INCREASED PRODUCTION OF THE ANTIBIOTIC AURODOX (X-5108) BY AURODOX-RESISTANT MUTANTS

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Conventional strain and media improvement techniques were of limited success in increasing yields of the antibiotic aurodox (X-5108) above 0.5 g/liter. Higher yields were obtained by reversion of a zero producer followed by the selection of mutants resistant to aurodox. Resistant strains of *Streptomyces goldiniensis* ATCC 21386 able to grow on 2 g/liter of aurodox produced greater than 2.5 g/liter of antibiotic. The rate of yield increase leveled off as the strains became resistant to greater than 2 g/liter of aurodox. These strains, in contrast to those sensitive to aurodox, gave a positive response to conventional mutagenic methods for further yield increases.

The antibiotic aurodox (X-5108) is produced by *Streptomyces goldiniensis*, ATCC 21386. Its production, structure, isolation and properties were described by BERGER *et al.*[1] and MAEHR *et al.*[2]. Its antibacterial spectrum is restricted mainly to Gram-positive bacteria. Aurodox is active as a poultry growth promotant[3].

Antibiotic yields by the original soil isolate were quite low, 20~40 mg/liter, but strain and media development raised yields to 350 mg/liter[1]. However, it became very difficult to obtain yields above this level. Over 50,000 survivors of mutagenesis were examined for yield increase, of which over 10,000 were tested in shake flask fermentations. The types of mutants examined included auxotrophs and their revertants, survivors of conventional mutagenesis, temperature sensitive mutants and a producer obtained by back mutation of a non-producer.

Most antibiotics are secondary metabolites, produced in idiophase (after growth has ceased). Therefore, their possible toxicity during the growth stage of the producing organism should have little or no effect on antibiotic yield[4]. There are, however, a few examples in the literature where the use of the antibiotic to select resistant cultures of the producing organism has resulted in higher yields, *i.e.* tetracycline[5], ristomycin[6] and streptomycin[7]. Our method for obtaining higher aurodox yielding mutants involved the selection of strains resistant to growth inhibition by aurodox. Our method for selecting aurodox resistant mutants is described in this paper.

Materials and Methods

Organism

Streptomyces goldiniensis and various mutant strains were used. Strains were maintained on Amidex agar slants containing (g/liter), 10 Amidex (Corn Products); 2 N–Z Amine A (Sheffield); 1 beef extract (Baltimore Biological); 0.02 $CoCl_2 \cdot 6H_2O$ (Fisher); 20 agar (Difco). The pH was adjusted to 7.3 before autoclaving. The inoculum medium contained (g/liter): 5 tomato pomace dried solids (Seaboard Supply); 5 Ardamine Z (Yeast Products); 10 cornstarch (Anheuser Busch); 1.0 $CaCO_3$; 1.0 K_2HPO_4; 0.1 silicone paste A (Dow Corning); 2 soybean oil (Welch, Holme & Clark, Harrison, New Jersey). The pH was adjusted to 7.0 before autoclaving.

Fermentation

The fermentation medium contained (g/liter): 7.5 yellow split peas; 30 soyalose (Central Soya); 1.0 K_2HPO_4; 12.5 cornstarch; 40 soybean oil. The pH was adjusted to 7.5 (with NaOH) before autoclaving. Fermentations were carried out in 500 ml Erlenmeyer flasks containing 100 ml of medium incubated at 28°C on a rotary shaker (250 rpm, 1-inch stroke). Flasks were sampled and assayed for aurodox concentrations after 6 and 8 days of fermentation.

Aurodox Assay

The assays on suitably diluted fermentation broths were carried out as described by LIU *et al.*[8]. Whole broth was assayed by an agar diffusion disc method with *Bacillus megaterium* ATCC 8011 as the test organism, or turbidimetrically, with *Streptococcus faecalis* ATCC 8043 as the test organism. Samples were diluted approximately 40: 1 in methanol containing 0.1% Tween 80 (Atlas Chemical Industries) for the turbidimetric assay. Aurodox sodium salt, 95% pure, was used as a standard with enough replicate assays performed to give a standard error of 15%.

Mutagenic Treatments

Spore suspensions were treated with the following mutagens under the standard conditions[5,9~13]: ethyl methane sulfonate, N-methyl-N-nitro nitrosoguanidine (Aldrich), ultraviolet light, nitrous acid (sodium nitrite, Fisher) and fast netrons (gamma irradiation, Radiation Technology Inc., Rockaway, New Jersey).

Overlay Assay

To screen for back mutation of a non-producer, spore suspensions were plated on mycophil agar medium containing 1% phytone (Baltimore Biological); 4% dextrose; 2% agar. After incubation for 3~4 days at 28°C, the resulting colonies were overlayed with molten mycophil agar at 48~50°C seeded with *Bacillus megaterium* ATCC 8011. Colonies capable of producing $\geq$100 mcg/ml of aurodox in shake flask fermentations were detected by the zone of inhibition occurring in the lawn of *B. megaterium*.

Selection of Aurodox Resistant Mutants

Vegetative growth of *S. goldiniensis* from a 6 or 8 day fermentation was spread on Amidex plates containing aurodox (up to 2 g/liter). After incubation of the inoculated plates for 10 days at 28°C, growth was removed and aseptically homogenized with a tissue grinder. The homogenates were diluted in sterile distilled water and spread on an agar medium containing the same or a higher concentration of aurodox. Isolated colonies appearing after 10 days of incubation were transferred to agar slants and the resulting strains evaluated for antibiotic yields. Strains obtained in this manner maintained their resistance to aurodox even when passed several times in the absence of the antibiotic.

Results

Conventional Mutagenic Treatment

A higher yielding strain was not obtained following mutagenic treatment of the "original culture". At least 1,000 colonies were examined after treatment by each of the mentioned mutagens. An interesting finding was the large numbers of non-producers following treatment with ultraviolet light (0.5% non-producers with UV doses giving a 90% or greater kill).

Reversion of the Non-Producers

One out of 5 non-producers examined reverted to production after treatment with ultraviolet light. The reversion frequency was low, one per 10,000 examined UV survivors. This revertant, strain 3296-102/9, produced 20% more aurodox than the parent strain. Conventional mutagenesis of this revertant failed to yield a higher producing strain.

Resistant Mutants

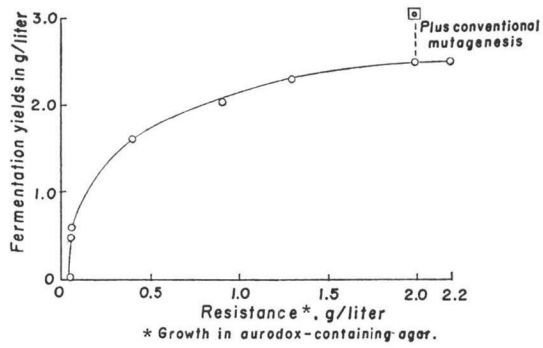
(1) Development of Strains Resistant to Low Levels of Aurodox

The original culture was unable to grow on agar containing 50 mcg/ml of aurodox. This sensitivity to aurodox was the same for the zero producer and the zero revertant. Spores or vegetative survivors of mutagenesis failed to grow on higher concentrations of aurodox. Only when vegetative growth from fermentation flasks producing aurodox was streaked on agar containing aurodox did resistance increase, as shown by growth on higher levels of aurodox, *i.e.*, 400 mcg/ml agar. Therefore, prior contact with the antibiotic was needed for resistance expression. Eight out of 34 colonies isolated from agar plates containing 400 mcg/ml aurodox produced yields of aurodox in shake flask fermentation that were 20~300% above that of the non-producer revertant. Strain 4081-104/79 which produced 1.6 g/liter of aurodox was used for further resistance development.

(2) Development of Resistance to Moderate Levels of Aurodox

Strains able to grow on mycophil agar containing 900 mcg/ml aurodox were obtained from strain 4081-104/79. The method used was identical to that used in section A above. It is interesting that all 240 colonies examined from this selection procedure gave yields higher than the parent strain, ranging from 1.8 to 2.1 g/liter. The strain chosen as superior, 4081-128-87 gave a consistant yield of 2.1 g/liter.

Fig. 1. Resistance to aurodox *versus* yield of aurodox



(3) Development of Strains Resistant to High Levels of Aurodox

Strains selected as resistant to 2 g/liter of aurodox gave yields of 2.5 g/liter of aurodox. This decrease in the rate of yield increase with higher levels of resistance is illustrated in Fig. 1 and Table 1. A promising preliminary finding

Table 1. Geneology, yields and aurodox resistance of *S. goldiniensis* strains

Geneology		Aurodox yield g/liter	Resistance level g/liter
2602–80–B_1	"Original" strain*	0.5	0.05
	↓ UV		
295–83–22E	Zero producer	0.0	0.05
	↓ UV		
3296/102/9	Revertant from zero producer	0.6	0.05
	↓ SR		
4081–104/79	Aurodox resistant	1.6	0.4
	↓ SR		
4081–128/87	Aurodox resistant	2.1	0.9
	↓ SR		
5187–44/102	Aurodox resistant	2.3	1.3
	↓ SR		
5187–74/53	Aurodox resistant	2.5	2.0

* Starting strain for present study. Considerable strain work had been conducted previously by Dr. J. BERGER.

UV=ultraviolet irradiation

SR=selection for resistance to aurodox

is that these highly resistant strains respond favorably to conventional mutagenesis as shown in Fig. 1. Thus, yields of greater than 3 g/liter have been encountered with aurodox resistant strains treated by conventional mutagenesis.

Discussion

The method needed to obtain yield increases for a given antibiotic-producing microorganism cannot be predicted. Morphological variants were useful in obtaining increased yields of penicillin[13]. Auxotrophs, zero producers and their revertants proved useful in obtaining high-yielding tetracycline strains[5]. THOMA (1971)[14] stressed that a wide variety of mutagens should be used, since each strain and mutagen may interact in a unique way. The above methods were not fruitful in increasing yields of aurodox above 0.5 g/liter. Significant yield increases were only obtained when strains resistant to aurodox were selected on antibiotic-containing agar.

If survivors from UV irradiation ($\geq 90\%$ kill) were plated on agar containing high levels of aurodox, no growth occurred. However, if the UV survivors were first passed through a flask fermentation broth, and then the vegetative growth from such a broth was plated on aurodox-containing agar, aurodox-resistant mutants did grow out, some of which on subsequent testing in new fermentation broth gave higher aurodox yields than the parent zero-producer revertant. Thus it appears that prior contact with antibiotic is necessary for expression of resistance to aurodox. The need for this prior contact was extended in further studies by LIU *et al.*[8] who demonstrated that our mutants, resistant to aurodox, overcame the feedback inhibition of aurodox biosynthesis faster and at a higher level, than did the non-resistant strains.

It is possible that mutations for yield increase in the aurodox-sensitive strains were masked by feedback inhibition of aurodox biosynthesis. The highly resistant strains, unhampered by feedback inhibition, have become amenable to conventional mutagenic procedures.

The time of production and number of enzymes needed for the biosynthesis of aurodox have not been examined. The production of aurodox by low producers before inhibition by feedback inhibition indicates the presence of the enzymes needed for biosynthesis. It has been shown* that aurodox inhibits both protein synthesis and the release of elongation factor Tu from the ribosome in the producing organism, in several other bacteria and in chloroplast cell-free systems. Several other hypotheses could explain the feedback inhibition of aurodox biosynthesis. If the enzymes of biosynthesis are labile, aurodox could act as a corepressor to specifically inhibit enzyme synthesis at the transcriptional or messenger RNA level. If the produced enzymes are stable, aurodox could inhibit by binding allosterically to one of the enzymes in the biosynthetic pathway. The induced resistance to feedback inhibition could also be due to a change in cell permeability analogous to the development of resistance to tetracycline's inhibition of protein synthesis[18].

The fact that our mutants resistant to growth inhibition by aurodox are resistant to feed-back inhibition of aurodox biosynthesis suggests a logical explanation for the observed increased antibiotic production.

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