INDUCTION OF RESISTANCE TO AURODOX BY AURODOX IN THE ANTIBIOTIC-PRODUCING CULTURE, *STREPTOMYCES GOLDINIENSIS*

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The sensitivity of protein and aurodox synthesis to aurodox was examined in relationship to the development of resistance to aurodox in *Streptomyces goldiniensis* during fermentation. It was found that the culture remains sensitive to the antibiotic as long as no aurodox is present in the medium. Resistance only develops when aurodox is present, either exogenously added or endogenously synthesized by the culture. These observations suggest that the development of resistance is an inducible process, and evidence is presented indicating that aurodox induces a specific resistance system in *S. goldiniensis*.

In the course of our studies on aurodox fermentation by *Streptomyces goldiniensis*, it was found that the antibiotic inhibits growth of the producing culture and also inhibits its own biosynthesis[1]. In order to gain a better understanding of how the antibiotic-producing culture copes with the adverse effect of its own product, the effect of aurodox on *S. goldiniensis* was further investigated. In this report, we show that *S. goldiniensis* develops specific resistance to aurodox, but only when aurodox is present in the medium, and that the development of this resistance appears to be induced by aurodox.

Materials and Methods

Organisms and Fermentation

S. coelicolor ATCC 23899 and the following mutants of *S. goldiniensis* ATCC 21387 were used: strain 3296-102-6, previously used in the study of feedback inhibition of aurodox synthesis[1], produces about 400~500 μg/ml aurodox in the fermentation medium described below; strain 3296-102-6NP, which produces less than 1 μg/ml aurodox in the fermentation medium, is a non-pigmented mutant derived from 3296-102-6; strain 5787-60-228, isolated on the basis of resistance to 2 mg/ml aurodox produces up to 1,500 μg/ml aurodox[2].

All cultures were started by growing in an inoculum medium which contained (g/liter): Ardamine Z (Yeast Product Corp., Paterson, N. J.) 5, tomato pomace (Seaboard Supply Company, Philadelphia, Pa.) 5, Cornstarch 10, calcium carbonate 1, potassium phosphate dibasic (anhydrous) 1, and soybean oil 10. The pH was adjusted to 7.0 before autoclaving. The cultures were grown for 48 hours at 28°C, then either used directly to inoculate a fermentation medium (3%, vol/vol), or preserved as a stock culture. The fermentation medium contained (g/liter): Phytone (pepsin digest of soybean meal, Baltimore Biological Laboratory Inc., Baltimore, Maryland) 15, soluble starch 20, yeast extract (Difco) 1, potassium phosphate dibasic (anhydrous) 1, calcium carbonate 1, $CoCl_2 \cdot 6H_2O$, 0.0238, and soybean oil 30. The pH was adjusted to 7.5 with NaOH before sterilization. In addition, Difco Thermoactinomyces fermentation medium (Difco Laboratories, Detroit, Michigan) was used. In this medium strain 5787-60-228 produces less than 5 μg/ml of aurodox. All fermentations were carried out at 28°C in the same manner as described previously[1].

A preliminary account of this study was presented at the Annual Meeting of the American Society for Microbiology 1977 (Abstr. paper 031, Ann. Meeting American Society for Microbiology, p. 250, 1977).

Aurodox Assay

The aurodox concentration in broth was determined by isotope dilution. A measured quantity of aurodox-^{14}C with known specific activity, prepared by fermentation from butyrate-1-^{14}C[3], was added to a culture of *S. goldiniensis*. At specific times thereafter, samples of broth were removed, adjusted to pH 7.0 with HCl, and then extracted with an equal volume of chloroform containing a known quantity of unlabelled aurodox. The antibiotic in the chloroform extract was purified by thin-layer chromatography (Merck Silica gel 60F-254) in a solvent system composed of chloroform, methanol and concentrated ammonium hydroxide (4: 1: 0.1, volume ratio). After chromatography, aurodox (Rf=0.23) was eluted with methanol and assayed spectrophotometrically at 325 nm ($E^{1\%}_{1cm}$ Na salt= 435). The radioactivity of the purified antibiotic was measured in an Intertechnique liquid scintillation spectrometer by counting 1 ml of the methanol eluant in 10 ml Aquasol (New England Nuclear, Boston, Mass.) at a counting efficiency of 71.5%. The amount of aurodox present in the broth was calculated from the ratio of the specific activity of the aurodox-^{14}C added to the broth to that isolated from the broth.

Protein and Aurodox Synthesis Measurements

Incorporation of L-valine-^{14}C or L-phenylalanine-^{14}C into the hot trichloroacetic acid (10%) precipitated fraction of a washed mycelial preparation was used as a measure of protein synthesis. Aurodox synthesis by washed mycelium was determined by measuring the incorporation of L-methionine-methyl-^{14}C into aurodox. The details for both assays have already been described[1]. The ^{14}C-labelled amino acids were purchased from New England Nuclear (Boston, Mass.).

Cell Growth

A 5-ml portion of fermentation broth was centrifuged and the insoluble fraction washed once with 5 ml *n*-hexane, then twice with distilled water. This washed mycelium was dried at 100°C for 24 hours and then weighed.

Results

Effect of Aurodox on Aurodox Fermentation

Fig. 1A and 1B depicts the overall effect of aurodox on an aurodox fermentation.

When antibiotic is added at 300 μg/ml, a level below the maximum that strain 3296-102-6 can produce, mycelial growth is initially inhibited but growth occurs after a prolonged incubation period. The length of lag is dependent on aurodox concentration and size of inoculum (Data not shown). Once mycelial growth begins, production of aurodox, as indicated by the decrease in specific activity of labelled aurodox-^{14}C, follows approximately two days later. However, the total *de novo* synthesis of aurodox by the culture is reduced by approximately the amount added, so that the final concentration of aurodox does not exceed the production potential for this strain, about 400 ~ 500 μg/ml.

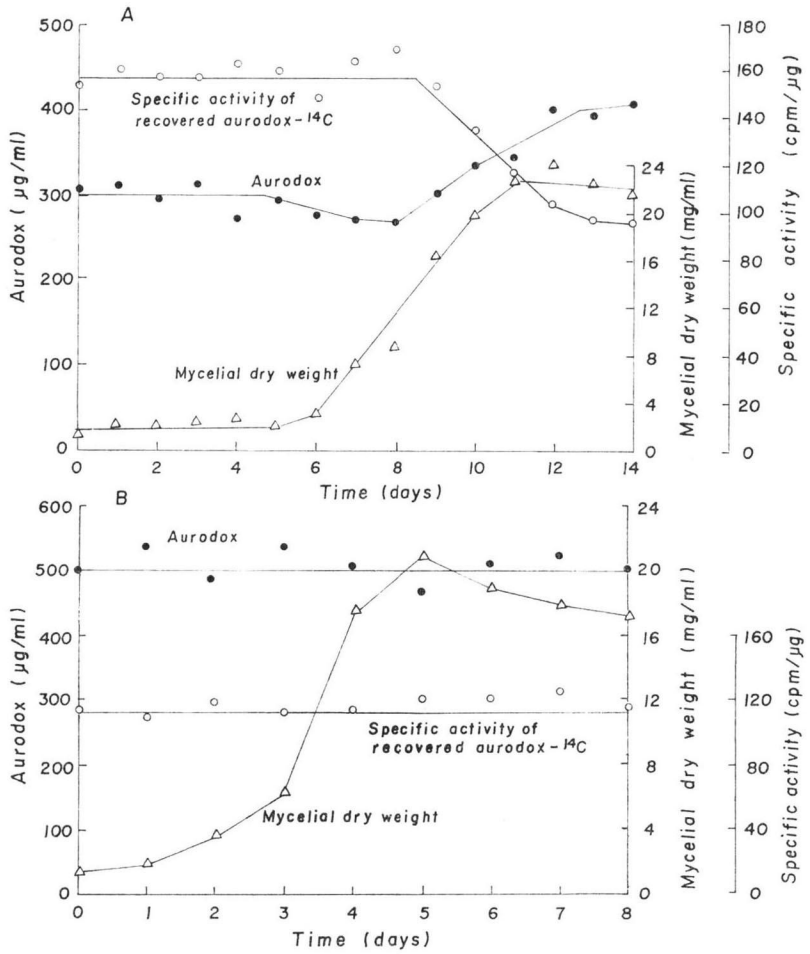
When mycelium that has been adapted to grow in the presence of aurodox is used as inoculum, the culture resumes its growth after a much shorter lag period even in the presence of 500 μg/ml of aurodox (Fig. 1B). In the presence of this high level of aurodox, aurodox biosynthesis is completely inhibited as is evident by the lack of isotope dilution of labelled aurodox added to the medium. However, the culture is fully capable of synthesizing aurodox, if it is transferred again into a fresh fermentation medium containing no aurodox (Data not shown).

Development of Resistance to Inhibition of Protein Synthesis by Aurodox

Since inhibition of *S. goldiniensis* growth by aurodox is likely a result of inhibition of protein synthesis[4,5], this relationship was followed during the course of a fermentation. Results shown in Table 1 indicate that with all strains used, protein synthesis in young cells is always sensitive to aurodox. The

Fig. 1. Effect of exogenous aurodox on aurodox fermentation.

The fermentation was started by inoculating a 2-day old culture of strain 3296-102-6 grown in inoculum medium to the fermentation medium containing 300 μg/ml aurodox-^{14}C. (A). A portion of broth samples were then withdrawn daily from the fermentation and the mycelial dry-weight, specific radioactivity of aurodox-^{14}C, and aurodox concentration in broth were determined as described in Materials and Methods. At the end of fermentation, 14 days after inoculation, a portion of the stationary phase culture (3%, v/v) was inoculated into fermentation medium containing 500 μg/ml of aurodox-^{14}C. (B). Again, the mycelial dry-weight, specific radioactivity of aurodox-^{14}C and aurodox concentration in broth from samples were measured daily.



development of resistance is observed as a culture grows older, but only with strains that produce aurodox. Resistance to aurodox develops to a higher level for the strain which produces a higher level of aurodox, 5187-60-228. The non-producing strains, 3296-102-6NP, is sensitive to aurodox irrespective of the age of mycelium.

Table 1 also shows that development of resistance to inhibition by aurodox is specific for aurodox, since the *S. goldiniensis* strains tested remain sensitive to other inhibitors of protein synthesis, chloramphenicol and puromycin (at 100 μg/ml). Furthermore, cultures of *S. goldiniensis* grown in the

Table 1. Inhibition of protein synthesis in different strains of *Streptomyces goldiniensis* by various antibiotics.

Strain	Inhibitor	Concentration (μg/ml)	% Inhibition at				
			Day 1	Day 2	Day 3	Day 4	Day 7
3296-102-6-NP	Control	—	0	0	0	0	0
	Chloramphenicol	100	100	100	100	100	100
	Puromycin	100	100	100	100	100	100
	Aurodox	50	100	100	100	100	100
		100	100	100	100	100	100
		200	100	100	100	100	100
3296-100-6	Control	—	0	0	0	0	0
	Chloramphenicol	100	100	100	100	100	100
	Puromycin	100	100	100	100	100	100
	Aurodox	50	22	8	20	0	3
		100	19	22	37	0	3
		200	56	54	68	36	12
		400	100	100	97	64	38
		600	100	100	100	90	59
5187-60-228	Control	—	0	0	0	0	0
	Chloramphenicol	100	100	100	100	100	100
	Puromycin	100	100	93	100	100	100
	Aurodox	50	21	21	3	4	7
		100	15	15	4	14	9
		200	34	61	52	24	5
		400	77	97	87	43	21
		600	98	100	95	56	28
		1000	100	100	100	83	40

Washed mycelium from 1, 2, 3, 4 and 7 days old culture grown in the fermentation medium was used for the assay. The mycelium was preincubated with the antibiotics for 30 minutes before the reaction was initiated by the addition of valine-^{14}C. All of the antibiotics were dissolved in an aqueous solution containing 20% dimethyl sulfoxide (DMSO) and 10% ethanol to give a final DMSO and ethanol concentration of 2.0 and 1.0% respectively in the reaction mixture. Incorporation of valine-^{14}C into the protein fraction of *S. goldiniensis* was determined by the procedure described in Materials and Methods.

Thermoactinomyces fermentation medium, a medium which supports little or no aurodox production, remain sensitive to aurodox at the end of fermentation, although some resistance to low levels of aurodox did develop in strain 5187-60-228 (Table 2).

Induction of Resistance to Aurodox by Aurodox

The results shown in Tables 1 and 2 suggest that either the synthesis of aurodox or the presence of the antibiotic is responsible for development of resistance to aurodox. To determine which of these is responsible, a non-producing mutant, 3296-102-6NP, was grown in fermentation medium and the inducibility of aurodox resistance by aurodox was examined. Since no *de novo* synthesis of aurodox occurs in the non-producing mutant, any development of resistance to aurodox can be attributed exclusively to the induction of resistance by the exogenously added aurodox. The results shown in Table 3 indicate that this is indeed the case. The induction of resistance by aurodox is a slow process; no

Table 2. Inhibition of protein synthesis by aurodox in different strains of *Streptomyces goldiniensis* growing in Difco Thermoactinomyces fermentation medium.

Strain	Aurodox concentration (μg/ml)	% Inhibition at				
		Day 1	Day 2	Day 3	Day 4	Day 7
3296-102-6	0	0	0	0	0	0
	50	78	100	100	80	92
	100	80	95	95	96	95
	200	83	98	98	97	98
	400	92	99	99	99	99
	600	100	100	100	100	100
5187-60-228	0	0	0	0	0	0
	50	94	87	87	56	47
	100	94	97	97	90	68
	200	95	98	98	98	84
	400	96	100	100	100	97
	600	97	100	100	100	99
	1000	100	100	100	100	99
3296-102-6NP	0	0	0	0	0	0
	50	100	95	100	96	100
	100	100	98	100	100	100
	200	100	100	100	100	100

The experiment was essentially the same as described in Table 1 except that all of the cultures were grown in Difco Thermoactinomyces fermentation medium and that phenylalanine-^{14}C was used to determine the activity of protein synthesis.

resistance develops after four hours of induction, but after overnight incubation (17 hours) resistance become evident. Induction of resistance is aurodox concentration dependent. As little as 1 μg/ml (1.25 μM) of aurodox induces a partial tolerance to 50 μg/ml aurodox. The observation that resistance to low levels of aurodox develops in 5187-60-228 (Table 2) is consistent with the small amount (<5 μg/ml) of antibiotic produced by the culture in Difco Thermoactinomyces fermentation medium. No resistance to aurodox can be demonstrated when *S. coelicolor*, grown in the same medium, is incubated with various concentrations of aurodox (Data not shown).

Table 3. Induction of resistance to aurodox inhibition by aurodox.

Concentration of inducer (aurodox) added to the culture (μg/ml)	% Inhibition of protein synthesis by aurodox	
	at 50 μg/ml	at 200 μg/ml
0	98	97
1	61	86
2	33	91
5	33	79
10	33	76
20	16	69
50	2	41
100	5	50

A zero-producer of aurodox, 3296-102-6NP was grown in the fermentation medium overnight. Aurodox (inducer) was then added aseptically at the concentrations indicated. After 17 hours of further incubation, inhibition of protein synthesis by aurodox at 50 and 200 μg/ml was determined as described in Table 1.

Development of Resistance to Inhibition of Aurodox Biosynthesis

As shown in Fig. 1A and 1B, the biosynthesis of aurodox is regulated by aurodox present in the medium. Further study reveals that the antibiotic synthesizing system later becomes insensitive

Fig. 2. Development of resistance to inhibition of aurodox biosynthesis.

The inhibition of aurodox biosynthesis by various concentrations of aurodox was measured as a function of culture age during antibiotic production phase. To determine the effect of aurodox on aurodox biosynthesis, washed mycelium suspended in distilled water was added to a mixture of methionine-CH_3-^{14}C (0.2 μCi/ml final volume) plus aurodox at the concentrations indicated. The incorporation of methionine-CH_3-^{14}C into aurodox was then determined (1). Both strains 3296-102-6 (A) and 5787-60-228 (B) were grown in the fermentation medium as described in Materials and Methods.

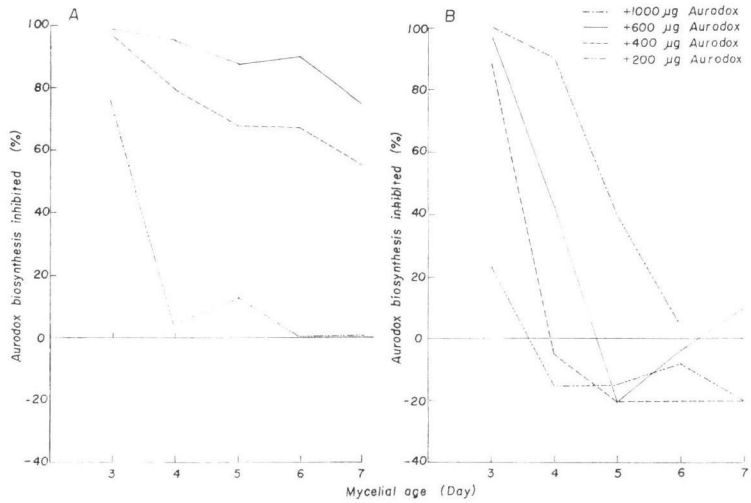


Table 4. Inhibitory effect of aurodox on protein and aurodox synthesizing systems in *S. goldiniensis* during fermentation.

Strain	Aurodox concentration (μg/ml)	% Synthesis inhibited at					
		Day 3		Day 4		Day 7	
		Aurodox	Protein	Aurodox	Protein	Aurodox	Protein
3296-102-6	0	0	0	0	0	0	0
	50	—*	20	—	0	—	3
	100	—	37	—	0	—	2
	200	74	68	15	36	0	12
	400	97	97	74	64	45	38
	600	100	100	95	90	75	59
5187-60-228	0	0	0	0	0	0	0
	50	—	3	—	4	—	7
	100	—	4	—	14	—	9
	200	23	52	0	24	0	5
	400	89	87	0	43	0	21
	600	95	95	43	56	10	28
	1,000	100	100	90	83	40	40
3296-102-6NP	0	100	0	100	0	100	0
	50	100	100	100	100	100	100
	100	100	100	100	100	100	100
	200	100	100	100	100	100	100

* Test not done

All the data are taken from Fig. 1 and Table 1.

to inhibition by aurodox (Fig. 2). The aurodox synthesizing system in both strain 5187-60-228 and 3296-102-6 are very sensitive to inhibition by aurodox at the early idiophase stage (day 3 after inoculation). As the antibiotic is accumulating, resistance to aurodox inhibition of the antibiotic synthesizing system increases. The ability of a culture to acquire resistance to the inhibition appears to be proportional to the antibiotic producing potential of the producing strain. Strain 3296-102-6 quickly acquires resistance to inhibition by aurodox at a 200 μg/ml level, but is sensitive to the inhibition when aurodox concentration is 400 μg/ml or higher. High yield strain 5187-60-228 is capable of developing resistance to aurodox self inhibition at correspondingly higher concentrations of aurodox; as much as 1,000 μg/ml of antibiotic is tolerated by this strain.

As shown in Table 4, a fairly close correlation exists between the development of resistance to inhibition of protein synthesis and the development of resistance to inhibition of aurodox biosynthesis in *S. goldiniensis*.

Discussion

Many *Streptomyces* have been shown to develop resistance to the inhibitory effects of the antibiotics they produce[6~9,12]. We have shown here that *S. goldiniensis* is similar in this respect, developing resistance to the inhibitory effects of aurodox on protein synthesis and synthesis of aurodox itself. Furthermore, we have shown that the synthesis of aurodox *per se* is not a prerequisite since in a non-producing mutant the addition of aurodox is sufficient to induce resistance. The resistance state is specifically directed to aurodox, since protein synthesis remains sensitive to other inhibitors of protein synthesis (Table 1). The potential for resistance appears to be genetically controlled since it did not occur in an unrelated species, *S. coelicolor*. The specificities observed suggest that resistance development in *S. goldiniensis* has evolved as a mechanism for protection against inhibition due to its own secondary metabolite.

Various mechanisms leading to antibiotic tolerance in antibiotic-producing microorganisms have been reported. Enzymatic modification of antibiotic occurs in *S. griseus*[10] and *S. venezuela*[11]. Enzymatic methylation of the antibiotic target site, ribosomal RNA, leads to thiostrepton tolerance in *S. azureus*[12,13]. Reduced ribosomal affinity for erythromycin is responsible for erythromycin tolerance in *S. erythreus*[14]. Rifamycin tolerance in *S. mediterranei* is thought to be due to an antibiotic-insensitive RNA polymerase[15]. Permeability barriers are thought to play a role in the resistance of *S. aureofaciens* to tetracyclines[9,16] and *S. parvulus* to actinomycin[8].

None of these reported mechanisms has been clearly identified in the development of resistance to aurodox in *S. goldiniensis*. In an experiment reported earlier[1], we have shown that gross inactivation of aurodox does not occur, but modification of a small amount of aurodox can not be ruled out (see Fig. 1A). A modified product could in turn act as an inducer to trigger the cell defense mechanism in a manner similar to that proposed for *Escherichia coli* carrying an R-factor resistance to aminoglycoside antibiotics[17]. A second possibility considered for the development of resistance to aurodox is that a permeability barrier develops during the course of incubation with the antibiotic. Although we have not been able to experimentally show a change in aurodox permeability, such a hypothesis is consistent with the fact that resistance to aurodox inhibition affects aurodox and protein synthesis simultaneously (Table 4). Accumulation of a metabolite or modification of elongation factor Tu[18] are other possibilities open to experimental evaluation. Whatever the mechanism, the presence of an inducible system for resistance provides *S. goldiniensis* with protection from the inhibitory effects of aurodox, allowing antibiotic synthesis to continue to its maximum potential.

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