MULTIPLE EFFECTS INDUCED BY UNSTABLE MUTATION IN *STREPTOMYCES LAVENDULAE*

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Arg mutants, isolated from *Streptomyces lavendulae* at unusually high frequencies, showed several phenotypic characteristics. The characteristics common to all *arg* mutants include: (1) repression of β-lactamase production, (2) inhibition of aerial mycelium formation, (3) development of acid pH, (4) low saturation density of growth in liquid culture, (5) a decrease in antibiotic production, (6) an increase in sensitivity to benzylpenicillin and (7) a decrease in production of pigment. These results suggest that the *arg* mutation concomitantly caused the depression of secondary metabolism in *S. lavendulae*.

We had previously reported the frequent isolation of *arg* mutants in *S. lavendulae*[1]. All of the *arg* mutants grew on the minimal medium supplemented with argininosuccinate but not on minimal medium supplemented with ornithine or citrulline. The parental strain was shown to produce β-lactamase constitutively and extracellularly like most of *Streptomyces* strains[2]. The *arg* mutants concomitantly showed no or very low extracellular β-lactamase activity and did not form aerial mycelium[1]. One possibility is that the gene for β-lactamase might transpose near or into argininosuccinate synthetase gene and cause the multiple effects. Another possible explanation we propose is that the transposable element might be a common regulatory gene necessary for secondary metabolism.

In this report we describe the detailed examination of common phenotypes induced by the mutation and present data which suggest that the mutation is caused not by the transposition of β-lactamase structural gene but by some common regulatory gene for differentiation.

Materials and Methods

Strains and media

The parental strain (S55-B1) is a single spore isolate derived from the original strain (*Streptomyces lavendulae* KCC S0055, which was derived from ATCC 8664) provided by Dr. A. SEINO of Kaken Chemical Co. This strain was maintained on slants of GAA agar as described earlier[1]. *Arg* mutants were derived by treatment with curing agents or UV. Other auxotrophic mutants were obtained by exposure to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine[1]. The mutants were maintained on slants of GPMY agar. GPMY agar consists of 1% galactose, 0.2% peptone, 0.1% meat extract, 0.1% yeast extract and 2% agar, pH 6.8~7.0. The auxotrophic markers of the mutant strains used in this paper are *arg* (22-24), *leu* (Y33-1) and *his* (F11-1). An *arg*$^+$ revertant (22-24R1) was isolated spontaneously from 22-24[1].

Assay method for β-lactamase activity

The assay method for β-lactamase activity has been described previously[1]. Intracellular β-lactamase activity was determined as follows: Cells were harvested, washed 3~4 times with 0.85% NaCl and then sonicated 4 times for 10 minutes in 10 mM phosphate buffer (pH 7.0) - 0.3 M KCl. The suspension was held in an ice bath for 30 minutes and then centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was the source of the intracellular enzyme.

Screening of sugars on aerial mycelium formation

Aerial mycelium formation of the parental and the mutant strains was examined by substituting glucose of BENNETT's agar with various sugars[1].

Measurement of growth

Cells were grown in 100 ml of PYG medium[3] in 500-ml Erlenmeyer flasks at 27°C for 2 days with shaking (210 rpm). The mycelia were harvested and resuspended in 0.85% NaCl. Short mycelial fragments were prepared by cutting with Polytron (Kinematica). The optical absorbance at 600 nm of the suspension was measured and an aliquot was transferred into 100 ml of PYG medium. At appropriate intervals the absorbance of cultures was measured at 600 nm. When the absorbance was above 0.8, the culture was diluted to ensure accurate measurement of growth.

Antibiotic-sensitivity spectrum

Mycelia were cut into fragments as described above. The cell fragments were mixed with BENNETT's agar; 10 ml of seeded agar was poured into a Petri dish (9 cm diameter). Paper discs (8 mm) containing antibiotics were placed on the seeded plates. The plates were incubated at 27°C overnight and the diameters of the inhibitory zones were measured.

Assay method of antibiotic production

Cells were grown in 100 ml of PYG medium at 27°C for 2 days on a rotary shaker (210 rpm). Cells were then transferred to the same medium. The culture broth was tested daily for antimicrobial activity against *Bacillus subtilis* PCI219 by the paper disc method. Streptomycin sulfate (1.2 μg/disc and 0.24 μg/disc) was used as a standard.

Results and Discussion

β-Lactamase Production in *arg* Strains

We have examined whether low extracellular β-lactamase activity of *arg* strains was caused by the production of inhibitor. Culture broths from strains B1 and 22-24 (*arg*) were mixed at a ratio of 1:1 and assayed for the enzyme activity. Fig. 1 shows that broth from strain 22-24 has no inhibitory activity against the β-lactamase produced by strain B1.

Inhibition of the excretion of β-lactamase into the broth is another possible cause of low activity. Fig. 2 shows intracellular β-lactamase activity per flask in strains B1 and 22-24. If the activity is ex-

Fig. 1. Effect of cultured medium of an *arg* mutant (22-24) on β-lactamase activity of B1.

Cultured media of B1 and 22-24 were mixed at daily intervals in a ratio of 1:1 and the enzyme activity was measured.

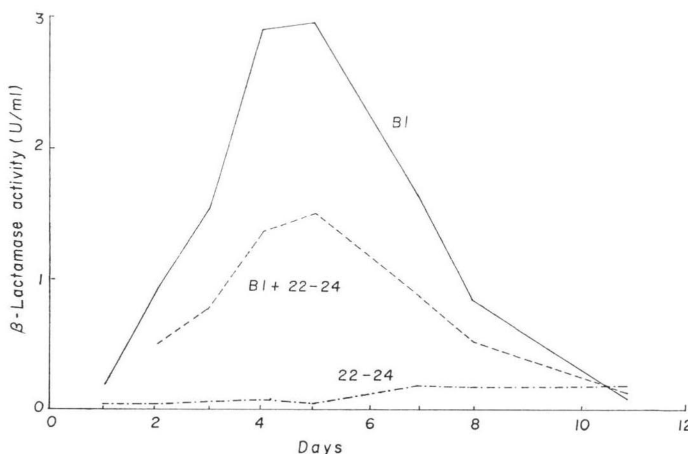
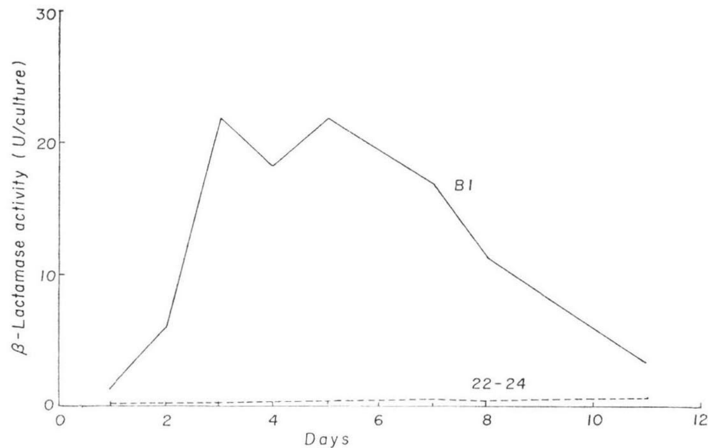


Fig. 2. Intracellular β-lactamase activity of B1 and an *arg* mutant (22-24). The preparation of intracellular enzyme is described in Materials and Methods.



pressed as units per mg protein, the enzyme activity of strain 22-24 is only 20~30% of the parent. Therefore there was no intracellular accumulation of β-lactamase in strain 22-24 and we can conclude that low β-lactamase activity in *arg* mutants is caused not by the inhibition of the enzyme excretion but by the decrease in enzyme production.

Morphological Studies

Table 1 shows the effect of sugars on the formation of aerial mycelium in various strains. Parental strain S55-B1 formed aerial mycelium and sporulated well. All *arg* mutants isolated in the previous paper[1] could not produce aerial mycelium and spores. An example from Table 1 is culture 22-24 which did not form aerial mycelium on the solid media containing any of the 13 sugars tested. The arg^+ revertants regained the ability to form aerial mycelium and to sporulate.

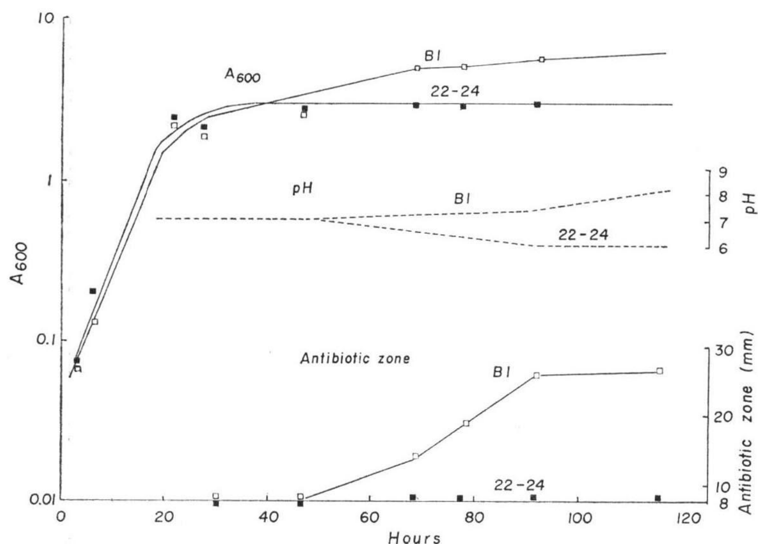
Table 1. Effect of sugars on aerial mycelium formation.

sugar	B1 (wild type)	Y33-1 (*leu*)	F11-1 (*his*)	22-24 (*arg*)	22-24R1 (arg^+ revertant)
Glucose	± to +	−	−	−	−
Sucrose	+	±	+	−	++
Maltose	++	− to ±	+	−	−
Starch	++	±	+	−	NT*
Glycerol	+	−	−	−	NT
Galactose	++	± to +	+	−	++
Fructose	+	±	+	−	+
Arabinose	± to +	−	±	−	NT
Mannitol	+	±	+	−	++
Xylose	± to +	±	−	−	NT
Inositol	+	±	−	−	NT
Lactose	+	± to +	+	−	++
Mannose	++	± to +	−	−	++

−: Aerial mycelium (−), spore (−), ±: Aerial mycelium (+), spore (−), +: Aerial mycelium (+), spore (+), ++: Aerial mycelium (+), dense spore, *: Not tested

Fig. 3. Growth curves, pH change and antibiotic production of B1 and *arg* mutant (22-24) cultured in PYG medium.

Growth was measured by absorbance at 600 nm. Antibiotic production was determined by the size of the inhibitory zone against *B. subtilis* PCI219. The diameter of disc was 8 mm.



On the other hand, other auxotrophic mutants could form aerial mycelium when the sugar of BENNETT's agar was replaced by other sugars. For example Y33-1 (*leu*) and F11-1 (*his*) could form aerial mycelium on the media containing galactose, lactose and so on.

Growth and pH Change of Cultured Medium

Another phenotype common to all *arg* mutants is the growth extent and the pH change of cultured broth. The *arg* mutants formed relatively small colonies with less pigment compared to the wild-type. Fig. 3 shows the growth curves of the wild-type (B1) and an *arg* mutant (22-24) in PYG medium. They grew well with doubling times of about 4 hours in log phase. At 40 hour, strain 22-24 stopped growing and showed a constant absorbance for the duration of the fermentation. On the other hand, strain B1 continued growing slowly but steadily to reach twice the absorbance value of strain 22-24.

The difference in pH change of the medium between the two strains was striking. The pH of the medium incubated with strain B1 increased as the cells grew. In strain 22-24, the pH of the medium began to decrease when cell growth stopped and remained acidic.

Sensitivity to Various Antibiotics

The sensitivity against various antibiotics of B1, 22-24 and its arg^+ revertant (22-24R1) was examined. Table 2 shows the size of the growth inhibitory zones caused by various antibiotics. The most distinct difference is the sensitivity of the three strains to benzylpenicillin. It was shown in the previous paper[1] that 22-24 had a low extracellular β-lactamase activity (about 10% of B1) and that 22-24R1 had negligible enzyme activity. The sensitivity to benzylpenicillin increased with the decrease in β-lactamase activity. In another experiment, mycelia were plated on an agar plate containing benzylpenicillin; this showed a similar significant difference in the sensitivity to benzylpenicillin (data not shown).

Table 2. Sensitivity of *S. lavendulae* to various antibiotics.

		Diameters (mm) of inhibitory zones					
B1	Conc.* (μg/disc)	1	1/2	1/4	1/8	1/16	1/32
Tetracycline	1.5	11.6	(9.0)**	—	—	—	—
Chloramphenicol	6.0	26.6	22.6	20.5	17.6	12.3	—
Penicillin	120.0	15.8	—	—	—	—	—
Erythromycin	0.3	20.0	17.2	15.3	9.2	—	—
Streptomycin	6.0	12.4	10.6	8.9	—	—	—
Kanamycin	30.0	16.1	14.6	13.1	12.0	10.6	(8.6)
22-24	Conc.* (μg/disc)	1	1/2	1/4	1/8	1/16	1/32
Tetracycline	1.5	12.1	(8.4)	—	—	—	—
Chloramphenicol	6.0	31.8	28.0	23.1	18.9	13.3	—
Penicillin	120.0	26.3	22.6	17.3	13.5	—	—
Erythromycin	0.3	27.2	18.9	16.4	12.4	—	—
Streptomycin	6.0	14.6	12.8	11.5	9.0	—	—
Kanamycin	30.0	17.3	15.7	13.6	12.8	11.4	(8.5)
22-24R1	Conc.* (μg/disc)	1	1/2	1/4	1/8	1/16	1/32
Tetracycline	1.5	10.3	—	—	—	—	—
Chloramphenicol	6.0	24.8	16.4	15.0	—	—	—
Penicillin	120.0	NT	NT	24.0	21.0	19.9	15.7
Erythromycin	0.3	23.4	20.8	16.8	15.9	10.8	—
Streptomycin	6.0	16.5	12.8	12.4	11.4	10.9	—
Kanamycin	30.0	22.4	19.9	16.6	13.5	11.4	(8.4)

* Conc. shows the maximum concentration of the each antibiotic. The serial dilution is shown by a fraction.
** Half-inhibitory zone
NT: Not tested. —: No discernible zone

Antibiotic Production in B1 and 22-24

S. lavendulae is known to produce several kinds of antibiotics including streptothricin[4]. We have examined whether the multiple effects of arginine auxotrophy altered antibiotic production. Fig. 3 shows the time course of antibiotic production of strains B1 and 22-24 cultured in PYG medium. B1 produced antimicrobial activity against *Bacillus subtilis* PCI219 from the beginning of the stationary phase but 22-24 produced no such activity during the prolonged incubation.

Our studies in this report have shown that an unusual mutation occurs at high frequencies in *S. lavendulae*, which is accompanied by arginine auxotrophy and the repression of several types of secondary metabolism. All *arg* mutants isolated from B1 showed the simultaneous loss of the various types of secondary metabolism. As for the arg^{+} revertants, only three clones were isolated because of the low reversion frequency[1]. All of the revertants, however, showed phenotypes similar to strain 22-24R1.

REDSHAW *et al*[5]. reported a similar kind of variant in *Streptomyces*. Aerial mycelium-negative variants isolated after the treatment with curing agents required arginine or argininosuccinate for growth on minimal medium. The variants also showed the simultaneous loss of the characteristic pigment and earthy odor of the wild-type. They also reported that the development of aerial mycelia in *S. alboniger*, *S. scabies* and *S. coelicolor* was sensitive to glucose repression[6]. They suggested that the accumulation

of undissociated organic acids appeared to be involved in glucose repression of aerial mycelia formation. The *arg* mutants described in this report also showed development of an acid pH but it is unknown yet whether the wide loss of secondary metabolic events was caused by catabolite regulation.

Streptomyces is a very attractive organism because of its wide use for antibiotic production in industry and also its unique differentiation for a prokaryote. Our *arg* mutants are now available for study of regulation of antibiotic production and differentiation, and the relationship between the two.

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