SAGAMICIN AND THE RELATED AMINOGLYCOSIDES: FERMENTATION AND BIOSYNTHESIS

III. ISOLATION AND CHARACTERIZATION OF *MICROMONOSPORA SAGAMIENSIS* MUTANTS BLOCKED IN GENTAMICIN C_1 PATHWAY

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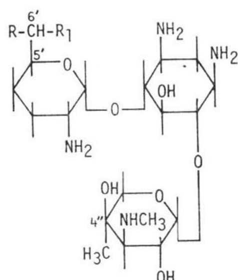
Production of sagamicin and gentamicin C_1 in *Micromonospora sagamiensis* was regulated by cobalt ion. In a parental strain, KY11510, cobalt ion stimulated gentamicin C_1 production and suppressed sagamicin production. By ultraviolet light or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment, six mutants blocked in gentamicin C_1 biosynthesis were obtained from KY11510. These mutants were classified into two types. The first type, four mutants, produced no gentamicin C_1 even when cobalt ion was added to the fermentation. The second type, two mutants, produced a small amount of gentamicin C_1 when a high concentration of cobalt ion was added. Based on biotransformation experiments, these mutants appeared to be blocked at the 6'-*C*-methylation step in the biosynthesis of gentamicin C_1. The mutants showed an increased production of sagamicin. In addition, cobalt ion stimulated sagamicin production in the mutants. The mechanism of cobalt regulation in the parent and the mutants is discussed.

Sagamicin (6'-*N*-methylgentamicin C_{1a}) is an aminoglycoside antibiotic produced by *Micromonospora sagamiensis* (Fig. 1)[1~3]. Sagamicin exhibits potent antibacterial activity against a broad range of Gram-negative and Gram-positive bacteria. The antibiotic is less ototoxic and nephrotoxic than the gentamicin C complex[4,5].

M. sagamiensis co-produced a small quantity of gentamicin C_1, as one of minor components, in a standard sagamicin fermentation. In the presence of cobalt ion, gentamicin C_1 was produced as the major component. During the course of improvement of sagamicin fermentation, we attempted to get a mutant strain producing an increased amount of sagamicin without producing gentamicin C_1. There have been several reports on the isolation of mutants producing increased amounts of one component among co-produced antibiotics. STARK *et al.*[6] isolated several types of mutants differing in the pro-

Fig. 1. The structure of sagamicin and gentamicins C.



	R	R_1
Gentamicin C_{1a}	H	NH_2
Gentamicin C_2	CH_3	NH_2
Gentamicin C_1	CH_3	$NHCH_3$
Sagamicin	H	$NHCH_3$

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duction of each nebramycin component in *Streptomyces tenebrarius*. Mutants producing increased levels of antimycin A_1 at the expense of A_3 and A_4 have been described by KLEUPFEL *et al.*[7].

This paper deals with the isolation of blocked mutants capable of yielding increased amounts of sagamicin without producing gentamicin C_1 in the presence of cobalt ion. The blocked step of these mutants in the biosynthetic pathway for sagamicin and gentamicin is postulated on the basis of the biotransformation experiments. The role of cobalt ions in the biosynthesis is also discussed.

Materials and Methods

Microorganisms

Micromonospora sagamiensis, KY11510, was used as the parent strain for the isolation of blocked mutants.

Media

Seed and fermentation media of the following compositions were used. The seed medium consisted of 1,000 ml of distilled water, 20 g Stabilose K (soluble starch), 5 g glucose, 5 g peptone, 5 g yeast extract, 3 g meat extract, and 2 g calcium carbonate (pH 8.0). The fermentation medium consisted of 1,000 ml of distilled water, 40 g Stabilose K, 10 g soy bean meal, 20 g Pharmamedia (cotton seed flour), 1 ml corn oil, 5 g casein, 2 g Ca-Mg_2-phytate, 150 mg $FeSO_4 \cdot 7H_2O$, 500 mg $MgSO_4 \cdot 7H_2O$, and 250 mg KH_2PO_4 (pH 8.0). ATCC No. 5 agar was used as the medium for the isolation of mutants.

Mutagenesis

The mutants blocked in gentamicin C_1 biosynthesis in *M. sagamiensis* were produced by exposing mycelial fragments to ultraviolet light or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). Mycelial suspensions for mutagenic treatment were prepared from liquid culture. The cultured mycelia in the seed medium were harvested by centrifugation and washed once with distilled water. The mycelial suspensions were treated with a homogenizer to break up large mycelial fragments, and these were filtered through cotton wool, centrifuged, and suspended in distilled water. Seventy five per cent of the mycelia in the resulting suspension were less than 10 μm in length.

Ultraviolet irradiation was performed by exposing 5 ml of a suspension of mycelial fragments in a Petri dish to a dose giving a survival of 1.0 to 0.1%, and the mycelial suspension was subsequently spread on ATCC No. 5 agar plates containing 500 μg/ml of caffeine[8,9].

Treatment with NTG was performed under the conditions recomended by DELIC *et al.*,[10] using 200 μg/ml of NTG in 0.05 M tris buffer (pH 9.0) for 60 minutes. The treated mycelial fragments were incubated overnight in seed medium, and an aliquot of appropriate dilution of the culture was spread on an ATCC No. 5 agar plate.

After about 3 weeks of incubation of the plate at 30°C, each of the colonies developed was picked up and the production of antibiotics was tested.

Test for Antibiotic Production

An 0.5-ml aliquot of the seed cultures of *M. sagamiensis* was added to 5 ml of the fermentation medium containing 1 μg of cobalt chloride per ml in a large test tube, and shaken at 30°C at 300 rpm. After ten days, the fermentation broth was acidified to pH 2.0 with oxalic acid and shaken for 30 minutes. After centrifugation, the supernatant was assayed for antibiotics. Sagamicin and gentamicin components were determined by thin-layer chromatography (TLC) on a Merck Silica Gel 60 plate using a solvent system composed of lower phase of chloroform - methanol - 17% ammonium hydroxide (2: 1: 1, v/v). The amounts of the individual products were assayed by fluorometric measurements of the 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole derivatives formed on silica gel TLC plates according to KABASAKALIAN *et al.*[11]. The TLC plates were scanned with a Simadzu CS-910 double-beam densitometer.

Biotransformation with Resting Cells[12]

M. sagamiensis was incubated in the fermentation medium with shaking. After incubation for 3 days, the mycelia were harvested and washed 3 times with 0.1 M tris-HCl buffer (pH 7.5). The washed mycelia were suspended in the same buffer and used for the biotransformation experiments. The reac-

tion mixture containing 150 mg (dry weight) cells and 500 μg substrate in 10 ml of tris-HCl buffer (pH 7.5) was incubated with shaking at 30°C for 20 hours. The biotransformation products in the reaction mixture were detected by bioautography against *Bacillus subtilis* KY4273 after paper chromatography (PPC). The PPC was carried out on Toyo No. 51 paper and developed in the lower phase of chloroform-methanol - 17% ammonium hydroxide (2: 1: 1, v/v).

Results and Discussion

The effect of cobalt ion on the production of sagamicin and gentamicin C_1 by *M. sagamiensis* KY-11510 was investigated. In the absence of cobalt ion, thc amount of gentamicin C_1 production was approximately one-fourth that of sagamicin production. In cobalt ion-supplemented cultures, the production of sagamicin decreased with concomitant increase in the yield of gentamicin C_1 (Fig. 2). Thus, the gentamicin C_1 production exceeded that of sagamicin production in the presence of more than 50 μg/liter of cobalt chloride.

Fig. 2. Effect of cobalt chloride on the production of sagamicin and gentamicin C_1 by *M. sagamiensis*, KY11510.

Strain KY11510 was cultured in a large test tube (2.5×19 cm) containing 10 ml of seed medium at 30°C. After 3 days of incubation with shaking, 3 ml of culture was transferred to 30 ml of the fermentation medium in a 300-ml flask containing various concentrations of cobalt chloride. Fermentation was carried out at 30°C on a rotary shaker for 10 days, and the yield of sagamicin and gentamicin C_1 was determined as described in Materials and Methods.

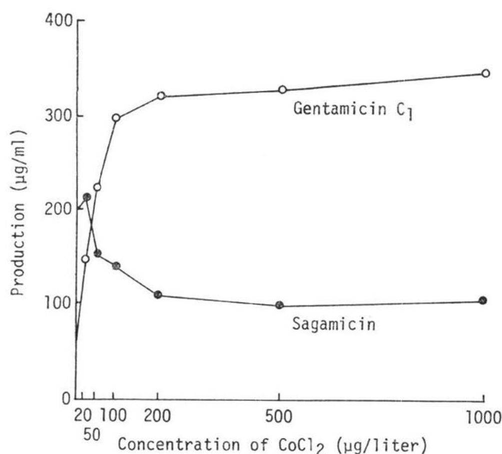
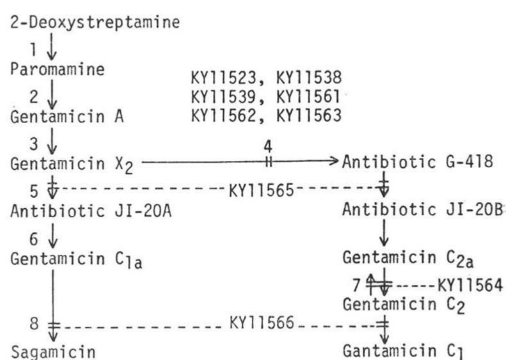


Fig. 3. The biosynthetic pathway of sagamicin and gentamicin and blocked steps in the mutants of *M. sagamiensis*[12].



1. Addition of D-glucosamine. 2. Addition of D-xylose, amino substitution and *N*-methylation at C-3″. 3. *C*-Methylation, with inversion of configuration, at C-4″. 4. *C*-Methylation at C-6′ with inversion of configuration. 5. Amino substitution at C-6′. 6. 3′,4′-Dehydroxylation. 7. Epimerization at C-6′. 8. *N*-Methylation at C-6′.

In a previous paper[12], mutants blocked in the production of sagamicin and gentamicin C_1 in *M. sagamiensis* have been described. Based on biotransformation experiments utilizing the compounds produced by the mutants and resting cells of these mutants and 2-deoxystreptamine (DOS) idiotrophs, a biosynthetic pathway for sagamicin and gentamicin was proposed (Fig. 3). In this pathway, two *C*-methylation steps, from gentamicin A to X_2 (4″-*C*-methylation) and from gentamicin X_2 to G-418 (6′-*C*-methylation), may be involved. TESTA and TILLEY proposed that both steps depended on cobalt ion[13]. Therefore, it is suggested that the stimulation of gentamicin C_1 production by cobalt is due to the activation of the *C*-methylation steps by cobalt.

In order to obtain mutants improved in sagamicin production, we attempted to isolate mutants

Fig. 4. Production of sagamicin and gentamicin C_1 by the mutants in flask fermentation.

Cobalt chloride (1 mg/liter) was added to a 300-ml flask each containing 30 ml of the production medium. Fermentation conditions were reported in the footnote of Fig. 2.

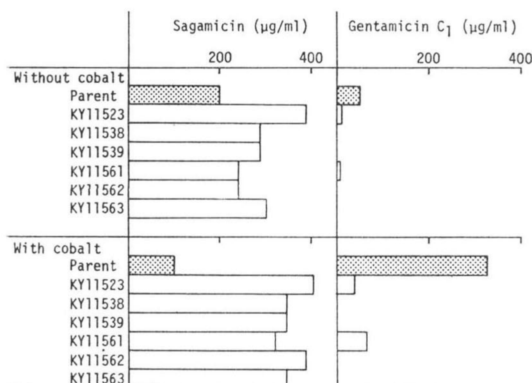
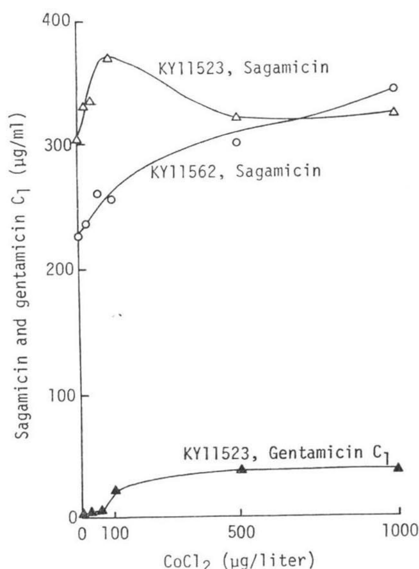


Fig. 5. Effect of cobalt chloride on the production of sagamicin and gentamicin C_1 in the mutants KY11523 and KY11562.

With fermentation conditions, see the footnote of Fig. 2.



blocked in the 6′-*C*-methylation step. The blocked mutants were screened by selecting those which produce no gentamicin C_1 even in the presence of 1 mg of cobalt chloride per liter in a fermentation medium.

Six mutants were isolated from about 9,000 colonies tested. Fig. 4 shows the production of sagamicin and gentamicin C_1 by the mutants in flask fermentation. Mutants KY11538, KY11539, KY11562 and KY11563 were unable to produce gentamicin C_1 even in the presence of cobalt. KY11523 and KY11561 produced a small amount of gentamicin C_1. These mutants were proved to be superior to the parent with respect to sagamicin production in the absence of cobalt. Moreover, the production of sagamicin by these mutants increased by the addition of cobalt. The results with the representative mutants KY11523 and KY11562, are shown in Fig. 5. The production of sagamicin in KY11562 increased by the addition of cobalt chloride and reached the maximum level at 1 mg/liter of cobalt chloride. The addition of $1 \sim 10$ mg/liter of cobalt chloride did not suppress sagamicin production. Thus, the maximum amount of sagamicin production in KY11562 was 1.6 fold that in KY11510. The production of sagamicin in KY11523 reached the maximum level at 100 μg/liter of cobalt chloride and was suppressed at higher levels of cobalt. The mutant also produced a small amount of gentamicin C_1. The production of gentamicin C_1 was stimulated by cobalt.

In order to clarify which step in gentamicin C_1 biosynthesis was blocked in the mutant, biotransformation of gentamicin precursors to gentamicin C_1 was investigated with the resting cells of the mutants (Table 1). As previously described[12], KY11525, a DOS idiotrophic mutant, transformed DOS, paromamine, or gentamicin A or X_2 to gentamicin C_{1a}, C_2, C_1 and sagamicin; G-418 or JI-20B to C_2 and C_1; C_2 to C_1; JI-20A to C_{1a} and sagamicin; C_{1a} to sagamicin. Non-gentamicin C_1-producing mutants KY11538, KY11539, KY11562 and KY11563, were able to transform G-418, JI-20B or gentamicin C_2 to C_1. However, DOS, paromamine, gentamicin A, and X_2 were not transformed to gentamicin C_2 or C_1. The formation of sagamicin from the precursors could not be determined, because the amount of anti-

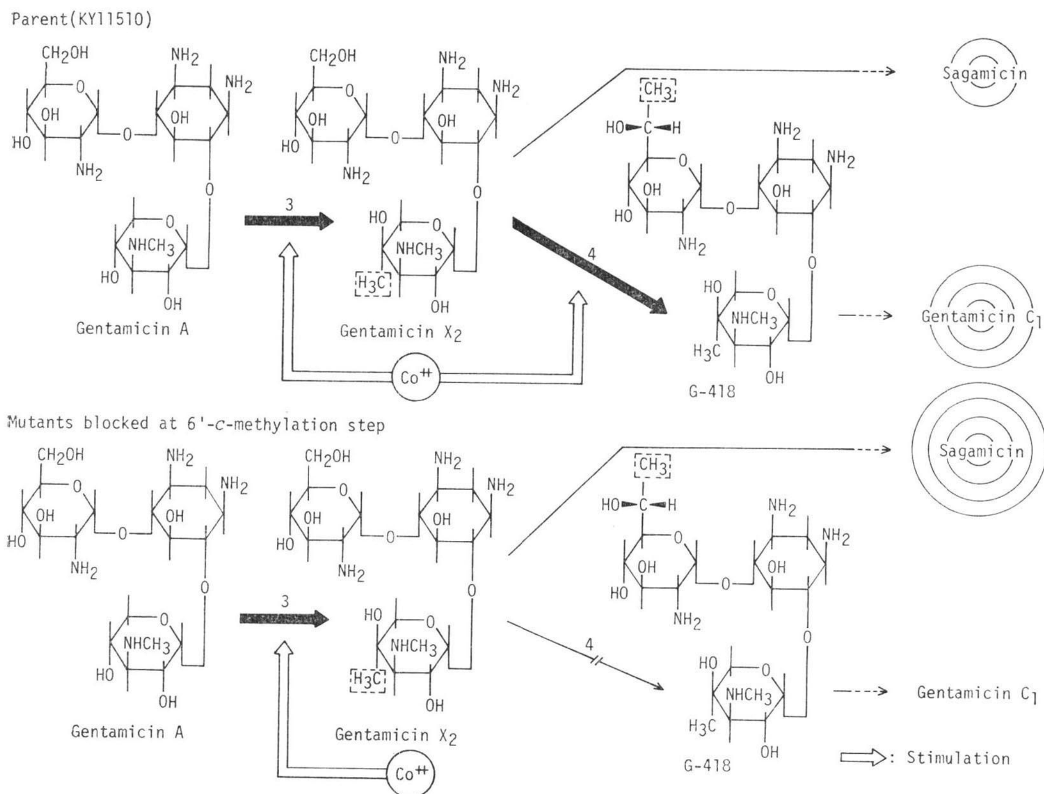
biotic originating from the washed cells of the mutants was too large to be distinguished from the antibiotic formed by transformation of precursor added. However, the result may indicate that the mutants possessed no activity to transform gentamicin X_2 to G-418. Thus, it is suggested that these mutants have been blocked at the 6′-*C*-methylation step in the biosynthesis of gentamicin C_1. The mutants KY11523 and KY11561, which produced a small amount of gentamicin C_1, may be leaky mutants with respect to the 6′-*C*-methylation step, because they possessed a small activity to transform gentamicin X_2 to G-418 (unpublished data).

The blocked mutants described above were prototrophic, and no changes were observed in the colonial morphology. Increase in sagamicin production in the mutants may be explained by overflow of gentamicin X_2, the common intermediate in both the sagamicin and gentamicin C_1

Table 1. Biotransformation of biosynthetic precursors to gentamicin C_1 with the resting cells of the blocked mutants and DOS idiotroph.

Substrate	Biotransformation activity to gentamicin C_1	
	KY11525 (DOS^-)	KY11538, KY11539, KY11562, KY11563
None	−	−
2-Deoxystreptamine	+	−
Paromamine	+	−
Gentamicin A	+	−
Gentamicin X_2	+	−
Antibiotic JI-20A	−	−
Gentamicin C_{1a}	−	−
Antibiotic G-418	+	+
Antibiotic JI-20B	+	+
Gentamicin C_2	+	+

Fig. 6. Effect of cobalt chloride on the production of sagamicin and gentamicin C_1.



pathways, into sagamicin, because of the blockage of the mutants at the 6′-*C*-methylation step. Moreover, the stimulation of the 4″-*C*-methylation activity by cobalt may lead to a further increment in sagamicin production in these mutants (Fig. 6).

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