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Evidence for a Chromosomal Location of the Genes Coding for Chloramphenicol Production in *Streptomyces venezuelae*

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Of seven chloramphenicol-producing actinomycetes examined, only *Streptomyces venezuelae* strain 13s contained extrachromosomal DNA detectable by agarose gel electrophoresis and cesium chloride-ethidium bromide density gradient centrifugation. The single 17-megadalton plasmid present in this strain was indistinguishable from plasmid pUC3 previously isolated from mutagenized cultures. Strains selected for their inability to produce chloramphenicol after treatment with acriflavine or ethidium bromide still contained a plasmid that had the same electrophoretic mobility as plasmid pUC3 and yielded similar fragments when digested with restriction endonucleases. By regenerating protoplasts of strain 13s and screening for isolates lacking extrachromosomal DNA, strain PC51-5 was obtained. The absence of plasmid pUC3 sequences in this strain was confirmed by Southern hybridization using ^{32}P-labeled plasmid as a probe. Since the plasmidless strain produced as much chloramphenicol as did the parent strain, pUC3 contains neither structural nor regulatory genes for antibiotic production. Evidence from electrophoretic analysis of *Bam*HI digests of total cellular DNA from wild-type and dye-treated nonproducing progeny indicated that acriflavine caused structural changes in the chromosome.

Initial attempts to map the genes involved in chloramphenicol production indicated that the determinants of antibiotic biosynthesis in *Streptomyces venezuelae* ISP5230 were not linked to chromosomal markers (1). Since antibiotic production was also lost with high frequency from cultures treated with agents known to cause elimination of plasmids (22), these results suggested that the genes for chloramphenicol biosynthesis were located on an extrachromosomal element. The evidence appeared to be strengthened by the detection in strain ISP5230, by sucrose density gradient centrifugation and electron microscopy, of a DNA fraction that contained relatively large circular molecules with a flower-shaped configuration. No comparable fraction was present in the derivatives obtained by treatment with acriflavine (22). Subsequent examination showed that the acriflavine-treated strains were not true nonproducers but made small amounts of chloramphenicol. Strains that had completely lost antibiotic production could be selected after exposing the parent strain to mutagens; these *cpp*$^-$ markers mapped in the chromosome. It was suggested that they identified structural genes for the biosynthetic pathway, whereas the plasmid-borne genes cured by dye treatment played a regulatory role (2).

Working with strain 13s, a different chloramphenicol-producing isolate of *S. venezuelae*, Michelson and Vining (17) observed that exposure to acriflavine or ethidium bromide also gave relatively large numbers of progeny that failed to produce the antibiotic or produced it in much smaller quantities. They suggested plasmid elimination as a possible explanation, but did not establish the presence of a plasmid in their strain. Convincing evidence of a plasmid in *S. venezuelae* was first provided by Malik (14), who isolated circular DNA forms of approximately 18 megadaltons (Mdal). The culture used was a derivative of strain 13s obtained by mutagenesis with nitrosoguanidine and selection for high chloramphenicol production. In a later study, Malik and Reusser (15) characterized a plasmid, designated pUC3, by the pattern of fragments obtained from restriction endonuclease digests. The strain from which pUC3 was isolated, although referred to as *Streptomyces* species 3022a, was in fact the same mutagenized derivative of *S. venezuelae* 13s used initially by Malik (V. S. Malik, personal communication).

We confirmed that the parent strain 13s contained a plasmid indistinguishable from plasmid pUC3. Examination of the chloramphenicol-nonproducing strains obtained from strain 13s by treatment with intercalating dyes indicated that they retained the same 18-Mdal DNA component as the parent strain. Moreover, eliminating the plasmid during regeneration of proto-

plasts had no effect on chloramphenicol production. We conclude that pUC3 codes for neither structural nor regulatory genes required for antibiotic biosynthesis.

MATERIALS AND METHODS

Culture. Strain 13s is a natural variant of *Streptomyces* sp. 3022a, selected for high chloramphenicol production (16). It was recently identified as *S. venezuelae* (T. Pridham, personal communication). *Streptomyces* sp. 3022a and *Kitasatoa kauaiensis* were obtained from the Upjohn Company, Kalamazoo, Mich., as strains UC2374 and UC5543, respectively. *S. omiyaensis* and *S. venezuelae* ISP5230 were obtained from A. Seino, Kaken Chemical Co., Tokyo, as strains KCC S-0124 and KCC S-0526, respectively; *S. venezuelae* NRRL B-902 was received from C. W. Hesseltine, Northern Regional Research Center, U.S. Department of Agriculture; *S. venezuelae* PD04828 and PD05080 were provided by J. Ehrlich, Parke, Davis & Co., Detroit, Mich. Strains of *S. venezuelae* that had lost the ability to produce chloramphenicol were obtained by treating strain 13s with acriflavine or ethidium bromide (17).

Cultures from which plasmid DNA was isolated were grown in GYM medium containing 0.4% glucose, 0.4% yeast extract, and 1% malt extract for 24 h at 27°C, beginning with a 1% vegetative inoculum prepared in glycerol-nutrient broth-yeast extract medium (16). Constant cell yields (absorbance measured at 600 nm [A_{600}]) were obtained. Mycelium was collected by centrifugation, washed in TES buffer (14), and stored at −20°C.

Isolation of total cellular DNA. The mycelium, suspended in 90 ml of TES buffer to give an A_{600} of 1.0 to 1.5, was incubated with lysozyme (1 mg · ml^{-1}) for 1 h at 37°C. Sarkosyl was added to a concentration of 0.25%, and the lysate was treated sequentially with RNase A (100 μg · ml^{-1}, 1.5 h) and pronase (200 μg · ml^{-1}, 2 h) and then extracted overnight with phenol. The aqueous phase was reextracted with phenol (1 h) and then with chloroform. The DNA was precipitated with 2 volumes of cold ethanol. The precipitate was collected immediately, washed in 70% ethanol, and air dried. It was dissolved in 10 ml of TE buffer (35) at 4°C.

Isolation of plasmid DNA. Plasmid DNA was separated from the bulk of the cellular DNA by the acid-phenol extraction method (35), except that the plasmid DNA was recovered from the aqueous phase by ethanol precipitation at −20°C. The precipitate was dissolved in water, dialyzed against a mixture of 1 mM Tris-hydrochloride (pH 8.0)–1 mM sodium chloride–0.01 mM sodium EDTA, freeze-dried, and reconstituted in water to give a concentration of 10 mM Tris–10 mM sodium chloride–0.1 mM sodium EDTA.

For the rapid isolation of plasmid DNA, actinomycete colonies were patched on GYM agar and incubated for 24 h at 27°C. Mycelium collected with a loop was treated with lysozyme (1 mg · ml^{-1}) for 1 h at 37°C and lysed with alkaline sodium dodecyl sulfate at room temperature as described by Birnboim and Doly (3), except that the RNase treatment was omitted.

Restriction endonuclease analysis. Approximately 5 μl of plasmid DNA solution, diluted to 20 μl with water, was mixed with 5 μl of 5× digestion buffer (15) and digested with 2 to 8 U of endonuclease *Eco*RI or *Bam*HI for 1 h at 37°C. The digests were examined by electrophoresis in a 0.7% agarose gel at 2.3 V · cm^{-1} for 18 h in a buffer containing 40 mM Tris-hydrochloride (pH 7.8), 5 mM sodium acetate, and 1 mM sodium EDTA. The molecular weights of the *Eco*RI and *Bam*HI fragments were determined by using *Eco*RI-generated fragments of phage λ DNA for calibration.

For restriction endonuclease analysis of total cellular DNA, the solution obtained after dissolving the total DNA precipitate was dialyzed. The purity of the sample was estimated from its A_{260}/A_{280} value; this was usually above 1.9. Approximately 0.7 μg of DNA was digested with 10 U of *Bam*HI for 3 h at 37°C. The digest was heated to 65°C and rapidly chilled in ice water before being electrophoresed in a 0.85% agarose gel at 3 V · cm^{-1} for 18 h.

Plasmid curing. Protoplasts were prepared from cultures of strain 13s grown for 24 h at 27°C in medium S containing 1% glycine (6). Mycelium from a 2.5-ml culture sample was treated with lysozyme (1 mg · ml^{-1}) for 2 h at 37°C. The frequency of protoplast formation as determined by the method of Hopwood et al. (7) was greater than 95%. Colonies obtained from regenerated protoplasts were screened for the presence of plasmid DNA.

Nick-translation and Southern blot hybridization. Approximately 1 μg of plasmid DNA, purified by subjecting the acid-phenol extract to cesium chloride-ethidium bromide density gradient centrifugation, was used to prepare a ^{32}P-labeled probe by nick-translation (27). Endonuclease *Bam*HI digests of total cellular and purified plasmid DNAs were electrophoresed in a 0.85% agarose gel. The resulting bands were transferred to a nitrocellulose filter by the Southern blot technique (33) and hybridized with denatured, ^{32}P-labeled probe. The filter was exposed to Kodak X-Omat film for 24 h at −70°C with a DuPont lighting-plus intensifying screen.

RESULTS

Plasmid of strain 13s. Plasmid DNA from strain 13s, prepared for electron microscopy by the Kleinschmidt technique as described by Krell and Stoltz (13), showed circular DNA molecules. The contour lengths of 37 molecules, measured on enlarged prints with a Zeiss Videoplan image analysis computer, averaged 9.21 ± 0.15 μm. Bacteriophage PM2 DNA (6.3 Mdal), present as an internal standard, measured 3.43 ± 0.18 μm, giving an approximate value of 17 Mdal for the plasmid. Agarose gel electrophoresis of the plasmid DNA preparation gave three bands (Fig. 1, lane a), corresponding to open circular, linear, and covalently closed circular molecules. Digestion with *Eco*RI gave two fragments of 14.2 and 5.3 Mdal (lane d), and digestion with *Bam*HI produced four fragments of 12.0, 3.5, 2.4, and 1.7 Mdal (lane g).

The patterns obtained by *Hin*dIII (data not shown) and *Bam*HI digestion of the plasmid DNA preparation from strain 13s were the same as those reported for plasmid pUC3 (15). How-

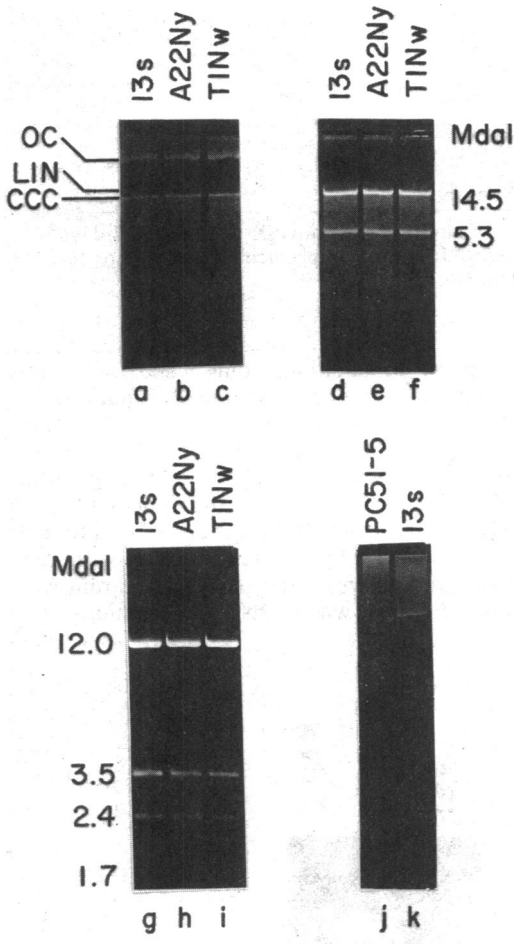

FIG. 1. Agarose gel electrophoresis of plasmid DNA preparations from strains 13s, A22Ny, and T1Nw. Lanes: a, b, and c, undigested samples; d, e, and f, *Eco*RI digests; g, h, and i, *Bam*HI digests; j and k, extracts obtained by the rapid plasmid isolation procedure from strains PC51-5 and 13s. DNA: OC, open circular; LIN, linear; CCC, covalently closed circular.

ever, with *Eco*RI Malik and Reusser (15) found three fragments of 10.8, 5.6, and 3.4 Mdal. The difference appears to be due to incomplete digestion of our preparation through failure of the 14.2-Mdal fragment to cleave further. With samples of plasmid DNA from strain 13s that had been further purified by cesium chloride-ethidium bromide density gradient centrifugation, we obtained three *Eco*RI fragments similar in size to those reported by Malik and Reusser.

Four other chloramphenicol-producing strains of *S. venezuelae*, one strain of *S. omiyaensis*, and one strain of *K. kauaiensis* were examined for the presence of extrachromosomal elements by agarose gel electrophoresis of DNA extracted by the rapid screening procedure. No evidence of plasmid components was found.

Nonproducing derivatives. Eight nonproducing derivatives of strain 13s obtained by treatment with acriflavine or ethidium bromide (17) were also examined for the presence of plasmid DNA by agarose gel electrophoresis of extracts prepared by the rapid screening procedure. All were found to contain a band with the same mobility as the covalently closed circular form of the plasmid from the parent strain. The identity of the bands was verified by digestion with *Eco*RI, which gave the two expected fragments. Two nonproducing strains, A22Ny and T1Nw, were selected for further study. Plasmid DNA obtained from these strains by the acid-phenol method gave patterns similar to that given by the plasmid DNA preparation from the parent strain (Fig. 1, lanes b and c). The digestion patterns obtained with *Eco*RI (lanes e and f) and *Bam*HI (lanes h and i) were the same for each preparation, and both preparations gave the linear form after digestion with *Hin*dIII.

The possibility of detecting small structural changes that might not be revealed by the digestion patterns obtained with endonucleases *Eco*RI and *Bam*HI was increased by also digesting the plasmid DNA preparations with restriction endonucleases having a larger number of cleavage sites in the plasmid. For these comparisons, plasmid DNA isolated by the acid-phenol procedure was further purified by cesium chloride-ethidium bromide density gradient centrifugation. When samples were digested and examined by electrophoresis, endonuclease *Pst*I gave 6 bands, *Bst*EII gave 13 bands, and *Sma*I and *Sst*II each produced 12 bands. The pattern of bands produced by each enzyme was the same for all of the plasmid DNA preparations (data not shown).

Plasmidless strain. Sixty-seven colonies obtained from regenerated protoplasts of strain 13s were screened for the presence of plasmid DNA by the rapid isolation procedure (3). One colony, designated strain PC51-5, lacked the pUC3 band (Fig. 1, lane j). Extraction of total DNA by the acid-phenol method followed by cesium chloride-ethidium bromide centrifugation of the extract also failed to reveal plasmid DNA in this isolate.

The possibility that pUC3 had become integrated into the chromosome was tested by Southern hybridization. Total cellular DNA was digested with restriction endonuclease *Bam*HI and electrophoresed in an agarose gel. The separated fragments were transferred to a nitrocellulose filter and hybridized to a ^{32}P-labeled probe prepared by nick-translation of pUC3. The probe did not hybridize with the DNA from strain PC51-5 (Fig. 2, lane b); it did hybridize

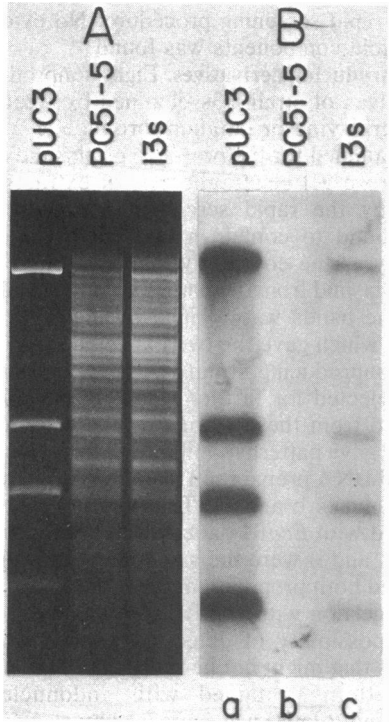

FIG. 2. (A) Agarose gel electrophoresis of endonuclease *Bam*HI digests of plasmid DNA isolated from strain 13s and of total chromosomal DNA obtained from strains PC51-5 and 13s. The DNA was denatured, transferred to a nitrocellulose filter, and hybridized to a ^{32}P-labeled probe obtained by nick-translation of the plasmid. (B) Hybridization patterns.

with fragments in the DNA from strain 13s (lane c) in regions of the gel corresponding to the *Bam*HI-generated fragments of the plasmid. Thus, strain PC51-5 did not have any pUC3 DNA integrated into its chromosome.

The plasmidless strain produced as much chloramphenicol as did the parent strain (Table 1).

Altered restriction endonuclease digestion profile of DNA from strain A22Ny. Samples of the total DNA from the parent strain 13s, the plasmidless strain PC51-5, strain A22Ny (obtained by acriflavine treatment), and strains T1Nw, T228, and T233 (obtained by treatment with ethidium bromide) were digested with restriction endonuclease *Bam*HI and examined by agarose gel electrophoresis. A large number of bands were visible above the background. Strains 13s, PC51-5, T1Nw, T228, and T223 gave identical patterns (Fig. 3). Strain A22Ny gave a banding pattern that was generally similar to those of the other strains, but lacked two major bands at 3.4 and 4.0 Mdal and had an extra band at 4.1 Mdal

TABLE 1. Production of chloramphenicol by *S. venezuelae* strains

Strains	Plasmid	Chloramphenicol[a] (mg/l)
13s	+	43
A22Ny	+	0
PC51-5	–	59

[a] Cultures grown in GSL medium for 7 days were assayed for chloramphenicol in the culture supernatant (16).

(lane b). In addition, some bands were less intense than the corresponding bands for the other strains, and some were relatively diffuse.

The banding patterns were highly reproducible. Since they did not vary with the age of the culture, it is unlikely that differences between A22Ny and the other strains are related to gene dosage caused by differences in growth rate. Constant banding patterns within a strain were also obtained when DNA preparations with

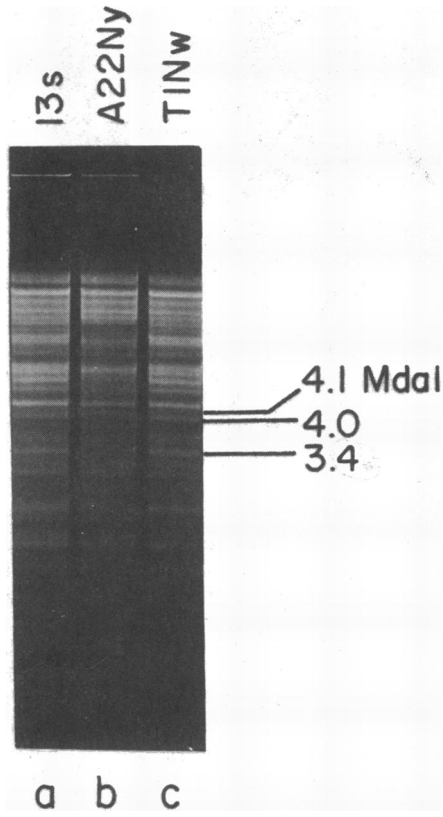

FIG. 3. Banding pattern produced by *Bam*HI digestion of total cellular DNA from strains 13s, A22Ny, and T1Nw. Marks on the right indicate where strain A22Ny differed from the other two strains.

A_{260}/A_{280} values between 1.75 and 2.0 were examined, indicating that any differences were unrelated to differing degrees of protein contamination.

DISCUSSION

The plasmid isolated from *S. venezuelae* 13s was similar in contour length and in the pattern of fragments obtained by digestion with endonucleases *Hin*dIII, *Eco*RI, and *Bam*HI to plasmid pUC3, obtained from a mutagenized derivative of this organism (15). Since a DNA component electrophoretically indistinguishable from pUC3 was retained in all eight nonproducing cultures obtained by ethidium bromide or acriflavine treatment that were examined, loss of antibiotic production was not due to curing of the plasmid. Moreover, the absence of any detectable difference in restriction enzyme fragment patterns between the plasmid in strain 13s and those isolated from two of the nonproducing strains makes it unlikely that the genes for antibiotic biosynthesis were altered in a way that destroyed their function without affecting the electrophoretic mobility of the plasmid. These results, and the ability of a plasmidless strain, PC51-5, to produce chloramphenicol in yields similar to those of the parent, argue against a role for pUC3 in antibiotic production.

Additional chloramphenicol-producing strains of *S. venezuelae* and several other chloramphenicol-producing actinomycetes examined showed no evidence of a plasmid component in their DNA. One of four chloramphenicol-producing strains of *Streptomyces phaeochromogenes* studied did contain a 9-Mdal plasmid, pJV1. As in *S. venezuelae* 13s, however, the plasmid was eliminated during protoplast regeneration without affecting chloramphenicol biosynthesis (J. Doull, L. C. Vining, and C. Stuttard, FEMS Microbiol. Lett., in press). In a similar survey of macrolide antibiotic producers, Omura and coworkers (23) found that only 5 of the 21 strains examined contained plasmids. The results suggest that antibiotic production need not be plasmid coded and, in particular, that the genes coding for chloramphenicol biosynthesis and its regulation are located in the chromosome. This contrasts with the conclusion from earlier studies with *S. venezuelae* ISP5230, which suggested that regulatory functions are located on an extrachromosomal element (2). We were unable to detect a plasmid component in the DNA from strain ISP5230, but spontaneous loss of an extrachromosomal element could have occurred during maintenance. A difference in spore color between strains NRRL 2277 and KCC S-0526, which are separately maintained subcultures of the *S. venezuelae* type culture, emphasizes the variability of this species.

Although it is clear that plasmids pUC3 and pJV1 carry neither structural nor regulatory genes for chloramphenicol biosynthesis, we cannot exclude the possibility that such genes are located on extrachromosomal DNA that could not be detected by the methods we used. In *Streptomyces coelicolor* A(3)2, the large plasmid, SCP1, coding for methylenomycin production proved to be extraordinarily difficult to detect despite overwhelming genetic evidence for its existence (8). However, without further strong support for the existence of extrachromosomal DNA that affects chloramphenicol production, this frequently quoted example (2, 22) of plasmid involvement in the regulation of antibiotic biosynthesis should be regarded cautiously.

Evidence that an antibiotic-producing strain and its nonproducing derivatives obtained by treatment with acriflavine contain the same plasmid was also obtained with *Streptomyces ribosidificus* (19). Thus, experiments showing loss of antibiotic production at high frequency in *Streptomyces* strains treated with curing agents (9, 10, 12, 20, 21, 24, 29, 32, 34; L. T. Chang, D. A. Behr, and R. P. Elander, Abstr. Int. Symp. Genet. Ind. Microorganisms 3rd, Madison, Wis., 1978, p. 36; S. T. Chung and R. L. Morris, Abstr. 3rd ISGIM, p. 39; H. Schrempf and W. Goebel, Abstr. 3rd ISGIM, p. 40; T. J. White and J. Davies, Abstr. 3rd ISGIM, p. 39) should not be taken as evidence of plasmid mediation until more extensive studies can be carried out. It is possible that curing agents affect antibiotic production by rearranging chromosomal DNA. Because the banding of *Bam*HI-digested total DNA from strain 13s was indistinguishable from that of DNA from the plasmidless strain, the different pattern given by digests of strain A22Ny DNA indicates that changes caused by acriflavine treatment reside in the chromosome. However, changes as distinct as those observed in strain A22Ny are not required for the loss of chloramphenicol production, since the three nonproducing ethidium bromide-treated strains examined were also indistinguishable in banding pattern from the parent.

Some or all of the distinct bands formed during electrophoresis of *Bam*HI digests may represent reiterated sequences. Schrempf (30) presented evidence for amplified regions of DNA in strains of *Streptomyces reticuli* and their involvement in chromosomal rearrangements that affect the production of secondary metabolites. Loss of reiterated sequences and altered restriction profiles have also been observed in strains of *Streptomyces* obtained by interspecific protoplast fusion (28). Hintermann and co-workers (5) obtained evidence of chromosomal rearrangement involving the loss of a

9-Mdal fragment in digests of DNA from *Streptomyces glaucescens* cells that had been treated with ethidium bromide. Although the nature of these chromosomal rearrangements and whether they are, indeed, a consequence of the treatments used has not been established conclusively, a number of reports (4, 11, 18, 25, 26, 31) suggest that transposition-like events are an important factor in the genetic instability of *Streptomyces* strains.

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