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Protoplast fusion in *Streptomyces avermitilis*

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SUMMARY

The power of protoplast fusion as a generally applicable method for obtaining genetic recombination is demonstrated by the recombination of genes involved in avermectin biosynthesis. A backcross of *Streptomyces avermitilis* strain MA6202, an improved mutant that had lost the ability to carry out the methylation of the C-5 hydroxyl of the avermectin molecule, with the original soil isolate MA4680 resulted in the recovery of at least one unambiguous recombinant class despite the instability of rifampicin resistance, one of two markers initially used for recombinant selection. Such intrinsic instability is frequently encountered in streptomycete genetics, and this result delineates the utility of protoplast fusion as a genetic tool. Other difficulties addressed include recovery of complementary recombinant classes, differences in recombination frequency due to colony density on regeneration medium, and alteration in plating efficiency on diagnostic media following protoplasting and regeneration. The results of a cross between a nicotinamide auxotroph MRG1003 and a lysine auxotroph MRG1004 are included to aid in the elucidation of these problems as well as to support the finding of homologous recombination in *S. avermitilis*.

INTRODUCTION

Avermectins are a novel class of natural products highly active against nematodes and arthropod parasites in a wide variety of hosts but lacking antibacterial or antifungal activities. Their mode of action, as discussed by Campbell [5], is to open GABA-regulated chloride ion channels causing inhibition of neurotransmission in nematodes and neuromuscular transmission in arthropods. The result of avermectin treatment is thus paralysis and death. Burg et al. [4] reported that this group of closely related, 16-membered macrocyclic lactones is produced by a previously unknown actinomycete, MA4680 (NRRL 8165), isolated from soil by the Kitasato Institute. This species was named *Streptomyces avermitilis*, which was intended to convey the idea that it creates a wormless or 'averminous' condition [5].

Avermectins are typical secondary metabolites in that biosynthesis commences after the cessation of growth and involves a novel biosynthetic pathway. Avermectins, as produced by the original soil

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isolate, are a mixture of eight closely related compounds (Fig. 1).

Genetic studies of avermectin biosynthesis are complicated by the absence of readily selectable markers or easily detectable biosynthetic intermediates. Protoplast fusion provides a generally applicable method of obtaining genetic recombination. The increased genetic variability so obtained should be useful in a strain improvement program, in particular, by eliminating deleterious mutations that are likely to accumulate in a classical strain development program. An efficient system of genetic recombination by protoplast fusion was established for *S. avermitilis* for use as a routine tool both for studying the biology of the organism and for constructing improved strains of this industrially important streptomycete.

MATERIALS AND METHODS

Strains

The strains used in these studies and their relevant phenotypes are listed in Table 1. MA6202 was derived by C. Ruby from a *S. avermitilis* strain that had undergone several rounds of mutation. MA4680 (NRRL 8165) is the original soil isolate.

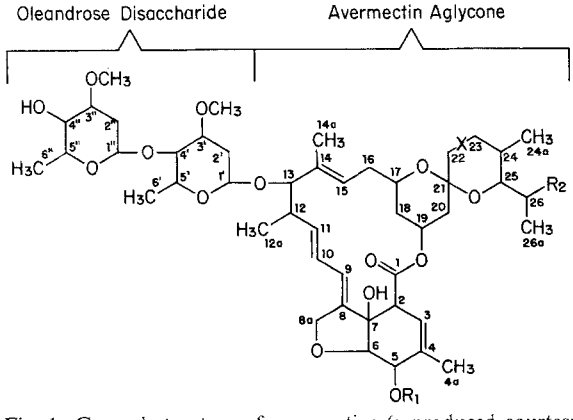

Fig. 1. General structure of avermectins (reproduced courtesy of M.D. Schulman [14]). Avermectin terminology is as follows: R_1 = H in B components; R_1 = CH_3 in A components; X = CH=CH in 1 components; H = CH_2CHOH in 2 components; R_2 = CH_2-CH_3 in a components; R_2 = CH_3 in b components.

Table 1

List of strains used in present study

Strain	Relevant phenotype[a]	Notes
MA6202	*Rif*^{S} *Grn Avr*$^+$ *Omt*$^-$ *Mal*$^-$	improved mutant
MA4680	*Rif*^{R} *Brn Avr*$^-$ *Omt*$^+$ *Mal*$^+$	original soil isolate
MRG1003	*Nic*	mutant of MA5080
MRG1004	*Lys*	mutant of MA5080

[a] Abbreviations: *Rif* = susceptibility to rifampicin (R or S); *Brn* = brown spores; *Grn* = green spores; *Nic* = requires nicotinamide; *Lys* = requires lysine; *Avr* = avermectin detectability on TLC (+ or −); *Omt* = presence or absence of *O*-methyltransferase; *Mal* = ability to use maltose as sole carbon source (+ or −).

MRG1003 and MRG1004 are auxotrophic derivatives of *S. avermitilis* MA5080 from the collection of R. Goldberg. Strains were maintained as frozen vegetative mycelia (FVM) at −80°C and as spore suspensions in 40% (w/v) glycerol at −20°C.

Chemicals

Lysozyme (hen egg white), 3 × -crystallized, with a specific activity of 14 870 Shugar units /mg was obtained from Calbiochem-Behring Corp., La Jolla, CA. Lysozyme of specific activity 25 000 Shugar units/mg obtained from Calbiochem-Behring Corp. may also be used. Polyethylene glycol MW 1000 was obtained from BDH Chemicals Ltd., Poole, U.K.

Protoplast formation and storage

Methods used were those of Hopwood et al. [11] except as listed. YEME mycelial growth medium contained 25% sucrose and 0.2–0.8% glycine depending on the strain. Flasks containing 30 ml were inoculated with 1.0 ml of FVM and incubated for 3 days at 27°C. P10 buffer was P buffer with 0.01 M MES, pH 6.5, substituted for TES. After harvest, mycelia were incubated with lysozyme solution for 60–90 min at 37°C. The protoplast pellets were resuspended in P20 buffer (P10 buffer that contained 20% sucrose). Protoplasts were frozen in a dry ice/ethanol bath prior to storage at −80°C.

Protoplast fusion

The method of Hopwood et al. [11] was used, except that stored protoplasts were mixed in equal ratios (approx. 2×10^7 protoplasts of each parent) based on pre-determined viable counts from RM14 medium. After fusion, protoplasts were diluted in P20 buffer.

Regeneration

RM14 medium was modified from RM medium described by Foor et al. [10]. It contained sucrose 250 g, K_2SO_4 0.25 g, $MgCl_2 \cdot 6H_2O$ 10.12 g, glucose 10 g, casamino acids 0.1 g, Bacto-oatmeal agar 3 g, L-proline 3 g, yeast extract 2 g, trace elements 2 ml, agar 20 g, and distilled water to 930 ml. Additions made after autoclaving were 10 ml of 0.5% KH_2PO_4, 10 ml of 2 M $CaCl_2 \cdot 2H_2O$, and 50 ml of 0.2 M MES buffer, pH 6.5. One liter of trace elements solution contained $ZnCl_2$ 40 mg, $FeCl_3 \cdot 6H_2O$ 200 mg, $CuCl_2 \cdot 2H_2O$ 10 mg, $MnCl_2 \cdot 4H_2O$ 10 mg, $Na_2B_4O_7 \cdot 10H_2O$ 10 mg, and $(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$ 10 mg. Ten-fold dilutions of fused protoplasts were gently spread on the agar surface, and plates were incubated for 14 days at 27°C and 90% relative humidity. RM14 without the osmotic stabilizer sucrose was used to monitor the presence of any non-protoplasted units in the samples.

Maturation of regenerated protoplasts

Regenerants remained bald on RM14 medium. To facilitate sporulation, the agar from regeneration plates was transferred intact to the surface of 2-fold-concentrated YME-TE agar medium to allow replacement by diffusion of regeneration medium constituents with those of a medium optimal for sporulation. YME-TE medium (L. Kaplan and M. Nallin, personal communication) consisted of glucose 0.4%, malt extract 1.0%, yeast extract (Difco) 0.4%, trace elements 5 ml, and agar 2.0%, pH 7.0. One liter of trace elements solution was prepared in 0.6 N HCl and contained $MgSO_4 \cdot 7H_2O$ 61.1 g, $CaCO_3$ 20 g, $FeCl_3 \cdot 6H_2O$ 5.4 g, $ZnSO_4 \cdot 7H_2O$ 1.44 g, $MnSO_4 \cdot H_2O$ 1.11 g, $CuSO_4 \cdot 5H_2O$ 0.25 g, $CoCl_2 \cdot 6H_2O$ 0.28 g, H_3BO_3 0.062 g, and $Na_2MoO_4 \cdot 2H_2O$ 0.49 g. Plates were incubated for an additional 5–7 days at 27°C by which time sporulation had occurred.

Isolation and identification of recombinants

Following fusion, regeneration and sporulation were performed under non-selective conditions; spores were harvested and analyzed by direct plating on selective and non-selective media. Alternatively, individual colonies from YME-TE were patched onto selective and non-selective media using sterile toothpicks. Minimal medium of Hopwood et al. [11] may be used. When required, minimal medium was supplemented with 2 μg/ml nicotinamide or 50 μg/ml L-lysine or both. For maltose utilization studies, glucose and L-asparagine were replaced by 0.2% maltose. YME-TE with and without 0.5 μg/ml rifampicin was used to detect differences in spore color and susceptibility to rifampicin.

Fermentation and extraction

Fermentation conditions were basically those of Burg et al. [4]. Fermentation medium B with minor modification was used. Avermectins were extracted from the cells by adding methanol, shaking for 10 min, and centrifuging to obtain a clear extract.

Thin layer chromatography (TLC)

Extracts were spotted on Kieselgel 60 F_{254} precoated silica gel TLC plates and developed for 12 min in ethyl acetate/methylene chloride/methanol (9:9:1) (R. Goegelman, personal communication). Bands were visualized by fluorescence quenching under a 254 nm lamp.

Fragmentation of vegetative mycelia

Vegetative mycelia from regeneration plates were placed in P20 buffer containing 4 mm glass beads and vortexed vigorously; 10 ml cells plus 40 ml P20 buffer were placed in a standard rosette flask packed in ice and sonicated at 52 W for 0, 5, 10, 20, 30, 40, 50, 60, 90 and 120 s using a Braun Sonic 2000 cell disrupter with a standard probe immersed approximately 1.5 cm into the liquid. Viable counts and spore color differences were determined on YME-TE agar medium. Aliquots of the suspen-

sion, following vortexing with glass beads, were frozen in a dry ice/ethanol bath and stored at −80°C.

RESULTS

Regeneration frequency

S. avermitilis protoplasts regenerate at a rate that may be considered to be quite good. Results for the four strains used in this study are presented in Table 2 and were calculated as the number of regenerants per ml obtained from RM14 medium compared to the number of protoplasts per ml determined using a hemacytometer counting chamber.

The relative concentration of osmotically stable forms in the protoplast preparations was low (Table 3), and these osmotically stable plating units represented less than $1/10^5$ of the total viable count ($\approx 2 \times 10^7$ cfu/ml). The numbers were calculated by determining the number of cfu's at the appropriate dilution on RM14 medium with and without the osmotic stabilizer sucrose. Approximately 2×10^7 viable protoplasts of each parent were combined and fused (1.0 ml total volume), diluted, and plated. At a 10^{-2} dilution of the fusion mixture, no cfu's were present on plates containing RM14 medium without sucrose. Further dilution indicated that the total viable count on RM14 with sucrose was 2×10^7 cfu/ml (data not shown).

Cross of MA6202 with MA4680

The protoplast fusion cross of these two strains employed two naturally occurring markers, spore color and rifampicin susceptibility. MA4680 produced brown spores and was resistant to rifampicin at 0.5 μg/ml; MA6202 produced green spores and was sensitive to rifampicin at 0.5 μg/ml. Colonies were initially classified on the basis of spore color and then tested for susceptibility to rifampicin by patching. The relevant recombinant classes were *Rif*^{R} *Grn* and *Rif*^{S} *Brn*. Regeneration plates (RP's) of different colony density were analyzed separately (Table 4), and the proportion of recombinants isolated appeared greatest for both classes at a density of approximately 2×10^4 cfu/RP.

Self-fused parents served as controls. In the case of MA6202, neither *Rif*^{R} *Grn*, *Rif*^{S} *Brn* nor *Rif*^{R} *Brn* isolates were detected.

Self-fusion of MA4680, however, resulted in 11.9% *Rif*^{S} *Brn* strains. Using the totals from all RP colony densities (Table 4), Chi-square analysis of the self-fusion of MA4680 with the actual cross indicates that the increase in recombinant phenotype is highly significant ($P < 0.001$). If one assumes that approx. 11.9% of the *Rif*^{S} *Brn* strains from the cross may have resulted from self-fusion, subtraction from the 22.1% *Rif*^{S} *Brn* obtained from the cross yields about 10% *Rif*^{S} *Brn* recombinants resulting from the cross of MA4680 with MA6202, compared to about 6% *Rif*^{R} *Grn*.

To answer the question of a possible mutational event having occurred during self-fusion of MA4680, a comparison was made among spores obtained from untreated cells, from regenerated

Table 2

Regeneration frequency of protoplasts, viable counts vs. hemacytometer counts

Strain	% Regeneration
MA6202	19
MA4680	27
MRG1003	27
MRG1004	28

Table 3

Comparison of osmotically fragile and osmotically stable forms in protoplast preparations

Strain	% Glycine in YEME growth medium	cfu/ml	
		osmotically fragile forms	osmotically stable forms
MA6202	0.8	4.5×10^8	none
MA4680	0.5	1.3×10^8	1.0×10^1
MRG1003	0.5	2.0×10^8	1.0×10^2
MRG1004	0.8	2.0×10^7	4.5×10^1

Table 4

Recombination analysis of a cross between MA6202 and MA4680

Cross	cfu/RP	Number of recombinants/total number tested (%)	
		Rif^R *Grn*	*Rif*^{S} *Brn*
MA4680 × MA4680	3×10^4	n.d.[a]	61/512 (11.9)[b]
MA6202 × MA6202	3×10^4	0/512	n.d.
MA4680 × MA6202	2×10^5	9/256 (3.5)	50/256 (19.5)
MA4680 × MA6202	2×10^4	46/512 (9.0)	131/512 (25.5)
MA4680 × MA6202	2×10^3	13/256 (5.1)	45/256 (17.6)
MA4680 × MA6202	total	68/1024 (6.6)	226/1024 (22.1)[b]

[a] None detected in more than 700 tested.
[b] $\chi^2 = 22.5$ ($P < 0.001$).

protoplasts, and from self-fused and regenerated protoplasts. Of 490 colonies obtained from native spores, no *Rif*^{S} strains were obtained. The frequency was increased to 29/490 and 40/490 by protoplasting and regeneration without and with self-fusion, respectively.

Clearly, other means had to be employed to further distinguish *Rif*^{S} *Brn* recombinants from possible parental types. The *S*-adenosylmethionine-dependent methylation of the C-5 hydroxyl is performed by avermectin B *O*-methyltransferase [14]. The original soil isolate, MA4680, possesses this enzyme (*Omt*$^+$); MA6202 is *Omt*$^-$. An additional 1027 potential recombinants were isolated (Table 5). From among 2313 *Brn* colonies tested, 613 (27%) were *Rif*^{S}. From among 4600 *Grn* colonies tested, 414 (9.0%) were *Rif*^{R}. These 1027 strains as well as 105 parental *Rif*^{S} *Grn* strains from the self-fusion of MA6202 and 170 *Rif*^{R} *Brn* parental types and 61 *Rif*^{S} *Brn* strains from the MA4680 self-fusion were fermented and extracted. Extracts were then tested for detectable levels of avermectin on TLC (*Avr*$^+$). It was interesting to note that 14/61 (23%) *Rif*^{S} strains from the self-fusion of MA4680

Table 5

Segregation of genes involved in the biosynthesis of avermectin: analysis of a cross of MA6202 with MA4680

Cross	Recombinant class	No. detected/ No. tested[a]	Unselected markers (No. detected/No. tested)			
			Avr$^-$	*Avr*$^+$	*Omt*$^-$	*Omt*$^+$
MA6202 × MA6202	*Rif*^{S} *Brn*	n.d.[b]				
	Rif^R *Grn*	0/105	9/105	96/105	96/96	0/96
MA4680 × MA4680	*Rif*^{S} *Brn*	61/513	47/61	14/61	0/14	14/14
	Rif^R *Grn*	n.d.				
MA4680 × MA6202	*Rif*^{S} *Brn*	613/2313	448/613	165/613	88/165	77/165
	Rif^R *Grn*	414/4600	162/414	252/414	152/252	100/252

[a] Initial selection and classification were based on spore color.
[b] n.d. = none detected. Relates to the inability to detect the indicated spore color.

showed ± or better avermectin production by TLC while only one strain (0.6%) was scored as ± from among the 170 *Rif*^{R} isolates from that self-fusion. The remaining 169 strains were negative by TLC. The presence of *Omt* can only be scored by TLC in strains classified as *Avr*$^+$. It should be emphasized that the *Avr* notation is at best a semi-quantitative marker; it is obvious that the original soil isolate has the biosynthetic pathway, and *Omt* activity has been demonstrated by other methods. Those strains with detectable avermectin production were further scored with respect to the presence or absence of *Omt* based on the detection of avermectin A components. All strains with detectable levels of avermectin by TLC isolated from the self-fusion of MA6202 were *Omt*$^-$, while all from the self-fusion of MA4680 were *Omt*$^+$. Among strains isolated from the cross, it was found that 162 (39%) of *Rif*^{R} *Grn* strains were *Avr*$^-$; 252 (61%) were *Avr*$^+$ and, of these, 100 (40%) were *Omt*$^+$. It was also found that 448 (73%) of *Rif*^{S} *Brn strains were Avr*$^-$; 165 (27%) were *Avr*$^+$ and, of these, 77 (47%) were *Omt*$^+$.

Retrospective analysis of the parental strains, MA4680 and MA6202, revealed that the latter was unable to grow on maltose (*Mal*$^-$) as sole carbon source while the original soil isolate grew slowly (*Mal*$^+$) and eventually sporulated. Selected *Avr*$^+$ recombinants were tested for maltose utilization, and complementary recombinants were found in all four groups, yielding the eight predicted recombinant classes (Table 6). *Avr*$^-$ strains were not tested.

Table 6

The ability of *Avr*$^+$ recombinants to grow on maltose as sole carbon source

Recombinant class	n[a]	Mal$^+$ [b]
Rif^R *Grn Avr*$^+$ *Omt*$^+$	66	31
Rif^R *Grn Avr*$^+$ *Omt*$^-$	19	1
Rif^S *Brn Avr*$^+$ *Omt*$^+$	42	10
Rif^S *Brn Avr*$^+$ *Omt*$^-$	33	11

[a] Total number of strains tested.
[b] Number of strains that grew on maltose.

Table 7

Frequency of prototrophic recombinants by direct plating of spores in a cross of MRG1003 with MRG1004: effect of RP colony density

Approximate cfu/RP	% Prototrophs
1.5×10^5	0.5
1.5×10^4	0.7
1.5×10^3	2.2
1.5×10^2	2.7

Cross of MRG1003 with MRG1004

MRG1003 is a nicotinamide auxotroph (*Nic*) with a spontaneous reversion frequency of 2×10^{-5}; MRG1004 is a lysine auxotroph (*Lys*) with a spontaneous reversion frequency of 4×10^{-6}. Analysis of the cross by direct plating of spores (Table 7) showed that the frequency of prototrophs increased from 0.5 to 2.7% as the RP colony density decreased from 1.5×10^5 to 1.5×10^2 cfu/RP. Although the prototrophs are stable, a heterocaryon cannot rigorously be excluded. To shed light on the nature of the recombinants, we elected to determine the frequency of double auxotrophs.

To test for the presence of double auxotrophs, individual colonies, selected at random and grown from spores obtained from RP plates at the indicated density, were tested by patching on diagnostic media (Table 8). The frequency of prototrophs from the 1.5×10^3 colony density RP's was 3.2% but no double auxotrophs were isolated. The frequency of prototrophs from the 1.5×10^4 colony density RP's was lower, 1.8%, but double auxotrophs were isolated at a frequency of 0.6%. Recovery of the *Nic* parent, an excellent sporulator, was considerably greater than that of the *Lys* parent.

When the direct plating method was compared to the patching method, it was found that the frequency of prototrophs obtained by the patching method was 1.5–2.5 times greater than that observed by direct plating. The difference was small, but it was subsequently found that the efficiency of plating (EOP) of spores from regenerated protoplasts of MA5080 was almost 10-fold lower than

Table 8

Isolation of markers in a cross of MRG1003 with MRG1004 demonstrated by patching of isolated colonies

Approximate cfu/RP	Phenotype: No. observed/No. tested (%)					
	prototrophs		*Nic Lys*		*Nic*	*Lys*
	ratio	(%)	ratio	(%)	ratio	ratio
1.5×10^4	9/490	(1.8)	3/490	(0.6)	472/490	6/490
1.5×10^3	19/588	(3.2)	0/588	(0)	557/588	12/588

that of native spores when plated on minimal medium. The possibility that protoplasting and regeneration had produced large numbers of auxotrophs was tested by patching, but no auxotrophs were found. The EOP for MRG1003 and MRG1004 was also tested (Table 9) and found to be decreased when spores were obtained from regenerated protoplasts.

Fragmentation of vegetative mycelia

Since the recovery of viable spores appears to be one of the factors involved in inconsistencies in the recovery of complementary recombinant classes, it was felt that any future mapping and linkage studies would be compromised by the continued use of spores. Therefore, an alternative method of harvest was sought. A subsequent cross of one strain that produces abundant brown spores with one that produces reduced quantities of green spores was made. Half of the RP's containing approximately 10^4 cfu/plate were transferred to obtain spores. The other half were harvested as vegetative mycelia and fragmented as described in Materials and Methods. Microscopic examination indicated that after 40–50 s only tiny fragments were detectable. Sonication for 30–60 s increased plating efficiency by two orders of magnitude but sonication for greater than 60 s resulted in a gradual decrease in viable count (data not shown). Both the spores and the fragmented mycelia were plated on YME-TE and spore color of the resulting colonies was used as an indication of the frequency of parental types obtained with the two methods. Table 10 shows that parental types were recovered in essentially equal ratios

Table 9

EOP of spores from regenerated and native sources on minimal medium compared to YME-TE medium

Strain	EOP[a]	
	native	regenerated
MA5080	1.0	0.12
MRG1003	1.0	0.12
MRG1004	0.75	0.01

[a] Minimal medium included nicotinamide or L-lysine as appropriate.

Table 10

Comparison of spores and fragmented vegetative mycelia for recovering equal ratios of parental types in a protoplast fusion cross

RP colony density was approximately 10^4 cfu/plate.

	Source of cfu	
	spores	fragmented vegetative mycelia[a]
Percent *Grn*	1.5	42
Percent *Brn*	98.5	58

[a] The proportion obtained from 40 s sonication is reported; however, all treatments gave similar results.

when fragmented mycelia were used but that the brown parent outnumbered the green parent by about 65 to 1 when spores were used. This indicates that, by using fragmented mycelia, sporulation differences will no longer play a role in discrepancies found in recombination frequencies.

DISCUSSION

Recombination in *S. avermitilis* elicited by protoplast fusion has been demonstrated with two sets of genetic markers. In both instances all classes of recombinants can be demonstrated or otherwise accounted for. Recovery of recombinants was dependent, however, on the RP colony density, the optimum for both crosses being approximately 2×10^4 cfu/RP (Tables 4 and 7). The fact that recombination frequencies were lower at very high RP colony density was not surprising. Regeneration is slow and asynchronous and, if recombinants are late in regenerating, the early regenerants may crowd them out either by production of antibiotic or of some lytic enzyme or perhaps by medium depletion. Crowding is believed by some [12] to be due to the presence of non-protoplasted units on RP's. This was not the case in the present study, since non-protoplasted units were absent from both fusions at all RP colony densities tested. On the other hand, when regeneration plates contain isolated colonies, these colonies have a greater opportunity to exhibit size differences. Differences in colony size will be reflected by unequal contributions to a pooled spore or mycelial preparation. This could explain the fact that prototrophs were found at the greatest frequency at the lowest RP colony density tested but no double auxotrophs were found at that same density, yet both classes were isolated from a higher RP colony density (Table 8). It is, therefore, wise to determine the optimal RP colony density for all recombinant classes using several sets of markers and to aim for that density in future crosses. Alternatively, although a great deal of extra work is involved, different dilution sets may be evaluated as separate experiments.

Differences in sporulation ability also played a role in the frequency of recovery of certain recombinant classes. For example, the double auxotrophs obtained from the cross of MRG1003 with MRG1004 were poor sporulators and would thus be underrepresented in the spore progeny consisting of prototrophs and parents. In fact, even though input of parental types was equal, the output from spores greatly favored the well-sporulating MRG1003 (*Nic*) parent (Table 8). The discrepancy between the frequency of prototrophs and double auxotrophs may be more apparent than real. It is likely that differences in colony size of regenerants as well as in efficiency of sporulation favored the isolation of prototrophs rather than double auxotrophs in the experiment described above. Spores are desirable for stability upon storage, ease of sampling, and reproducibility as well as for inspiring confidence that each cfu is haploid. However, the problems presented by use of spores far outweigh the benefits. The use of fragmented vegetative mycelia (Table 10), combined with attainment of proper RP colony density, is expected to solve the problems presented by the use of spores.

The direct plating of spores as a method of recombinant identification in the cross of MRG1003 with MRG1004 demonstrated another potential hazard. The differences in prototrophic recombination frequencies were small (1.5–2.5-fold) when the patching method (Table 8) was compared to the direct plating method (Table 7). However, the decrease in EOP on minimal medium of spores obtained from protoplasted and regenerated MRG1003 and MRG1004 and their prototrophic parent MA5080 compared to untreated cells (Table 9) suggests that protoplasting and regeneration may cause some deficiency in germination ability and, thus, recombinants may be lost. We attempted to circumvent this problem by first plating under nonselective conditions and then testing individual colonies for desired traits. Fragmentation of vegetative mycelia appears to be an alternative approach to the problem.

Differences in sporulation efficiency cannot account for the difference in the frequency of Rif^R *Grn* and Rif^S *Brn* recombinants in the cross of MA6202 with MA4680, since spore color was selected prior to testing for susceptibility to rifampicin.

In the cross of MA6202 with MA4680, the possibility of plasmid involvement in *Rif*^{R} is being considered. Self-fusion of MA6202 yielded only *Rif*^{S} *Grn* parental types. MA4680 spores from untreated cells yielded only *Rif*^{R} *Brn* parental clones. However, regenerants of both self-fused and non-fused protoplasts of MA4680 produced *Rif*^{S} *Brn* strains at a frequency of 6–12%, suggesting the possibility of plasmid curing during protoplasting and/or regeneration [1]. Initial attempts to physically isolate plasmid DNA from *S. avermitilis* were unsuccessful, although more rigorous conditions have not yet been tried (P. Gibbons, personal communication). Thus, conjugal matings to investigate the possible existence of a self-transmissible element are indicated. Stonesifer et al. [15], although unable to isolate plasmid DNA from wild-type *S. fradiae,* have evidence that structural genes encoding enzymes for biosynthesis of tylosin (a 16-membered lactone with three sugars produced by *S. fradiae*), genes involved in the expression of resistance to tylosin and several other antibiotics, and a single copy of an amplifiable unit of DNA were jointly transferred by conjugation from several different *S. fradiae* strains to two different mutants of *S. fradiae*. Also, tylosin biosynthesis and resistance could be lost following protoplast regeneration. This, added to differences in phenotype between the two recipients, suggested to them that loss of tylosin biosynthesis and resistance might be due to deletions of either plasmid or chromosomal DNA spanning different but overlapping regions of DNA rather than to simple plasmid curing.

Self-fusion of MA4680 resulted in a single *Rif*^{R} isolate (from among 170 tested) yielding detectable levels of avermectin by TLC. *Rif*^{S} isolates from the same experiment gave detectable avermectin in 23% (14/61) of the cases. In addition, band intensities on TLC from a number of the *Rif*^{S} isolates were greater than the one *Rif*^{R} strain with detectable levels of avermectin. Genetic instability in *Streptomyces* is not uncommon, and it may be possible that DNA deletion and amplification are involved in loss of expression of rifampicin resistance and in an increase in the level of avermectin biosynthesis [2,3,7,8,9,13,15].

An intriguing possibility relates to the unproven mechanism of rifampicin resistance in *S. avermitilis*. Since *Rif*^{R} mutants in bacteria usually have an altered RNA polymerase [16], it is quite possible that loss of resistance to rifampicin in *S. avermitilis* may be due to a modified RNA polymerase. Chater [6] has shown that streptomycetes possess rifampicin-sensitive RNA polymerase as do other procaryotes, with sensitivity ranging from 0.01 μg/ml for complete inhibition of *S. albus* to 10 μg/ml for 50% inhibition of the enzyme in *S. coelicolor* A3(2) and in *S. lividans* 1326. The concentration of rifampicin necessary for complete inhibition of growth in wild-type *S. avermitilis* was determined to be about 0.5 μg/ml. If an altered RNA polymerase does exist in *S. avermitilis,* it may be more efficient in transcription of genes involved in avermectin biosynthesis. It will be interesting to test the effect on avermectin production when *Rif*^{R} *Grn* recombinant strains (all of which produce lower levels of avermectin than their high-producing *Rif*^{S} *Grn* parent) are made *Rif*^{S} by protoplasting and regeneration.

One of the complications in analyzing this cross is the observation that a significant fraction of the *Rif*^{S} *Brn* strains may not be the result of recombination but of self-fusion or of protoplasting and regeneration. Further separation into other classes based on avermectin production, *O*-methyltransferase activity, and maltose utilization showed that at least some *Rif*^{S} *Brn* strains from the cross were indeed the result of recombination. For example, the only class of *Rif*^{S} *Brn Avr*$^+$ strains resulting from self-fusion or protoplasting and regeneration alone was also *Omt*$^+$ and *Mal*$^+$, consistent with loss of a single genetic locus. This class represents a minority of the *Rif*^{S} *Brn* recombinants tested, and all *Rif*^{R} *Grn* strains are clearly the result of recombination.

Protoplast fusion, although an imperfect system, has been shown to generate different phenotypes in *S. avermitilis*; in crosses involving pairs of genetically marked strains, all recombinant phenotypes sought were recovered. The utility of this technique as a general means of genetic recombination is further emphasized by the fact that, even with an unstable marker, at least one unambiguous recombinant class was isolated easily and at high frequency,

and the ability to restore *O*-methyltransferase biosynthetic activity to an improved mutant that had lost it has important implications for a strain improvement program.

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