THE OCCURRENCE OF STREPTOMYCIN-PHOSPHORYLATING ENZYMES IN STREPTOMYCIN-PRODUCING STREPTOMYCETES

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WALKER *et al.*[1,2], NOMI *et al.*[3,4], and DAVIES *et al.*[5,6] have reported that streptomycin-producing strains of *Streptomyces griseus* and *Streptomyces bikiniensis* can phosphorylate streptomycin. We have studied the occurrence of this ability in an actinophage-resistant streptomycin-producing survivor obtained from the non-streptomycin-producing *S. griseus* NRRL B-2926 and found that this strain does not have the streptomycin-phosphorylating system.

Materials and Methods

S. griseus strains ATCC 27,001 (a streptomycin producer) and NRRL B-2926 (a streptomycin non-producer) were maintained on slants of a glucose - yeast extract - potassium phosphate buffer medium. Shaken flask cultures were started by transferring spores from the slants to cotton-plugged 250 ml Erlenmeyer flasks containing 100 ml of soybean meal - glucose - $CaCO_3$ medium. The inoculated flasks were placed on a rotary shaker operating at 300 rpm (1 inch displacement) in an incubator maintained at 30°C. Samples were removed periodically during the 5-day incubation period for antibiotic determination using *Bacillus subtilis* Marburg as test organism in an agar-diffusion bioassay with streptomycin sulfate as standard, pH determination, and evaluation of the cells for streptomycin-phosphorylating capability. The test system for the streptomycin-phosphorylating capability contained: 0.5 ml quantities of 1 mM streptomycin sulfate, 600 mM KCl, 100 mM magnesium acetate, 50 mM dithiothreitol, 40 mM ATP, 1 M potassium phosphate buffer (pH 7.0), and the supernatant solution obtained by grinding 1 g wet cells with 1 ml of M/15 potassium phosphate buffer and centrifuging at 10,000×*g* for 20 minutes. The total volume was adjusted to 5.0 ml by adding 1.5 ml (or less) of M/15 potassium phosphate buffer (pH 7.0). The mixture was incubated at 30°C for 4 hours and the residual streptomycin determined by bioassay (after placing the sample in a boiling water bath for 3 minutes). The streptomycin-phosphate was isolated from a mixture of this type by adsorption on IRC-50 resin (Na^+ cycle), elution with 1 N HCl, and treatment of the neutralized eluate with active charcoal to remove the inorganic salts.

The streptomycin-producing variant from *S. griseus* NRRL B-2926 was obtained by exposure of vegetative cells of this culture to an actinophage grown on *S. griseus* ATCC 27,001. The surviving population was plated on a glucose - yeast extract - phosphate buffer agar medium and after 6 days' incubation the plates were over-laid with agar containing the *B. subtilis* Marburg culture. Those streptomycete colonies showing inhibition of the *B. subtilis* were grown in the soybean meal - glucose - $CaCO_3$ medium in shaken Erlenmeyer flasks. The fermentations were sampled periodically and the samples analyzed for streptomycin titer, ability of the cells to phosphorylate streptomycin, and the presence of the anti-fungal polyene reported to be produced by *S. griseus* NRRL B-2926[8]. This polyene was purified by extraction of the fermentation sample with *n*-butanol. The *n*-butanol was removed by evaporation *in vacuo* and the residue dissolved in metanol. The u.v. spectrum was determined over the range 240 to 450 nm.

Results and Discussion

Study of the fermentations inoculated with the *S. griseus* ATCC 27,001 showed that after 5 days' incubation at 30°C streptomycin titer of the order of 200 mcg/ml were obtained and the pH of the fermentations was pH 8.3. These cells contained the enzyme system which phosphorylated streptomycin with the product being identified as streptomycin-phosphate by paper ionophoresis at pH 1.9 (where it had a mobility of 0.7 relative to streptomycin), a positive test with the HANES reagent,[7] and regeneration to streptomycin by treatment with alkaline phosphatase (Sigma Chemical Company) at pH 8.5 for 1~24 hours at 37°C.

Study of similar samples from *S. griseus* NRRL

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B–2926 fermentations showed no antibacterial antibiotic activity nor did the cells phosphorylate streptomycin in the test system mentioned above. Traces of a polyene antibiotic inhibiting *Candida albicans* in agar diffusion bioassay were noted in the *n*-butanol extracts of the broth. This polyene had significant absorption at 337,360,380, and 404 nm and appeared to be a heptaene.

The phage resistant, antibiotic-producing variant obtained from *S. griseus* NRRL B–2926 and designated as 'D' was grown in the soybean meal - glucose - $CaCO_3$ medium in shaken Erlenmeyer flasks. The antibiotic produced in this medium was identified as streptomycin on the basis of mobility in paper chromatography (using a *p*-toluenesulfonic acid - *n*-butanol - water system as developing solvent and development at 25° and 35°C), mobility in paper ionophoresis at pH 1.9, and by positive maltol and nitroprusside tests. Antibiotic titers in the fermentations were of the order of 400 mcg/ml (calculated as streptomycin). The heptaene antifungal agent was also noted in the *n*-butanol extracts of the fermentation samples. No evidence of mannosidostreptomycin was noted in the paper ionophoresis and paper chromatography studies. (*S. griseus* ATCC 27,001 produces considerable mannosidostreptomycinase[9] and no mannosidostreptomycin was detected in fermentations inoculated with this culture, either). No streptomycin inactivation was noted with cell-free extracts from strain 'D' prepared under conditions where similar extracts from *S. griseus* ATCC 27,001 inactivated the streptomycin by converting it to the streptomycin-phosphate.

These studies suggest that under rather special circumstances it is possible to obtain streptomycin-producing cultures which do not have the ability to inactivate the antibiotic by phosphorylation, and these cultures may be the exception to the observation of DOWDING and DAVIES[6] that most aminoglycoside-producing streptomycetes have abilities for inactivating the antibiotic.

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