *S. aureofaciens* and *S. rimosus* was analyzed. The interactions were classified, by simple macroscopic inspection, into three different classes: ++, abundant growth at the point of contact between the two drops; +, poor growth but still clearly evident; −, no growth observed at the point of contact. In Table 2 are reported some examples of these types of interaction.

The validity of our scoring method was proved by transferring the mycelium at the point of contact between two drops that did not show interaction into selective media. When the score was −, no recombinants were isolated.

Frequencies of revertants in the auxotrophic mutants used in crosses. The frequencies of revertants are reported in Table 3.

Results of crosses. The spores at the point of contact between the drops (see Materials and Methods) were harvested and used for subsequent analysis. It is well known that in these organisms it is very difficult to prepare a suspension containing only spores and no mycelium fragments, especially when sporulation is not very abundant as in our case. When we refer to a spore suspension, we mean not only a suspension containing a great number of spores but also a certain amount of mycelial fragments which in some instances can also reach the order of magnitude of 5 to 10% of the spores.

Four examples of the analysis of spores produced in crosses are reported in Table 4. The spore suspension was seeded in complete medium TSSA for estimating the total viable count and on selective media containing only two or none of the nutrilites required by the parental strains.

TABLE 2. *Growth interaction in crosses of Streptomyces aureofaciens × S. rimosus*

Cross	Interaction*
DR 11 × R 17	++
DR 11 × R 171	++
DR 11 × R 164	−
DR 12 × R 152	+
DR 12 × R 17	−
DR 12 × R 171	−
DR 32 × R 17	+
DR 32 × R 171	+
DR 32 × R 152	+
DR 352 × R 161	++
DR 352 × R 17	+
DR 352 × R 164	−
DR 321 × R 16	++
DR 321 × R 151	++
DR 321 × R 152	+
1361/1C × R 171	++
1361/1C × R 182	++
1361/1C × R 183	+
1361/1C × R 187	−

* For the scoring criteria, see Results.

In principle, the overgrowth at the point of contact between two drops could be due to reversion of the genetic markers in either one or both parental strains, to syntrophism, to heterokaryosis, or to genetic recombination. All these possibilities were analyzed.

Reversion of genetic markers. The frequencies of spores from the crosses growing in selective media or on minimal medium argues strongly against the hypothesis of a reversion process. The frequencies of reversion among the parental strains reported in Table 3 support an expectation of double or triple mutational events with frequencies many orders of magnitude lower than the observed frequencies of colonies capable of growing in selective or minimal medium.

Syntrophism or heterokaryosis. The data reported in Table 4 suggest that syntrophism or heterokaryosis should not play a major role in the interaction between *S. aureofaciens* and *S. rimosus* because of significant differences of colonies growing in different selective media.

Experiments were planned to obtain evidence of the importance of these two phenomena for the observed interaction. Thirty-nine prototrophs selected on minimal medium from the cross DR 11 × R 171 were tested for their genetic stability. Each colony was picked up directly from the selective medium and transferred to a slant with complete medium TSSA. After sporulation, conidia were harvested, and filtered through

TABLE 3. Frequencies of revertants in spore population of auxotrophic mutants used in crosses *Streptomyces aureofaciens* × *S. rimosus*

Strain	Frequency of revertants for individual markers										
	arg	*bio*	*cys*	*his*	*isol+val*	*leu*	*met*	*pan*	*phe+tyr+try*	*thr*	*ura*
S. aureofaciens											
1361/1C *met⁻ bio⁻*	—	1.1×10^{-8}					5×10^{-9}			—	
DR 11 *met⁻ arg⁻*	1.3×10^{-8}	—					3×10^{-8}			—	
Dr 321 *arg⁻ met⁻ thr⁻*	1.6×10^{-8}	—					2.5×10^{-8}			1.1×10^{-8}	
S. rimosus											
R 16 *cys⁻ ura⁻*	—		2.4×10^{-9}	—	—	—		—	—		9×10^{-9}
R 151 *cys⁻ leu⁻ his⁻*	—		1.2×10^{-9}	4×10^{-9}	—	2.7×10^{-9}		—	—		—
R 152 *cys⁻ leu⁻ (isol+ val)⁻*	—		3×10^{-9}	—	2.3×10^{-8}	1.9×10^{-9}		—	—		—
R 171 *his⁻ (isol+val)⁻ pan⁻*	—		—	6.5×10^{-9}	1.5×10^{-8}	—		2.8×10^{-7}	—		—
R 182 *his⁻ arg⁻ (phe+ tyr+try)⁻*	1.4×10^{-7}		—	1.3×10^{-8}	—	—		—	1.9×10^{-8}		—

TABLE 4. *Analysis of spores produced in crosses of Streptomyces aureofaciens × S. rimosus*

Cross	No. of spores	No. of colonies growing on selective media containing two nutrilites									
		a d	a e	a f	b d	b e	b f	c d	c e	c f	None
1361/1C R 182 *met*⁻(a) *bio*⁻(b) × *his*⁻(d) *arg*⁻(e) (*tyr*+*try*+*phe*)⁻ (f)	5 × 10⁷	1	1	4	16	11	105	—	—	—	1
DR 11 R 171 *arg*⁻(a) *met*⁻(b) × *his*⁻(d) (*isol*+*val*)⁻ (e) *pan*⁻(f)	4 × 10⁶	101	91	79	194	163	201	—	—	—	80
DR 321 R 16 *arg*⁻(a) *thr*⁻(b) *met*⁻(c) × *cys*⁻(d) *ura*⁻(e)	2 × 10⁸	6	12	—	0	0	—	4	125	—	1
DR 321 R 151 *arg*⁻(a) *thr*⁻(b) *met*⁻(c) × *cys*⁻(d) *leu*⁻(e) *his*⁻(f)	7 × 10⁸	0	0	0	101	176	57	4	27	12	0

Whatman no. 2 filter paper to eliminate the greater part of the mycelial fragments. Diluted suspensions were then seeded on complete medium, yielding 15 to 30 colonies per plate. After sporulation, the colonies were replicated on minimal medium. Colonies which failed to grow on minimal medium were tested for phenotype.

Data reported in Table 5 show that most prototrophs are stable, and that a few markers segregate in a small number of crosses, but parental combinations are never recovered, thus disproving the hypothesis of syntrophism and of heterokaryosis. In Table 5 one can see that 4 of 39 colonies have segregated *met*⁻ auxotrophs and 2 of 39 segregated (*isol* + *val*)⁻ auxotrophs. The frequency of segregation of *met*⁻ auxotrophs varied among the four colonies from a maximum of 30% to a minimum of 6%. The frequency of (*isol* + *val*)⁻ segregants in the two colonies was 7 and 13%, respectively.

We can, therefore, conclude that syntrophism can be excluded as cause of the observed interaction and also that heterokaryosis does not play an important role. The fact that 6 colonies of 39 segregate for one marker indicates that we are certainly not dealing with conventional heterokaryosis.

Genetic recombination. The proof that true genetic recombination occurs in crosses is provided by the isolation of stable recombinants, i.e., stable strains carrying new combinations of genes provided by both parents. In two crosses, such proof was obtained (Table 6).

TABLE 5. *Segregation analysis of 39 prototrophic colonies from cross of DR 11 × R 171*

No. of prototrophs selected on minimal medium	No. of replicated colonies	Phenotype of replicated colonies	
		No. of prototrophs	No. of auxotrophs
33	996	996	—
1	39	34	5 *met*⁻
1	33	30	3 *met*⁻
1	49	46	3 *met*⁻
1	40	28	12 *met*⁻
1	43	40	3 (*isol* + *val*)⁻
1	38	33	5 (*isol* + *val*)⁻

As shown in Table 6, all 61 colonies tested in the first cross and all 8 colonies of the second cross carry genetic markers coming from both parental strains, confirming that true recombination is taking place.

DISCUSSION

The existence of an interaction between *S. aureofaciens* and *S. rimosus* seems to be clearly shown. Most of our strains of *S. aureofaciens* have a common origin from strain DR, and all of the mutants of *S. rimosus* were derived from a common ancestor. The clear difference of interaction in crosses should therefore be due to changes induced by the subsequent mutagenic treatment with ultraviolet radiation or to some sort of incompatibility due to the specific genotypes involved in the crosses. We do not intend to

TABLE 6. *Crosses providing evidence of recombination*

Cross	Selected on medium containing	No.	Phenotype
DR 321 *arg⁻thr⁻met⁻* × R 16 *cys⁻ura⁻*	*met ura*	61	*met⁻ura⁻*
DR 321 *arg⁻thr⁻met⁻* × R 152 *cys⁻leu⁻ (isol+val)⁻*	*thr met leu isol val*	8	*leu⁻(isol+val)⁻met⁻*

discuss here in detail the nature of cross compatibility, as this problem needs much more information than our data provide, and the purpose of this paper is to investigate the possibility of genetic recombination rather than to analyze its mechanism.

From the above data, it can be concluded that the interaction between the strains of *S. rimosus* and *S. aureofaciens* is mainly due to genetic recombination, even if one cannot exclude that a minor fraction of the new phenotypes could be accounted for by heterokaryosis. Some of the colonies selected on minimal medium have segregated, even after two transfers, *met⁻* or *(isol + val)⁻* auxotrophs; this fact could be explained by assuming that the interaction resulted first in the formation of partial heterozygotes with a subsequent segregation. This type of segregation recalls the "heteroclones" described by Sermonti et al. (16) in *S. coelicolor*.

It should be added that the great majority of the recombinants showed the typical morphological aspects of *S. rimosus*; recombinants morphologically similar to *S. aureofaciens* were a small fraction (2 to 5%). This suggests the possibility that one of the two strains, *S. rimosus*, could be the recipient and the other strain, *S. aureofaciens*, the donor. In this respect, it is worthwhile to mention that sexual polarity has been described in *S. coelicolor* (14).

ACKNOWLEDGMENTS

We wish to acknowledge the helpful advice of L. L. Cavalli-Sforza and G. E. Magni.

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