GENETICS AND BIOCHEMICAL STUDIES OF CHLORAMPHENICOL-NONPRODUCING MUTANTS OF *STREPTOMYCES VENEZUELAE* CARRYING PLASMID

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Chloramphenicol-nonproducing and plasmid-less mutants obtained previously by treatment with acriflavine still produced a small amount of chloramphenicol in a medium. To study the role of plasmid in chloramphenicol production, 70 chloramphenicol-nonproducing mutants were isolated by acriflavine treatment, high-temperature incubation, UV-irradiation or nitrosoguanidine treatment, starting from a producer (SVM2). Most of them did not produce any amount of chloramphenicol. One mutant, SVM2-2A7 was found to produce 1-deoxychloramphenicol instead of chloramphenicol. The mutations (*cpp*) affecting chloramphenicol production were analyzed by crosses with a producing strain carrying the complementing auxotrophic markers. Except for the plasmid-less strains, all Cpp mutations including the 1-deoxychloramphenicol-producing mutation were mapped between *met* and *ilv* on the chromosome. Additional crosses indicated that these chromosomal *cpp* mutants still carried the plasmids which had a role in increasing chloramphenicol production. Therefore, it can be concluded that the structural genes for all or most steps of chloramphenicol biosynthesis including the 3-hydroxylation of *p*-aminophenylalanine are located between *met* and *ilv* on the chromosome of *S. venezuelae* and that the plasmid plays an important role in increasing the chloramphenicol production. The activity of arylamine synthetase involved in the initial step of the chloramphenicol biosynthesis was unrelated to the presence or absence of plasmid. Moreover, the presence of plasmids was not required for host resistance to chloramphenicol.

In 1970, we reported a preliminary study suggesting a possibility of plasmid involvement in the antibiotic production[1]. Subsequently, this has been confirmed by many investigators. Plasmid involvement has been reported in the production of antimycin A[2], aureothricin[3], cephamycin[4], chloramphenicol[3,5], holomycin[6], kanamycin[7,8], leucomycin[9], methylenomycin A[10,11], neomycin[12,13], oxytetracycline[14], paromomycin[15], puromycin[16], streptomycin[17] and turimycin[18]. The genes for the biosynthesis of methylenomycin A in *Streptomyces coelicolor* A3(2) were carried by plasmid SCP-1[10,11]. The genes for a multienzyme complex involved in the biosynthesis of leupeptin (a protease inhibitor produced by many streptomycetes) were found to be transferred from a producing mutant to a nonproducing mutant[19]. It is also possible that plasmids are involved in the regulation of antibiotic production, or in resistance to antibiotics but not directly in biosynthetic steps to secondary metabolites.

The production of chloramphenicol in *Streptomyces venezuelae* ISP 5230 is lost with high frequency by treatment with acriflavine[3,5]. Genetic evidence for plasmid involvement in chloramphenicol production was confirmed as follows: When an auxotrophically-marked, nonproducing mutant SVM3 was crossed with a chloramphenicol-producing mutant, SVM1, with different auxo-

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trophic markers, the chloramphenicol-nonproducing mutation did not show linkage with a set of chromosomal markers[3,5]. However, it was found that this and other most plasmid-less and chloramphenicol-nonproducing mutants still produced a very small amounts of chloramphenicol. Mutants which had completely lost the ability to produce chloramphenicol could be obtained by UV irradiation or NTG treatment. In this paper, further genetic and biochemical studies on chloramphenicol biosynthesis are reported.

Materials and Methods

Organisms and media

Mutant strains derived from *Streptomyces venezuelae* ISP 5230 used in the experiments are shown in Table 1. *Escherichia coli* NIHJ, *E. coli* K12 and *E. coli* K12 ML4079 (carrying R factor expressing resistance to chloramphenicol) were used as the test organisms for antibiotic activity. OPY medium for the maintenance of streptomycete strains, minimal medium (MM) as basal medium, and SP medium for production of chloramphenicol were prepared as described previously[5]. The following synthetic medium, GNa, was also used for chloramphenicol production: 30 ml of glycerin, 2.0 g $NaNO_3$, 8.0 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 1.4 g KH_2PO_4, 1.98 g K_2HPO_4, 0.2 g amino acids required, 0.04 g adenine hydrochloride, 20 g agar, 2.0 ml of trace element solution[20] in 1 liter of deionized water (KH_2PO_4 and K_2HPO_4 were dissolved and autoclaved separately and then added to media). The GPY medium used for measuring resistance to chloramphenicol consisted of 10 g glucose, 4.0 g peptone, 4.0 g yeast extract (Difco), 4.0 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g K_2HPO_4, 18 g agar in 1 liter of deionized water.

Isolation of chloramphenicol-nonproducing strains

The chloramphenicol-producing strain (SVM2) was treated with acriflavine, incubation at high temperature, UV irradiation, or N-methyl-N′-nitro-N-nitrosoguanidine (NTG). The procedures used here are virtually the same as those reported previously[1,5,21], except that mycelia were cut by means of a Polytron (Kinematica Gnbh, Switzerland) instead of by sonic oscillation. Antibiotic production was examined by the agar-piece or cylinder plate method using *E. coli* NIHJ as the test organism. The antibiotic produced was identified by thin-layer chromatography, using *E. coli* K12 or *E. coli* K12 ML4079 as test organisms.

Genetic cross and mapping procedures

These procedures were carried out as described in previous papers[3,5].

Extraction and identification of chloramphenicol and *p*-aminophenylalanine

Seed cultures prepared by shaking in medium SP were transferred into 500 ml flasks containing 100 ml of medium GNa and shake-cultured at 27°C for 7 days. The mycelium was harvested and washed with 0.5% NaCl contain-

Table 1. Mutants of *Streptomyces venezuelae* ISP 5230.

Strain No.	Genotype*	Remarks
SVM1	*lys ilv str pro met*	Derived from ISP 5230
SVM4	*lys ilv str pro met cpp*P	Derived from SVM1
SVM2	*his leu ade*	Derived from ISP 5230
SVM3	*his leu ade cpp*P	Derived from SVM2
SVM2-HT8	*his leu ade cpp*P	Derived from SVM2
SVM2-1A1	*his leu ade cpp*	Derived from SVM2
SVM2-HT3	*his leu ade cpp*A	Derived from SVM2
SVM2-2A7	*his leu ade cpp*C	Derived from SVM2
SVM2-U26	*his leu ade cpp*B	Derived from SVM2
SVM2-N7	*his leu ade cpp*D	Derived from SVM2
HT3R1	*his lys met str cpp*A	Derived from SVM2

* *Lys, ilv, pro, met, his, leu* and *ade* indicate nutritional requirements for lysine, isoleucine-valine, proline, methionine, leucine and adenine, respectively; *str* indicates resistance to 400 μg/ml of streptomycin; *cpp* indicates mutation of chloramphenicol-producing ability.

ing 0.05% $MgSO_4 \cdot 7H_2O$ and the amount of mycelia was measured by dry weight or optical density at 660 nm. Chloramphenicol and related substances in fermentation filtrates were extracted twice with ethylacetate. The extract was evaporated, and the dried residue dissolved in a small volume of ethylacetate and washed with water. A sample was analyzed by thin-layer chromatography (TLC) and tested for antibiotic activity. TLC was carried out on pre-coated TLC plates of silica gel (Kodak No. 6060) using solvent systems Nos. 4, 5 and 6: Solvent system No. 4: chloroform - methanol (93: 7 or 87.5: 12.5); solvent system No. 5: *n*-butanol - chloroform - acetic acid (10: 90: 0.5); solvent system No. 6: ethyl acetate saturated with water. The chromatogram was sprayed with a solution of 3 ml of 15% stannous chloride, 15 ml of conc. HCl and 180 ml of water. After drying, it was analyzed by spraying with 1 g *p*-dimethylaminobenzaldehyde dissolved in a mixture of 30 ml ethanol, 30 ml of conc. HCl and 180 ml of *n*-butanol[22].

In order to extract *p*-aminophenylalanine and related substances, 20~30 g of the wet mycelium was suspended in 70% ethanol (5 ml/g wet mycelium) and kept overnight at room temperature. The extract was evaporated to dryness at 40°C, and *p*-aminophenylalanine was isolated by using alginic acid-column chromatography according to JONES and WESTLAKE[22]. The *p*-aminophenylalanine fraction was neutralized with 1 N NaOH and passed through a Dowex 50 W-X4 column (1.5×15 cm, 200~400 mesh, pyrimidium form) with 0.1 N pyrimidine. The *p*-aminophenylalanine fraction was evaporated to dryness at 40°C and dissolved in a drop of water for TLC analysis. The TLC was carried out on precoated silica plates (Kodak No. 6060) using solvent system Nos. 1, 2 and 3. The solvent systems were No. 1: *i*-propanol - *t*-butanol - *n*-butanol - ammonia - water (4: 4: 4: 2: 4); No. 2: *n*-butanol - ethanol - 4 N ammonium hydroxide (6: 2: 3); and No. 3: *n*-butanol - acetic acid - water (12: 3: 5). The chromatograms were sprayed with the solutions as described by JONES and WESTLAKE[22].

Arylamine synthetase activity

Cells cultured in GNa medium were harvested during 2 to 6 days of incubation. The extraction and assay of arylamine synthetase were carried out by the methods described by JONES and WESTLAKE[22]. The protein content was measured by the method of LOWRY *et al.*[23] with bovine serum albumin as reference.

Resistance to chloramphenicol

Cultures incubated in GPY medium with or without chloramphenicol (20 μg/ml) for 2 days were used as inoculum to measure resistance to chloramphenicol, by the usual agar dilution method.

Chemicals

Chorismic acid, *p*-amino-DL-phenylalanine, and nicotinamide adenine dinucleotide were purchased from Sigma. An authentic sample of chloramphenicol was obtained from Sankyo Co. Ltd. Corynecin I, II and III were gifts from Dr. TOMITA of Kyowa Hakko Kogyo Co., Ltd.

Results

Mutations Affecting the Capacity to Produce Chloramphenicol

The auxotrophic strains SVM1 and SVM2 produced 20 μg/ml of chloramphenicol in the medium SP and 100 μg/ml in the synthetic medium GNa. The strains, SVM3, SVM4, and SVM2-T8 obtained by acriflavine treatment or by incubation at high temperature were indicated to be any one of plasmid loss, plasmid mutation or integration in the chromosome with subsequent inactivation. SVM3 and SVM4 were described in a previous paper[3,5] as chloramphenicol-nonproducing mutants. Subsequently these strains and also the strain SVM2-T8 were found to produce a very small amount of chloramphenicol (about 1% amount of the strain SVM1 or SVM2) in medium SP. Moreover they produced about 10% amount of the producing strains (Table 2) in the synthetic medium GNa. The antibiotic produced by these mutants were identified as chloramphenicol by TLC (Fig. 1). These cured strains still might be contaminated with cells containing plasmids, and accordingly we prepared

Table 2. Genetic characterization of Cpp mutants in *Streptomyces venezuelae*.

	Typical mutant obtained	Original strain	Mutagen used	Medium for selection of *cpp*	Freq. (%)	CAP produced (μg/ml) SP	GNa	*cpp* locus mapped	*cpp*$^+$ strains among recombinants with SVM4 HT3R1**** (or 3)***	
		SVM1*				20~25	100~120			
		SVM2*				20~25	100~120			
I	SVM3	SVM2	AF	SP	3~9	0.2	5~10	plasmid loss	0/601	
	SVM4	SVM1	AF	SP	3~9	0.2	5~10	plasmid loss	0/601	
	SVM2-HT8	SVM2	HT	SP	1.5~2.9	0.2	5~10	plasmid loss	0/108	
II	SVM2-1A1	SVM2	AF	GNa	0.1~0.2	0	0.5	*met-ilv*	NT	
	SVM2-HT3	SVM2	HT	GNa	0.1~0.3	0	0.5	*met-ilv*	3/42	0/302
III	SVM2-2A7	SVM2	AF	SP		0**	0**	*met-ilv*	33/422	0/231
IV	SVM2-U26	SVM2	UV	SP	0.2~0.3	0	0	*met-ilv*	55/216	0/74
	SVM2-N7	SVM2	NTG	SP	0.2~0.5	0	0	*met-ilv*	5/80	108/400

* SVM1: *lys, ilv, pro, met, str*^{r}. SVM2: *his, leu, ade.*

** Chloramphenicol was not produced but 1-deoxychloramphenicol was produced.

*** Recombinants were selected with *his*$^+$/*lys*$^+$ or *leu*$^+$/*pro*$^+$.

**** Recombinants were selected with *ade*$^+$/*lys*$^+$. Strain HT3R1 derived from the cross between SVM1×SVM2-HT3 has the marker of *his, lys, met, str*^{r}, and *cpp*.

Abbreviation: AF; acriflavine, HT; high temperature, UV; ultraviolet irradiation, NTG; N-methyl-N′-nitro-N-nitrosoguanidine, NT; not tested.

Fig. 1. Thin-layer chromatograms of the samples extracted with ethylacetate from the cultured broth of plasmid-less strain (SVM3) and 1-deoxychloramphenicol-producing strain (SVM2-2A7).

The chromatograms developed were sprayed with an acidic stannous chloride solution to reduce the nitro compounds to amines. After drying, the plates were sprayed with a solution containing *p*-dimethylaminobenzaldehyde.

Abbreviations CAP: Chloramphenicol, Deoxy-CAP: 1-Deoxychloramphenicol, Samp: Extract from plasmid-less strain SVM3, Cory I, II, III: Corynecine I, II, and III, respectively, F: Front of solvent.

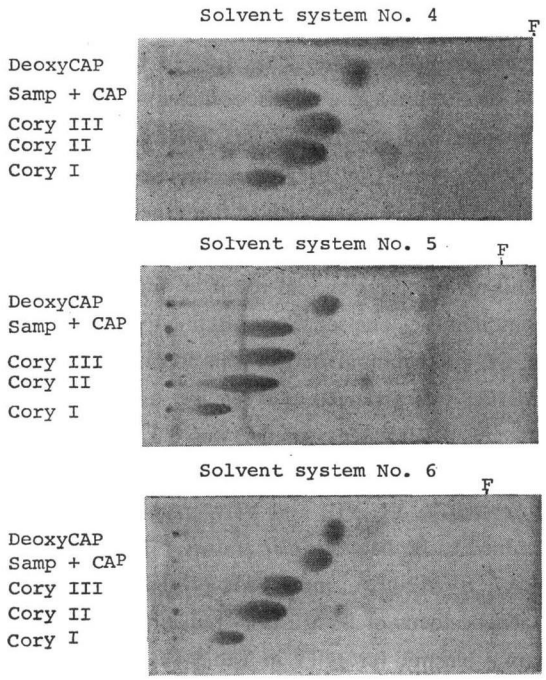
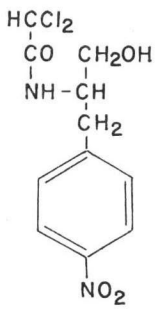


Fig. 2. Structure of 1-deoxychloramphenicol.



other chloramphenicol-nonproducing strains from the original producing strain SVM2 by acriflavine treatment, high-temperature incubation, UV irradiation or NTG treatment. The chloramphenicol-nonproducing mutants (Cpp) thus obtained were classified into 4 groups on the basis of ability to produce chloramphenicol or related products (Table 2). The plasmid-less mutants obtained by acriflavine treatment or high temperature incubation belong to group I. They all produced a very small amount of chloramphenicol. The Cpp mutants of group II also produced a very small amount (0.5 μg/ml) of chloramphenicol in GNa medium. Among Cpp mutants selected on the natural medium SP, a novel mutant SVM2-2A7 which did not produce any chloramphenicol but produced 1-deoxychloramphenicol (Fig. 2) was obtained by acriflavine treatment. It was classified in group III. Cpp mutants which did not produce any chloramphenicol were obtained with a frequency of 0.2~0.5% after treatment with ultraviolet irradiation or nitrosoguanidine and were classified in group IV.

Identification of 1-Deoxychloramphenicol

A mutant SVM2-2A7 of group III produced a weak antibiotic activity which showed Rf values different from those of chloramphenicol in TLC using solvent systems Nos. 4, 5 and 6. The antibiotic was also distinguished from corynecin I, II and III by TLC (Fig. 1). This antibiotic was isolated and identified as 1-deoxychloramphenicol (Fig. 2) by mass spectrometry, and proton and C^{13} magnetic resonance spectrometry.

^{14}C-Chloramphenicol added to growing cultures or cell-free systems of mutant SVM2-2A7 was not transformed to 1-deoxychloramphenicol.

Genetic Mapping Analysis of Cpp Mutations

The *cpp* loci in the non or minute producers (SVM2-HT8, SVM2-HT3, SVM2-2A7, SVM2-U26 and SVM2-N7) were analyzed by mapping. Each mutant (*his, ade, leu*, and *cpp*) was crossed with SVM1 (*lys, met, ilv, pro*, and *str* and *cpp*$^+$). Recombinants were selected by the use of different pairs of markers, except for *cpp* alleles. The recombinants were purified and scored for unselected markers. Their marker sequences were arranged to make a circular linkage map according to the methods described in previous papers[3,4]. A typical example of the linkage analysis obtained is shown in Table 3. Strain SVM1 (*lys, met, ilv, pro, str*^{r}) was crossed with SVM2-2A7 (*his, leu, ade, cpp*), and recombinants were selected for *his*$^+$/*lys*$^+$, *str*$^+$/*lys*$^+$, *leu*$^+$/*ilv*$^+$, or *ade*$^+$/*ilv*$^+$. The recombinants obtained were classified into 33 genotypes. The underlined markers indicate those derived from the SVM2-2A7 parent. The sequence of loci shown in Table 3 was arranged in such a way that the underlined markers do not separate each other on a circle. This arrangement should result in minimizing the number of the quadruple crossovers (QC) in the recombinants. The sequence obtained was same as that previously reported by us[5]. The frequency of QC of recombinants for this marker sequence was 6.5% among 308 recombinants, when the *cpp* marker was arranged between *met* and *ilv* in the sequence. When the sequence was arranged without *cpp*, the frequency of QC was 3.3% as shown in Table 3. Table 4 shows the frequency of QC-recombinants for the marker sequence in which *cpp* was placed between every pair of markers. In the crosses V, VI, VII, and VIII, map positions of *cpp* which gave the lowest QC frequency were determined to be between *met* and *ilv*. These results indicate that the *cpp* locus in SVM2-HT3, SVM2-2A7, SVM2-U26, and SVM2-N7 exists between *met* and *ilv*. As reported in a previous paper, the genetic locus of SVM2-HT8 reducing the ability to produce chloramphenicol was again shown to lie on a plasmid (cross IV in Table 4).

Table 3. Analysis of a cross between strains SVM1 and SVM2-2A7.

Genotype of SVM1: *his*$^+$ *ade*$^+$ *str leu*$^+$ *lys met cpp*$^+$ *ilv pro* (CAP producer)
Genotype of SVM2-2A7: *his ade str*$^+$ *leu lys*$^+$ *met*$^+$ *cppC ilv*$^+$ *pro*$^+$ (1-deoxyCAP producer).
Selection: *his*$^+$/*lys*$^+$, *str*^{r}/*lys*$^+$, *leu*$^+$/*ilv*$^+$ and *ade*$^+$/*ilv*$^+$

Genotype	Arrangement of marker sequence (markers derived from SVM2-2A7 are underlined) -*his*—*ade*—*str*—*leu*—*lys*—*met*—(*cppC*)—*ilv*—*pro*-									Recombinants obtained	No. of crossover	
1	+	+	*str*	+	*lys*	*met*	(*cppC*)	+	+	73	2	
2	*his*	+	*str*	+	*lys*	*met*	(*cppC*)	+	+	47	2	
3	+	+	*str*	*leu*	+	+	(+)	*ilv*	*pro*	63	2	
4	+	+	*str*	*leu*	+	+	(*cppC*)	*ilv*	*pro*	32	2	
5	+	+	+	*leu*	+	+	(*cppC*)	+	+	26	2	
6	*his*	*ade*	+	+	*lys*	+	(*cppC*)	+	+	12	2	
7	+	+	*str*	*leu*	+	+	(*cppC*)	+	+	6	2	
8	+	+	*str*	*leu*	+	*met*	(+)	*ilv*	*pro*	5	2	
9	+	+	*str*	+	*lys*	*met*	(+)	+	+	6	2	
10	*his*	+	*str*	+	*lys*	*met*	(+)	+	+	5	2	
11	+	+	*str*	*leu*	+	+	(+)	*ilv*	+	3	4	
12	*his*	+	*str*	+	*lys*	+	(*cppC*)	+	+	3	2	
13	+	+	*str*	+	+	+	(*cppC*)	+	+	2	2	
14	*his*	+	*str*	+	*lys*	+	(+)	+	+	2	2	*
15	*his*	*ade*	*str*	+	+	+	(*cppC*)	+	+	2	2	
16	+	+	+	*leu*	+	+	(+)	+	+	2	2	*
17	*his*	+	*str*	*leu*	+	*met*	(*cppC*)	+	+	2	4	
18	+	+	*str*	+	+	+	(+)	+	+	2	2	*
19	+	+	*str*	+	*lys*	+	(+)	+	*pro*	1	2	*
20	+	+	*str*	+	*lys*	+	(*cppC*)	+	*pro*	1	2	
21	+	+	*str*	*leu*	+	*met*	(*cppC*)	*ilv*	*pro*	1	2	*
22	+	+	*str*	+	+	+	(+)	+	*pro*	1	2	*
23	+	+	*str*	+	+	*met*	(+)	+	+	1	4	
24	+	+	*str*	+	*lys*	+	(*cppC*)	+	+	1	2	
25	+	+	*str*	+	+	+	(*cppC*)	+	+	1	2	
26	+	+	*str*	*leu*	+	+	(*cppC*)	+	+	1	2	
27	+	+	+	+	+	+	(*cppC*)	+	+	1	4	
28	*his*	*ade*	*str*	+	*lys*	+	(*cppC*)	+	+	1	2	
29	+	+	+	*leu*	+	*met*	(*cppC*)	+	+	1	4	
30	+	+	+	*leu*	+	+	(*cppC*)	*ilv*	+	1	4	
31	*his*	+	*str*	*leu*	+	+	(+)	+	+	1	2	*
32	*his*	+	*str*	*leu*	+	+	(*cppC*)	+	+	1	2	
33	*his*	+	+	+	*lys*	+	(*cppC*)	+	+	1	4	
	Total									308		

Quadruple crossover (Q.C.) in the marker sequence mapped without *cppC*: 3.3%
Q.C. in the marker sequence with *cppC*: 6.5%
Q.C. in the secondary possible marker sequence with *cppC*: 15.6%
*: Additional Q.C. when *cppC* was placed in the sequence minimizing Q.C.

Plasmids in Chloramphenicol-Nonproducing Mutants

The presence or absence of plasmids in the mutants which had chromosomal *cpp* mutations was examined by crossing them with the plasmid-less and chloramphenicol-minute-producing mutant,

Table 4. Frequency of quadruple crossover (Q.C.) in the marker sequence with *cpp* between other pairs of markers.

Cross		Total No. of recombinants	Frequency of Q.C. in the marker sequence								*cpp* locus mapped
			when *cpp* was placed between adjacent markers (%)							without *cpp*	
			his	*ade*	*str*	*leu*	*lys*	*met*	*ilv*	*pro* (*his*)	
I	(SVM1×SVM3)*	127	15.8	18.9	17.3	35.4	40.2	40.9	46.5	38.5	3.2	plasmid loss
II	(SVM1×SVM5)*	123	33.3	23.6	19.5	29.3	54.5	52.9	47.2	39.0	4.9	plasmid loss
III	(SVM2×SVM4)*	140	55.7	31.4	18.6	39.3	15.7	38.6	60.7	62.1	3.6	plasmid loss
IV	(SVM1×SVM2-HT8)	126	47.6	32.5	15.9	46.8	27.8	24.6	49.2	49.2	2.4	plasmid loss
V	(SVM1×SVM2-HT3)	241	36.0	38.9	59.7	78.8	48.9	5.7	22.4	10.7	2.4	between *met-ilv*
VI	(SVM1×SVM2-2A7)	308	50.0	58.1	46.4	71.1	64.6	6.5	19.8	17.5	3.3	between *met-ilv*
VII	(SVM1×SVM2-U26)	133	6.8	17.3	36.1	85.0	38.6	2.3	6.0	12.0	2.3	between *met-ilv*
VIII	(SVM1×SVM2-N7)	113	21.2	16.8	31.9	59.3	23.0	2.7	17.7	32.7	0	between *met-ilv*

* Data for cross I, II and III have been reported previously[5].

SVM4. If a *cpp* mutant carries the plasmid, strains which have a high capacity for chloramphenicol production should be found in the nutritional recombinants obtained. After the *cpp* mutants had been crossed with SVM4, the *his*$^+$/*lys*$^+$ (or *leu*$^+$/*ilv*$^+$) recombinants were selected and the chloramphenicol production was tested in each recombinant. As shown in Table 2, high-producing recombinants were found in all cases of chromosomal *cpp* mutants. However, in recombination between the plasmid-less variants themselves (such as SVM3 *vs* SVM4 and SVM4 *vs* SVM2-HT8), no high-producing recombinants were obtained.

Genetic Separation of *cpp* Loci

The *cpp* mutations mapped between *met* and *ilv*, were studied in further detail. All of the *cpp* mutants described above, however, had the same auxotrophic markers, and therefore a mutant carrying other markers was prepared by crossing SVM2-HT3 with SVM1. A recombinant (HT3R1) was obtained with the markers of *his, lys, met, str*^{r}, and *cpp*. HT3R1 was crossed with each *cpp* mutant, and *ade*$^+$/*lys*$^+$ recombinants were selected. Their ability to produce chloramphenicol was examined in the recombinants obtained. Although high-producing recombinants were not found in the crosses between HT3R1 and SVM2-HT3, SVM2-2A7, or SVM2-U26, recombination between HT3R1 and SVM2-N7 gave high-producing strains with a high frequency (Table 2). This indicates that the locus of the *cpp* mutation in SVM2-N7 is separated from the loci of the *cpp* mutations in other strains used in these crosses. The *cpp* locus of SVM2-N7 was designated *cpp* D.

p-Aminophenylalanine and Arylamine Synthetase in *S. venezuelae*

In order to clarify the role of the plasmid in chloramphenicol production, *p*-aminophenylalanine and arylamine synthetase in cells were examined. *p*-Aminophenylalanine is known to be

an intermediate in chloramphenicol biosynthesis[24,25]. A *p*-aminophenylalanine-like substance extracted from various cells was subjected to TLC using the solvent systems Nos. 1, 2 and 3. Cell extracts from strains of group I and III contained a compound which had Rf values identical with those of authentic DL-*p*-aminophenylalanine: Rf, 0.50, 0.41 and 0.28 on No. 1, 2 and 3 solvents, respectively.

Fig. 3. Content of *p*-aminophenylalanine (*p*-APA), activity of arylamine synthetase (AASase) and chloramphenicol production (CAP) during the course of fermentation in strain SVM2.

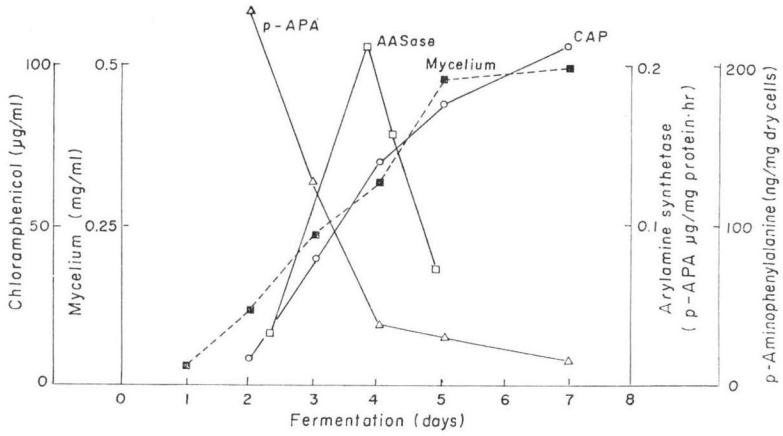
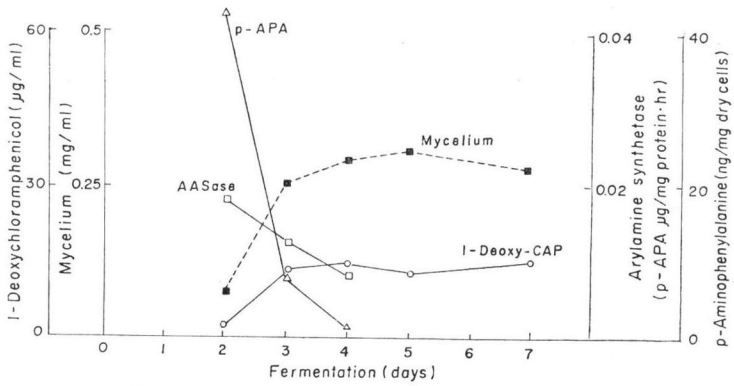


Fig. 4. Contents of *p*-aminophenylalanine (*p*-APA), activity of arylamine synthetase (AASase) and chloramphenicol production (CAP) during the course of fermentation in the 1-deoxychloramphenicol producing strain SVM2-2A7.



The amount of *p*-aminophenylalanine, activity of arylamine synthetase, and chloramphenicol production were measured during the course of growth in medium GNa. In the case of strain SVM2, chloramphenicol production paralleled growth (Fig. 3). The amount of *p*-aminophenylalanine was high in early growth phase, and decreased rapidly following increase in chloramphenicol production (Fig. 3). The activity of arylamine synthetase was highest at the time when chloramphenicol production reached about half maximum (Fig. 3). In the case of the 1-deoxychloramphenicol-producing mutant SVM2-2A7, cell growth was the same as that of the chloramphenicol producer up to 3 days, but soon thereafter reached a plateau which corresponded to 60% growth of the chloramphenicol producer (Fig. 4). 1-Deoxychloramphenicol was also produced parallel to growth. *p*-Aminophenylalanine was detected in this mutant at an early phase of growth, and the amount was about one tenth of that found in the chloramphenicol producer. The activity of arylamine synthetase was also about one tenth of that of the chloramphenicol producing strain (Fig. 4). These measurements were carried out in other strains. As shown in Table 5, the original strains (SVM1 and SVM2) and mutants of group I and III contained 230 ng and 20~40 ng of *p*-aminophenylalanine per mg of dry cells, respectively. No *p*-aminophenylalanine was detected in

Table 5. Amount of *p*-aminophenylalanine and activity of arylamine synthetase in Cpp mutants of *Streptomyces venezuelae*

Typical mutant obtained	*p*-Aminophenyl-alanine (ng/mg dry cells)	Arylamine synthetase (*p*-APA ng/min mg protein)
SVM1	190	NT
SVM2	230	0.21
SVM3	17	0.01
I SVM4	NT	NT
SVM2-HT8	20	0.01
II SVM2-1A1	—	—
SVM2-HT3	—	—
III SVM2-2A7	40	0.02
VI SVM2-U26	—	—
SVM2-N7	—	—

Table 6. Resistance to chloramphenicol in chloramphenicol-nonproducing strains.

Mutants tested	Maximal chloramphenicol concentration (μg/ml)* Preincubation steps**			
	None	1^c	1^c2^n	$1^c2^n3^n4^n$
(SVM1)	200	200	100	100
(SVM2)	200	200	200	200
SVM3	50	200	50	50
SVM4	100	200	100	50
SVM2-HT8	50	200	50	50
SVM2-2A1	50	200	50	50
SVM2-HT3	50	200	50	50
SVM2-2A7	50	200	50	50
SVM2-U26	50	200	50	50
SVM2-N7	50	200	50	50

* Strains grew well at this concentration of chloramphenicol, but grew poorly on the twice concentration of chloramphenicol.

** 1^c2^n indicates preincubation with chloramphenicol followed by second preincubation without chloramphenicol, and so on.

mutants of group II and IV. The activity of arylamine synthetase behaved similarly (Table 5). Therefore it is apparent that the content of *p*-aminophenylalanine and the activity of arylamine synthetase correlate with the level of production of chloramphenicol or 1-deoxychloramphenicol.

Chloramphenicol Resistance of Cpp Mutants

The maximum concentrations of chloramphenicol which allow growth of the mutants are shown in Table 6. Chloramphenicol-nonproducing mutants had a lower level of resistance (50 μg/ml of chloramphenicol) than that of chloramphenicol producers (200 μg/ml), when preincubated in medium without chloramphenicol. However, they acquired a higher level of resistance (200 μg/ml of chloramphenicol) as that of the producers, when preincubated in medium containing 20 μg/ml of chloramphenicol which did not otherwise affect growth. The high resistance level of 200 μg/ml was lost after incubation in medium without chloramphenicol.

Discussion

Treatment with acriflavine or incubation at high temperature have been frequently used for curing plasmids. These procedures however, have been found to cause mutations in some cases.

A mutant of *S. venezuelae*, SVM2-2A7, produced 1-deoxychloramphenicol instead of chloramphenicol. The addition of ^{14}C-chloramphenicol to the growing culture or cell-free system of this mutant did not produce ^{14}C-labelled 1-deoxychloramphenicol. The production of 1-deoxychloramphenicol is consistent with the pathway for chloramphenicol biosynthesis as proposed by WESTLAKE and VINING[25]. It is reasonable to propose that the hydroxylase which oxidizes the C3-position of *p*-aminophenylalanine to produce *p*-aminophenylserine is deficient in mutant SVM2-2A7.

All of the *cpp* mutations including the 1-deoxychloramphenicol-producing mutation, were mapped between *met* and *ilv*. However, *cpp* loci were found in at least 2 genetically separable positions between *met* and *ilv*, as shown when the recombinants obtained from crosses between a *cpp* mutant HT3R1 and other *cpp* mutants were examined for chloramphenicol production.

The Cpp mutations of SVM3, SVM4, and SVM2-T8 belonging to group I had been believed

due to either plasmid loss or plasmid mutation. When these mutants were crossed, no recombinants which produced chloramphenicol to the same extent as the original producer were found in the nutritional recombinants obtained. These results suggest that the reduction of chloramphenicol production in the strains belonging to group I is not due to a plasmid mutation but to a plasmid loss. On the other hand, chloramphenicol-high-producing strains were detected with high frequency among the recombinants obtained by a cross between a chromosomal *cpp* mutant and a plasmid-less mutant SVM4. These results indicate that all or most of the structural genes for the chloramphenicol biosynthetic steps including the hydroxylation of *p*-aminophenylalanine are located between *met* and *ilv* on the chromosome, and that the plasmid has a role to increase (regulate) chloramphenicol production.

The production of chloramphenicol by mixed cultures of chromosomal *cpp* mutants in various combinations was also studied (data were not indicated). Antibiotic synthesis could not be detected even in a pair of chromosomal *cpp* mutants with different chromosomal *cpp* loci (SVM2-U26 *vs* SVM2-N7). These results may indicate a permeability barrier to the intermediate products.

VINING and his colaborators have found that arylamine synthetase in *Streptomyces* sp. 3022a converts chorismic acid to *p*-aminophenylalanine at the initial step in chloramphenicol biosynthesis[22,24]. We have studied the relation between arylamine synthetase activity (or the intracellular *p*-aminophenylalanine content) and chloramphenicol production. Arylamine synthetase reached its peak of activity before maximal production of chloramphenicol, and thereafter activity decreased following the increase in production of chloramphenicol. Arylamine synthetase activity and intracellular *p*-aminophenylalanine content in all of the strains tested correlated with their production of chloramphenicol or 1-deoxychloramphenicol irrespective of the presence or absence of plasmids. These results also indicate that the presence of plasmids is not related to the synthesis and activity of arylamine synthetase.

All of the chromosomal or plasmid-less Cpp mutants displayed a lower level of resistance to chloramphenicol than the producers. However, following preincubation in medium containing 20 μg/ml of chloramphenicol, their resistance level increased to the same level as in the producer. These results indicate that the plasmid is not involved in resistance to chloramphenicol, the mechanism of which is unknown.

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