GENETIC AND BIOCHEMICAL FEATURES OF SPIRAMYCIN BIOSYNTHESIS IN *STREPTOMYCES AMBOFACIENS*

—— CURING, PROTOPLAST REGENERATION AND PLASMID TRANSFER ——

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Spiramycin-producing *Streptomyces ambofaciens* KA-1028 harboring the pSA1 plasmid gave rise to spiramycin non-producing variants at high frequencies by various curing treatments. However, a number of the spiramycin non-producing progeny obtained by treatment with acridine dyes, still harbored plasmid DNAs which could not be differentiated from plasmid pSA1 by contour length, cleavage patterns and heteroduplex analysis. By treatment with mitomycin C, plasmid pSA1 was cured at high efficiency and spiramycin non-producing strains were obtained.

Strain U-1717R obtained by regeneration of protoplasts of plasmid-cured strain U-1717 regained spiramycin production on growth on solid medium only. Furthermore, transconjugants obtained by mating between strain KA-1028 and U-1717R-24 (streptomycin-resistant) regained spiramycin production in both liquid and solid media.

We conclude that the genes for the biosynthesis of spiramycin are encoded in a replicon other than plasmid pSA1 but that this plasmid plays a role in the regulation of spiramycin production.

OKANISHI *et al.*[1] have suggested that plasmids are involved in antibiotic production in actinomycetes, subsequently the relationship between plasmids and antibiotic production has been studied in various antibiotic-producing actinomycetes.

In the methylenomycin producer *Streptomyces coelicolor* A3 (2), the genes for methylenomycin biosynthesis are encoded by plasmid SCP1[2]. Plasmids have been shown to be involved in the regulation of the production of chloramphenicol[3,4] and aureothricin[5].

In previous papers, we reported that the spiramycin producer *S. ambofaciens* KA-1028 harbored a plasmid, pSA1, possessing a molecular weight of 53.1×10^6 [6], and that spiramycin non-producing strains could be isolated from the strain KA-1028 by treatment with acriflavine[7]. Since no plasmid DNAs could be detected in the cleared lysate fractions from spiramycin non-producing strains, we speculated that pSA1 DNA was involved in spiramycin production[7]. Thereafter, we found, however, that the spiramycin non-producing strains AF-11 and QN-25 obtained from strain KA-1028 by treatment with acridine dyes harbored a chromosomal and membrane-associated plasmid which could not be detected in the cleared lysate fractions[8]. The plasmid DNAs from the strains AF-11 and QN-25 are the same molecular weight as that of pSA1 DNA from the spiramycin producer KA-1028[8].

The present paper deals with treatment of the spiramycin producer with various curing agents, ultraviolet and high temperature, comparison of plasmid DNAs from spiramycin non-producing strains and pSA1 DNA, partial restoration of spiramycin production by protoplast regeneration of a spiramycin non-producing strain, and the transfer of pSA1 DNA by mating. The possible roles of the plasmid in spiramycin production are discussed.

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Materials and Methods

Organisms

The spiramycin producer *Streptomyces ambofaciens* KA-1028 (ISP-5053) and its mutant strains devoid of spiramycin production (shown in Table 2) were used. These organisms were maintained in ISP medium No. 2 (yeast extract–malt extract agar)[9].

Curing Experiments

The spiramycin-producing strain KA-1028 was incubated with aeration in Tryptic Soy Broth (TSB, BBL) supplemented with acriflavine (10 μg/ml), quinacrine (100 μg/ml) or mitomycin C (0.125 μg/ml), or grown in TSB at the highest temperature (40°C) permitting growth. After incubation for 3 days, the culture was treated for 2 minutes with an Ultra Turrax homogenizer (Ika-Werk Janke & Kunkel KG, West Germany) to cut the mycelia, diluted with sterile saline, and inoculated onto a plate containing ISP medium No. 2.

For ultraviolet treatment, after a mycelium suspension of strain KA-1028 (about 10^5 colony forming units) was spread on solid media (ISP medium No. 2), the plate was irradiated with ultraviolet rays (15W ultraviolet lamp) at a height of 45 cm for 30 seconds.

Spiramycin Production

Colonies obtained from curing treatment were inoculated onto GY agar medium containing 1% glucose, 1% dried yeasts, 0.5% NaCl, 0.1% $NaNO_3$, 1% $CaCO_3$, and 1.5% agar (adjusted to pH 7.5 with 2 N NaOH before autoclaving), and then incubated at 27°C for 3 days. Production of spiramycin by each colony was examined by the agar-piece method using *Micrococcus luteus* PCI 1001 as the test organism. Fermentation in a liquid medium was performed as follows: a culture grown in a liquid medium was transferred to GY liquid medium (agar was omitted from GY agar medium) and shake-cultured on a reciprocal shaker at 27°C for 3 days. The cultured broth was centrifuged at 2,000$\times g$ for 10 minutes and the supernatant fluid assayed for the presence of spiramycin by a paper disc method using *Micrococcus luteus* PCI 1001 as the test organism. Strain KA-1028 produced about 100 μg/ml of spiramycin under these conditions.

Formation of Soluble Pigment and Aerial Mycelium

The formation of soluble pigment was examined on ISP medium No. 3 (oatmeal agar)[9]. Aerial mycelium formation was observed on ISP medium No. 4 (inorganic salts - starch agar)[9] supplemented with 50 μg of arginine-HCl per ml.

Carbon Utilization

Utilization of several carbon sources was examined on ISP medium No. 9 (carbon utilization medium)[9] supplemented with appropriate carbon sources (1.0%) and 50 μg of arginine-HCl per ml.

Sensitivity to Antibiotics

Minimal inhibitory concentrations (MIC) of tetracycline and spiramycin against the spiramycin-producing and non-producing strains were determined by the conventional agar dilution method using Tryptic Soy Agar (TSA, BBL).

Assay for Spiramycin I-3-hydroxyl Acylase and TDP-Mycarose Synthetase

Mycelia were incubated in GY liquid medium for 65 hours at 27°C and disrupted with twice the weight of quartz sand. The debris was removed by centrifugation at 25,000$\times g$ for 30 minutes and the supernatant fluid was used as a crude enzyme preparation. The assay of spiramycin I-3-hydroxyl acylase was as described in the previous paper[10]. The reaction mixture (50 μl) contained 0.5 mM spiramycin I, 1.0 mM [1-^{14}C]acetyl-CoA (0.05 μCi), 50 mM tris-HCl (pH 8.5), 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, and 25 μl of the crude enzyme solution. TDP-mycarose synthetase was assayed as described by PAPE & BRILLINGER[11].

Bioconversion of Forocidin I to Spiramycin III

The bioconversion of forocidin I to spiramycin III was performed as described by KITAO *et al.*[12].

Preparation of Plasmid DNA

Plasmid DNA was extracted and partially purified as described by ŌMURA *et al.*[6]. The partially

purified plasmid DNA fraction was applied to a Sepharose 4B (Pharmacia) column to remove RNAs. The void volume fractions containing high molecular weight DNA were combined and concentrated by ethanol precipitation and the plasmid DNA purified by dye-buoyant centrifugation as described by IKEDA *et al.*[5].

Digestion of DNA with Restriction Endonucleases and Agarose Gel Electrophoresis

The digestion of plasmid DNA with restriction endonucleases and agarose gel electrophoresis was carried out as described previously[8]. Lambda *c*I 857 *Sam* 7 phage DNA was used as a standard molecule.

Cleavage of DNA Heteroduplexes with S1 Nuclease

^{3}H-Labeled plasmid DNA (0.06 μg) from strain AF-11 or QN-25 and pSA1 DNA (1.0 μg) were mixed and cleaved to linear duplexes by digestion with *Hin*d III. The DNAs were denatured in 0.1 N NaOH at room temperature for 20 minutes, and the solution neutralized to pH 7 with 1 N HCl. The NaCl concentration was then raised to 300 mM. The DNA was reannealed at 75°C for 24 hours. The reannealed DNA was treated with S1 nuclease as described by SHENK *et al.*[13]. S1 nuclease-generated DNA fragments were analyzed by neutral sucrose density gradient centrifugation as described previously[8]. *Hin*d III endonuclease-generated linear ^{14}C-pSA1 DNA and ^{32}P-λ *c*I 857 *Sam* 7 (linear DNA) were added to each sample as internal standards (45 *S* and 35 *S*, respectively). S1 nuclease was prepared by the procedure of VOGT[14].

DNA-DNA Membrane Filter Hybridization

The total DNA fraction prepared by the method of OKANISHI and GREGORY[15] was subjected to digestion with *Eco* R1 and extracted with phenol. The DNA was precipitated by addition of 2 volumes of ethanol to the aqueous layer and dissolved in 0.1×SSC (standard saline citrate: 0.15 M NaCl and 0.015 M sodium citrate) and the salt concentration was increased to 1×SSC. ^{3}H-Labeled pSA1 DNA was prepared by dye-buoyant centrifugation as described previously[8]. DNA fixation and reassociation on membrane filters was performed by the method of OKANISHI and GREGORY[15].

Preparation and Regeneration of Protoplasts

Mycelia were shake-cultured at 27°C for 48 hours in TSB medium. The culture was transferred at 2% inoculum size into fresh TSB medium supplemented with 0.4% glycine, and incubated with aeration at 32°C for 24~30 hours. Mycelia harvested from a 10-ml culture by centrifugation at 1,500×*g* for 7 minutes were washed with 0.4 M sucrose solution, and suspended in 5 ml of P3 medium (SHIRAHAMA *et al.*[16]) that *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) was substituted for *N*-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES). Egg-white lysozyme was added to a concentration of 1.0 mg per ml, followed by incubation at 28°C for 50 minutes. The lysozyme-treated suspension was centrifuged at 300×*g* for 5 minutes to remove intact mycelia, and the supernatant containing the protoplasts recentrifuged at 1,000×*g* for 10 minutes. After the sedimented protoplasts were gently suspended in 5 ml of a modified PWP[16] medium (HEPES was substituted for TES), the suspension was centrifuged at 1,000×*g* for 10 minutes and the sedimented protoplasts resuspended in the modified PWP medium. The protoplast suspension was diluted with modified PWP medium to a density 2~5×10^{8} protoplasts per ml, and 0.1 ml of the diluted suspension was placed on the surface of protoplast regeneration medium RM2 (basal layer 15 ml) containing 2.2% noble agar (Difco). Three milliliter of melted RM2 medium (kept at 37°C) containing 0.55% low gelling temperature agarose (Sigma Chemical Co.) was overlaid and the soft agar surface was dried by keeping the plate open for 1 hour at room temperature, and the plate was incubated at 26°C for 10 days. The regeneration medium RM2 consisted of 17% sucrose, 1.0% glucose, 0.5% Casamino Acids (low phosphate content[8]), 0.3% L-asparagine·H_2O, 0.1% $NaNO_3$, 0.4% trace elements solution[15], 0.81% $MgCl_2 \cdot 6H_2O$, 0.22% $CaCl_2 \cdot 2H_2O$, 0.005% K_2HPO_4 and 25 mM HEPES buffer (pH 7.2).

Mating

Streptomycin (5 μg/ml)-resistant strains obtained by treatment of a plasmid-cured strain with ultraviolet rays were used as recipients.

Donor and recipient strains were incubated with aeration in TSB medium at 27°C for 2 days, the

mycelia were cut with an Ultra Turrax homogenizer and passed through cotton wool to remove the aggregated mycelia. About 10^5 colony-forming units from donor and recipient strains were mixed and incubated on a slant of ISP medium No. 2 at 27°C for 14 days. The spores and mycelia in the mixed culture were scraped off, suspended in 10 ml of sterile saline, and homogenized with an Ultra Turrax homogenizer. The homogenized suspension was placed on a TSA plate supplemented with 5 μg of streptomycin per ml and incubated at 27°C for 7 days. The plasmid-containing strains were screened by electrophoretic analysis of partially purified ccc DNA fractions obtained by alkaline denaturation method[6].

Chemicals

Mitomycin C and spiramycin were gifts from Kyowa Hakko Kogyo Co., Ltd., Tokyo. Acriflavine, quinacrine, ethidium bromide and CsCl were purchased from Nakarai Chemicals Ltd., Kyoto. Restriction endonucleases *Eco* R1, *Hind* III and *Bam* H1 were obtained from Takara Shuzo Co. Ltd., Kyoto, [methyl-^{3}H]thymidine (41 Ci/mmole), [2-^{14}C]thymidine (42 mCi/mmole), [1-^{14}C]acetyl-CoA (52 mCi/mmole) were from New England Nuclear and ^{32}P-orthophosphate (carrier-free) from Japan Atomic Energy Research Institute.

Results

Curing Treatments

To examine the relationship between the presence of plasmid pSA1 and spiramycin production in *S. ambofaciens* KA-1028, we treated the organism with a variety of curing agents, ultraviolet rays and high temperature. The frequencies of appearance of spiramycin non-producing strains and plasmid-deficient strains from strain KA-1028 by these treatments are summarized in Table 1. No correlation was seen between the occurrence of spiramycin non-producing progeny and plasmid-deficient progeny from the spiramycin-producing strain KA-1028 harboring plasmid pSA1.

Table 1. Frequencies of occurrence of spiramycin non-producing progeny and plasmid-deficient progeny by various curing treatments of *Streptomyces ambofaciens*.

Treatment	Frequency of spp$^-$ progeny	Frequency of plasmid-deficient progeny
Acriflavine (10 μg/ml)	10.1% (43/ 426)	0% (0/200)
Quinacrine (100 μg/ml)	40.3% (423/1050)	0% (0/200)
Ultraviolet (survival 1%)	1.0% (19/2000)	0.5% (1/200)
Mitomycin C (0.125 μg/ml)	100% (313/ 313)	8.0% (16/200)
High temperature (40°C)	0% (0/ 251)	N. D.*

* Not determined.

Table 2. Properties of *S. ambofaciens* KA-1028 and its variant strains devoid of spiramycin productivity.

Strain	Treatment	Spiramycin production	pSA1 plasmid (copy number)	Pigment formation	Aerial mycelium formation	Amino acid requirement for growth	Rhamnose utilization	MIC (μg/ml)	
								SPM	TC
KA-1028	—	+	+(2)	+	+	—	+	>1000	6.25
AF-11	Acriflavine	—	+(1)	—	—	Arg	—	>1000	6.25
QN-25	Quinacrine	—	+(1)	—	—	Arg	—	>1000	6.25
U-1030	Ultraviolet	—	—	+	+	Arg	—	>1000	6.25
U-1717	Ultraviolet	—	—	—	—	Arg	—	>1000	6.25
MMC-2	Mitomycin C	—	—	—	+	—	+	>1000	6.25
MMC-8	Mitomycin C	—	—	+	+	—	+	>1000	6.25

* MIC, minimal inhibitory concentration; SPM, spiramycin; TC, tetracycline.

Properties of Spiramycin Non-producing Strains Obtained by Various Curing Treatments

Tables 2 and 3 show the properties of the spiramycin non-producing strains. Spiramycin non-producing strains AF-11 and QN-25 which were obtained from the strain KA-1028 by treatments with acriflavine and quinacrine, respectively, still harbored plasmid DNA of about 53×10^6 daltons[8], but other strains U-1030, U-1717, MMC-2 and MMC-8 harbored no plasmid (closed circular form).

Table 3. Activities of enzymes involved in spiramycin production in *S. ambofaciens* KA-1028 and its non-producing variant strains.

Strain	3-OH acylase (mU/mg)	TDP-mycarose synthetase (mU/mg)	Bioconversion of forocidin I to spiramycin III
KA-1028 (spp$^+$)	0.86	0.16	+
AF-11 (spp$^-$)	0.75	0.13	+
QN-25 (spp$^-$)	0.78	0.14	+
U-1030 (spp$^-$)	0.68	0.13	+
U-1717 (spp$^-$)	0.70	0.12	+
MMC-2 (spp$^-$)	0.65	0.04	±
MMC-8 (spp$^-$)	0.69	0.05	±

The activities of spiramycin I-3-hydroxyl acylase in spiramycin non-producing strains were similar to that in strain KA-1028 as shown in Table 3. The activities of TDP-mycarose synthetase in strains MMC-2 and MMC-8 were one-third to one-fourth of that in strain KA-1028. The bioconversion of forocidin I (an intermediate in spiramycin biosynthesis) to spiramycin III in strains AF-11, QN-25, U-1030, and U-1717 also were almost the same as that in strain KA-1028, although in strains MMC-2 and MMC-8 the activity was decreased: this may be reflected by the decrease of TDP-mycarose synthetase.

Identity of Plasmid DNAs from Strains AF-11 and QN-25 with pSA1 DNA from Strain KA-1028

We have reported that the sizes of the plasmid DNAs from spiramycin non-producing strains AF-11 and QN-25 agreed with that of pSA1 DNA (53.1×10^6 daltons) when the mobilities were determined in agarose gel electrophoresis[8].

The contour lengths of plasmid DNAs from strains AF-11 and QN-25 by electron microscopy were equal to that of pSA1 (data not shown). As shown in Fig. 1, digestion of the plasmid DNAs with the restriction endonucleases *Bam* H1, *Eco* R1 and *Hin*d III produced twenty-eight, three and one fragments, respectively. The cleavage patterns were identical with that of pSA1 DNA.

Heteroduplexes between ^{3}H-labeled plasmid DNA from strain AF-11 or QN-25 and unlabelled pSA1 DNA prepared by reassociation of the linear plasmid DNAs after cleaved by *Hin*d III, digested with S1 nuclease and analyzed by sucrose density gradient centrifugation. The S1 nuclease-treated heteroduplex DNAs were shown to be homogeneous and to be identical with *Hin*d III endonuclease-generated linear duplex pSA1 DNA (^{14}C-labeled) (data not shown).

DNA-DNA Hybridization of pSA1 with Total DNAs from Variant Strains

To determine whether the plasmid DNA molecule is integrated into chromosomes of the "cured" strains, DNA-DNA hybridization was carried out between ^{3}H-labeled pSA1 DNA and the total DNAs of strains AF-11, QN-25, U-1030, U-1717, MMC-2 and MMC-8. No hybridization was detected between U-1030, U-1717, MMC-2 and MMC-8 and ^{3}H-pSA1 DNA (Fig. 2). On the other hand, DNAs from strains KA-1028, AF-11 and QN-25 hybridized with ^{3}H-pSA1 DNA; the rates of the hybridization of the total DNAs of strains AF-11 and QN-25 with ^{3}H-pSA1 DNA were about 50% that of the total DNA of strain KA-1028 with ^{3}H-pSA1 DNA.

Fig. 1. Agarose gel electrophoretic analysis of purified plasmid DNAs from strains KA-1028 (1, 4 and 7), AF-11 (2, 5 and 8) and QN-25 (3, 6 and 9) digested with *Bam* H1 (1, 2 and 3), *Eco* R1 (4, 5 and 6) and *Hin*d III (7, 8 and 9).

A and B are λ cI 857 *Sam* 7 DNA digested with *Eco* R1 and *Hin*d III, respectively.

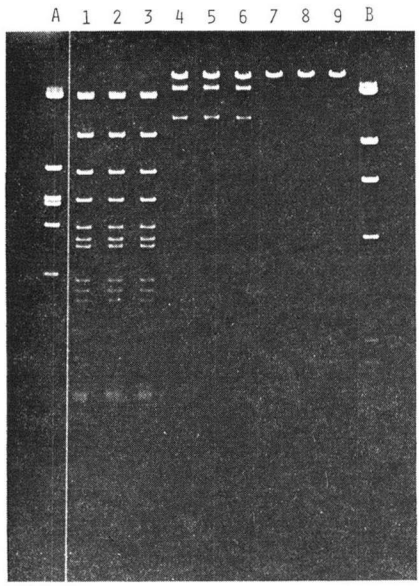

Fig. 2. DNA-DNA hybridization profiles between ^{3}H-pSA1 DNA and total DNAs from the parent and its variant strains.

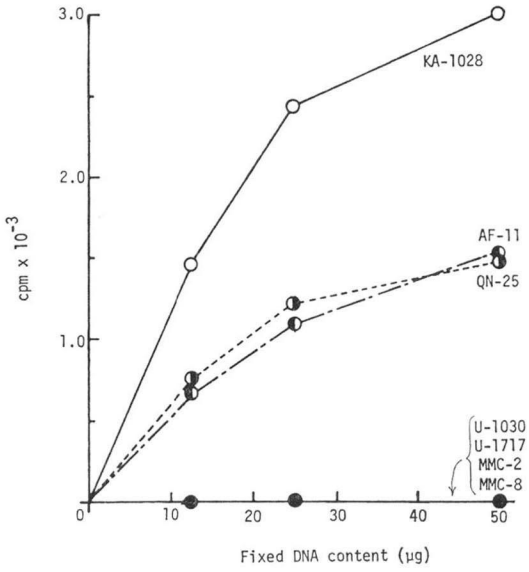

Table 4. Frequency of restoration of spiramycin production by regeneration of protoplasts from spiramycin non-producing strains.

Strain	spp^{+} Progeny after protoplast regeneration
AF-11 (spp^{-}, pSA1^{+})	0 / 386
U-1030 (spp^{-}, pSA1^{-})	0 / 500
U-1717 (spp^{-}, pSA1^{-})	522 / 523
MMC-2 (spp^{-}, pSA1^{-})	0 / 325
MMC-8 (spp^{-}, pSA1^{-})	0 / 387

Table 5. The comparison of strain U-1717R obtained by regeneration of protoplasts with its parent strains.

	KA-1028 (original)	U-1717	U-1717R
ccc DNA (pSA1)	+	−	−
Spiramycin productivity			
Solid medium	+	−	+
Liquid medium	+	−	−
Aerial mycelium formation	+	−	+
Arg requirement	−	+	+
Rhamnose utilization	+	−	−

These results are consistent with the finding that strain KA-1028 harbors two copies of pSA1 DNA per chromosome, while strains AF-11 and QN-25 one copy[8].

Partial Restoration of Spiramycin Productivity by Protoplast Regeneration

The properties of the progeny of protoplast regeneration of "cured" spiramycin non-producing strains U-1030, U-1717, MMC-2 and MMC-8 were examined. Protoplasts were regenerated on regeneration medium RM 2 at a frequency of about 30%. U-1030, MMC-2 and MMC-8 did not change in morphology and spiramycin production after regeneration. On the other hand, more than 99% of the regenerated progeny of the non-producing strain U-1717 produced spiramycin (Table 4). Spiramycin production similar to that of strain KA-1028 was observed only when strain U-1717R, obtained from U-1717, was incubated on solid medium (Table 5). All of the strains in which spiramycin pro-

duction was partially restored formed aerial mycelium on inorganic salts - starch agar.

Since U-1717R does not harbor pSA1 DNA, the fact that it produced spiramycin in a solid medium (identified by mass spectroscopy) indicates that the genes coding enzymes involved in spiramycin biosynthesis are contained not in pSA1 DNA but in an other replicon.

Plasmid Transfer by Mating

In order to examine the function of plasmid pSA1, we attempted the transfer of the plasmid into plasmid-cured strain by mating. Streptomycin-resistant strains U-1717R-24 and MMC-2-7 obtained from plasmid-cured strains U-1717R and MMC-2 were used as recipients. The plasmid containing strains were screened among streptomycin-resistant colonies after mating.

In crosses of strains KA-1028 × U-1717R-24, QN-25 × U-1717R-24, and KA-1028 × MMC-2-7, pSA1 DNA was transferred at a frequency of $1 \sim 5 \times 10^{-3}$ and transconjugants derived from KA-1028 × U-1717R-24 produced spiramycin in liquid medium (Table 6). The mobilities of plasmid DNAs from the three transconjugants were identical with that of pSA1 DNA as shown

Table 6. Frequency of plasmid pSA1 transfer.

Components of mating		Frequency of plasmid transfer	spp$^+$ Progeny
Donor	Recipient		
KA-1028 (pSA1) × U-1717R-24		3/621	*3/621
QN-25 (pSA1) × U-1717R-24		1/752	**N.D.
KA-1028 (pSA1) × MMC-2-7		2/996	***0/996

* liquid medium, ** not determined, ***solid medium.

Fig. 3. Agarose gel electrophoresis of (A) partially purified plasmid DNA fractions from strains KA-1028 (1) and QN-25 (5) and transconjugants TC-171 (2), TC-569 (3) and TC-748 (4) and of (B) their purified DNAs digested with restriction endonuclease *Bam* H1.

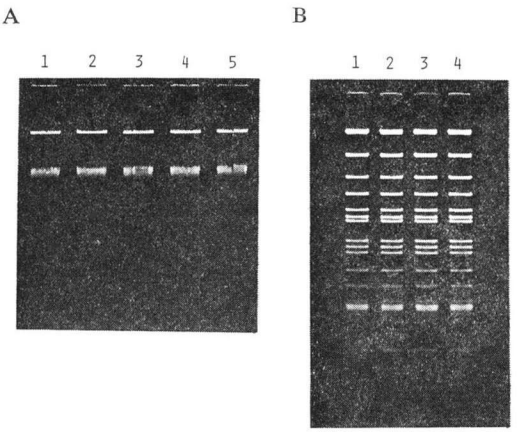

Table 7. Properties of pSA1-harboring strains obtained by mating between pSA1-harboring strain KA-1028 or QN-25 (as donor) and pSA1 non-harboring strain U-1717R-24 or MMC-2-7 (as recipient).

	Donor		Recipient		Transconjugant		
	KA-1028 (original)	QN-25	U-1717R-24	MMC-2-7	TC-171	TC-748	TC-569
Parent	—	KA-1028	U-1717R	MMC-2	—	—	—
Donor	—	—	—	—	KA-1028	QN-25	KA-1028
Recipient	—	—	—	—	U-1717R-24	U-1717R-24	MMC-2-7
Treatment	—	Quinacrine	Ultra-violet	Ultra-violet	mating	mating	mating
ccc DNA (pSA1)	+	+	−	−	+	+	+
Spiramycin productivity							
Solid medium	+	−	+	−	+	+	−
Liquid medium	+	−	−	−	+	+	−
Aerial mycelium formation	+	−	+	+	+	+	+
Arg requirement	−	+	+	−	+	+	−
Streptomycin resistance	−	−	+	+	+	+	+

in Fig. 3A. The plasmid DNAs from the three transconjugants were further purified by dye-buoyant centrifugation and were digested with restriction endonucleases. The cleavage patterns of *Bam* H1-digested plasmid DNAs from the transconjugants were good agreement with that of pSA1 DNA as shown in Fig. 3B. The transconjugants were examined for spiramycin production in solid and liquid media. The transconjugants derived from crosses KA-1028×U-1717R-24 and QN-25×U-1717R-24 produced spiramycin in both solid and liquid media (Table 7). However, the transconjugant derived from the cross KA-1028×MMC-2-7 produced no spiramycin (Table 7).

Discussion

In a number of actinomycetes, loss of antibiotic productivity has been observed to be associated with the "curing" of plasmids[1,5,17~19]. Spiramycin non-producing progeny could be obtained from the spiramycin-producing strain *S. ambofaciens* KA-1028 which harbored pSA1 plasmid by the treatment with acridine dyes. The spiramycin non-producing progeny thus obtained were found to harbor the pSA1 plasmid. NOJIRI *et al.*[20] has observed that the plasmid DNA (52×10^6 daltons) of the ribostamycin-producing *S. ribosidificus* ATCC 21294 was not eliminated by treatment with acriflavine although antibiotic production was lost at high frequency. And we have found that plasmid pSA1 of *S. ambofaciens* KA-1028 could be easily eliminated by treatment with mitomycin C. It has been reported that the plasmid DNAs in *Pseudomonas* are eliminated inefficiently with acridine dyes but eliminated a treatment with mitomycin C[21,22].

It has been reported that "curing" often causes changes in several phenotypic characteristics accompanied with the loss of antibiotic productivity (pleiotropic effects)[1,5,17,23,24]. In *S. ambofaciens*, some spiramycin non-producing strains obtained after curing possessed arginine requirements and deficiencies in aerial mycelium formation, soluble pigment production and rhamnose utilization. However, these changes were not always accompanied with the loss of spiramycin production and with loss of plasmid pSA1. Since both *S. ambofaciens* KA-1028 and the spiramycin non-producing strains (U-1030, U-1717, MMC-2 and MMC-8) which did not harbor pSA1 plasmid were shown to be spiramycin-resistant, we conclude that the gene (s) for spiramycin resistance is not encoded by plasmid pSA1.

Comparison of plasmid DNAs from spiramycin producing and non-producing strains with respect to size, endonuclease cleavage and heteroduplex formation indicates that plasmid DNA from non-producing strains AF-11 and QN-25 were identical with pSA1 DNA. However we cannot exclude the possibility that plasmid DNAs from strains AF-11 and QN-25 were generated from pSA1 DNA by point mutation, or a small deletion or insertion.

Regeneration of protoplasts of plasmid-cured spiramycin non-producing strains U-1030, U-1717, MMC-2 and MMC-8 partially restored spiramycin production in strain U-1717 alone. Strain U-1717R regenerated from strain U-1717 produced spiramycin on a solid medium. The restoration of antibiotic production after regeneration from protoplasts has also been reported by OKANISHI[25]. After transfer of plasmid pSA1 by mating, strain U-1717R produced an amount of spiramycin similar to that produced by the original strain KA-1028 in both solid and liquid media. On the other hand, the progeny regenerated from protoplasts of plasmid-cured strains other than U-1717 did not produce spiramycin even after the transfer of pSA1 DNA by mating.

From the above results, we conclude the following: (1) Since strain U-1717R does not harbor pSA1 DNA but produced spiramycin on a solid medium, the structural genes of enzymes for spiramycin biosynthesis must be encoded on a replicon other than plasmid pSA1. (2) Since the infectious transfer of spiramycin production to a spiramycin non-producing strain was not oberved (Table 6, KA-1028×MMC-2-7), the genes for spiramycin biosynthesis must be encoded by the chromosome. (3) Plasmid pSA1 is necessary for production of large amounts of spiramycin in liquid medium. We suggest that the plasmid pSA1 plays some role(s) in the regulation of spiramycin production. The true function of the plasmid and mechanism of the regulation remain to be clarified.

It is of interest that the spiramycin producer *S. ambofaciens* KA-1028 gives rise to spiramycin non-

producing variants at high frequencies following various "curing" treatments even though the genes for spiramycin production are not encoded on a plasmid. The frequent occurrence of such phenotypic variations cannot be explained by mutation. FREEMAN *et al.*[26] reported that chloramphenicol resistant *S. coelicolor* A3 (2), which lacked chloramphenicol acetyltransferase, produced spontaneous and UV-induced chloramphenicol sensitive variants at high frequencies, and suggested that a transposable element[27,28] might be involved. The ribostamycin producer *S. ribosidificus* ATCC 21294 gave rise to ribostamycin non-producing strains at high frequencies on curing but the endogenous plasmid was not cured[20].

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