PHENOTYPIC CHANGES ASSOCIATED WITH LOSS OF EXPRESSION OF TYLOSIN BIOSYNTHESIS AND RESISTANCE GENES IN *STREPTOMYCES FRADIAE*

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Two mutants of the tylosin-producing *Streptomyces fradiae* defective in the biosynthesis of the macrolide antibiotic tylosin were isolated from colonies derived from regenerated protoplasts. Both strains were unable to carry out any of at least seven tylosin biosynthetic steps and were sensitive to tylosin. One strain, JS82, was also more sensitive to chloramphenicol (Cm), mitomycin C (Mc), hygromycin B (Hm) and kanamycin (Km) than its parent strain. The other strain, JS87, was also more sensitive to Cm than wild type but expressed normal levels of resistance to Mc and Hm. Both strains expressed genetic instabilities associated with auxotrophy or expression of antibiotic resistance. Since the genetic instabilities were not due to defective error-free or error-prone DNA repair, they appear to be due to genetic rearrangements associated with the deletion or amplification of sequences linked to and perhaps encompassing tylosin biosynthesis genes.

Streptomyces fradiae produces tylosin[1,2] an economically important macrolide antibiotic. Recent studies with mutants blocked in different steps of tylosin biosynthesis have elucidated the biosynthetic pathway from tylactone to tylosin[1,2,3] and have identified a rate limiting step in the biosynthetic pathway suitable for applied genetic manipulation[4,5].

We have been interested in defining the genetic locations and possible physical linkage of tylosin biosynthesis genes in *S. fradiae*. Since in conjugal matings we were not able to demonstrate recombination between mutants of *S. fradiae* carrying auxotrophic mutations or antibiotic resistance mutations (which were presumably chromosomal)[6], we developed a protoplast fusion procedure to carry out genetic recombination and mapping[7,8]. In preliminary experiments to map the tylosin biosynthesis genes, it was observed that production of tylosin or tylosin-like intermediates or branch products of specific mutants was lost at high frequencies after genetic recombination mediated by protoplast fusion[6]. Since protoplast formation and regeneration can cause loss of plasmids in *Streptomyces*[9,10,11] and in other Gram-positive bacteria[12,13] we initiated studies to determine if the tylosin biosynthesis genes were located on a plasmid or plasmid-like element. To approach this question, we first attempted to obtain strains of *S. fradiae* cured of plasmid by protoplast regeneration.

Two strains, JS82 and JS87, which were obtained by protoplast regeneration of wild type and high tylosin producing strains, respectively, were unable to produce tylosin and were sensitive to tylosin. In conjugation experiments with various strains proficient in the production of tylosin or blocked in specific steps of tylosin biosynthesis used as donors, JS82 was shown to be a high frequency recipient for expression of tylosin resistance and tylosin structural genes, but not for other (presumably chromosomal) genes[14]. Attempts to isolate plasmid DNA from JS82, JS87 and the parental strains, however, were unsuccessful[15] (MATSUSHIMA and BALTZ, unpublished). In experiments to determine if the loss of tylosin production might be due to a loss of plasmid DNA in *S. fradiae* JS82, it was ob-

served that JS82 contained a 10.5 kb DNA sequence amplified in tandem about 500-fold[15]. The amplifiable unit of DNA is present in a single copy in wild type strains of *S. fradiae* and is bounded by 2.2 kb direct repeat sequences[16]. Wild type strains also contain two more sequences homologous to the direct repeat sequences.

Unlike JS82, JS87 did not contain amplified DNA. Instead, JS87 was deleted for the amplifiable unit of DNA and for all four homologous sequences which are present in single copies in wild type cells[17]. It therefore appeared that both deletion and amplification was associated with the loss of tylosin production and may have been triggered by the process of protoplast regeneration.

In this report we describe additional genetic properties of JS82 and JS87. We found that deletion or amplification is associated with a variety of phenotypic changes in these strains. None of seven measurable tylosin biosynthetic enzyme conversions (and probably many more) can occur in either of the strains and several other antibiotic resistance phenotypes normally expressed in wild type *S. fradiae* are not expressed in these strains. Also, JS82 and JS87 display genetic instabilities that are not associated with defects in normal pathways of error-free or error-prone repair of damaged DNA.

Materials and Methods

Chemicals

Rifampin (Rif) and mitomycin C (Mc) were purchased from Calbiochem-Behring Corporation; chloramphenicol (Cm) was purchased from Aldrich Chemical Company; spectinomycin hydrochloride (Spc) was kindly supplied by the Upjohn Company; and hygromycin B (Hm) and tylosin (Tyl) were supplied by Eli Lilly and Company.

Streptomyces Strains

The *Streptomyces* strains used in this study are listed in Table 1. The spontaneous Rif resistant and Spc resistant mutants were isolated on AS-1 agar containing 50 μg per ml of the respective antibiotics.

Media and Growth Conditions

S. fradiae strains were grown in Trypticase soy (TS) broth and fragmented by ultrasound as described previously[7]. NB agar contained 8 g nutrient broth (Difco) and 15 g agar per liter distilled water. CD medium contained 35 g Czapek Dox broth (Difco) and 15 g Difco agar per liter of distilled water. The following media were prepared as described: AS-1 and CDA agar media[6]; modified R2 medium[7]; P media[18]; hypertonic soft agar overlays[8] and complex fermentation and vegetative media[1].

Isolation of Mutants Defective in Tylosin Production

Protoplasts were prepared from *S. fradiae* T59235, diluted and plated on modified R2 agar as described[7]. Protoplasts of *S. fradiae* JS51 were prepared and regenerated as described[8]. Well isolated colonies derived from regenerated protoplasts were picked and tested for tylosin production as described[1]. They were also patched onto AS-1 agar plus 400 μg/ml tylosin to score for tylosin resistance.

Assay for Tylosin Biosynthetic Enzymes by *In Vivo* Bioconversions

Bioconversion tests were carried out *in vivo* with biosynthetic intermediates to tylosin as described[2]. Bioconversion of tylosin to relomycin was determined as described[1].

Determination of Antibiotic Resistance

Mycelia were grown to late exponential or early stationary phase, homogenized and sonicated. Cells were swabbed onto NB or AS-1 agar plates containing various levels of antibiotics for qualitative tests. For quantitative efficiency of plating tests, cells were serially diluted and plated on plates containing varying concentrations of antibiotics. All antibiotics were sterilized by filtration.

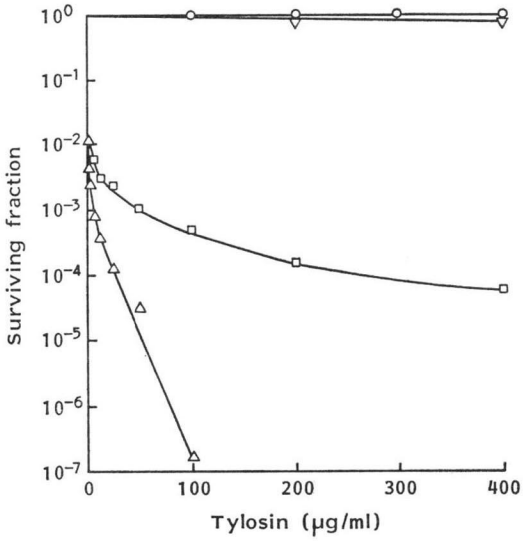
Table 1. *Streptomyces fradiae* strains.

Strain	Phenotype[a]	Derivation	Ref or source
C4	Tyl^r Tyl^+ Nar^- Hm^r Cm^r Mc^r Aud^+ Rif^s Spc^s His^+/Met^+	Sequential mutation of T59235	5)
JS51	Tyl^r Tyl^+ Nar^- Hm^r Cm^r Mc^r Aud^+ Rif^r Spc^s His^+/Met^+	Spontaneous Rif^r mutant of C4	14); 15); this study
JS87	Tyl^s Tyl^- Nar^- Hm^r Cm^s Mc^r Aud^- Rif^r Spc^s His^+/Met^+	Regenerated protoplast of JS51	This study; 15)
JS102	Tyl^s Tyl^- Nar^- Hm^r Cm^s Mc^r Aud^- Rif^r Spc^s His^-/Met^-	Spontaneous auxotrophic sector of JS87	This study
JS103	Tyl^s Tyl^- Nar^- Hm^r Cm^s Mc^r Aud^- Rif^r Spc^s His^-/Met^-	Spontaneous auxotrophic sector of JS87	This study
JS104	Tyl^s Tyl^- Nar^- Hm^r Cm^s Mc^{rr} Aud^- Rif^r Spc^s His^-/Met^-	Spontaneous Mc^{rr} mutant of JS102	This study
JS105	Tyl^s Tyl^- Nar^- Hm^r Cm^s Mc^{rr} Aud^- Rif^r Spc^s His^-/Met^-	Spontaneous Mc^{rr} mutant of JS103	This study
T59235	Tyl^r Tyl^+ Nar^+ Hm^r Cm^r Mc^r Aud^+ Rif^s Spc^s His^+/Met^+	Wild type strain	3)
JS82	Tyl^s Tyl^- Nar^+ Hm^s Cm^s Mc^s Aud^{500+} Rif^s Spc^s His^+/Met^+	Regenerated protoplast of T59235	14); 15), this study
JS85	Tyl^s Tyl^- Nar^+ Hm^s Cm^s Mc^s Aud^{500+} Rif^s Spc^r His^+/Met^+	Spontaneous Spc^r mutant of JS82	3); 15)
JS98	Tyl^s Tyl^- Nar^+ Hm^r Cm^s Mc^s Aud^{500+} Rif^s Spc^s His^+/Met^+	Spontaneous Hm^r mutant of JS82	This study
JS99	Tyl^s Tyl^- Nar^+ Hm^{rr} Cm^s Mc^s Aud^{500+} Rif^s Spc^s His^+/Met^+	Spontaneous Hm^{rr} mutant of JS98	This study
JS101	Tyl^s Tyl^- Nar^+ Hm^{rrr} Cm^s Mc^s Aud^{500+} Rif^s Spc^s His^+/Met^+	Spontaneous Hm^{rrr} mutant of JS99	This study

[a] Tyl^r, resistance to 400 μg/ml tylosin; Tyl^+, production of tylosin; Nar^-, nitrate reductase auxotrophy; Hm^r, resistance to 5 μg/ml hygromycin B; Hm^{rr}, resistance to 10 μg/ml; Hm^{rrr}, resistance to 20 μg/ml; Cm^r, resistance to 5 μg/ml chloramphenicol; Mc^r resistance to 1 μg/ml mitomycin C (EOP$\sim 10^{-2}$); Mc^{rr}, resistance to 4 μg/ml Mc (EOP$\sim 10^{-2}$); Aud^+, single copy of amplifiable DNA; Aud^{500+}, $\sim$500 copies of amplifiable DNA; Rif^r, resistance to 50 μg/ml rifampin; Spc^r, resistance to 50 μg/ml spectinomycin; His^-/Met^-, requirement for histidine or methionine. Unless stated otherwise, antibiotic resistance phenotypes indicate EOP's of 10^{-1} or higher.

EOP: Efficacy of plating.

Fig. 1. Sensitivity of *Streptomyces fradiae* strains to tylosin.
○ T59235, ▽ JS51, □ JS82, △ JS87.



Treatment of Cells with Mutagenic Agents

Inactivation of *S. fradiae* strains by ultraviolet light (UV), methyl methanesulfonate (MMS) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and mutagenesis by MNNG to spectinomycin resistance were determined as described[1,19].

Results

Isolation and Properties of *S. fradiae* JS82 and JS87

Protoplasts were prepared from two tylosin-producing *S. fradiae* strains, T59235, a wild type strain, and JS51, a Rif^r derivative of a high tylosin producing strain, C4 (Table 1). Twelve of 200 regenerated clones of T59235 produced little or no tylosin. Two of the 12 clones produced no detectable tylosin and were more sensitive to tylosin than T59235. One of the Tyl^-, Tyl^s clones, JS81, was auxotrophic and required methionine for growth. The other, JS82, was prototrophic and was chosen for further analysis. Fig. 1 shows the level of sensitivity of JS82 to tylosin. While JS82 was much more sensitive to tylosin than T59235, a subpopulation of the culture (about 1 in 10^4) formed colonies on media containing up to 400 μg/ml tylosin. Several of these (phenotypically) Tyl^r clones were picked, grown in TS broth and replated on tylosin. They expressed the same phenotype on tylosin as JS82 [*i.e.* a subpopulation of the culture (about 1 in 10^4) formed colonies].

When protoplasts of JS51 were regenerated on modified R2 agar, one of 250 clones produced no detectable tylosin and was much more sensitive to tylosin than JS51 (Fig. 1). This clone, JS87, was prototrophic but differed from JS82 in that it did not contain a subpopulation of cells phenotypically resistant to relatively high concentrations of tylosin (Fig. 1).

Bioconversion of Tylosin Intermediates

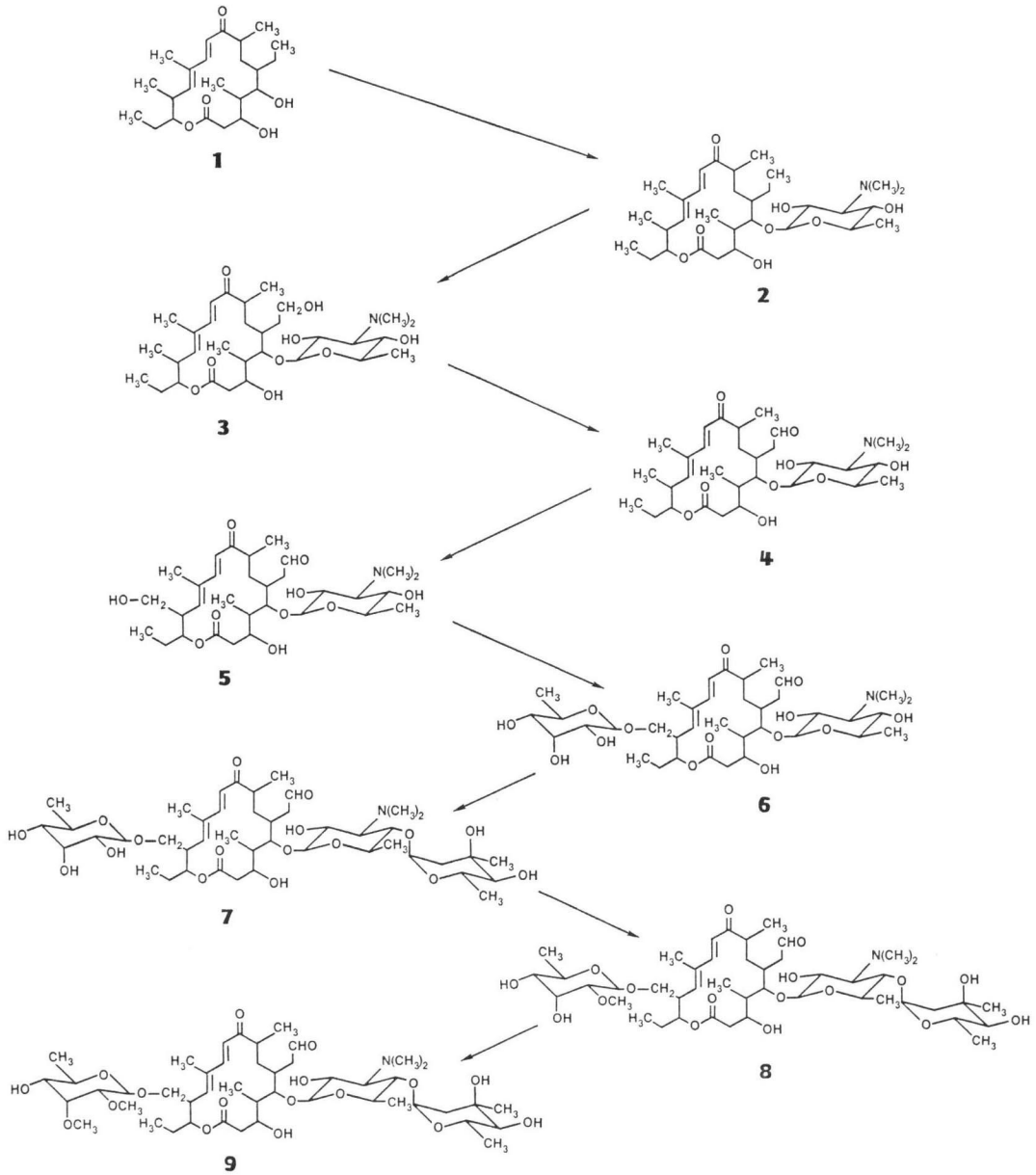
The preferred biosynthetic pathway from tylactone to tylosin is shown in Fig. 2. When the intermediates in tylosin biosynthesis are added to typical *tylG* mutants of *S. fradiae* they are bioconverted to tylosin or to intermediates to tylosin at efficiencies easily quantified by thin-layer chromatography and UV absorption analysis[2]. When compounds **1**, **3**, **4**, **5**, **6**, **7** and **8** (see Fig. 2) were added to cultures of JS82 and JS87 under conditions for optimal bioconversion in complex fermentation media, no bioconversion to any subsequent intermediates or to tylosin was observed (data not shown). However, compounds **7**, **8** and **9** were converted to 20-dihydrodemethylmacrocin, 20-dihydromacrocin and relomycin, respectively, at about 10% efficiency, indicating that both JS82 and JS87 produce normal levels of the macrolide reductase enzyme which plays no apparent role in tylosin biosynthesis[1].

Resistance of JS82 and JS87 to Other Antibiotics

Since plasmids often confer antibiotic resistance to cells harboring them, we surveyed JS82 and JS87 for several antibiotic resistance phenotypes as compared to their immediate parents. JS82 was

Fig. 2. The preferred biosynthetic pathway from tylactone to tylosin.

The macrolide structures shown are tylactone (**1**), *O*-mycaminosyltylactone (**2**), 20-dihydro-23-deoxy-*O*-mycaminosyltylonolide (**3**), 23-deoxy-*O*-mycaminosyltylonolide (**4**), *O*-mycaminosyltylonolide (**5**), demethyllactenocin (**6**), demethylmacrocin (**7**), macrocin (**8**) and tylosin (**9**)[2].



more sensitive to Mc than its parent T59235, while JS87 showed essentially the same level of Mc resistance as C4 and T59235 (Fig. 3a). Both JS82 and JS87 were more sensitive to Cm than T59235 or JS51 (Fig. 3b), while only JS82 was more sensitive to Hm and Km than the wild type strains (Figs. 3c and 4a). JS82 and JS87 showed wild type levels of resistance to several other antibiotics tested, including neomycin (Nm) (Fig. 4b).

Fig. 3. Sensitivity of *Streptomyces fradiae* strains to mitomycin C (Mc), chloramphenicol (Cm) and hygromycin B (Hm).
○ T59235, ▽ JS51, □ JS82, △ JS87, ● C4.

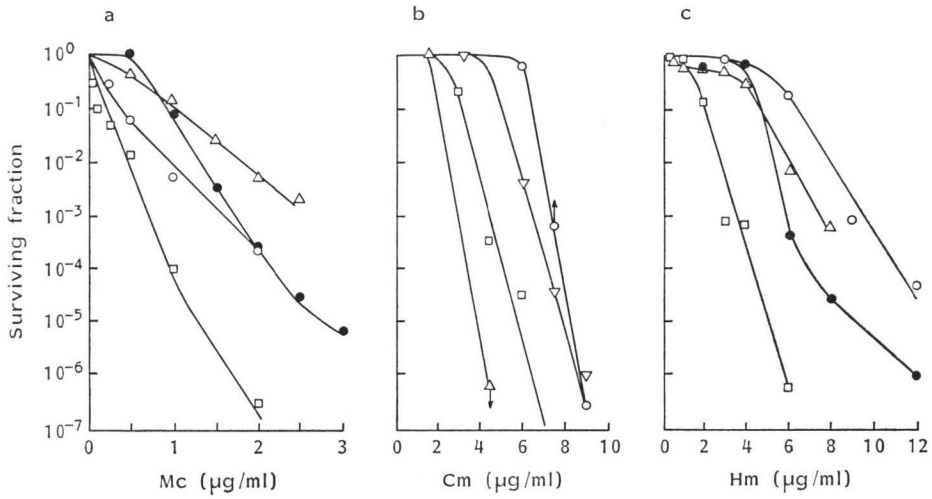
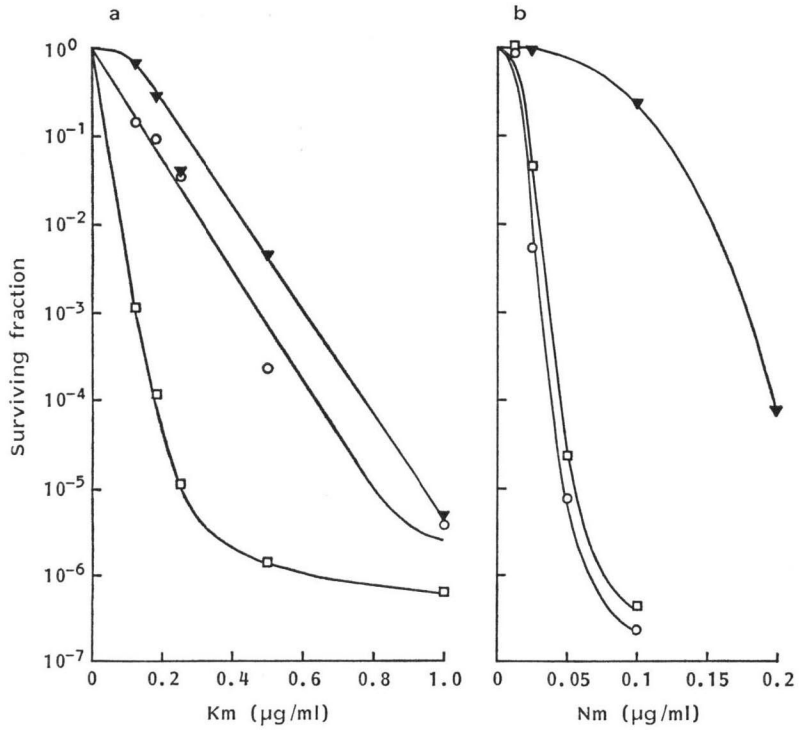


Fig. 4. Sensitivity of *Streptomyces fradiae* strains to kanamycin (Km) and neomycin (Nm).
○ T59235, □ JS82, ▼ JS98.



Genetic Instabilities in JS82 and JS87

While JS82 is normally more sensitive to Hm than its parent strain, it segregates clones resistant to Hm at high frequency. For instance, when JS82 was plated on 4.0 μg/ml Hm, about 5 to 10% of

Fig. 5. Sensitivity of *Streptomyces fradiae* strains derived from JS82 to hygromycin B (Hm).

Dashed line, JS82 from Fig. 3; ▼ JS98, ■ JS99, ▲ JS101.

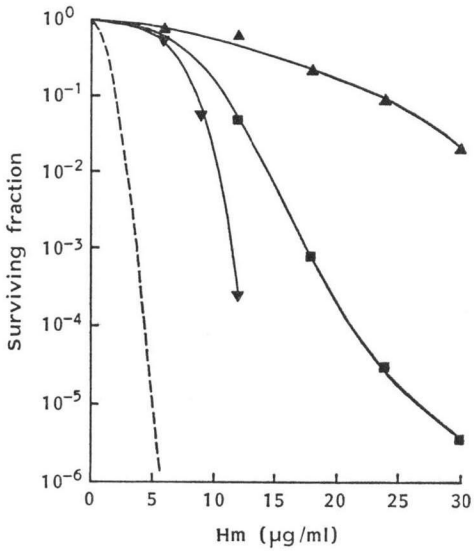
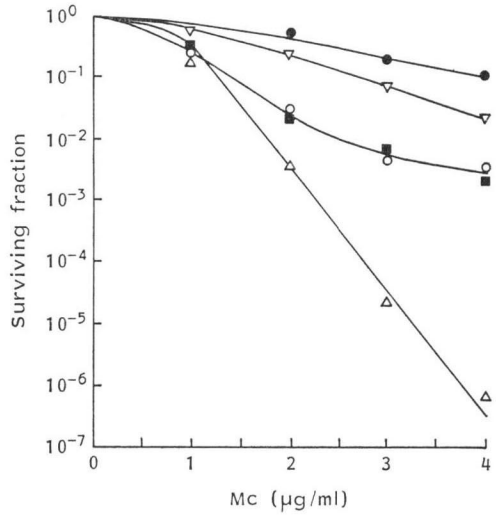


Fig. 6. Sensitivity of derivatives of JS87 to mitomycin C (Mc).

△, JS87, ○, JS102, ■ JS103, ▽ JS104, ● JS105.



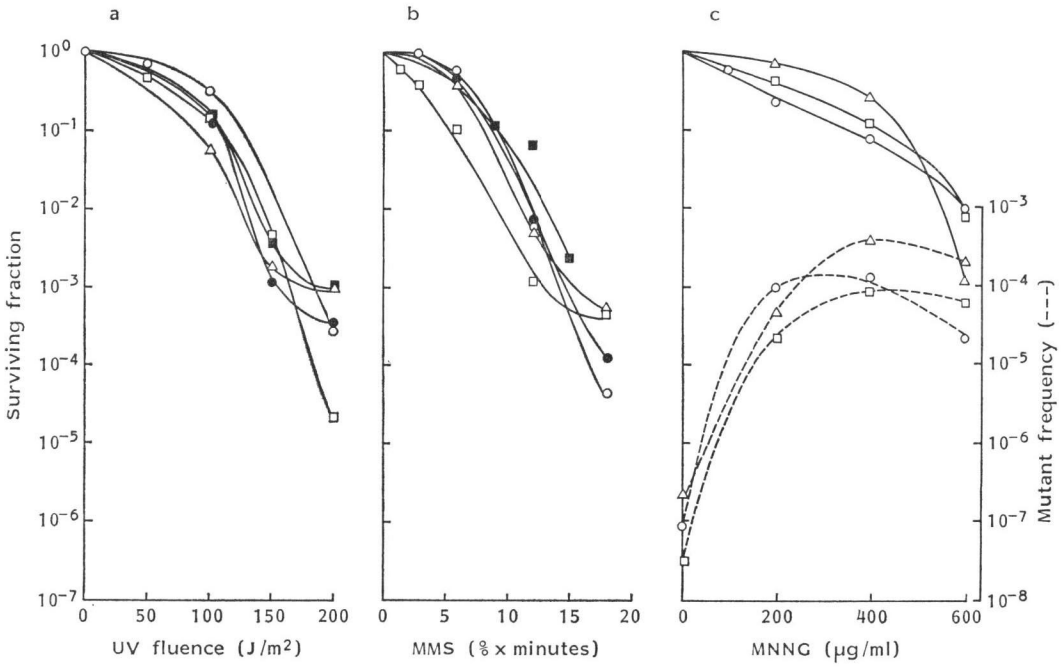
colonies which developed were much larger than the others. Large colonies were isolated, grown in liquid medium and retested for Hm^r. JS98 was a typical Hm^r clone derived from JS82 (Fig. 5). JS98 expressed wild type levels of Hm resistance, wild type resistance to Km (unlike JS82, Fig. 4a), and substantially increased resistance to Nm relative to JS82 and its wild type parent (Fig. 4b). JS98 segregated large colonies on Hm at 10 to 15 μg/ml; the wild type strain, T59235, however, did not segregate large colonies under these conditions. A large colony (JS99) isolated from JS98 was shown to be more resistant to Hm than JS98 (Fig. 5). JS99 in turn segregated large colonies when grown on 18 μg/ml Hm. A large colony (JS101) was isolated and retested for Hm^r. JS101 was more resistant to Hm than JS99 (Fig. 5). JS101 did not appear to be substantially more resistant to Km and Nm in patch tests. This genetic instability involving Hm^r was associated only with JS82.

JS87 colonies growing on AS-1 agar show a high degree of sectoring of morphological variants uncharacteristic of *S. fradiae*. Fifty-four sectors were picked and patched on CDA agar and 27% were auxotrophic. Several of these auxotrophs were further analyzed, and all were found to contain the same requirements; they all grew well on minimal media supplemented with either methionine or histidine, but not with homocysteine or histidinol, the immediate precursors of methionine and histidine. Two independently isolated mutants, JS102 and JS103, were retained for further studies.

JS87 shows wild type levels of resistance to Mc. However, the two auxotrophic variants, JS102 and JS103, were also tested for the Mc^r phenotype and both exhibited identical biphasic dose responses (Fig. 6). Two clones (JS104 and JS105) apparently more resistant to Mc than JS87 were isolated and verified to be much more resistant to Mc than JS87. The auxotrophic mutation in JS102 reverted spontaneously to prototrophy at a frequency of about 10^{-7}. However, JS105 reverted spontaneously to prototrophy at a frequency of about 10^{-10}. The one prototrophic revertant clone obtained after reversion of JS105 had also reverted to the Mc sensitivity phenotype of JS82 (*i.e.*, EOP$\sim 10^{-5}$ at 2 μg/ml Mc).

Fig. 7. Inactivation of *Streptomyces fradiae* strains by UV, MMS and MNNG and mutagenesis to spectinomycin resistance by MNNG.

○ T59235, □ JS82, △ JS87, ▲ JS101, ■ JS103, ● JS105.



None of the various spontaneous mutants or revertants derived from JS82 or JS87 produced tylosin.

DNA Repair and Mutagenesis

Since the level of sensitivity or resistance to Mc and the other genetic instabilities observed in JS82 and JS87 might reflect differences in expression of either error-free or error-prone DNA repair pathways[19~21], we measured the levels of resistance in these strains to mutagenic agents which produce bulky lesions (UV light) or small lesions (MMS and MNNG) in DNA. JS82 and JS87 showed wild type levels of resistance to UV and MMS (Figs. 7a and 7b). Both JS82 and JS87 showed normal levels of resistance to MNNG and normal spontaneous and MNNG-induced resistance to Spc (Fig. 7c).

JS103 and JS105 were also tested for their levels of resistance to UV and MMS and both gave essentially the same responses as JS87, JS82 and T59235 (Figs. 7a and 7b).

Discussion

It was shown previously that during genetic recombination by protoplast fusion in *S. fradiae*, the production of tylosin is lost at high frequencies[6]. Since formation and regeneration of *Streptomyces* protoplasts can cause loss of plasmid DNA[9~11], our data suggested a possible plasmid involvement in tylosin biosynthesis. We have shown here that expression of tylosin resistance and tylosin production were lost in two strains which had undergone protoplast formation and regeneration. One strain, JS82, lost the genetic capability to express at least seven different tylosin biosynthetic enzymes, and lost expression of resistance to tylosin, Cm, Mc, Hm and Km. The loss of expression of tylosin resistance in JS82 was different from that observed in JS87. With JS82 a subpopulation of phenotypically

sensitive cells was able to grow on media containing up to at least 400 μg/ml tylosin. This phenotype was also observed with *S. fradiae* GS40, a mutant induced by MNNG[1]. Like JS82, GS40 was defective in tylosin biosynthesis[1]. The relatively high frequency of cells (~ 1 in 10^4) expressing tylosin resistance in JS82 and GS40 suggests that the genetic capability to express tylosin resistance was not lost, but that regulation of expression was altered.

While JS82 was more sensitive to Hm, Km and Nm than its parent strain, it segregated Hm^r clones at high frequencies. One clone also regained normal levels of Km resistance and was much more resistant to Nm than the wild type parent of JS82. Thus the three resistances might be associated with the same mechanism, perhaps at the level of transport; the separate resistance genes might be linked and regulated by a common mechanism; or the resistance patterns may reflect more complicated possibilities involving several partially cross-reacting resistance mechanisms. The data on JS82 and JS98 may suggest the latter since JS82 lost Km^r and Hm^r but not Nm^r, while JS98 showed reversion to wild type levels of Km^r and Hm^r but substantially increased levels of Nm^r. Since the strains expressing high-level resistance to Hm did not produce detectable levels of hygromycin B phosphotransferase (N. ALLEN, personal communication), the Hm resistance phenotype in *S. fradiae* must be due to a mechanism different from those previously observed[22,23].

An interesting aspect of the complicated pattern of expression of antibiotic resistance in JS82 was the repeated high frequency of spontaneous mutation to even higher levels of Hm^r. It is not known if the Hm^r phenotype is associated with the amplified DNA in JS82[15]. However, our data suggest that the genetic event(s) associated with loss of tylosin biosynthesis, loss of multiple antibiotic resistance and DNA amplification in JS82 may be associated with the genetic instability in the expression of Hm^r. The high-frequency mutation to Hm^r is not due to aberrant error-free or error-prone DNA repair since JS82 expresses normal levels of resistance to UV, MMS and MNNG. Mutants of *S. fradiae* defective in error-free repair of bulky lesions such as pyrimidine dimers are more sensitive to UV-light than wild type strains[20,21], while mutants defective in error-prone DNA repair are more sensitive to the lethal effects of UV, MMS and MNNG[19,21]. Also, JS82 showed normal mutation induction by MNNG indicating that it possesses normal error-prone repair of damaged DNA[19]. Thus the genetic instability observed in JS82 is probably not due to increased levels of base pair substitution mutagenesis due to defective DNA repair, but must be associated with more complex DNA rearrangements.

Coupled deletion and amplification of genes has been observed previously in *Streptomyces reticuli*[24,25] and *Streptomyces glaucescens*[26~28]. In both cases, deletion and amplification was often associated with loss of certain antibiotic resistance phenotypes and antibiotic production. Chloramphenicol sensitive mutants of *S. lividans* 66 also arise spontaneously at high frequency. These mutants segregate Arg^- derivatives containing amplified DNA at very high frequencies[29]. JS82 may share some common features with mutants of these species.

A second strain isolated after protoplast regeneration, *S. fradiae* JS87, also lost expression of multiple tylosin biosynthetic genes, tylosin resistance, and Cm resistance, but retained normal levels of expression of Hm^r and Mc^r. Unlike JS82, it was deleted for the amplifiable unit of DNA and two other sequences homologous to the direct repeats bounding the amplifiable unit of DNA in the wild type strains[17]. Like JS82, JS87 was prototrophic and sporulated so the deletion does not include functions required for normal growth and differentiation.

JS87 was also genetically unstable and segregated auxotrophic mutants with the unusual requirement for either histidine or methionine; thus it appears that these mutations affect cross pathway regulation. Auxotrophic mutants segregated mutants more resistant to Mc than JS87 at high frequency, and one of the auxotrophs reverted to prototrophy at a much higher frequency than its Mc resistant derivative. Since reversion of the auxotrophic marker in the Mc resistant derivative was associated with the loss of the Mc^r phenotype, it appears that the deletion in JS87 may have triggered two sequential events; mutation to His/Met auxotrophy followed by mutation to high level Mc resistance. Since reversion from auxotrophy to prototrophy has not been shown to occur from the high level Mc resistance state without loss of the Mc resistance phenotype, two genetic events may be required to revert Mc^r auxotrophs to prototrophy. Since JS87 expresses normal error-free and error-prone DNA repair phenotypes, the high frequency mutation events associated with forward mutation to Met/

His auxotrophy and Mc resistance are not due to spontaneous base pair substitutions but to DNA rearrangements. The lack of cross resistance to MMS and UV in the Mc^r derivative of JS87 also suggests that this strain does not express a general enhanced level of DNA repair enzymes for damaged DNA, but that the Mc^r phenotype hyperexpressed in JS105 is due to a specific resistance mechanism for Mc.

Since both deletion (as in JS87) and DNA amplification (as in JS82) involve some common DNA sequences that are associated with loss of tylosin production and loss of other antibiotic resistances, the genes conferring these phenotypes may be genetically linked. However, the substantial phenotypic differences between JS82 and JS87 indicate that simple plasmid curing cannot account for the loss of tylosin production in these strains. Deletion, amplification and rearrangement of plasmid DNA, chromosomal DNA or both remain open as possible explanations for these results.

Acknowledgments

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