Arch. Pharm. Res. **15**(1), 35-40 (1992)

Genetic Analysis on Bioconversion of Aniline to Acetaminophen in *Streptomyces fradiae*

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(Received December 18, 1991)

Abstract □ *S. fradiae* showed the highest acetanilide p-hydroxylation activity in the tested strains. And *S. fradiae* was well characterized genetically, especially with respect to tylosin production. Two mutants, which lost hydroxylation, were isolated in 140 regenerated colonies from protoplasts. In restriction enzyme digesion of total DNAs, isolation of giant linear plasmid DNA and determination of antibiotic resistances to chloramphenicol, tylosin, hygromycin B and mitomycin C, any differences among mutants and a wild type strain were not detected. These facts suggest that lesion on 6,000 Kb chromosomal DNA was responsible for the lack of p-hydroxylation activity induced by protoplast formation and regeneration.

Keywords □ Acetanilide p-hydroxylation, protoplast formation and regeneration, genetic instability, giant linear plasmid.

Regio- and stereospecific microbial hydroxylations are attractive for modification of organic compounds to biologically active fine chemicals or pharmaceuticals. Because of their versatile biosynthetic capabilities, *Streptomyces* are suited for the effective bioconversion of economically important molecules[1]. Among the various *Streptomyces* spp. screened for acetanilide p-hydroxylation activity, *S. fradiae* showed the strongest activity[2]. Furthermore, its activity can be increased to 130 μg/ml-150 μg/ml (26-30% bioconversion activity) by proper combination of medium components[3]. In general, the formation of protoplast and regeneration of *Streptomyces* protoplasts can be associated with genetic instabilites and macrolesion[4]. *S. fradiae*, a tylosin producer and well characterized example, derived two mutants with these mutations from regenerated protoplasts[5]. Two mutant strains (JS85 and JS87) showed lack of both tylosin production and resistance but DNA amplication (10.5 kb DNA to 500 copies) and genetic instability on hygromycin resistance (JS 85), DNA deletion of Aud (amplifiable unit of DNA) and direct repeats of the four homologous sequences in Aud and segregation of auxotrophic variants at high frequencies (JS87) respectively[5]. In these regards, we attempted to obtain the mutant losing p-hydroxylation activity among the regenerated colonies from protoplasts and investigate the relationship of p-hydroxylation gene to the genetic instabilities mentioned above.

EXPERIMENTAL METHODS

Microorganisms and media

The microorganism used was *Streptomyces fradiae* NRRL 2702, which produces the macrolide antibiotic tylosin. *S. fradiae* JS85 and JS87 were obtained from R. H. Baltz in Eli Lilly and Company. Both strains are sensitive to tylosin and blocked in tylosin biosynthesis. Mycelia for preparation of protoplasts were cultivated in Trypticase soy broth (30g/*l*. TS broth) containing 0.4% glycine. Glycine was prepared at 4% solution, sterilized separately and then added aseptically. P medium and R2 medium were as described by Okanishi *et al.*[6]. Modified R2 medium has same composition except that 1.8g L-asparagine was substitued for proline as the nitrogen source. Hypertonic soft agar overlay contained sucrose, $MgCl_2$, $CaCl_2$, and TES buffer at the concentrations used in R2 medium, plus 0.65% (w/v) bacto agar. NB agar was used for determination of antibiotic resistances and contained nutrient broth (8g/*l*)

and bactoagar (15g/l). Modified Theriault's screening medium (7% sucrose,0.5% soybean flour, 0.5% yeast extract, 0.5% NaCl, 0.295% K_2HPO_4, 0.162% K_3PO_4) was used for detecting acetanilide p-hydroxylation activity.

Protoplast formation and regeneration

This procedure was performed according to Baltz *et al.*[7] with slight modification. A flask containing TS broth was inoculated with frozen vegetative mycelia and grown until mycelial masses developed (usually more than 48 hrs). Cell grown fully were homogenized and diluted twofold in TS broth and 10 m*l* was added to glass scintillation vial. Mycelia were fractured for 5 to 10s at 30 μ with MSE MK-2 sonicator, transferred to TS broth supplemented with 0.4% (w/v) glycine and incubated 20-24 hrs. Myccelia were then homogenized, fragmented ultrasonically (3 sec) again, transferred to the fresh same medium and further incubated 19-20 hrs. Growth was followed by monitoring the absorbance at 600 nm (A_{600}). Mycelia to be protoplasted were washed three times with ice cold P medium, suspended and treated with 1 mg lysozyme ml^{-1} in P medium for 1.5 hrs at 37℃. Occasionally, the mixture was pipetted up and down. The reaction was terminated with the addition of equal volume of P medium, filtered through cotton to remove the unreacted mycelia. Protoplasts were quantified by direct counts in a haemacytometer. Protoplasts were washed two times by centrifugation (3,000×g, 10 min), resuspened, serially diluted tenfold in P medium, mixed with soft agar overlay at 37℃, plated on modified R2 medium and incubated at 29℃ for about 2 weeks for viable counts to determine cell regeneration.

Detection of acetanilide p-hydroxylation activity

Colonies from regenerated protoplasts were inoculated in modified Theriault's screening medium and grown at 29℃, 180 rpm until mycelial masses developed. The culture was transferred (10% v/v) to the same fresh medium and saturated acetanilide solution (5 mg/m*l*) was added after 48 hr incubation to the final concentration of 500 μg/m*l*. 5 m*l* of culture was withdrawn 5 days later and analysed with TLC.

Isolation of DNA

The same conditions were used for culturing mycelia as were described for the protoplast formation and regeneration. *S. fradiae* total DNA was isolated according to Hershberger *et al.*[8]. 1g of wet packed mycelia was washed with 0.3M sucrose three times and suspended in 10 m*l* of buffer containing 10 mM Tris (pH 8.0), 1 mM EDTA and 25% (w/v) sucrose (Tris-EDTA-sucrose). The suspension was supplemented with 5 m*l* of 0.25M EDTA, 5 m*l* of lysozyme solution (10 mg/m*l* in Tris-EDTA-sucrose) and 20 μ*l* diethylpyrocarbonate solution, and incubated at 30℃ for 30 min. Sodium dodecyl sulfate was added to a final concentration of 1%. The lysate was incubated at 55℃ for 30 min, mixed with 10 m*l* of 5M NaCl, incubated on ice for 3 hrs and centrifuged at 42,000×g for 45 min. The supernatant was precipitated with 0.64 volume of isopropanol. The nucleic acid precipitate was collected with glass rod. The residual isopropanol was blown off with nitrogen gas. The precipitate was dissolved in 5 m*l* of TE buffer treated with RNase (40 μg/m*l*) and digested with proteinase K (50 μg/m*l*) at 37℃ for 15 hrs. The DNA solution was extracted with phenol saturated with 50 mM Tris (pH 8.0), precipitated with ethanol and redissolved in 1 m*l* of TE buffer. Giant linear plasmid DNA was detected in *S. fradiae* by *in situ* lysis of protoplasts embedded in agarose gel[9] and electrophoresed in 0.1% agarose gel[10,11]. Protoplasts prepared in P3 buffer (70 mM NaCl, 5mM $MgCl_2$, $CaCl_2$, 0.25M N-Tris (hydroxymethyl)-methyl-2-aminoethanesulphonic acid (TES, pH 7.2) and 0.5M sucrose) was washed with the same buffer and suspended in a mixture of 0.5 m*l* of 0.5M sucrose in TSE (30 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM EDTA) and 0.1 m*l* of 0.5M EDTA pH 8.0). Then 1.2 m*l* of 1.5% low-melting-point agarose in 0.5M sucrose in TSE kept at 37℃ was added and mixed. After solidification in 3 mm thickness on 2 cm×3 cm plate, 0.5 m*l* of 50 μg/ml^{-1} proteinase K in TSE and 0.5 m*l* of 10% SDS were overlaid, and the plate sealed with parafilm and incubated at 37℃ for 16 hrs. The proteinase K-SDS solution was withdrawn and replaced with 0.5M EDTA (pH 8.0) and the plate stored at 4℃. For electrophoresis, 1.5% agarose frame (outside 15 cm× 13 cm, inside 10 cm×8 cm) was constructed and 0.1% agarsose was poured and solidified at 4℃. A small piece (0.8 cm×0.15 cm×0.3 cm) was cut out from the sample gel and applied to 0.1% agarose gel in 0.5×TBE (89 mM Tris, 89 mM boric acid,

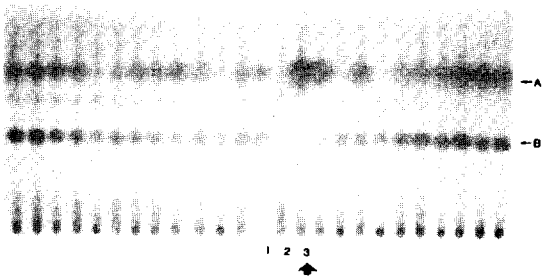

Fig. 1. Selection of mutant lost the acetanilide p-hydroxylation activity through protoplast formation and regeneration of *S. fradiae*.

A and B represent acetanilide and acetaminophen respectively. Lane 1, standards; lane 2, metabolites of *S. fradiae* wild type with acetanilide; lane 3, metabolites of mutant lost acetanilide p-hydroxylation activity.

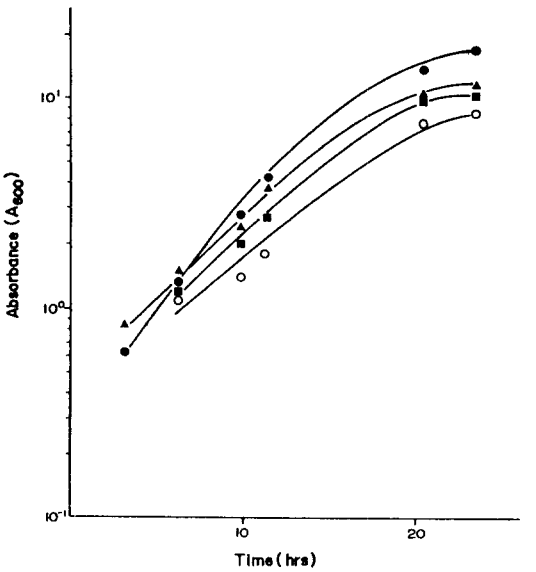

Fig. 2. Growth of *S. fradiae* in TS broth containing 0% (●), 0.2% (▲), 0.3% (■) and 0.4% (○) glycine.

2 mM EDTA). Electrophoresis was conducted in 0.5×TBE at 2.5 Vcm^{-1} for 1 hr and then 0.1 Vcm^{-1} for 40 hrs.

Restriction enzyme digestion

Restriction enzymes (*Sal*I, *Sma*I, *Bam*HI and *Kpn*I) were purchased from Jecheol Chemical Co., and used according to the recommendations of the manufacturer. The fragments from *Sal*I and *Sma*I digestion of *S. fradiae* total DNA were electrophoresed in 1.4% agarose slab gel at 5.5 Vcm^{-1} for 2.5 hrs and the fragments from *Kpn*I and *Bam*HI were in 0.7% agarose gel at 2 Vcm^{-1} for 4 hrs. Electrophoresis buffer was TAE buffer.

Determination of antibiotic resistance

Fully grown mycelia in TS broth at 29℃, 180 rpm were homogenized and treated ultrasonically described in protoplast formation and regeneration. Cells were transferred to fresh TS broth, further incubated for 19-20 hrs, homogenized and sonicated. And then cells were serially diluted tenfold and plated on NB agar containing various antibiotics, tylosin (400 μg/m*l*), hygromycin B (6 μg/m*l*), chloramphenicol (1.5 μg/m*l*) and mitomycin C (1.5 μg/m*l*). When no more colony was formed (10-12 days later), the number of colonies was counted and compared with control grown in NB agar containing no antibiotics. Sensitivity was determined by dividing the number of colony on antibiotic-containing medium by the number of colony on normal medium. All antibiotics were sterilized by filtration.

RESULTS AND DISCUSSION

S. fradiae showed the highest hydroxylation activity of *Streptomyces* strains tested and its activity could be increased to 130-150 μg/m*l* by the proper combination of medium composition. About 140 colonies from regenerated protoplasts appeared last (after 10 to 14 day incubation) were tested in liquid culture whether they had lost hydroxylation activity, two mutants losing the hydroxylation activity were isolated (Fig. 1). Both strains were able to form aerial mycelium and spores as wild type but nearly could produce red-orange pigment. The optimal conditions for the preparation of protoplasts are as follows. On trasferring the culture, the mycelia were fragmented uniformly with ultrasound for 5-10 sec at 30 μ. For the mycelia more susceptible to lysozyme treatment, *S. fradiae* was cultivated in TS broth containing 0.4% glycine, partially growth-inhibitory concentration of glycine (Fig. 2). Complete growth inhibition was achieved at 0.6% glycine. The cultivation time was adapted to 19-20 hrs, the transition phase between the exponential and stationary growth phases for efficient regeneration of protoplasts (Fig. 2). The formation and regeneration of pro-

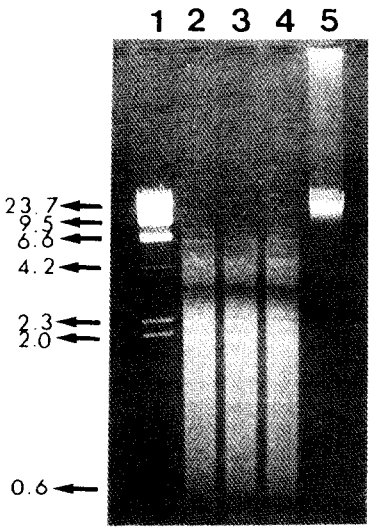

Fig. 3. Analysis of *S. fradiae* total DNA with restriction enzyme.

The numbers and arrow heads denote the sizes and positions of the marker bands. Lane 1, *Hin*dIII digested phase λ DNA; lane 2, wild type total DNA digested with *Sma*I; lane 3, mutant 1 total DNA digested with *Sma*I; lane 4, mutant 2 total DNA digested with *Sma*I; lane 5, total DNA.

toplasts of *Streptomyces* had been regarded as a mild procedure to cure the plasmid of tested strains simply. But in *S. fradiae*, a tylosin producer, this procedure caused more complicated DNA rearrangements: both high frequencies of amplification and high frequencies of deamplification or deletion associated with phenotypic changes involving loss of expression of many antibiotic biosynthetic enzyme reactions and several antibiotic resistances (frequency was 0.4-6%)[12]. For example, one mutant, *S. fradiae* JS85, lost expression of at least seven tylosin biosynthetic enzyme reactions and lost expression of resistance to tylosin(tyl), mitomycin C(mc), hygromycin B(hm), and chloramphenicol(cm)[12], and contains a 10.5 kb DNA sequence reiterated in tandem to about 500 copies representing approximately 50% of the normal genomic DNA content of *Streptomyces* spp. or one-third of total DNA in the mutants[8]. The wild type parent of this strain contained a single copy of the amplifiable unit of DNA bounded by direct repeats of a 2.2 kb homologous sequence[13]. JS85 also displayed genetic instability associated with hygromycin resistance. While it originally lost expression of hygromycin resistance, it segregated hygromycin resistant clones at high frequencies. Another Tyl$^-$, Tyl^s mutant strain, *S. fradiae* JS87, was also derived from regenerated protoplasts but showed a different pattern of antibiotic resistances and was deleted for the Aud sequence and the four homologous sequences[12,13]. The information on *S. fradiae* JS85 and JS87 suggests that the stress exerted by protoplast formation and regeneration may trigger deletion and amplification of DNA and perhaps other genetic instabilites. These genetic alterations may in turn trigger additional genetic event associated with auxotrophy and expression of antibiotic resistances. Furthermore, *S. fradiae* was found to contain a 420 kb linear plasmid, designated as GLP (giant linear plasmid)[14]. Field Inversion Gel Electrophoresis (FIGE) was used to determine if the contained copies of Aud or the cloned tylosin biosynthetic genes and if GLP had the relationship with tylosin biosynthesis. But the deletions, structural rearrangements and unstable genes were not determined to be part of GLP[15]. On the basis of these facts mentioned above, the approaches were carried out with two mutants to determine if the genetic events caused by the protoplasts formation and regeneration in *S. fradiae* were related with the loss of p-hydroxylation activity and its relationship with GLP. But neither amplification of Aud (amplifiable unit of DNA) nor deletion was not identified with restriction enzyme digestion analysis (*Kpn*I, *Bam*HI, *Sma*I and *Sal*I, Fig. 3). Furthermore, resistances to antibiotics stated above were not reduced significantly (Table I). And the putative large linear plasmid DNA bands could be detected in two mutants as well as *S. fradiae* wild type (Fig. 4) with electrophoresis on 0.1% agarose gel following *in situ* lysis of

Table I. Sensitivity of *S. fradiae* strains to antibiotics

	S. fradiae wild type	*S. fradiae* mutant 1	*S. fradiae* mutant 2
Tylosin (400 μg/ml)	10°	1.5×10^{-2}	2.3×10^{-2}
Chloramphenicol (1.5 μg/ml)	7×10^{-2}	2.7×10^{-2}	1.7×10^{-2}
Mitomycin C (1.5 μg/ml)	4×10^{-3}	1.7×10^{-3}	1.1×10^{-4}
Hygromycin B (6 μg/ml)	less than 10^{-6}	less than 10^{-6}	less than 10^{-6}

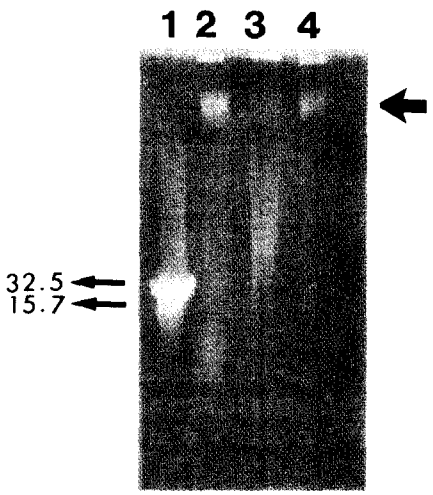

Fig. 4. Detection of large linear plasmid DNA in *S. fradiae* wild type and mutants lost acetanilide p-hydroxylation activity.
Lane 1, *Sal*I digested phage λ DNA; lane 2, DNA of *S. fradiae* wild type; lane 3, DNA of mutant 1; lane 4, DNA of mutant 2.

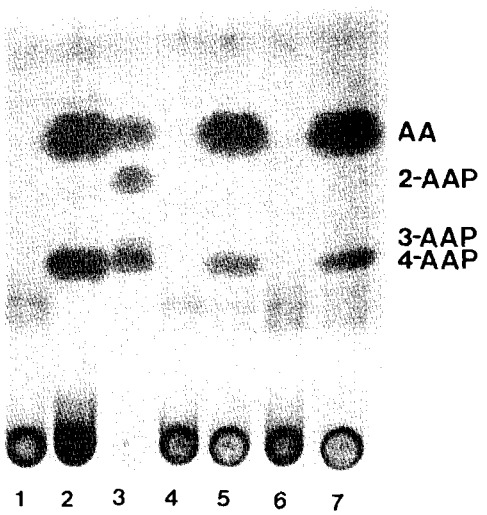

Fig. 5. Detection of acetanilide p-hydroxylation activity in *S. fradiae* JS85 and JS87.
AA, acetanilide; 2-, 3-, 4-AAP, 2-, 3-, 4-acetamidophenol; Lane 1, *S. fradiae* wild type blank; lane 2, wild type; lane 3, standards; lane 4, *S. fradiae* JS85 blank; lane 5, *S. fradiae* JS85; lane 6, *S. fradiae* JS87 blank; lane 7, *S. fradiae* JS87.

protoplasts embedded in agarose gel. Therefore, GLP and genetic instability associated with DNA rearrangement, amplification and deletion did not show any relationship with the loss of p-hydroxylation activity. Based on these results, it could be suggested that the lesion on 6,000 kb chromosomal DNA having no relationship with genetic instabilities or tylosin synthesis and resistances verified until now was responsible for the lack of p-hydroxylation activity induced by protoplast formation and regeneration. This conclusion is supported strongly by the fact that both *S. fradiae* JS85 harboring GLP and *S. fradiae* devoid of GLP[5] showed the strong p-hydroxylation activities (Fig. 5).

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