INDUCTION OF ANTIBIOTIC PRODUCTION WITH ETHIDIUM BROMIDE IN *STREPTOMYCES HYGROSCOPICUS*

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Protoplast regeneration carried out in a carriomycin producing organism, *Streptomyces hygroscopicus* 358 AV2, lose carriomycin productivity without loss of carriomycin-resistance and the ability of formation of aerial mycelium. Ethidium bromide treatment on the 358 AV2 strain generated a bald mutant that produced carriomycin and a new antibiotic curromycin. In some other media, however, the parent strain produced curromycin, indicating that the ethidium bromide treatment altered the regulation of antibiotic production. Ethidium bromide treatment on a protoplast-regenerated strain derived from the parent strain resulted in derivatives capable of producing carriomycin and curromycin. These strains were unstable and tended to lose the recovered antibiotic productivity easily.

The regulation of antibiotic production has been reported to be correlated with the presence of plasmids, the appearance of reiterated DNA sequences or the presence of a regulation factor. All of the genes for methylenomycin production were found on the SCP1 plasmid carried in *Streptomyces coelicolor* A3(2)[1,2], and the enhancement of chloramphenicol production was found to be governed by a plasmid in *Streptomyces venezuelae*[3~5]. The appearance of reiterated DNA sequences[6~13] induced by protoplast regeneration or treatment with intercalating dyes has been implicated in the disappearance of some characteristics like melanin formation, pigment production or antibiotic production. Finally, A-factor was found to induce streptomycin production in *Streptomyces griseus*[14,15].

In this paper we report that ethidium bromide treatment enhanced the production of curromycin[16,17] in a carriomycin[18,19] producing strain, *Streptomyces hygroscopicus* 358 AV2, which produced only low levels or no curromycin depending on the media used.

Materials and Methods

Strain

The parental strain *Streptomyces hygroscopicus* 358 AV2 was isolated by the authors from a soil sample taken from grassland at Oyabe town (Toyama prefecture, Japan).

Protoplast Formation and Regeneration

Protoplast formation and regeneration were done according to OKANISHI's method[20] except that 0.3 M sucrose was used as osmotic stabilizer in the regeneration medium.

Dye Treatment

The parental strain was grown in YM medium consisting of yeast extract 0.4%, malt extract 1.0% and glucose 0.4% for 48 hours at 27°C in the presence of various concentrations of dyes. The mycelia that grew poorly at an appropriate concentration of dyes were cut by a Polytron and plated on YM agar plates. After growth, individual colonies were transferred to YM agar pieces by long

tooth-sticks. After the agar pieces were incubated at 27°C for 7 days, antibiotic productivity was examined against *Bacillus subtilis* IAM 1026.

Culture Conditions and Media

For antibiotic production strains were grown for 96 hours at 27°C in K or Q medium. K medium consisted of soluble starch 2.5%, soy bean meal 1.5%, dry yeast 0.2% and calcium carbonate 0.4%, while Q medium consisted of glycerol 2.0%, casein 0.5%, molasses 1.0%, Polypepton 0.1% and calcium carbonate 0.4%.

Determination of the Quantity of Carriomycin and Curromycin Produced

One hundred ml of the cultured broth was extracted by EtOAc. After evaporation of EtOAc the concd extracts were applied to silica gel TLC plates and developed with $CHCl_3$ - MeOH (98: 2). Antibiotics were extracted from the silica gel by MeOH, and the concentration of the antibiotics was determined by the comparison of the diameter of inhibitory zone against *B. subtilis* with that of the authentic samples. Distinction between curromycins A and B was done by reversed phase HPLC (Radial-pak C18, THF - H_2O, 35 : 65) because curromycins A and B were not separated by silica gel chromatography.

Detection of Reiterated DNA Sequences

Extraction of total DNA was done by HERSHBERGER's method[9]. Restriction endonucleases were purchased from Toyobo Co., Ltd. and used according to the manufacturer's recommendation. Agarose gel electrophoresis was performed using 0.7% (w/v) agarose gel in E buffer (Tris-acetate 0.04 M, EDTA·2Na 0.002 M, pH 8.0). The gels were stained in 0.5 μg/ml of ethidium bromide for 20 minutes and the DNA bands visualized under UV light (254 nm).

Results and Discussion

Effect of Protoplast Regeneration

The *S. hygroscopicus* 358 AV2 strain produces a polyether antibiotic, carriomycin, whose production is stable after successive cultivation. When this strain was subjected to protoplast regeneration, more than 90% of the resultant colonies were found to have lost the carriomycin productivity, as shown by the agar piece method (data not shown). All the regenerated strains formed aerial mycelia, and their physiological properties like starch hydrolysis, gelatin liquefaction, peptonization and coagulation of skim milk, and carbon utilization were identical with those of the parental strain. But the appearance of their spore surface was changed from rough to smooth. The regenerated strains produced a purple diffusible pigment which was not produced by the parental strain in glycerol - asparagine or oatmeal agar.

Addition of the cultured broth of the parental strain did not restore carriomycin production in the regenerated strains. This rules out the possibility that an inducible, diffusible regulatory substance was involved in antibiotic production in the parental strain.

It has been reported that plasmids can be eliminated from *Streptomyces* cells by protoplast regeneration[21,22] which has resulted in some alteration of phenotypes like enhancement of antibiotic resistance[9,23,24], antibiotic productivity[9,24~27] and nutrition requirement[26]. One interpretation of our results is that a plasmid might be involved in the production of carriomycin. We obtained variable results for the presence of a plasmid in the parental strain; *i.e.*, a plasmid band was observed by cesium chloride - ethidium bromide (CsCl - EB) centrifugation in a lysate of the parental strain, which was prepared from 15 ml of cultured broth in GPY medium after protoplasting the cells with lysozyme and pronase followed by lysis with sodium *N*-lauroyl sarcocinate. But this result was not reproducible. This result was interpreted as that the plasmid(s) tended to be integrated in the host chromosome.

Table 1. Effect of various DNA intercalating dyes on the parent strain.

	Amy^+Car^+	Amy^+Car^-	$Amy^-Car^+Cur^-$	$Amy^-Car^+Cur^+$	Amy^-Car^-
			(%)		
AF 0.5 μg/ml	88	0	0	7	5
AO 4.0 μg/ml	66	0	0	32	2
EB 2.0 μg/ml	7	0	0	93	0

Amy; Ability of aerial mycelium formation, Car; production of carriomycin, Cur; production of curromycin.

AF; Acriflavine, AO; acridine orage, EB; ethidium bromide.

The parent strain 358 AV2 shows Amy^+ Car^+. The numbers indicate the percentage of the colonies which show the phenotypes described. Car^- and Cur^- strains show no observable inhibitory zones of *B. subtilis* by the colonies grown on agar pieces.

In some streptomycetes, such interaction between free form of a plasmid and the chromosome has been reported[28~32]. Alternatively this might be that the plasmid was unstable in our culture or isolaton condition, and lost during culture or storage of the strain or during isolation. It is also possible that the procedure we employed for the plasmid extraction is not suitable for this particular plasmid. Therefore, the possible relationship between carriomycin production and the presence of a plasmid is unknown. As a similar phenomenon has been reported for the loss of resistance and production of tylosin in *Streptomyces fradiae*; *i.e.*, after protoplast regeneration. No extrachromosomal DNA was detectable in this case[9]. The authors suggested that tylosin biosynthetic genes were present on a self-transmissible element[33].

Effect of Intercalating Dyes

To test how intercalating dyes known to eliminate plasmids from host cells affect the production of the antibiotic, we treated *S. hygroscopicus* 358 AV2 with various concentrations of dyes. Addition of dyes at concentrations that allowed only poor growth in YM medium *i.e.*, acriflavine (AF) 0.5 μg/ml, acridine orange (AO) 4 μg/ml, and ethidium bromide (EB) 2 μg/ml resulted in generation of colonies with various phenotypes. The results are shown in Table 1. When AF and AO were used, most of the colonies showed the wild-type phenotypes, *i.e.*, aerial mycelium positive (Amy^+) and carriomycin producing (Car^+) phenotype, whereas the rest were bald mutants (Amy^-). The bald mutants we obtained did not show auxotrophy. It should be noted that some relationship exists between loss of aerial mycelium formation and arginine auxotrophy[28,34~37]. Among the bald mutants some showed a reduced carriomycin production (0.4 μg/ml in the K medium) compared with the parental strain (10 μg/ml in the K medium). The ratio of the bald mutants to the wild-type colonies was 93% in the case of treatment with EB, showing that this drug was the most effective for producing bald mutants under the conditions used. Among the Amy^- mutants, 7%, 32% and 93% obtained by the AF, AO and EB treatment, respectively, were found to produce another inhibitory substance effective against *B. subtilis*. We have determined the chemical structures of the active principles, to be curromycins A and B in one of the bald mutants (EB 32), and found that the antibiotics are unrelated in structure to carriomycin[18,19]. Curromycins A and B were produced by the mutant, EB 32, at a concentration of 16 and 0 μg/ml, respectively, in the K medium, whereas the parental strain produced only a trace or an undetectable amount of the curromycins in the same medium. The parental strain, however, was found to produce only curromycin A in Q medium, whereas the bald mutant strain produced both

Table 2. Effect of media on carriomycin and curromycin production.

	K medium		Q medium	
	Car*	Cur*	Car*	Cur*
Parent	10	0	40	16 (A)
Regenerated-strain	0	0	0	0
EB 32	0.4	16 (A)	5.5	24 (A and B)
A-4	0.1	1.3 (NT)	0.6	45 (NT)

A and B in the parentheses show curromycins A and B, respectively. Car and Cur denote carriomycin and curromycin, respectively. For the A-4 strain, distinction between curromycins A and B was not tested (NT).

* μg/ml.

Table 3. Production of carriomycin and curromycin in modified K medium by the parental strain.

	Carriomycin*	Curromycin*
K medium	10.0	0
KG medium (starch → glycerol)	0.7	7.0
KC medium (starch → casein)	0.3	0
KP medium (dry yeast → Polypepton)	0.6	0
KM medium (soy bean meal → molasses)	0.3	t

The arrows in the parentheses indicate that each component of the K medium shown on the left was replaced by the other component shown on the right.

* μg/ml.

t: Trace.

Table 4. Regulation of antibiotic production by carbon source.

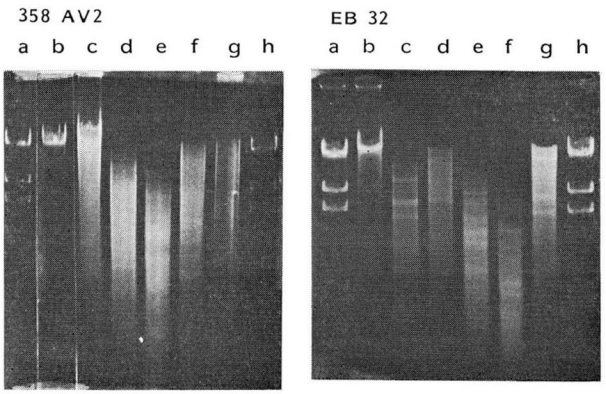
	Glucose	Sucrose	Glycerol	Maltose	Inositol	Lactose	Galactose	Sorbitol
Parent								
Carriomycin	1.4	1.3	0.7	1.1	1.5	1.4	1.4	1.4
Curromycin	0	0	7	0	0	0	0	0
EB 32								
Carriomycin	0	0	t	t	0	t	0	t
Curromycin	0	25	5	0	0	0	0	0

Numbers designate the amounts of antibiotics produced (μg/ml), and t indicates only a trace amount (<1 μg/ml) produced.

curromycins A and B in the same medium (Table 2). To learn how the culture conditions affect the antibiotic production, the parental and EB 32 strains were cultured in media in which each medium component of the K medium was exchanged for that of the Q medium. The antibiotic production patterns were investigated and the results show that the parental strain produced curromycin in the KG medium whose carbon source was glycerol (Table 3).

The influence of various carbon sources in the K medium on production of both antibiotics was investigated. In the parent strain carriomycin was produced with all carbon sources tested, whereas the EB 32 strain produced a trace amount (less than 1 μg/ml) of the antibiotic in glycerol, maltose, lactose and sorbitol media (Table 4). Curromycin was produced in the sucrose and glycerol media by the EB 32 strain, whereas only in the glycerol medium by the parental strain (Table 4). These facts suggest that regulation of both carriomycin and curromycin production was altered by the EB treatment.

Fig. 1. Digestion patterns of DNA isolated from *S. hygroscopicus* 358 AV2 and EB 32.
a and h: λDNA digested with *Hind* III, b: total DNA uncut, c: total DNA digested with *Bcl* I, d: *Pst* I, e: *Pvu* II, f: *Sma* I, g: *Bgl* II.



Detection of Reiterated DNA Sequence

As in the case of the strains regenerated from protoplasts, the dye-treated bald mutants did not show significant physiological changes compared with the parental strain (data not shown), and extrachromosomal elements were not detectable in the bald mutant by CsCl - EB density gradient centrifugation. To know if any chromosomal rearrangement had occured in the EB 32 strain, and if it might correlate with alteration of the antibiotic production pattern, we examined DNA from the strain for reiterated DNA sequences. Digestion with restriction enzymes of total DNA from the parental and regenerated strains (data not shown) did not show distinct bands in agarose gel electrophoresis. In comparison with the digestion patterns of the parental strain, however, those of the EB 32 strain showed several distinct but not very prominent bands that were not seen in the parental strain (Fig. 1), suggesting that the EB 32 strain had aquired reiterated DNA sequences with a small copy number. EB treatment is known to give rise to amplification or deletion of chromosomal DNA[13]. The DNA reiteration seen in our results, therefore, might be related to carriomycin production.

Protoplast Regeneration of EB 32 Mutant

The EB 32 mutant was subjected to protoplast regeneration, and the effect of this process on carriomycin productivity was examined. The regeneration efficiency of the bald mutant was less than 1% and the carriomycin productivity was not lost after regeneration, which is in contrast to the loss of carriomycin production in the regenerated strains derived from the parental strain. The production of carriomycin and curromycin was at the same level in both the EB 32 and its regenerated mutant (data not shown).

Recovery of Antibiotic Productivity by EB Treatment in the Protoplast-regenerated Parental Strain

The protoplast-regenerated strains which were derived from the parental strain and had lost the ability to produce antibiotics were found to maintain resistance against carriomycin (100 μg/ml) but showed reduced resistance to curromycin compared with the parental strain. The parental strain grew in the presence of curromycin (100 μg/ml) in the liquid YM medium, whereas the growth of the re-

generated strain was retarded by curromycin (5 μg/ml) in the same medium.

It was of interest to test if EB treatment of the regenerated strain induced curromycin production since it had been found that EB treatment of the parental strain induced the production of this antibiotic (see above). After EB treatment of one of the regenerated strains (Amy^+), colonies were examined for the production of carriomycin and curromycin. In contrast to the case of the EB treatment on the parental strain 358 AV2, Amy^- colonies were not obtained. Some of the colonies showed antibacterial activity against *B. subtilis*, but most of the active strains gradually lost their antibiotic production ability. Only one out of ten assayed strains, A-4, could produce antibiotics in liquid culture. The active substances produced in the K medium turned out to be carriomycin (0.1 μg/ml) and curromycin (1.3 μg/ml) as shown in Table 2. In the Q medium, the A-4 strain showed a different antibiotic production pattern compared with those of the parent or its protoplast-regenerated strains. It is interesting that production of carriomycin and curromycin was synchronously induced. Digestion with restriction enzymes of the total DNA from the A-4 strain did not show reiterated sequences (data not shown). Although reiterated DNA sequences were not observed, alteration of the chromosome might have occurred and influenced the antibiotic production, since reiterated sequences are often unstable and their appearance is reported to be linked to chromosomal rearrangement[13].

At present, it is not known how the intercalating dyes induce curromycin production, and, in certain cases, carriomycin production, and what kind of DNA rearrangement the drugs induce. However, these results suggest that certain intercalating dyes can activate silent genes which are involved in antibiotic production.

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