FUNCTION OF PLASMIDS IN THE PRODUCTION OF AUREOTHRICIN

I. ELIMINATION OF PLASMIDS AND ALTERATION OF PHENOTYPES CAUSED BY PROTOPLAST REGENERATION IN *STREPTOMYCES KASUGAENSIS*

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The spontaneous mutant 18a derived from *Streptomyces kasugaensis* MB273 exhibited pleiotropic effect such as loss of aerial mycelium formation, aureothricin (AT) production, and of citrullin biosynthesis, as well as changes in plasmid; the mutant required cystine for production of aureothricin. An improved method of protoplast regeneration was applied to *S. kasugaensis* MB 273-18a and a regeneration efficiency of 90% or more was obtained. Sixty to ninety percent of the colonies regenerated from the 18a protoplasts exhibited reversion of the pleiotropic mutation in 18a. Moreover, of 13 regenerated strains which showed these drastic phenotypic variations, it was found that their plasmid types varied. These types could be divided into two groups; the RI type (5 strains) which contained a large amount of pSK2, a small amount of pSK3 and no pSK1, and the RII type (8 strains) in which no closed-circular DNA was detected. From these results, the following conclusions were obtained. First, plasmid curing in RII type strains and also the variation of plasmid copy in the RI type strains occurred as the result of protoplast regeneration. Second, the structural genes for biosynthesis of AT probably exist on chromosome. Third, regeneration of 18a protoplasts causes the reversion of pleiotropic mutation with high frequency. A working hypothesis was proposed to explain these complex phenomena.

Recent studies of genetic determinants for antibiotic biosynthesis are making it clear that plasmids are often involved. However, the role of plasmids in antibiotic production is still not clear, except for the plasmid SCP1 carrying the structural genes of methylenomycin in *Streptomyces coelicolor* A3(2)[1,2].

Streptomyces kasugaensis strain M338 and the newly isolated strain MB273 have been used in our laboratory; both produce aureothricin (AT), thiolutin and kasugamycin. AT and thiolutin are *N*-propionylpyrrothine and *N*-acetylpyrrothine, respectively.

The results of our early experiments showed that L-cystine was the precursor of AT biosynthesis and that pyrrothine was an intermediate in the pathway from L-cystine to AT[3,4]. AT-non-producing mutants were induced by acriflavine treatment. When these non-producers were incubated in synthetic liquid medium supplemented with cystine, neither AT nor pyrrothine was detected. However, when pyrrothine was added in place of cystine, all non-producers produced AT[4,5] even "natural" AT-non-producers such as *S. venezuelae* or *S. erythreus* produced AT from added pyrrothine[4,5]. Acylation of pyrrothine was also confirmed in cell-free systems prepared from these non-producing mutants[5].

Because of the facts mentioned above, we thought that plasmid-borne genes might play a role in the biosynthetic pathway from cystine to pyrrothine, and that chromosomal genes might be involved in the acylation of pyrrothine to AT. In this paper, it is shown that this conclusion is in need of revision. *S. kasugaensis* MB273 carries three kinds of plasmids; pSK1, pSK2 and pSK3. Both pSK1 and pSK2 are 6.8Md, and pSK3 is 14.5Md. The pSK1 molecule has one cleavage site for *Sal* I, *Eco* RI and *Bam*

HI, and three sites for *Bgl* II[6]. The pSK2 molecule has no cleavage site for *Sal* I, one site for *Eco* RI and *Bgl* II, and two sites for *Bam* HI[6]. The pSK3 is not a simple dimer of pSK1 or pSK2 since it is cleaved once by *Sal* I. It is possible to clarify plasmid function by introduction of each plasmid into recipient cells. But in the case of *S. kasugaensis*, good methods for formation and regeneration of protoplasts did not exist. Accordingly, we have developed a method for protoplast regeneration applicable to many actinomycetes[7]. In this paper, we describe the complex phenotypic alteration of a mutant 18a of *S. kasugaensis* MB273, accompanying protoplast regeneration.

Materials and Methods

Microorganisms

Streptomyces kasugaensis MB273 supplied from Dr. M. HAMADA, Institute of Microbial Chemistry, was used in the experiments instead of strain M338, because strain MB273 sporulated well and plasmid DNA could be more easily isolated. *Escherichia coli* NIHJ was used as the test organism for the assay of AT production.

Media

GPY medium for seed cultures, GPYG medium for growing cultures, P3 medium for protoplast formation, PWP medium for washing protoplasts and R3 medium for protoplast regeneration have been described elsewhere[7]. The soft agar medium for the upper layer of protoplast regeneration contained 0.4% Low Melting Point Agarose (BRL) in place of Noble agar in the lower layer of R3 medium. AT production medium contained glucose 1 g, maltose 15 g, Soytone 5 g, yeast extract 1 g, Na_2SO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, K_2HPO_4 2 g, $Na_2HPO_4 \cdot 12H_2O$ 8 g, and agar 20 g per 1 liter of deionized water. MY medium for aerial mycelium formation contained maltose 10 g, yeast extract 3 g, ashes from wood 0.3 g and agar 18 g per 1 liter of deionized water.

Formation and Regeneration of Protoplasts

Streptomyces species were grown in GPY medium at 27°C for 2 days, and transferred into fresh growing culture medium, GPYG, with 2% inoculum size, followed by shaking at 27°C for 26~28 hours. The mycelia obtained from 8 ml of the growing culture were washed with 0.35 M sucrose solution by centrifugation, resuspended in 8 ml of P3 medium, and treated with 0.3 to 1.0 mg lysozyme ml^{-1} at 28°C for 30 to 60 minutes. The protoplasts were washed two times with the same volume of PWP medium, after intact cells were removed by filtration through cotton and diluted in PWP medium. 0.1 ml of the diluted sample and 4 ml of soft agar of R3 medium kept at below 38°C were plated on the underlayer of medium R3 by gentle mixing and incubated at 26~27°C after drying the surface of the plate.

Viable Counts to Determine Intact Cells in Protoplast Suspension

Intact cells in the protoplast suspension were determined by counting the colonies grown on the reference R3 medium, in which disodium succinate, Mg^{2+} and Ca^{2+} were omitted.

Isolation of Plasmid

General procedures for the isolation of closed-circular DNA from *S. kasugaensis* and its derivatives were as described previously[8].

Distinction of Plasmids by Agarose Gel Electrophoresis

The digestion of plasmid DNA with restriction endonucleases and agarose gel electrophoresis of the digested samples were carried out as described previously[6]. From the previous report[6], closed-circular plasmids pSK1 and pSK2 which run as a single band in the agarose gel electrophoresis were differentiated by digestion with *Sal* I or *Bam* HI. *Sal* I cleaves one site of pSK1 but not in pSK2. *Bam* HI cleaves at one site in pSK1 and two sites in pSK2, which give rise to a slightly smaller fragment than a full-length fragment of pSK1 and a minute fragment. *Eco* RI cleaved pSK1 and pSK2 once each. Closed-circular pSK3 was identified by it's one cleavage site with *Sal* I and 2 sites with *Eco* RI or *Bam* HI, and by the full-length fragment of 14.5×10^6 daltons.

Assay of AT

AT was determined by the disc assay method or agar cylinder method using *E. coli* as a test organism (kasugamycin produced together with AT from *S. kasugaensis* did not show activity against *E. coli*).

Aerial Mycelium Formation

Aerial mycelium formation was observed on strains incubated on MY agar and Bennett agar at 28°C for 14 days.

Results and Discussion

As Table 1 shows, the original strain MB273 has three plasmids designated pSK1, pSK2 and pSK3. This strain produces aureothricin (AT) in both liquid and agar media. AT-non-producing mutants were isolated spontaneously from strain MB273; one of them was designated 18a and AT was detected in neither liquid nor agar medium in this variant. This variant requires external cystine for AT production, although this strain can grow well without the addition of cystine. Therefore, 18a must possess all the genes involved in the biosynthesis of AT from cystine. Moreover, addition of palmitate or oleate to the agar medium also caused AT production without supplemental cystine. However, this effect of cystine or specific fatty acids was not found in liquid medium. Strain 18a has a pleiotropic mutation, it produces no aerial mycelium and requires citrulline for growth, in addition to its inability to produce AT. The plasmids, pSK1 and pSK2, were not detected in the mutant 18a in earlier studies, however, very small amounts of pSK1 and pSK2 have now been isolated from the strain as the result of improved plasmid-isolation procedures (to be published).

We attempted to transform plasmid DNAs pSK1 and pSK2, into 18a protoplasts to clarify their function. At the time, strain 18a had been considered not to have pSK1 and pSK2. For these transformation experiments, an improved regeneration method had been developed since the regeneration efficiency of protoplasts of some streptomycetes such as *S. kasugaensis* was low in the regeneration

Table 1. Characterization of *S. kasugaensis* MB 273 and its mutants.

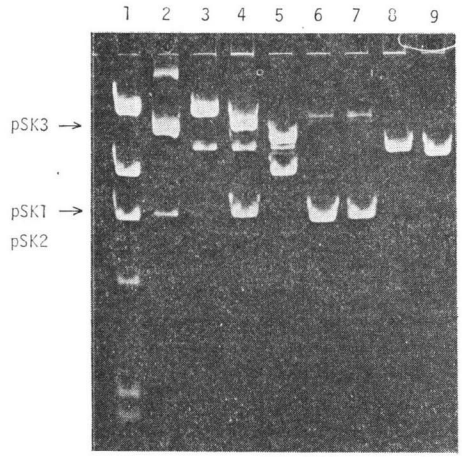
	Parent MB273	Mutant 18a	Mutant 18a-RI type (R5)	Mutant 18a-RII type (M518)
Method		spontaneous from MB 273	regeneration from 18a protoplasts	regeneration from 18a protoplasts
ccc DNA				
pSK1	+++	+	−	−
pSK2	++	+	+++	−
pSK3	+	+++	+	−
AT production				
Liquid medium	++	−	−	−
Agar medium	++	−	++	++
Supplement required for AT production on agar medium	none	L-cystine palmitate oleate	none	none
Aerial mycelium formation	++++	−	++	++
Nutritional requirement	−	citrulline	−	−
TC resistance (μg/ml)	50	12.5	12.5	12.5

medium R2[9]. The regeneration method described in Materials and Methods was applied successfully to protoplasts of mutants of *S. kasugaensis* MB273 with a regeneration frequency of more than 80% after incubation for 8 to 10 days. However, unexpected results were obtained. About half of the colonies regenerated from the protoplasts without supplemental plasmid DNA showed a drastic alteration in their phenotype. They formed aerial mycelia and grew well without addition of citrulline.

The control experiments were repeated with the following result. Regeneration occurred with a frequency of more than 90%. As Table 1 shows, 60~90% of the regenerated colonies showed intermediate phenotypes between MB273 and 18a. They produced AT on agar medium without cystine, recovered the ability to form aerial mycelium and they were capable of citrulline biosynthesis. However, they did not produce AT when incubated in liquid medium. Among the AT-producing colonies which regenerated, 13 strains were used for plasmid analysis. Five strains designated 18a-RI type showed increased copy number of pSK2, little pSK3, and no detectable pSK1. The other 8 strains (18a-RII type) had no detectable plasmid. However, both types of mutants obtained showed the same phenotype (Table 1). In another control experiments, 50 colonies were isolated from the mycelia of 18a, and all showed identical phenotypes to 18a.

Fig. 1 shows agarose gel electrophoretic patterns of plasmids of strain 18a and the 18a-RI type strain. The plasmids of strain 18a are shown in column 2, an increased amount of pSK3 (14.5 Md), a small amount of pSK1 and pSK2 (6.8 Md) as a single band, and the open circular form of pSK3 are observed. After *Sal* I digestion, pSK3 and pSK1 were cleaved once, and pSK2 remained undigested (column 3). With *Eco* RI, pSK3 was cleaved twice, and pSK1 and pSK2 once (column 4). *Bam* HI digestion gave 2 and 1 cleavages with pSK2 and pSK3, and pSK1, respectively (column 5). Since the top band of column 2 disappeared on digestion with *Sal* I and *Bam* HI, it is presumed to be the open circular form of pSK3.

Fig. 1. Digestion pattern of the 18a and 18a-RI plasmid by restriction endonucleases.
1: λ+*Hind* III (reference). 2: 18a plasmids. 3: 18a plasmids+*Sal* I. 4: 18a plasmids+*Eco* RI. 5: 18a plasmids+*Bam* HI. 6: 18a-RI plasmids. 7: 18a-RI plasmids+*Sal* I. 8: 18a-RI plasmids +*Eco* RI. 9: 18a-RI plasmids+*Bam* HI.



The plasmid pattern of the RI type strain is different from that of strain 18a: a large amount of plasmid of 6.8 Md and a band which seems to be the linear DNA of pSK3 were observed (column 6). *Sal* I digestion did not cleave the 18a-RI plasmids (column 7), and *Eco* RI and *Bam* HI cleaved the major plasmid once and twice, respectively (columns 8 and 9). Therefore, the major plasmid of the RI type strain was identified as pSK2.

AT production in agar media was tested using *E. coli* as the test organism, as shown in Fig. 2. All of the colonies which acquired AT production showed concomitant reversion of the pleiotropic mutations in 18a strain. The ability to form aerial mycelia in regenerated colonies was tested on MY agar medium plates (see Fig. 3), and most of the regenerated 18a colonies had recovered this ability (Fig. 3).

Fig. 2. Recovered AT production in agar cylinder by the protoplast-regenerated cells of *S. kasugaensis* MB273-18a.

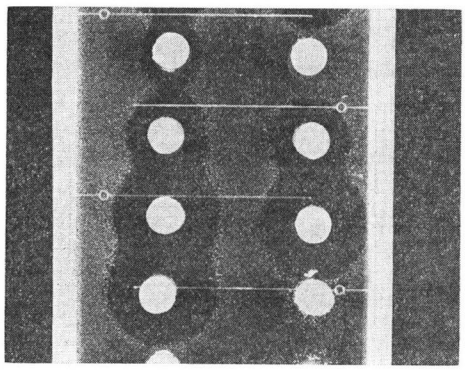
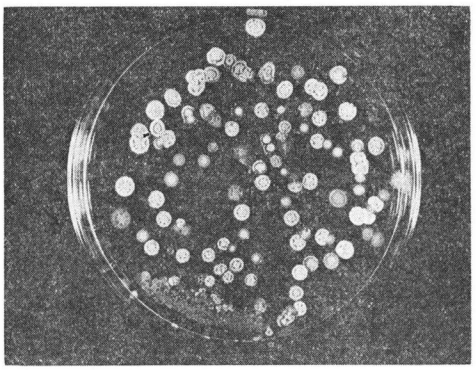


Fig. 3. Recovered aerial mycelia formation of the protoplast-regenerated cells of *S. kasugaensis* MB273-18a.

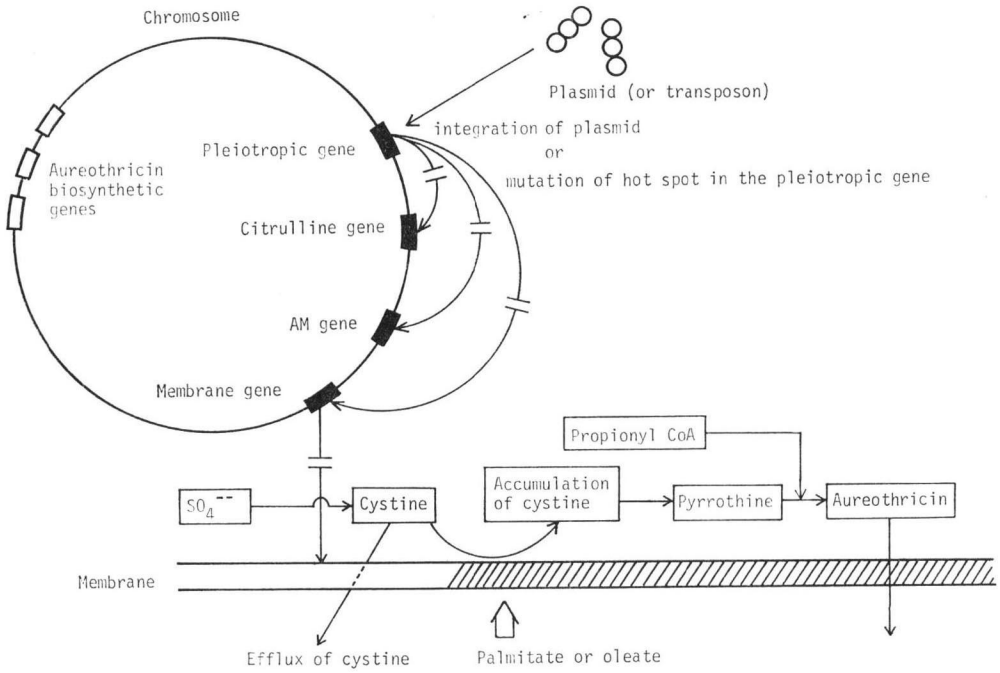


MY agar medium: Maltose 1.5%, yeast extract 0.3%, ash 0.01%, agar 1.8%.

We can conclude at this point that 1) plasmid curing as shown in RII type strains and also the variation of plasmid copy number in the RI type strains occurred as the result of protoplast regeneration. A cured strain of MB273 strain had not been obtained previously. The same RII type strains were also obtained with 10% frequency by protoplast regeneration of a RI-type strain derived from strain 18a (data not shown), 2), since the RII-type strains having no plasmid produced AT in agar medium, the structural genes for the biosynthesis of aureothricin probably exist on the chromosome of this strain, and 3) the regeneration of 18a protoplasts causes the reversion of the pleiotropic mutation with high frequency.

What are relationships between variation of plasmid copy number, AT productivity in agar medium and citrulline biosynthesis in *S. kasugaensis*? It is difficult to explain these phenotypic alterations by the loss of plasmids or by variation in plasmid copy number. To explain these complex phenomena, we propose a working hypothesis as shown in Fig. 4. A multifunctional (pleiotropic) gene is presumed to exist on the chromosome of *S. kasugaensis*. When this gene in strain MB273 is inactivated, pleiotropic mutations as shown in strain 18a will appear. The inactivation of this gene may be caused by the integration of a plasmid or transposon, or by mutation at hot spot that may be present in the gene. The gene must control at least 3 functional genes: that is, citrulline biosynthesis, aerial mycelium formation and AT production in agar medium (which may be related to membrane function). Therefore, inactivation of the proposed multifunctional gene causes the pleiotropic effects of citrulline requirement, no formation of aerial mycelium, and no production of AT by the change in membranous function. On the other hand, mutant 18a can produce AT by supplementation with cystine, palmitate or oleate in agar medium. Since cystine is a precursor of AT biosynthesis, strain 18a may release the intracellular cystine as a result of the membrane permeability defect, and thereby it may require supplemental cystine for producing AT. Palmitate or oleate may function either by activation of cystine biosynthesis or accumulation of cystine in cells by preventing the release of cystine from cells. The accumulated cystine is used to synthesize AT; the effect of the specific fatty acids was found only in the growth on agar medium. This interpretation is supported by results reported by OKAZAKI *et al.*[10,11] in which the alteration of fatty acid components in the cell membrane caused the excretion of as intracellular precursor of neomycin or streptothricin, with a concomitant loss of antibiotic production. We intend to test

Fig. 4. Working hypothesis on pleiotropic mutation and fatty acid effect found in *Streptomyces kasugaensis* MB273-18a.



this working hypothesis by integration of pSK1, pSK2 or pSK3 into chromosomal DNA, or reiteration of a certain chromosomal DNA, or by introducing mutation to obtain the pleiotropic mutant from RII type of strain.

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