Isolation and Characterization of *Streptomyces venezuelae* Mutants Blocked in Chloramphenicol Biosynthesis

By J. DOULL,¹ Z. AHMED,² C. STUTTARD^{1*} AND L. C. VINING²

Departments of Microbiology¹ and Biology², Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7

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Twelve *Streptomyces venezuelae* mutants blocked in chloramphenicol biosynthesis were isolated. Two of these (Cm1-1 and Cm1-12) were apparently blocked in the conversion of chorismic acid to *p*-aminophenylalanine and three (Cm1-4, Cm1-5 and Cm1-8) accumulated *p*-aminophenylalanine and may have been blocked in the hydroxylation reaction that converted this intermediate to *p*-aminophenylserine. One mutant (Cm1-2) accumulated *D*-threo-1-*p*-nitrophenyl-2-propionamido-1,3-propanediol and *D*-threo-1-*p*-nitrophenyl-2-isobutyramido-1,3-propanediol, indicating that chlorination of the α -*N*-acyl group of chloramphenicol was blocked. The remaining six strains did not excrete any detectable chloramphenicol pathway intermediates.

INTRODUCTION

Extrachromosomal DNA appears to play a role in the production of antibiotics by some *Streptomyces* species. There is strong genetic evidence that structural genes coding for the biosynthesis of methylenomycin A in *Streptomyces coelicolor* A3(2) and *Streptomyces violaceus-ruber* are borne on plasmids SCP1 and pSV1, respectively (Kirby & Hopwood, 1977; Aguilar & Hopwood, 1982). Less conclusive evidence indicates that genes specifying the biosynthesis of tylosin in *Streptomyces fradiae* (Baltz *et al.*, 1981), erythromycin in *Streptomyces erythraeus* (Weber *et al.*, 1982), lankacidin in *Streptomyces* sp. 7434-AN (Hayakawa *et al.*, 1979), streptomycin in *Streptomyces griseus* (Xue *et al.*, 1978), and actinomycin in *Streptomyces parvulus* (Ochi & Katz, 1980) are carried on plasmids, while Zippel *et al.* (1983) have invoked plasmid involvement in the regulation of turimycin biosynthesis in *Streptomyces hygroscopicus*. Another frequently quoted example is the presumed plasmid involvement in regulating chloramphenicol biosynthesis in *Streptomyces venezuelae* (Akagawa *et al.*, 1979). In this organism, genes coding for chloramphenicol biosynthetic enzymes were reported to be linked to chromosomal markers; only regulatory genes were believed to be plasmid-borne. We have detected extrachromosomal DNA in only one of four strains of *S. venezuelae* examined, and elimination of the plasmid (pUC3) from this strain failed to alter antibiotic production (Ahmed & Vining, 1983). We have also examined a chloramphenicol-producing strain of *Streptomyces phaeochromogenes* that contains plasmid pJV1, and have shown that the extrachromosomal DNA could be eliminated during protoplast formation and regeneration. Loss of the plasmid had no effect on chloramphenicol production (Doull *et al.*, 1983). These results show that neither structural nor regulatory genes for chloramphenicol biosynthesis are located on the detectable plasmids of these organisms. However, the possibility remains that such genes are associated with plasmid DNA that was not detected by the procedures used in these experiments. The most decisive evidence on this question should be provided by demonstrating consistent linkage between genetic markers for antibiotic production and genes for primary pathways or other chromosomally-

Abbreviations: FUV, far ultraviolet light (254 nm); NUV, near ultraviolet light (366 nm); MOP, 8-methoxypsoralen.

located markers. Therefore, we have isolated chloramphenicol-nonproducing mutants of *S. venezuelae* for use in mapping studies. This paper describes the isolation and characterization of such strains.

METHODS

Strains and growth conditions. *Streptomyces venezuelae* wild-type (prototrophic) strain ATCC 10712 (ISP 5230) was originally received from E. Wellington (Stuttard, 1982). Mutagenized spores were plated on MYM agar and minimal agar medium was used to test for growth factor requirements (Stuttard, 1982). Vegetative mycelial suspensions used as culture inocula were prepared by transferring a loopful of spores from a stock spore suspension in glycerol (20%, v/v) to 50 ml GNY medium and growing the culture for 48 h (Malik & Vining, 1970). For chloramphenicol production, a 1% (v/v) vegetative inoculum was added aseptically to medium containing glucose (3%, w/v), isoleucine (0.75%) plus basal salts, and incubated at 27 °C on a rotary shaker (Chatterjee *et al.*, 1983).

Chemicals. *p*-Aminophenylalanine was obtained from Sigma. Chloramphenicol and *D*-threo-1-*p*-nitrophenyl-2-acetamido-1,3-propanediol were gifts from Parke-Davis. The synthesis and properties of 2(*S*)-dichloroacetamido-3-(*p*-acetamidophenyl)propan-1-ol have been described (Wat *et al.*, 1971). *N*-Acetyl-*p*-nitrophenylalanine was synthesized as described by McGrath *et al.* (1968).

D-threo-1-*p*-Nitrophenyl-2-propionamido-1,3-propanediol was synthesized as described by Shirahata *et al.* (1972). The crude product in ethyl acetate was freed of unchanged *D*-threo-1-*p*-nitrophenyl-2-amino-1,3-propanediol by extraction with 2 M-HCl. It was purified by preparative thin-layer chromatography (TLC) and crystallization from dichloroethane to give colourless needles, m.p. 108–110 °C; R_F (TLC, system A) 0.24. The ^1H -NMR spectrum, recorded in deuteriochloroform at 80 MHz on a Varian model FT-80 spectrometer with tetramethylsilane as the internal reference, showed signals at δ 1.04 (t, 3H, CH_3 , $J_{\text{CH}_3, \text{CH}_2}$ 7.6 Hz), 2.15 (q, 2H, CH_2 , $J_{\text{CH}_2, \text{CH}_3}$ 7.6 Hz), 3.00 (bs, 1H, OH), 3.78–4.30 (ABC, 3H, H-1', H-2'), 5.21 (d, 1H, H-3', $J_{2',3'}$ 3.3 Hz), 6.15 (bd, 1H, NH, $J_{\text{NH}, 2'}$ 7.6 Hz), 7.88 (AA'BB', 4H, H-2, H-3, H-5, H-6, $\Delta\nu_{\text{AB}}$ 51.1 Hz, $N = J_{\text{AB}} + J_{\text{AB}'} = 8.8$ Hz).

D-threo-1-*p*-Nitrophenyl-2-isobutyramido-1,3-propanediol was synthesized as follows. Isobutyric acid (88 mg) in dry acetone (2 ml) containing triethylamine (0.14 ml) was cooled to –10 °C in an ice-salt bath. Ethyl chloroformate (0.7 ml) was added slowly with stirring and was followed, 25 min later, by a solution of *D*-threo-1-*p*-nitrophenyl-2-amino-1,3-propanediol (212 mg) in acetone (4 ml). The mixture was allowed to come to room temperature and, after 4 h, was evaporated to dryness. The residue was dissolved in a mixture of ethyl acetate (25 ml) and water (5 ml). The product in the ethyl acetate layer was recovered and crystallized from water to give colourless needles, m.p. 130–131 °C; R_F (TLC, system A) 0.30. The ^1H -NMR spectrum, recorded as above, showed signals at δ 1.01 (d, 3H, CH_3 , $J_{\text{CH}_3, \text{H}}$ 6.9 Hz), 1.06 (d, 3H, CH_3 , $J_{\text{CH}_3, \text{H}}$ 6.9 Hz), 1.62 (bs, 1H, OH), 2.27 (qn, 1H, CH, $J_{\text{H}, \text{CH}_3}$ 6.9 Hz), 3.8–4.3 (ABC, 3H, H-1, H-2'), 5.22 (d, 1H, H-3', $J_{2',3'}$ 3.4 Hz), 6.06 (bd, 1H, NH, $J_{\text{NH}, 2'}$ 7.5 Hz), 7.88 (AA'BB', 4H, H-2, H-3, H-5, H-6, $\Delta\nu_{\text{AB}}$ 51.1 Hz, $N = J_{\text{AB}} + J_{\text{AB}'} = 8.8$ Hz).

Thin-layer chromatography. Samples of chloramphenicol, its analogues and the ethyl acetate extracts of cultures before or after column chromatography, were applied to thin layers of silica gel F254 on glass plates; the chromatograms were developed in chloroform/methanol (9:1, v/v; system A) or *n*-butanol/acetic acid/water (12:3:5, by vol.; system B). They were dried and sprayed in turn with aqueous stannous chloride and with a solution of *p*-dimethylaminobenzaldehyde in 2 M-HCl. Zones containing aromatic nitro- or amino-compounds became yellow. For preparative chromatograms, 20 × 20 cm plates of silica gel F254 (0.2 mm thickness) were used. The solvent was again chloroform/methanol (9:1, v/v) but chromatograms were developed twice. Samples of *p*-aminophenylalanine and polar fractions from cultures were chromatographed on thin layers of cellulose MN300. The chromatograms were developed with *n*-butanol/ethanol/4 M-ammonium hydroxide (8:1:3, by vol.; system C) or with *n*-butanol/acetic acid/water (12:3:5, by vol.; system D).

Mutagenesis. Four methods were used. (a) Irradiation with short-wavelength (254 nm) ultraviolet light (FUV): spore suspensions in 0.1 M-phosphate buffer, pH 7.0, were given sufficient exposure to produce approximately 99.9% killing and then plated on (i) MYM agar or (ii) MYM agar containing caffeine (500 mg ml⁻¹).

(b) Irradiation with long wavelength (366 nm) ultraviolet light (NUV) in the presence of 8-methoxypsoralen (MOP): spores suspended for 30 min in 0.015 M-phosphate buffer, pH 7.0 containing MOP (0.1 mg ml⁻¹) were irradiated to give about 1% c.f.u. survival and plated to obtain isolated colonies on MYM medium.

(c) Treatment with ethyl methanesulphonate (EMS): EMS (final concentration 70 mg ml⁻¹) was added to a suspension of spores in 0.1 M-phosphate buffer and the mixture was shaken for 2 h at 27 °C. Spores were then collected on a membrane filter, washed with 250 ml phosphate buffer and resuspended in a small volume of the buffer. Finally, they were plated to obtain isolated colonies on MYM agar.

Isolation of chloramphenicol-nonproducing mutants. Colonies that grew from mutagenized spores were allowed to sporulate and then streaked in patches on fresh MYM agar (five equally spaced patches per 9.0 cm diameter plate). The patched colonies were grown at 27 °C until they sporulated (3–4 d); the plates were then carefully overlaid between the patches with soft (0.5%, w/v) GNY agar seeded with *Micrococcus luteus*. The overlaid plates were

incubated overnight and examined for patches showing no inhibition of growth of the indicator bacteria. Inocula from such patches were restreaked on MYM agar to obtain single colonies which were again tested using the bioassay procedure. Isolates that still failed to inhibit the growth of *M. luteus* were then grown in chloramphenicol-production medium for 7 d and mycelia were removed by centrifugation. The supernatant fluids were examined for the presence of aromatic nitro- or amino-compounds by the colorimetric procedure of Levine & Fischbach (1951). Those giving positive tests were extracted with ethyl acetate, initially at neutral pH and again after acidification to pH 2. The extracts were concentrated separately and examined by TLC.

Compounds A, B and C from mutant Cml-2. The supernatant fluid (2 l) from cultures of mutant Cml-2 grown in chloramphenicol-production medium was extracted with 3 half-volumes of ethyl acetate; the combined extracts were washed once with water and then evaporated. The residue was dissolved in chloroform (40 ml) and applied to a silicic acid column (1.4 × 15 cm). The chromatogram was developed with successive 40 ml portions of chloroform/ethyl acetate (9:1, v/v), (4:1, v/v), (1:1, v/v); ethyl acetate; ethyl acetate/acetone (9:1, v/v), (4:1, v/v), (1:1, v/v) and acetone. The eluate was collected in 10 ml fractions. Examination of the fractions by TLC (system A) showed three aromatic nitro-compounds, A, B and C with R_F values of 0.31, 0.24 and 0.17, respectively. The R_F value of chloramphenicol was 0.29. Fractions containing a single compound were pooled and chromatographed again on preparative thin-layer plates. From each plate the predominant fluorescence-quenching band was removed; the product was extracted into acetone and recovered by evaporation.

Compound D from mutants Cml-4, Cml-5 and Cml-8. The supernatant fluid from a 750 ml culture of the mutant was applied to a column (2.5 × 30 cm) of Dowex 50 × 8 (H⁺) 50–100 mesh and the resin was washed with water (300 ml). Amino acids were eluted with 2 M-ammonium hydroxide and fractions that gave a positive reaction for aromatic amines were combined and evaporated. The residue was applied in water (80 ml) to a column (1.5 × 20 cm) of Dowex-50 (pyridinium). The column was developed successively with water (200 ml), 0.1 M-pyridine (100 ml) and 0.5 M-pyridine (100 ml) while 5 ml fractions were collected. Aromatic amines were detected by placing a drop from each fraction on filter paper and spraying with *p*-dimethylaminobenzaldehyde. Positive fractions were grouped as a single peak and lyophilized. TLC of the residue gave a single zone that reacted with dimethylaminobenzaldehyde; R_F values were 0.23 and 0.27 in systems C and D, respectively. Mutants Cml-4, Cml-5 and Cml-8 each accumulated the same substance which was identical in mobility to authentic *p*-aminophenylalanine. The ¹H-NMR spectrum of this substance, recorded in deuterated water at 80 MHz on a Varian model FT-80 spectrometer with tetramethylsilane in deuteriochloroform as the external reference, contained signals matching those given by *p*-aminophenylalanine, at δ 2.90, 3.06 and 3.80 (ABM, 3H, H-2'a, H-2'b, H-3', $J_{2'a,2'b} = 15.0$, $J_{2'b,3'} = 5.1$ Hz) and at 6.88 (AA'BB', 4H, H-2, H-3, H-5, H-6, $\Delta\nu_{AB} = 22.2$ Hz, $N = J_{AB} + J_{AB'} = 8.5$ Hz).

Ethanol extraction of mutant cells. Cells were grown in chloramphenicol-production medium for 7 d, harvested by centrifugation and washed with 0.1 M-phosphate buffer, pH 7.0. They were then resuspended in 70% ethanol and heated in a water bath at 80 °C for 10 min. The resulting extract was filtered and examined by TLC in system C.

Hydrolysis of culture supernatant fluids. Concentrated HCl (0.3 ml) was added to 10 ml test tubes containing supernatant fluid (2 ml) from cultures of each mutant grown in chloramphenicol-production medium. The tubes were sealed and placed at 100 °C for 4 h. After cooling, 1 ml portions were assayed for aromatic amines using the colorimetric procedure of Levine & Fischbach (1951). The validity of this procedure for measuring *N*-acylated aromatic amines in culture supernatant fluids was established with known amounts of 2(*S*)-dichloroacetamido-3-(*p*-acetamidophenyl)propan-1-ol.

***p*-Aminophenylalanine supplementation.** An aqueous solution of *p*-aminophenylalanine was sterilized by filtration and added at a final concentration of 1 mM to 50 ml of chloramphenicol-production medium. The medium then received a vegetative inoculum (0.5 ml) of a mutant strain and the culture was incubated for 7 d. The clarified broth was extracted with ethyl acetate and the extract was examined for chloramphenicol using the colorimetric assay.

Cosynthesis of chloramphenicol. To observe cosynthesis on solid medium, spores from pairs of mutant strains were used to inoculate mixed patches on MYM agar. The cultures were grown until sporulation occurred and the plates were then overlaid with *M. luteus* as described above. To test for cosynthesis in cultures grown in liquid medium, a vegetative inoculum (0.25 ml) of each member of a mutant pair was added to 50 ml of production medium. The cultures were incubated for 7 d and assayed for chloramphenicol colorimetrically.

RESULTS

Isolation of mutants

Approximately 25000 colonies arising from variously mutagenized spores were screened for their ability to produce antibiotics on MYM agar. Twelve showed absolutely no inhibition of the growth of *M. luteus*. These completely nonproducing mutants were isolated at frequencies of 1 in

Fig. 1. Biosynthesis of chloramphenicol. Reactions: A, glutaminase, arylamine synthetase; B, aminotransferase; C, hydroxylase; D, acyl transferase; E, reductase; F, oxidase. Lesions in mutants Cml-1 and Cml-12 affect reactions A or B.

Table 1. *Production of arylamines and aryl nitro-compounds by chloramphenicol-nonproducing (Cml⁻) mutants*

Cml mutant		<i>p</i> -Aminophenyl- alanine	Corynecins	Arylamino-/nitro- compounds excreted
No.	Mutagen*			
1	EMS	—	—	—
2	EMS	+	+	+
3	EMS	+	—	—
4	FUV + C	+	—	+
5	FUV	+	—	+
6	MOP-NUV	—	—	—
7	MOP-NUV	—	—	—
8	FUV	+	—	+
9	FUV	—	—	—
10	MOP-NUV	+	—	—
11	MOP-NUV	—	—	—
12	MOP-NUV	—	—	—

* FUV, far UV, spores plated on MYM agar; FUV + C, far UV, spores plated on MYM + caffeine agar; EMS, ethyl methanesulphonate; MOP-NUV, near UV in the presence of 8-methoxypsoralen; +, synthesis; —, no synthesis.

approximately 3000 c.f.u. (0.03%) surviving after mutagenesis with FUV and caffeine; 3 in about 8000 c.f.u. (0.04%) after EMS treatment; 5 in about 9000 (0.05%) after NUV + MOP mutagenesis; and 3 in 4500 (0.07%) after exposure to FUV alone. Leaky mutants producing small amounts of chloramphenicol were isolated at frequencies of approximately 0.3% with each method of mutagenesis. Each of the 12 nonproducing mutants was still prototrophic and grew well on minimal agar. When grown in chloramphenicol-production medium, eight showed no colorimetric reaction for aromatic amino- or nitro-compounds in the clarified culture fluids

(Table 1). In addition, concentrated extracts of the culture fluids of these eight mutants gave no zones that reacted with *p*-dimethylaminobenzaldehyde after thin-layer silica-gel chromatography. Absorbent paper assay disks soaked with concentrated extracts of the eight culture fluids showed no antibiotic activity against the indicator bacteria.

Cml-1 and Cml-12

To determine whether any of these eight strains was blocked in the conversion of chorismic acid to *p*-aminophenylalanine [the arylamine synthetase-catalysed aromatization step that initiates chloramphenicol biosynthesis (Fig. 1)], *p*-aminophenylalanine was added to the production medium. Two of the strains, Cml-1 and Cml-12, then produced chloramphenicol and were presumed to be deficient in arylamine synthetase activity (Jones & Westlake, 1974).

Cml-3 and Cml-10

The possibility was considered that some of the six strains (Cml-3, -6, -7, -9, -10, -11) that did not excrete chloramphenicol or any of its aromatic precursors might accumulate an intermediate that was modified by shunt metabolism so that it escaped detection as an aromatic amino- or nitro-compound. Such a compound, in which the *p*-amino group is acylated, has been described by Wat *et al.* (1971). We have ascertained that this metabolite has no inhibitory activity against *M. luteus*. To remove acyl groups which might prevent an aromatic amine from being detected, culture fluids were heated under acid conditions. Subsequent colorimetric assay of the hydrolysate failed to detect any aromatic amines excreted by these six mutants.

To determine whether any chloramphenicol intermediates were accumulated intracellularly, ethanol extracts of washed mycelia were examined by thin-layer cellulose chromatography. Cells of two mutant strains, Cml-3 and Cml-10, contained small amounts of an arylamine which had an R_F value similar to that of authentic *p*-aminophenylalanine on the same chromatogram. Accumulation of this intermediate, and its failure to promote the formation of chloramphenicol when added as a medium supplement, implicates a pathway lesion after the arylamine synthetase step.

Cml-6, Cml-7, Cml-9 and Cml-11

Extracts of cells of strains Cml-6, Cml-7, Cml-9 and Cml-11 failed to show any aromatic amino- or nitro-compounds; and, as mentioned above, none excreted any detectable aromatic amine. The possible nature of these mutants is considered in the Discussion.

Cml-2

Disks permeated with concentrated extracts of culture fluids of strain Cml-2 showed very weak inhibition of *M. luteus*. This mutant which, along with Cml-4, Cml-5 and Cml-8 (see below), gave a positive response when culture fluids were examined colorimetrically for aromatic nitro- or amino-compounds, was confirmed as an excretor by TLC. Extracts of culture fluids showed three zones that reacted as aromatic nitro-compounds with stannous chloride and *p*-dimethylaminobenzaldehyde and differed in R_F value from the zone given by chloramphenicol. Three aromatic nitro-compounds were isolated by silica-gel chromatography of the extracts from culture fluids of Cml-2. The structures of two of the metabolites were identified by comparing their chromatographic behaviour and $^1\text{H-NMR}$ spectra with those of authentic specimens as *D-threo*-1-*p*-nitrophenyl-2-propionamido-1,3-propanediol and *D-threo*-1-*p*-nitrophenyl-2-isobutyramido-1,3-propanediol. The third product was identical chromatographically to *D-threo*-1-*p*-nitrophenyl-2-acetamido-1,3-propanediol (R_F 0.18 by TLC) but the amount and purity of the sample were insufficient for NMR spectral identification. Relatively small amounts of *p*-aminophenylalanine were excreted by Cml-2. Mutant Cml-2 therefore appeared to be defective in the reaction that chlorinates the *N*-acyl group of chloramphenicol (Fig. 1).

Cml-4, Cml-5 and Cml-8

Culture filtrates of strains Cml-4, Cml-5 and Cml-8 contained a single polar arylamine that could not be extracted into organic solvents. The chromatographic properties of the compound

accumulated by each mutant were identical and coincided with those of authentic *p*-aminophenylalanine. No *p*-aminophenylserine could be detected. The product from Cm1-4 was isolated and was identified as *p*-aminophenylalanine from its NMR spectrum by comparison with an authentic sample. Thus these mutants were probably blocked in the hydroxylation of *p*-aminophenylalanine to *p*-aminophenylserine. It is interesting to note that Akagawa *et al.* (1979) isolated a mutant which appeared to be blocked in this hydroxylation step but which accumulated deoxychloramphenicol instead of *p*-aminophenylalanine. Our efforts to detect such a compound in cultures of mutants Cm1-4, Cm1-5 and Cm1-8 were unsuccessful. However, broths of mutants Cm1-4 and Cm1-5 clarified by centrifugation and then acidified before extraction did yield an aromatic nitro-compound with R_f values of 0.55 (system B) and 0.50 (system C) by TLC. This product was tentatively identified as *N*-acetyl-*p*-nitrophenylalanine by comparison with an authentic specimen (R_f values 0.55 and 0.51, respectively). Clarified broths of Cm1-5 showed an increase in aromatic amine content after acid hydrolysis, suggesting that acylation of the aromatic amino group of *p*-aminophenylalanine had also occurred. Since 2(*S*)-dichloroacetamido-3-(*p*-acetamidophenyl)propan-1-ol was not detected by TLC of culture extracts, the product was probably *p*-acetamidophenylalanine or *N*-acetyl-*p*-acetamidophenylalanine.

Cosynthesis

Attempts were made to detect cosynthesis of chloramphenicol by growing mixtures of mutants in all possible pairwise combinations on agar or in liquid medium. Any chloramphenicol biosynthesis which occurred was expected to result from cosynthesis rather than recombination since mixed cultures of these strains gave recombinant frequencies of 10^{-7} per parental c.f.u. or lower (unpublished observation). In agreement with the preceding findings, mutants Cm1-4, Cm1-5 and Cm1-8 (which accumulated *p*-aminophenylalanine) promoted chloramphenicol synthesis when grown with mutants Cm1-1 and Cm1-12 (blocked in the conversion of chorismic acid to *p*-aminophenylalanine). No other instances of cosynthesis were observed.

DISCUSSION

Radioactive feeding experiments (Westlake & Vining, 1969) indicated that the sequence of reactions in chloramphenicol biosynthesis following the formation of *p*-aminophenylalanine consists of: hydroxylation at the β carbon; substitution of the α -amino group; reduction of the carboxyl group to a hydroxymethyl group; and finally, oxidation of the *p*-amino to a *p*-nitro group (Fig. 1). The timing of the chlorination reaction is uncertain but existing evidence has suggested that the halogen atoms are introduced after acylation of the α -amino group (Simonsen *et al.*, 1978). Our isolation of two chloramphenicol-nonproducing mutants (Cm1-1, Cm1-12) blocked in the conversion of chorismic acid to *p*-aminophenylalanine, three (Cm1-4, Cm1-5, Cm1-8) accumulating *p*-aminophenylalanine, and one (Cm1-2) accumulating *D*-threo-1-*p*-nitrophenyl-2-propionamido-1,3-propanediol and *D*-threo-1-*p*-nitrophenyl-2-isobutyramido-1,3-propanediol is consistent with the overall pathway. However, the Cm1-2 products are more readily explained if the acyl substituent is chlorinated before attachment to the phenyl propanoid intermediate. In this mutant, absence of the halogenated acylation substrate would allow a low specificity transferase catalysing the acylation reaction to accept less preferred substrates such as acetyl-, propionyl- and isobutyryl-coenzyme A. Shirahata *et al.* (1972) isolated *D*-threo-1-*p*-nitrophenyl-2-acetamido-1,3-propanediol, *D*-threo-1-*p*-nitrophenyl-2-propionamido-1,3-propanediol, and *D*-threo-1-*p*-nitrophenyl-2-isobutyramido-1,3-propanediol (corynecins 1, 2 and 3, respectively) from cultures of *Corynebacterium hydrocarboclastus*. Trace amounts of these same compounds have also been reported to accompany chloramphenicol under conditions of antibiotic synthesis by *S. venezuelae* (Stratton & Rebstock, 1963).

The failure to obtain mutants accumulating other intermediates might be due to the low specificity of additional enzymes in the pathway. Mutants of *Streptomyces aureofaciens* blocked at early steps in the pathway of tetracycline biosynthesis produced congeners that retained a high

level of antibiotic activity (Vaněk *et al.*, 1971). A similar series of events in *S. venezuelae* might have yielded mutant strains that escaped our attention since the initial screening was for absence of antibiotic activity.

Although the four strains (Cm1-6, -7, -9, -11) that neither accumulated aromatic intermediates in the chloramphenicol pathway nor responded to *p*-aminophenylalanine might be classified as regulatory mutants, it is possible that some had suffered multi-site mutations. Their phenotype could result from one or more lesions affecting both the reaction sequence catalysed by arylamine synthetase and the reactions converting *p*-aminophenylalanine to chloramphenicol. However, the simultaneous acquisition of two or more independent mutations would be unlikely to give the relatively high proportion of nonexcretors observed. The high incidence of variants altered in chloramphenicol synthesis detected after mutagenic treatments has been seen previously in another strain of *S. venezuelae*, both with and without exposure to intercalating dyes (Michelson & Vining, 1978). In other streptomycetes high frequency changes in secondary metabolic processes, such as melanin formation, have been shown to accompany changes in DNA sequence organization, especially amplification of specific sequences (Schrempf, 1983). Whether such changes sometimes accompany loss of chloramphenicol production in *S. venezuelae* remains to be seen. Another suggested explanation for the phenotype of these mutants is that they fail to synthesize *p*-aminophenylpyruvic acid which might be an inducer for synthesis of other chloramphenicol biosynthetic enzymes. We could not test this possibility since the putative inducer is too chemically unstable to be synthesized.

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