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LA THÈSE A ÉTÉ  
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GENETIC STUDIES  
OF CHLORAMPHENICOL BIOSYNTHESIS  
IN STREPTOMYCES

by

© Janice Laura Doull

A Thesis

submitted to the Faculty of Graduate Studies  
in partial fulfillment of the requirements

for the degree

Doctor of Philosophy

Dalhousie University

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ABSTRACT

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Of eight chloramphenicol-producing Streptomyces screened for the presence of ccc DNA only Streptomyces phaeochromogenes NRRLB-3559 was found to harbour a plasmid. The elimination of this plasmid, pJV1, from the host strain had no effect on chloramphenicol production.

Twelve Streptomyces venezuelae mutants blocked in chloramphenicol biosynthesis were isolated. Two of these appeared to be blocked in the conversion of chorismic acid to p-aminophenylalanine and three accumulated p-aminophenylalanine and may be blocked in the hydroxylation reaction that converts this intermediate to p-aminophenylserine. One mutant 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  $\alpha$ -N-acyl group of chloramphenicol is blocked. Two other mutants accumulated p-aminophenylalanine intracellularly but not in culture broths. The final four mutants did not accumulate aromatic amino- or nitro-compounds either extracellularly or intracellularly nor did they produce any "masked" aromatic amino-compounds. The possibility exists therefore that some or all of these are regulatory mutants.

All of the chloramphenicol-nonproducing mutants were more sensitive to chloramphenicol than the wild-type strain but became resistant after exposure to the antibiotic. Unlike that in the wild-type strain, this resistance was not reversible.

All of the cml mutations included in crosses mapped to the chromosome of S. venezuelae in the arc between the markers his-6 and strA6 opposite to the ade-10 region. It appears therefore that at least three of the chloramphenicol biosynthetic genes, those coding for p-aminophenylalanine  $\beta$ -hydroxylase, arylamine synthetase and the enzyme responsible for chlorination are located on the chromosome in the same arc.

ABBREVIATIONS

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ade	adenine
ccc	covalently closed circular
*c.f.u.	colony forming units
cml	chloramphenicol
dCTP	deoxycytidine 5'-triphosphate
$\delta$	chemical shift
DAHP	3- deoxy-D-arabino-heptulosonate-7-phosphate
DAPP	2-dichloroacetamido-3-(p-acetamidophenyl)propan-1-ol
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
EDTA	ethylenediaminetetraacetic acid
EMS	ethyl methanesulfonate
dGTP	deoxyguanosine 5'-triphosphate
Hfr	high frequency of recombination
his	histidine
kb	kilobases
lys	lysine
Md	megadalton
met	methionine
nic	nicotinamide
NMR	nuclear magnetic resonance
OD	optical density
str	streptomycin
Tris	tris-hydroxymethylaminomethane
trp	tryptophan

dTTP deoxythymidine 5'-triphosphate

v/v volume per volume

w/v weight per volume

## Abbreviations Used in Genetic Studies\*

<u>ade</u>	adenine
<u>cml</u>	chloramphenicol
<u>cys</u>	cysteine
<u>his</u>	histidine
<u>hom</u>	homoserine
<u>ltz</u>	lethal zygosis ( <u>ltz</u> <sup>+</sup> indicates the ability to elicit lethal zygosis, <u>ltz</u> <sup>s</sup> indicates sensitivity to lethal zygosis)
<u>lys</u>	lysine
<u>met</u>	methionine
<u>nic</u>	nicotinamide
<u>r</u>	resistance
<u>s</u>	sensitivity
<u>str</u>	streptomycin
<u>thr</u>	threonine
<u>trp</u>	tryptophan
<u>ura</u>	uracil

\*When the three letter abbreviations are underlined and the first letter is not capitalized the designation refers to a mutant allele unless it is followed by a superscript + in which case the wild type allele is indicated. When the abbreviation is not underlined and the first letter is capitalized a phenotype is designated. Cml followed by a dash and a number indicates the strain designation for a chloramphenicol nonproducer.

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Finally, I must thank John who insists that husbands must go last, for

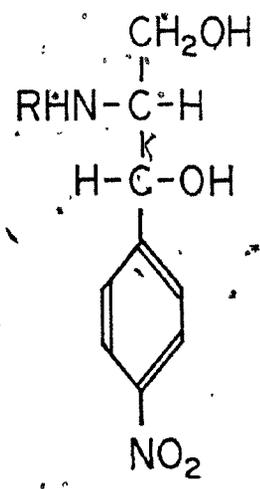
kindness and patience and for setting himself to such tasks as page numbering and reference sorting with remarkable enthusiasm.

Deo-optimo maximo

Chloramphenicol is a broad-spectrum antibiotic used in the treatment of a variety of bacterial and Rickettsial infections. It inhibits protein synthesis by binding to the 50-S ribosomal subunit and blocking the formation of peptide bonds (Pongs et al., 1973). Chloramphenicol was discovered independently in three laboratories (Ehrlich et al., 1947; Gottlieb et al., 1948; Umezawa et al., 1948) and is produced by Streptomyces venezuelae (Okami et al., 1948), Streptomyces omiyaensis (Umezawa et al., 1949) and Streptosporangium viridogriseum (Tamura et al., 1971). Its simple chemical structure, first described by Rebstock et al. (1949), is unusual in that it contains a nitro and a dichloroacetyl group (Figure 1). Corynebacterium hydrocarboclastus produces a group of compounds called corynecins (Figure 1) which differ from chloramphenicol only in the N-acyl group (Suzuki et al., 1972).

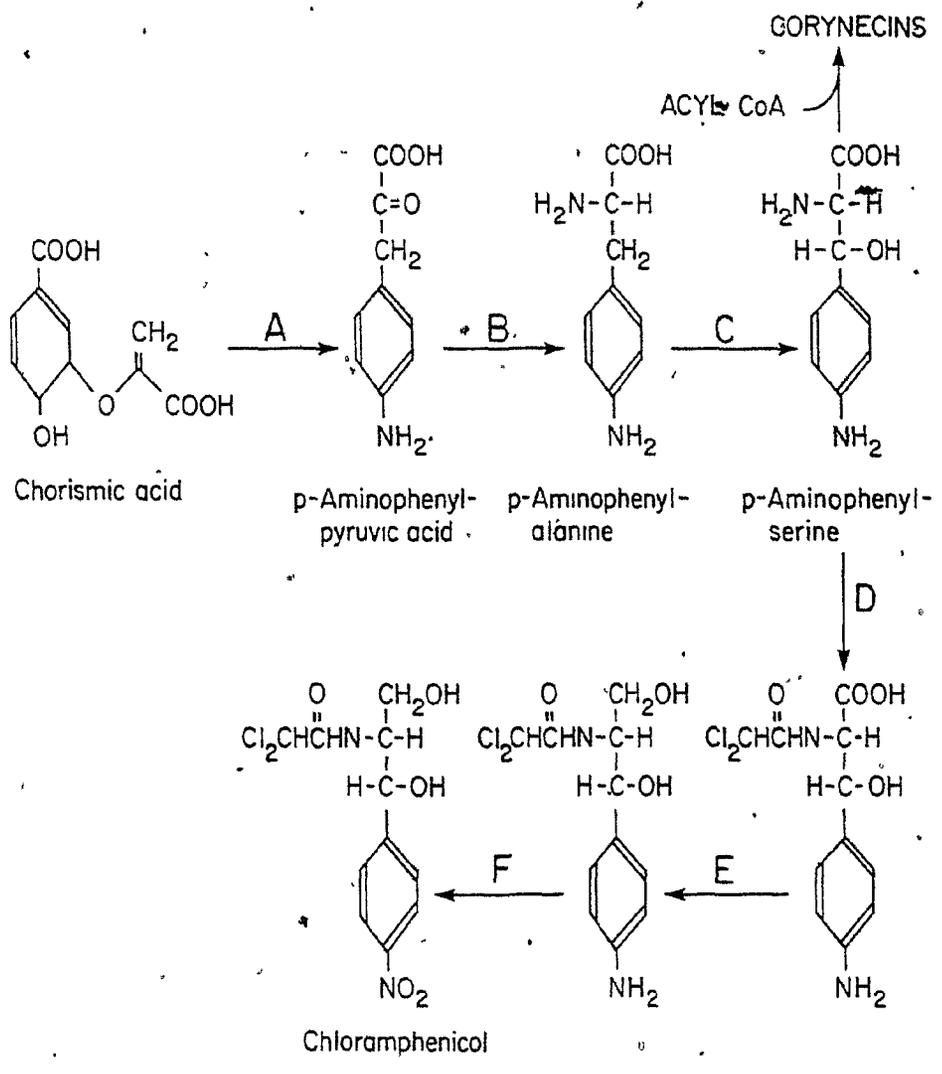
Studies using radiolabeled chloramphenicol precursors have provided much information on the biosynthesis of this antibiotic. Vining and Westlake (1964) demonstrated that while neither phenylalanine nor tyrosine act as direct precursors, chloramphenicol is synthesized via the shikimate pathway. Siddiqueullah et al. (1967) established that p-aminophenylalanine is a direct precursor, as are p-aminophenylserine and N-dichloroacetyl-p-aminophenylserinol (McGrath et al., 1968). From these and other data, it was proposed (Westlake and Vining, 1969) that the sequence of reactions in chloramphenicol biosynthesis following the formation of p-aminophenylalanine is as follows: hydroxylation at the  $\beta$ -carbon, substitution at the  $\alpha$ -amino group, reduction of the carboxyl to a hydroxymethyl group, and oxidation of the p-amino to a p-nitro group (Figure 2). Although studies using labeled precursors have

Figure 1. Structures of chloramphenicol and the corynecins.



	<u>R</u>
Corynecin I	COCH <sub>3</sub>
Corynecin II	COCH <sub>2</sub> CH <sub>3</sub>
Corynecin III	COCH(CH <sub>3</sub> ) <sub>2</sub>
Chloramphenicol	COCHCl <sub>2</sub>

Figure 2. Pathway for the biosynthesis of chloramphenicol.



provided considerable information on the biosynthesis of chloramphenicol, much remains to be learned concerning the regulation of this antibiotic and the specific enzymes involved in its biosynthesis. To date only one such enzyme, arylamine synthetase, which catalyzes the conversion of chorismic acid to p-aminophenylalanine, has been described (Jones and Vining, 1976). Mutants blocked in the chloramphenicol biosynthetic pathway would provide valuable tools for further study.

In recent years, great interest has been generated in the possibility of engineering industrial strains of microorganisms that produce increased antibiotic yields or that synthesize new antibiotics. Since the majority of antibiotics used clinically are produced by streptomycetes, most of the attention has turned to the study of their genetics and, in particular, the genetics of antibiotic production.

In many early studies, it was noted that antibiotic-nonproducing strains could be isolated at a high frequency following treatment of producers with various agents known to eliminate plasmids from Escherichia coli (Okanishi et al., 1970; Hotta et al., 1977; Omura et al., 1979). From this it was inferred that plasmids are involved in the synthesis of many antibiotics. Such was the case with chloramphenicol production in S. venezuelae; Akagawa et al. (1975) and Michelson and Vining (1978) reported a high frequency of nonproducers when a producing strain was exposed to intercalating dyes. Furthermore, Akagawa et al. (1975) demonstrated that none of the mutations thus produced showed linkage to chromosomal markers. A later study (Akagawa et al., 1979) conceded that some of the structural genes for chloramphenicol biosynthesis may be chromosomally-located but suggested that a plasmid is involved in the regulation of chloramphenicol production.

7  
With the development of improved plasmid isolation techniques, many studies now report a lack of correlation between plasmid loss and antibiotic nonproduction (Nojiri et al., 1980; Ikeda et al., 1981b). This observation, along with recent demonstrations that many of the phenotypic changes in Streptomyces caused by traditional plasmid curing agents are associated with DNA rearrangements rather than plasmid loss (Schrempf, 1983), requires that much of the earlier work implicating plasmid involvement in antibiotic production be reevaluated.

The purpose of this study was fourfold: i) to examine the possible role of plasmids in chloramphenicol production by surveying a number of producing strains for plasmid DNA and by determining the effect of plasmid loss on chloramphenicol production in any plasmid-containing strains identified; ii) to obtain through mutagenesis, chloramphenicol-nonproducing strains and by examining the nature of defects in these strains, to learn more about the chloramphenicol biosynthetic pathway; iii) to isolate chloramphenicol-pathway specific enzymes that may be altered in mutant strains; iv) using the mutations in strains blocked in chloramphenicol biosynthesis as genetic markers, to assign map locations to genes coding for pathway enzymes and, in particular, to determine whether or not any mutations are located on extrachromosomal elements.

## I. Chloramphenicol

### a. Biosynthesis

The structure of chloramphenicol suggests a biosynthetic relationship to the phenylpropanoid amino acids. However, when radiolabeled phenylalanine and tyrosine were added to chloramphenicol-producing cultures of Streptomyces sp. 3022a, subsequently classified as a S. venezuelae strain, these compounds were not incorporated intact into the p-nitrophenylserinol moiety of the antibiotic (Vining and Westlake, 1964). Although the biosynthetic route to chloramphenicol does not pass through phenylalanine or tyrosine, several lines of evidence suggested that these compounds share a common biosynthetic origin; [U-<sup>14</sup>C]glucose was incorporated into each with similar efficiency, and when cultures were fed [1-<sup>14</sup>C]glucose, [2-<sup>14</sup>C]glucose or [2-<sup>14</sup>C]glycine, labeling patterns in cellular phenylalanine and in chloramphenicol were similar. Labeled shikimic acid was incorporated selectively into the aromatic portions of chloramphenicol and into the phenylpropanoid amino acids. These data indicated that the pathways for chloramphenicol and the aromatic amino acids branched after a common reaction sequence resembling the shikimic acid - chorismic acid route found in other organisms. This conclusion was supported by analysis of the <sup>14</sup>C distribution in chloramphenicol obtained by supplementing cultures with [6-<sup>14</sup>C]glucose (O'Neill et al., 1973). Carbons -1, -2 and +6 accounted for 93% of the label, a pattern expected for compounds formed via the shikimate route. Similarly, Munroe et al. (1975) demonstrated by <sup>13</sup>C nuclear magnetic resonance (NMR) spectroscopy that chloramphenicol

produced when [6-<sup>13</sup>C]glucose was supplied as a precursor had an isotopic distribution consistent with biosynthesis via the shikimate pathway.

Siddiqueullah et al. (1967) reported that, of a wide variety of <sup>14</sup>C-labeled potential precursors tested, only p-aminophenylalanine was efficiently incorporated into chloramphenicol. Further studies (McGrath et al., 1968) established that p-aminophenyl[ $\alpha$ -<sup>14</sup>C,  $\alpha$ -<sup>15</sup>N]alanine was incorporated into the p-nitrophenylserinol portion of chloramphenicol with only a small change in the <sup>14</sup>C:<sup>15</sup>N ratio, demonstrating that the intact molecule was used for chloramphenicol biosynthesis. This conclusion was supported by the detection of p-aminophenylalanine in chloramphenicol-producing cultures of Streptomyces sp. 3022a.

Since p-aminophenylalanine served as a direct precursor of chloramphenicol, it seemed likely that the p-nitro group was derived by oxidation of the p-amino group: Studies showing that <sup>15</sup>N-labeled nitrate was not incorporated directly into the nitro group of chloramphenicol (Westlake and Vining, 1969), and the failure of p-nitrophenylalanine to serve as a precursor, provided additional evidence and suggested further that modifications elsewhere in the p-aminophenylalanine molecule precede oxidation of the p-amino group.

The finding that radioactivity was incorporated from p-aminophenyl[carboxyl-<sup>14</sup>C]serine specifically into the hydroxymethyl group of chloramphenicol suggested that  $\beta$ -hydroxylation follows the formation of p-aminophenylalanine (McGrath et al., 1968). This supported an earlier conclusion, based on the poor incorporation of radioactivity from <sup>14</sup>C-labeled phenylserine, that the  $\beta$ -hydroxy group of chloramphenicol is introduced later than the p-substituent. (Siddiqueullah et al., 1967). In addition, lack of specific incorporation of radioactivity from p-

nitrophenyl[carboxyl- $^{14}\text{C}$ ]serine indicated that oxidation of the *p*-amino group does not precede  $\beta$ -hydroxylation, while evidence that *p*-aminophenylserinol is not a precursor excluded prior reduction of the carboxyl group and indicated that  $\beta$ -hydroxylation is followed by *N*-acylation of the  $\alpha$ -amino group.

Efficient and specific conversion of dichloroacetyl-*p*-aminophenylserinol into chloramphenicol suggested that oxidation of the *p*-amino group is the ultimate step in chloramphenicol biosynthesis. Isolation of the *p*-amino analogue of chloramphenicol from cultures of *S. venezuelae* PD0745 (Stratton and Rebstock, 1963) supports this conclusion. The above data are consistent with the chloramphenicol biosynthetic scheme depicted in Figure 2.

Several studies have been conducted to determine the origin of the dichloroacetyl moiety of chloramphenicol. Wang et al. (1959) reported that dichloroacetic acid added to the medium of a producing culture inhibited incorporation of ionic  $^{36}\text{Cl}$  into chloramphenicol and suggested that the acid was incorporated via an intermediate into the antibiotic. However, these findings were not confirmed by Simonsen et al. (1978) who reported negligible incorporation of isotopically labeled dichloroacetic acid and favoured a pathway in which the free acid is not an intermediate.  $^{13}\text{C}$ -NMR spectroscopic analysis of the labeling pattern in chloramphenicol after feeding [1,2- $^{13}\text{C}$ ]acetate indicated that most of the precursor had been incorporated after  $^{13}\text{C}$ - $^{13}\text{C}$  bond fission. Since [2,3- $^{13}\text{C}$ ]succinic acid enriched only the carbonyl carbon of chloramphenicol, it was unlikely that acetate or a Krebs cycle intermediate acted as a direct precursor of the dichloroacetyl group. Mechanisms involving malonylcoenzyme A or malonyl-*p*-aminophenylserine as substrates

for the chlorination reaction were presented.

The intermediate at which the chloramphenicol pathway branches from the shikimate pathway was established to be chorismic acid by Jones and Westlake (1974). Cell extracts of Streptomyces sp. 3022a were found to catalyze the conversion of chorismic acid to an aromatic amine, later identified as p-aminophenylalanine (Jones and Vining, 1976). Prephenic and anthranilic acids did not serve as substrates for this enzyme, which was named arylamine synthetase. Activity peaked at the same time as, or just prior to, the appearance of chloramphenicol in culture fluids and was absent in chloramphenicol-nonproducing mutants.

b. Regulation of the Shikimate pathway

Phenylalanine, tyrosine, shikimic acid pathway intermediates and chloramphenicol do not inhibit the first specific enzyme, 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthetase, nor do any of these compounds repress its synthesis. However, inhibition was observed with D-ribose-5-phosphate (Lowe and Westlake, 1971).

Since DAHP synthetase did not appear to be subject to normal end product control, attention was directed to chorismate mutase, anthranilate synthetase and prephenate dehydratase, which function at the branch points of the pathway (Lowe and Westlake, 1972). Chorismate mutase activity was not inhibited by any of the compounds tested against DAHP synthetase, nor by mixtures of the three aromatic amino acids. Prephenate dehydratase, the branch-point enzyme leading specifically to phenylalanine, was inhibited by phenylalanine while anthranilate synthetase, the branch point enzyme leading specifically to tryptophan, was inhibited by tryptophan. Prephenate dehydrogenase, the enzyme

specific for tyrosine biosynthesis, could not be detected. The combined evidence indicates that the shikimate pathway in Streptomyces sp. 3022a is loosely controlled by feedback inhibition at the branch points. Failure to control by feedback the activity of the main shikimic acid pathway probably allows an accumulation of intermediates which can be alleviated by channelling them via chorismic acid into chloramphenicol biosynthesis (Lowe and Westlake, 1972).

c. Control of chloramphenicol biosynthesis.

To investigate the effect of chloramphenicol on its own biosynthesis, Malik and Vining (1972a) added  $^{14}\text{C}$ -labeled antibiotic to cultures of Streptomyces sp. 3022a growing in a medium containing  $^3\text{H}$ -labeled glucose and measured  $^{14}\text{C}$  and  $^3\text{H}$  in chloramphenicol recovered from the cultures. Estimates from these data of the amount of exogenously supplied antibiotic remaining and the amount formed by endogenous synthesis demonstrated clearly that chloramphenicol in the medium reduced the quantity of chloramphenicol produced.

Chloramphenicol added to producing cultures in concentrations that did not affect growth repressed arylamine synthetase but did not inhibit the enzyme (Jones and Westlake, 1974). The mechanism by which chloramphenicol controls its own biosynthesis differs, therefore, from that exerted by other shikimate pathway end products. This is not surprising in light of the observation (Legator and Gottlieb, 1953) that chloramphenicol does not accumulate intracellularly and also of the possibility (Malik and Vining, 1972b) that the producing organism develops resistance to chloramphenicol by introducing a permeability barrier at the cell envelope. It is, therefore, unlikely that chloramphenicol itself is an effector molecule for arylamine synthetase. Control is more

likely exerted through a buildup of intermediates within the cell. Support for this suggestion can be adduced from the observation that *p*-aminophenylalanine is a strong inhibitor of arylamine synthetase activity (Jones and Westlake, 1974).

d. Metabolic control of chloramphenicol biosynthesis

Antibiotics are secondary metabolites, not required for growth of the producing organism. In nutritionally rich media they are usually produced at high levels only after most of the cellular growth has occurred; their production often coincides with depletion of an essential nutrient (Martín and Demain, 1980). This suggests that system's regulating nutrient utilization may also be involved in controlling pathways leading to antibiotic production. However, Chatterjee and Vining (1981, 1982a and b) have demonstrated that, although carbon sources such as glucose are utilized preferentially by cultures of *S. venezuelae* strain 13s, mechanisms that control their utilization do not play a dual role in chloramphenicol regulation.

Optimum yields of chloramphenicol are obtained in media where the source of nitrogen is an amino acid supporting slow growth (Westlake et al., 1968), suggesting that biosynthesis of chloramphenicol might be controlled by nitrogen catabolite repression. Shapiro and Vining (1983), using stirred-jar cultures of *S. venezuelae* strain 13s under pH control, confirmed that nitrogen sources such as urea and ammonium sulfate which yielded the highest growth rates supported the poorest yields of antibiotic. Of the enzymes involved in nitrogen metabolism, glutamine synthetase and alanine dehydrogenase showed activities that were insensitive to changes in growth rate and nitrogen availability

whereas glutamate dehydrogenase and glutamate synthase were more active in cultures assimilating ammonium than in cultures growing on slowly-utilized nitrogen sources. The latter two enzymes are, therefore, possible links between the regulation of nitrogen assimilation and control of chloramphenicol biosynthesis.

e. Producer tolerance

Malik and Vining (1972b) reported that ribosomes from Streptomyces sp. 3022a bound chloramphenicol to the same extent as ribosomes from a sensitive strain of E. coli and demonstrated that the antibiotic inhibited incorporation of [U-<sup>14</sup>C]phenylalanine into polyphenylalanine in in vitro systems containing S. venezuelae strain 13s cell extracts. Similarly, addition of chloramphenicol to cultures growing in rich medium and producing little endogenous antibiotic inhibited the incorporation of [U-<sup>14</sup>C]leucine into mycelial protein. Addition of chloramphenicol to cultures producing comparable amounts of the antibiotic had no effect on protein synthesis.

When chloramphenicol was added to cultures in the early growth stage preceding rapid antibiotic biosynthesis, or to cultures growing in rich medium producing little antibiotic, a lag phase ensued; the length of the lag was proportional to the amount of antibiotic added. When growth resumed, inocula from the now-resistant cultures grew without a lag when transferred to fresh medium containing the same concentration of chloramphenicol. If the fresh medium contained a higher concentration of chloramphenicol than that to which a culture had previously been exposed, a growth delay was again encountered. Resistant cultures grown in the absence of chloramphenicol became fully sensitive in one transfer. These results indicate that chloramphenicol

tolerance in the producing organism is reversible and graded to the exposure level.

Chloramphenicol is metabolized by the producing organism, the first step being hydrolytic removal of the N-dichloroacetyl group, followed by reacylation to give N-acetyl p-nitrophenylserinol (Malik and Vining, 1970). Catabolism was fastest during rapid growth on a medium supporting low chloramphenicol production but was suppressed during the biosynthetic phase in cultures producing high levels of the antibiotic.

Cell extracts of S. venezuelae strain 13s contain an enzyme that catalyzes the hydrolysis of chloramphenicol (Malik and Vining, 1971). Chloramphenicol hydrolase activity was present in chloramphenicol-sensitive cultures and it did not increase during the development of resistance. It was concluded therefore that resistance in the producing organism is not solely due to inactivation of the antibiotic. Malik and Vining (1972b) postulated that exposure to chloramphenicol results in the development of a permeability barrier which bars the antibiotic from the sites of protein synthesis. Growth is inhibited until chloramphenicol hydrolase has inactivated chloramphenicol that entered the cells before establishment of the barrier. Since the inactivating enzyme is constitutive, the duration of the growth delay depends upon the initial concentration of antibiotic in the cells, which in turn relates to that in the medium.

## II. Plasmid Involvement in Antibiotic Production

Two features of antibiotic production in streptomycetes make the idea of plasmid involvement attractive: i) like such plasmid-deter-

mined features as antibiotic resistance, conjugational fertility and pathogenicity, the ability to produce an antibiotic is a dispensable function. ii) antibiotic production has frequently been observed to be an unstable trait (Sala and Westlake, 1966).

Plasmids have been implicated in the production of many antibiotics. Unfortunately the evidence is often limited to observations that treatment of antibiotic-producing strains with agents such as ethidium bromide, acriflavin dyes or high temperature, known to promote plasmid loss in E. coli, generates nonproducing strains at a high frequency. Such findings have been made for kasugamycin and aureothricin biosynthesis in Streptomyces kasugaensis (Okanishi et al., 1970), kanamycin biosynthesis in Streptomyces kanamyceticus (Hotta et al., 1977; Chang et al., 1978), paromomycin biosynthesis in Streptomyces rimosus forma paromomycinus (White and Davies, 1978) and streptomycin in Streptomyces bikiniensis (Shaw and Piwowarski, 1977). In light of evidence that agents such as ethidium bromide or acriflavin dyes frequently cause DNA rearrangements as well as plasmid elimination in Streptomyces (Schrempf, 1983) claims for plasmid involvement in the biosynthesis of an antibiotic based solely on curing data must be viewed with caution until more rigorous studies have been carried out.

### III. Plasmid Curing Studies

#### a. Spiramycin in Streptomyces ambofaciens

The unreliability of data from plasmid curing studies is evident in the case of spiramycin production by S. ambofaciens. Nonproducing strains were obtained at a frequency of about 10% by treatment with acriflavin (Omura et al., 1979). When a plasmid, pSA1, was detected in

cleared lysates of producing strains but not in nonproducers, it was presumed to be involved in spiramycin biosynthesis. However, an alternative plasmid isolation technique revealed that the spiramycin nonproducers harboured plasmid DNA indistinguishable from pSA1 in contour length and restriction cleavage pattern (Ikeda et al., 1981a). When the same producing strain was treated with presumptive curing agents, no correlation was seen between the generation of spiramycin-nonproducing progeny and those that lacked plasmid pSA1 (Ikeda et al., 1981b). It was concluded that the genes for spiramycin biosynthesis were not carried on this plasmid.

b. Neomycin production in *Streptomyces fradiae*

When plasmids pSF1 and pSF2 were isolated from a neomycin-producing strain of *S. fradiae* and nonproducing variants obtained by acridine dye treatment appeared to lack pSF1 and pSF2, the plasmids were suspected to have a role in the biosynthesis of this antibiotic (Yagisawa et al., 1978). However, closer examination of these strains showed that the parent and one of the "cured" nonproducers, A083, each harboured a single plasmid (Komatsu et al., 1981). Comparison of restriction endonuclease fragment patterns of the plasmids from A083, the parental strain and a high producing variant, H3, revealed that the plasmids from both H3 and A083 differed from the parental plasmid DNA in one small region of the plasmid map. The suggestion was made that this region might represent a "hot spot" for DNA rearrangements induced with curing agents, and these rearrangements were implicated in altered antibiotic production and resistance as well as in the plasmid copy number of the two mutant strains. As an alternative, the possibility that the plasmid

changes were merely coincidental to operative chromosomal mutations was not excluded.

c. Ribostamycin production in *Streptomyces ribosidificus*

The ribostamycin producer, *S. ribosidificus*, provides yet another example in which treatment with acriflavin yielded a high frequency of nonproducers yet failed to eliminate a resident plasmid (Nojiri et al., 1980). Comparison using restriction endonuclease digestion of plasmids from the producer and two acriflavin-derived nonproducer strains failed to reveal any differences.

d. Erythromycin production in *Streptomyces erythraeus*

Inconclusive data were derived from attempts to cure the erythromycin-producing organism, *S. erythraeus*, of the multiple plasmids detected in this strain (Yi-guang and Davies, 1981). Treatment with acridine or ethidium bromide yielded two variants, one blocked in erythromycin biosynthesis and one that produced high levels of the antibiotic. While the plasmid profiles of these two variants differed from the wild-type strain, it was not possible to conclude that any of the genes for erythromycin biosynthesis were located on the plasmids.

IV. Examples of Possible Plasmid-Determined Antibiotic Production

More compelling evidence of plasmid involvement in antibiotic production has been presented in a number of reports. This is of three main types: i) demonstration that antibiotic production determinants can be transferred at a frequency several orders of magnitude higher than that for known chromosomal markers, ii) demonstration of a lack of linkage between antibiotic determinants and known chromosomal

markers, and iii) demonstration by DNA hybridization using cloned determinants of antibiotic production as probes that these sequences have been lost from nonproducing strains.

a. Leupeptin production in *Streptomyces roseus*

Although leupeptin is not strictly an antibiotic, it is a closely related peptide secondary metabolite. Umezawa et al. (1978) observed that the ability to produce it was transferred from producing to non-producing strains in mixed cultures at a rate significantly higher than arginine or methionine chromosomal markers were transferred.

b. Tylosin production in *Streptomyces fradiae*

Preliminary studies indicated that some of the genes for tylosin biosynthesis in *S. fradiae* might be plasmid-encoded; in mixed cultures of two strains differing in their antibiotic sensitivities, auxotrophic requirements and ability to produce tylosin, tylosin production and resistance were transferred to about 8% of the tylosin-sensitive non-producers (Baltz et al., 1981). No chromosomal recombination was observed. Furthermore, in mixed cultures of tylosin-sensitive nonproducers and resistant mutants that accumulated tylosin precursors, many recipients of the resistance trait accumulated the corresponding precursors of the donor strains. These data suggests that some of the tylosin structural genes are located on a self-transmissible plasmid which also controls tylosin resistance. However, the putative plasmid has not yet been isolated.

c. Turimycin production in *Streptomyces hygrosopicus*

Following earlier studies (Kähler and Noack, 1974) with plasmid

curing agents that had indicated a possible plasmid involvement in turimycin production, Zippel et al. (1983) performed conjugational matings using auxotrophically-marked turimycin producers and non-producers. Auxotrophic markers were arranged in an order that minimized the proportion of recombinant genotypes requiring quadruple crossovers. When the turimycin marker was included in this sequence the frequency of the resulting recombinant genotypes requiring quadruple crossovers increased significantly. Moreover, protoplast fusion studies using the same parental strains assigned the turimycin genes to a different sector of the linkage map. These data were inconsistent with a single chromosomal location for turimycin genes and strengthened the suggestion of plasmid involvement in turimycin production. However, preliminary studies indicated the presence of a 6-Md plasmid in both the wild-type and nonproducing strains and the plasmid postulated to encode turimycin production was not clearly identified.

d. Actinomycin production in Streptomyces parvulus and Streptomyces antibioticus

Treatment of S. parvulus and S. antibioticus with acriflavin or novobiocin caused a high-frequency loss of the ability to produce actinomycin (Ochi and Katz, 1978). Following protoplast fusion between singly-auxotrophic producers and nonproducers, up to 95% of prototrophic progeny were actinomycin producers, whereas no actinomycin-producing progeny were detected following fusions between two nonproducing strains. When protoplasts of doubly-auxotrophic actinomycin-producing and nonproducing variants of S. parvulus were fused, 80-90% of the progeny in each recombinant class were actinomycin producers (Ochi and Katz, 1980). In addition, approximately 70% of regenerated colonies

possessing the nutritional markers of the nonproducing parent were producers. Thus, the ability to produce actinomycin was transferred independently of any nutritional marker. When the auxotrophic markers were ordered so as to minimize the proportion of progeny genotypes requiring quadruple crossovers, inclusion of the actinomycin marker in the sequence significantly increased the quadruple crossover frequency in each cross and resulted in an ambiguous map position for this marker. This observation was taken as further evidence for plasmid involvement in actinomycin production.

High frequency transfer of the ability to produce actinomycin was also seen following protoplast fusion using auxotrophic producing and nonproducing strains of S. antibioticus (Ochi, 1982). However, the determinant for actinomycin production was not transferred in conjugational matings, suggesting that it was located on a nonconjugative plasmid. When protoplasts prepared from an auxotrophic nonproducing strain of S. parvulus were transformed with DNA from lysed protoplasts of an actinomycin-producing strain, the frequency of transformation for chromosomal determinants was 20-fold higher than for the actinomycin-producing determinant. Ochi accounted for the low transformation frequency of the actinomycin determinant by suggesting that it was damaged by protoplast lysis. These results were considered to provide further evidence for the nonchromosomal location of the actinomycin genes.

e. Methylenomycin production in Streptomyces coelicolor A3(2) and Streptomyces violaceus-ruber SANK95570

Perhaps the most widely cited example of plasmid involvement in antibiotic production is that of SCPl in methylenomycin biosynthesis in

S. coelicolor A3(2). Kirby et al. (1975) noted that strains containing the plasmid SCP1 produced a diffusible inhibitor that retarded the growth of SCP1<sup>-</sup> strains as well as many other Streptomyces species and a wide spectrum of Gram-positive and Gram-negative genera. The inhibitory substance was identified by mass spectral analysis as methylenomycin A (Wright and Hopwood, 1976). Mutant strains unable to produce methylenomycin (Mmy<sup>-</sup>) but retaining antibiotic resistance (Mmy<sup>r</sup>) were isolated from a strain bearing the SCP1<sup>-</sup> plasmid carrying a cysB<sup>+</sup> gene (Kirby et al., 1975). Transfer of the plasmid into a cysB<sup>-</sup> SCP1<sup>-</sup> (Mmy<sup>-</sup> Mmy<sup>S</sup>) strain resulted in the recipients acquiring antibiotic resistance (Mmy<sup>r</sup>) and cysteine independence but remaining antibiotic nonproducers (Mmy<sup>-</sup>).

Further evidence that SCP1 carries the determinants for both methylenomycin biosynthesis and resistance was obtained when 16 antibiotic nonproducing strains were isolated. These fell into at least five phenotypic classes on the basis of cosynthesis, pigmentation, and examination of culture extracts by thin-layer chromatography (Kirby and Hopwood, 1976). The phenotype conferred by each mutation could be transferred with SCP1 to new recipients. Moreover, it was shown that two methylenomycin nonproducing species, Streptomyces lividans 66 and Streptomyces parvulus, gained the ability to produce methylenomycin upon interspecific conjugational transfer of SCP1.

Although SCP1 has never been isolated from S. coelicolor A3(2), Aguilar and Hopwood (1982) have isolated a large (110 Md) plasmid, pSV1, from S. violaceus-ruber SANK95570, the organism in which methylenomycin production was first discovered (Haneishi et al., 1974) and which was reported by Okanishi et al., (1980) to contain covalently closed circular DNA. That pSV1 coded for methylenomycin production and resis-

tance was suggested when all of the pSV1<sup>-</sup> colonies arising from regenerated protoplasts proved to be methylenomycin sensitive nonproducers. Supporting evidence came from interspecific crosses between S. lividans 66 and S. violaceus-ruber SANK95570. These gave methylenomycin resistant S. lividans progeny containing pSV1 and producing the antibiotic. Similarly, when pSV1 was used to transform protoplasts of S. lividans, all methylenomycin-resistant producers contained pSV1.

Further evidence that methylenomycin production genes are encoded by SCPI was provided by the mutational cloning experiments of Chater and Bruton (1983) in which attachment site-deleted phage  $\phi$ C31 KC400, carrying a viomycin resistance determinant, was used. This mutant phage transduces recipients to viomycin resistance only when its DNA contains a segment homologous to one in the recipient genome. If this condition is met the phage can integrate into the recipient genome at the site of homology.

To supply the required homologous DNA segment, fragments of the total DNA from an SCPI<sup>+</sup> strain of S. parvulus were inserted into  $\phi$ C31 KC400. Some of the recombinant phage were shown to harbour SCPI fragments by their ability to transduce viomycin resistance into SCPI<sup>+</sup> but not into an SCPI<sup>-</sup> strain of S. parvulus. Furthermore, hybridization could be detected between the DNA from two such phages and restriction endonuclease-generated fragments of total DNA from SCPI<sup>+</sup> but not SCPI<sup>-</sup> strains.

Chater and Bruton (1983) predicted that phage carrying DNA fragments of methylenomycin production genes would integrate into a recipient's methylenomycin production genes and thereby disrupt their expression. The recovery of methylenomycin-nonproducing mutants among

colonies transduced to viomycin resistance by phage carrying SCP1 fragments suggests that methylenomycin determinants are encoded by SCP1.

f. A-factor in *Streptomyces bikiniensis* and *Streptomyces griseus*

Cloning studies (Horinouchi et al., 1984) have provided evidence that A-factor, the autoregulator essential for streptomycin production and resistance as well as sporulation in *S. griseus* and *S. bikiniensis* (Hara and Beppu, 1982a and b), is encoded by a plasmid. Fragments of DNA from an A-factor-producing strain of *S. bikiniensis* were cloned into plasmid pIJ385. Transformation of A-factor-deficient mutants of *S. bikiniensis* or *S. griseus* with recombinant-plasmid DNA restored the ability of mutant strains to synthesize A-factor and concomitantly restored streptomycin production, streptomycin resistance and sporulation. A 3.8-kb fragment cloned in one recombinant plasmid contained the A-factor determinant. When this plasmid was nick-translated and used as a probe, it was found to hybridize with the total DNA from A-factor producing strains but not with DNA from A-factor nonproducing mutants, many of which were obtained by treatment with plasmid curing agents (Hara and Beppu, 1982a). This indicates that loss of A-factor in these strains is due to a deletion of DNA, most likely by plasmid elimination.

g. Chloramphenicol in *Streptomyces venezuelae*

Evidence for plasmid involvement in chloramphenicol production in *S. venezuelae* ISP5230 was presented by Akagawa et al. (1975). Chloramphenicol nonproducers were derived at a frequency of 2-5% after treatment with acriflavin. Crosses were carried out using multiply auxotrophic producers and nonproducers but recombinant frequencies were too low to exclude the possibility that progeny differing from parental

types in only one marker were revertants. A procedure was therefore devised for the determination of marker sequences which allowed such genotypes to be omitted. Eight markers were arranged in a circular sequence such that the frequency of recombinants requiring a quadruple crossover was minimized. When the chloramphenicol production (cpp) marker was placed between each adjacent pair of markers, the frequency of recombinants requiring quadruple crossovers increased significantly. Moreover, the location of cpp giving the minimum quadruple crossover frequency varied from cross to cross. Such results imply that the cpp marker is not located on the chromosomal linkage map. As Akagawa et al. (1975) pointed out, however, the possibility that the two might be nonallelic weakens this conclusion. The cpp<sup>+</sup> marker transferred to cpp<sup>-</sup> progeny only in crosses where chromosomal markers also transferred but this would be accounted for if the cpp markers were encoded by a non-conjugative plasmid.

In a later report (Akagawa et al., 1979), two of the apparent nonproducers used in the previous study were reported to actually produce chloramphenicol at low levels (up to 10  $\mu\text{g}\cdot\text{mL}^{-1}$ ). New nonproducing strains were therefore derived by treating an auxotrophic producer, SVM2, with a variety of alternative agents: acriflavin, high temperature, ultraviolet irradiation or nitrosoguanidine. The Cpp<sup>-</sup> derivatives obtained were divided into two groups: i) those obtained with potential plasmid curing agents and believed to be plasmidless (the two low producers from the previous study were added to this group); and ii) those generated only at a low frequency using "curing" agents or obtained by the use of nitrosoguanidine. Mutants in this latter group were believed to harbour chromosomal mutations. Unfortunately the

probable location of the structural block could be identified in only one of these mutants. Crosses between "plasmidless" strains and an auxotrophic producing strain failed to indicate a chromosomal location for the cpp marker, while crosses between mutants from the second group and the same producing strain did indicate a discrete chromosomal location for the cpp marker. Furthermore, crosses between nonproducing mutants bearing assigned chromosomal mutations and a "plasmidless" low producer yielded high producers among the progeny whereas crosses between pairs of "plasmidless" strains yielded only low producers. It was concluded, therefore, that most of the structural genes for the chloramphenicol pathway are located on the chromosome while a plasmid plays a role in increasing production levels. However, no plasmid has yet been isolated from this strain (Okanishi et al., 1980 ; Kirby et al., 1982).

Possible plasmid involvement in chloramphenicol production was also investigated by Michelson and Vining (1978) using S. venezuelae strain 13s. Treatment with intercalating dyes accelerated the high spontaneous generation of low and nonproducing variants. When eight of the nonproducing derivatives were subsequently examined for the presence of extrachromosomal DNA, all contained a plasmid with the same electrophoretic mobility as that of plasmid pUC3 (Malik and Reusser, 1979), which was present in the parental type (Ahmed and Vining, 1983). Furthermore, when a plasmidless derivative of strain 13s obtained by protoplast formation and regeneration was tested for chloramphenicol production, it produced titres comparable to those of the parent strain. These results indicate that pUC3 cannot be involved in chloramphenicol production.

When restriction endonuclease banding patterns of total DNA

extracts from four of the above nonproducing strains were compared with those from strain 13s; one strain showed a slightly different pattern, suggesting that curing agents affected antibiotic production in this strain by promoting chromosomal rearrangements.

## V. Chromosomally-determined Synthesis of Antibiotics

### a. Oxytetracycline production in *Streptomyces rimosus*

One of the first well-documented examples of chromosomally-determined antibiotic production was that of oxytetracycline biosynthesis in *S. rimosus* (Rhodes et al., 1981; Boronin and Mindlin, 1971; Alačević, 1973). Fifty-seven mutants blocked in production of this antibiotic were grouped into two classes on the basis of cosynthesis tests and their ability to convert intermediates to oxytetracycline. One class of mutants was unable to produce a cofactor (CSFI) essential for the reduction of oxydehydrotetracycline, the final step of the pathway. Mutants of the other class were blocked in the tetracycline biosynthetic pathway. Crosses between auxotrophic oxytetracycline producers and nonproducers indicated that the oxytetracycline and CSFI pathway genes were clustered in two chromosomal locations. Mutations in the oxytetracycline pathway before the formation of 4-aminodimethylaminoanhydrotetracycline mapped to a single region, and mutations in the pathway after this intermediate and all CSFI mutations mapped to a second region, diametrically opposite to the first. No evidence of plasmid involvement was obtained.

### b. Actinorhodin in *Streptomyces coelicolor* A3(2)

In addition to methylenomycin, *S. coelicolor* A3(2) produces an

antibiotic, actinorhodin, which acts as a pH indicator (blue at alkaline pH, red at acid pH). Seventy-six actinorhodin-nonproducers were grouped into seven types on the basis of the color of diffusible pigments formed, antibiotic activity and cosynthesis reactions (Rudd and Hopwood, 1979). Crosses between multiply marked representatives of each mutant class and auxotrophic producers indicated that the actinorhodin genes are located in a short chromosomal segment between hisD and guaA on the genetic map. The chromosomal location of the act genes has been confirmed by the recent cloning of genes for the entire biosynthetic pathway using S. coelicolor A3(2) as the source of DNA (Malpartida and Hopwood, 1984).

c. Undecylprodigiosin in Streptomyces coelicolor A3(2)

A second pigmented antibiotic, the biosynthesis of which is unrelated to that of actinorhodin, is also produced by S. coelicolor A3(2) (Rudd and Hopwood, 1980). This has been tentatively identified as undecylprodigiosin (Feitelson and Hopwood, 1983). Because actinorhodin or related compounds obscured this red pigment, nonproducing mutants (red) were isolated from a class I actinorhodin nonproducer in which synthesis of all pigments related to this product is blocked.

The 37 red mutants were grouped into five classes on the basis of cosynthesis tests. When multiply-marked representatives from each class were crossed with an actinorhodin-nonproducing, undecylprodigiosin-producing strain, all five classes of red genes were located in a short chromosomal region between cysD and leuB.

d. CDA in Streptomyces coelicolor A3(2)

A fourth antibiotic, called CDA (Calcium Dependent Antibiotic),

produced by S. coelicolor A3(2) inhibits Gram-positive bacteria by producing transmembrane channels that conduct monovalent cations in the presence of calcium (Lakey et al., 1983). Twenty-six CDA-nonproducing mutants were isolated, several of which were also defective in the production of aerial mycelium (Hopwood and Wright, 1983). Mapping studies indicated that all nonpleiotropic cda mutations were located in one region of the S. coelicolor map. Mutations that rendered strains aerial mycelium negative and CDA-nonproducing mapped to the same chromosomal regions as previously studied bald mutations variously designated bld or amy (aerial mycelium-less).

#### VI. Genetic Recombination in Streptomyces

Genetic recombination was first shown to occur in Streptomyces by Sermonti and Spada-Sermonti (1955). By growing two doubly-auxotrophic strains of S. coelicolor in mixed culture, then harvesting and plating the resultant spores on selective media, prototrophs could be isolated as well as progeny with mutant characteristics of both parental strains. Reports of similar findings in S. rimosus (Alikhanian and Mindlin, 1957) and Streptomyces griseoflavus (Saito, 1958) soon followed.

Hopwood (1959) was the first to demonstrate that quantitative data obtained from crosses between doubly auxotrophic strains of S. coelicolor A3(2) (actually an S. violaceus-ruber strain and therefore different from Sermonti's strain) could be used to establish linkage relationships. In his procedure, spores from a mixed culture of two mutant strains were plated on four selective media. The phenotypes of recombinants that appeared on each medium were determined by replica plating to diagnostic media. In general there was good agreement

between estimates of the frequency of a given phenotype recovered on different selective media. Also, where they could be recovered, both members of a pair of complementary genotypes had approximately the same frequency. The relative frequencies of each recombinant class were therefore considered to be free of possible bias caused by differential viability, and could be used to determine the arrangement of the auxotrophic markers.

Preliminary mapping data suggested two linkage groups in S. coelicolor A3(2) (Hopwood, 1959; Hopwood and Sermonti, 1962). These two groups were later found to be linked, forming a single circular linkage group (Hopwood, 1965 and 1966b). A detailed map of the chromosome of S. coelicolor A3(2) has been constructed (Hopwood and Sermonti, 1962; Hopwood, 1965, 1966a and 1967).

Mapping studies were soon extended to other Streptomyces species. The linkage maps of Streptomyces glaucescens (Baumann et al., 1974), S. rimosus (Friend and Hopwood, 1971; Alačević et al., 1973), Streptomyces bikiniensis var. zorbonensis (Coats and Roeser, 1971) and S. venezuelae 3022a (Francis et al., 1975) all showed notable similarity to that of S. coelicolor A3(2).

Although details of the events underlying the process of genetic exchange in Streptomyces remain obscure, Hopwood's work with S. coelicolor A3(2) has established several points (Hopwood, 1967): i) mating occurs between hyphae and the process resembles conjugation in gram-negative bacteria in that it results in the transfer of long segments of linked genes; ii) mating results in the production of merozygotes, each containing a complete chromosome from one parent and a chromosome fragment from the other; iii) when mating occurs, the formation of a

haploid recombinant can be interpreted as the consequence of an even number of crossover events between the whole chromosome and the chromosome fragment within the merozygote.

## VII. Fertility Systems

Sermonti and Casciano (1963) published the first description of different fertility types in S. coelicolor A3(2). In crosses between auxotrophically marked mutants, they observed a group of strains ( $R^-$ ) which gave no recombinants when crossed with each other, but which were fertile when crossed with any other ( $R^+$ ) strains. Crosses between  $R^+$  strains were also fertile.

Hopwood et al. (1969) found that fertility variants of S. coelicolor A3(2) could be isolated after treatment with ultraviolet light. When these variant ultrafertile (UF) strains were crossed with strains of the initial fertility (IF) type (corresponding to that of the wild type S. coelicolor A3(2)), virtually all of the progeny were the IF type. Transfer of this IF trait occurred at a frequency 10 000 times higher than that observed for chromosomal markers (Vivian, 1971). This led to the hypothesis that the factor responsible for the difference in fertility must be inherited independently of the chromosome. It was proposed that the change from IF to UF involved loss of a plasmid designated SCP1.

A third fertility type, called NF, was subsequently identified when crosses in which it was mated with UF (SCP1<sup>-</sup>) strains gave virtually all recombinant progeny (Hopwood et al., 1969). In contrast to previous crosses where the frequencies of complementary recombinant genotypes

were equal (that is, there was a statistically equal contribution of genetic material by the two parents), crosses between NF and SCP1<sup>-</sup> strains were polarized, the NF strain always donating a population of fragments and the SCP1<sup>-</sup> strain always acting as a recipient. Furthermore, a fragment from the 9 o'clock region of the NF parent was obligately inherited by all haploid progeny, NF marker frequencies declining in both clockwise and counterclockwise directions from the 9 o'clock position. Crosses between NF strains and IF (SCP1<sup>+</sup>) strains were also polar, with the NF strains donating a nonrandom population of fragments to the progeny (Vivian, 1970). The segregation of fertility in NF X IF crosses indicated that the fertility determinants in the 9 o'clock map region were located on the chromosome. From these observations, Vivian (1971) proposed that although NF strains harbour SCP1, the plasmid is not autonomous but probably associated with the chromosome in the 9 o'clock position.

Hopwood and Wright (1973) isolated a strain of S. coelicolor that donated the marker cysB<sup>+</sup> with a very high frequency. Donation of alleles at loci known to be closely linked to cysB occurred at a much lower rate. This strain was taken to be a fourth fertility type wherein SCP1 is an autonomous plasmid that carries a region of the chromosome. Thus SCP1 bears some resemblance to the F plasmid of E. coli K-12 (Curtiss, 1969) in that it can be lost to yield SCP1<sup>-</sup> strains, it can carry a chromosomal insertion or it can become integrated into the chromosome.

An attempt to isolate SCP1 from an SCP1<sup>+</sup> strain gave a 31.6-kb plasmid (Schrempf et al., 1975) but the same plasmid was isolated from SCP1<sup>-</sup> strains as well as from strains in which SCP1 appeared to have

integrated into the chromosome or acquired a chromosomal insertion (Schrempf and Goebel, 1977). On the other hand, no plasmid DNA could be isolated from S. lividans strains into which SCP1 had been transferred by conjugation. Consequently, the 31.6-kb DNA component was considered to be a second plasmid, SCP2. It was proposed that SCP2 accounted for the appreciable fertility of SCP1<sup>-</sup> X SCP1<sup>-</sup> matings. Repeated attempts to isolate SCP1 have been unsuccessful.

With one SCP1<sup>-</sup> strain an unusually high frequency of recombinants was obtained in crosses with other SCP1<sup>-</sup> strains (Bibb et al., 1977). This enhanced fertility could be transferred infectiously and was attributed to a variant form of SCP2 designated SCP2\*<sup>-</sup>. SCP2\*<sup>-</sup> X SCP2<sup>+</sup> crosses produced about 500 times as many recombinants as SCP2<sup>+</sup> X SCP2<sup>+</sup> crosses. SCP2\*<sup>-</sup> did not promote the exchange of any preferred region of the chromosome but simply enhanced generalized recombination.

By screening SCP2\*<sup>-</sup> colonies for spontaneous loss of their enhanced fertility when crossed with an SCP2<sup>+</sup> tester strain, SCP2<sup>-</sup> strains lacking the autonomous 31.6-kb plasmid were isolated. SCP2<sup>-</sup> X SCP2<sup>-</sup> matings were almost completely sterile while SCP2<sup>+</sup> X SCP2<sup>-</sup> were more fertile than SCP2<sup>+</sup> X SCP2<sup>+</sup> matings. Transfer of SCP2 or SCP2\*<sup>-</sup> to SCP2<sup>-</sup> strains in mixed cultures was almost 100% efficient.

Although plasmids appear to play an important role in recombination in S. coelicolor A3(2), the actual mechanisms by which they promote chromosome exchange remain obscure. The existence of fertility plasmids in other species has now been demonstrated (Hopwood et al., 1983; Bibb et al., 1981; Kieser et al., 1982) and it may be that plasmids are necessary for gene exchange at significant frequencies in Streptomyces.

Friend et al. (1978) attributed to plasmid loss the high fertility

of certain S. rimosus strains that arose spontaneously at a rate of 0.2 to 0.8% from the wild type population. In crosses involving one highly fertile parent, such as R559 (ade<sup>-</sup> cys<sup>-</sup> rib<sup>-</sup>), and one of low (normal) fertility, such as R276 (his<sup>-</sup>), about 50% of progeny showing the auxotrophic phenotype of the R559 parent now had low fertility when tested again in a cross with R276. The fact that strain R559 was infectiously converted to low fertility rather than strain R276 being converted to high fertility suggested that the original high fertility of R559 resulted from loss of plasmid that was retained in R276, rather than mutation of a low fertility plasmid to a high fertility variant. Fertility in S. rimosus was therefore proposed to be governed by a plasmid SRP1. The importance of this putative plasmid in promoting gene recombination in S. rimosus was emphasized by the very low frequency at which SRP1<sup>-</sup> X SRP1<sup>-</sup> crosses yielded recombinants.

It is interesting to note that SRP1<sup>+</sup> X SRP1<sup>-</sup> crosses were up to 100-fold more fertile than SRP1<sup>+</sup> X SRP1<sup>+</sup> crosses. In this, SRP1 seems to resemble SCP2. Although the phenomenon is as yet unexplained, Friend et al. (1978) have suggested that it is an example of the surface exclusion described for eubacterial plasmids such as F, where F<sup>+</sup> X F<sup>+</sup> crosses give few recombinants compared with F<sup>+</sup> X F<sup>-</sup> crosses (Hayes, 1968).

#### VIII. Lethal Zygotis

Bibb et al. (1977) observed that in SCP2<sup>\*</sup> X SCP2<sup>-</sup> matings, transfer of the SCP2<sup>\*</sup> plasmid was attended by retardation of growth of a portion of the plasmid-negative hyphae. This could be seen as a narrow zone of retarded development of the SCP2<sup>-</sup> culture when an SCP2<sup>\*</sup> patch was

replicated to a background lawn of spores of an SCP2<sup>-</sup> strain. This zone followed the contours of SCP2<sup>\*</sup> patch suggesting a requirement for hyphal contact rather than diffusion of an exogenous inhibitor. In sporulating lawns made by mixing a minority of SCP2<sup>\*</sup> spores with a majority of SCP2<sup>-</sup> spores, narrow circular zones surrounded each SCP2<sup>\*</sup> colony and were referred to as "pocks". The inhibition reaction was called "lethal zygosis" by analogy with the lethal zygosis reaction observed in *E. coli* (Skurray and Reeves, 1973). In the latter organism lethal zygosis is thought to result from simultaneous mating between one F<sup>-</sup> cell and several Hfr cells.

The phenomenon of lethal zygosis appears to be widespread among the streptomycetes and, although its cause is unclear, it has been correlated in many instances with plasmid transfer (Murakami et al., 1983; Ohnuki et al., 1983). Because of this, it has been useful in detecting transformants in molecular cloning experiments (Thompson et al., 1982; Katz et al., 1983).

Observations of lethal zygosis have also led to the discovery of new plasmids. Spores of *S. lividans* 66 isolated from mating mixtures with *S. coelicolor* A3(2) elicited lethal zygosis (i.e. were Ltz<sup>+</sup>) on confluent lawns of Ltz-sensitive *S. lividans* 66 (Bibb et al., 1981). A series of covalently closed circular DNA molecules, designated SLP1.1, SLP1.2, etc., ranging in size from 9.37 to 12.96 kb and sharing a large common fragment, could be isolated from the Ltz<sup>+</sup> *S. lividans* isolates whereas no DNA corresponding to these plasmids could be detected in the original parental strains. Labeled probes of SLP1.1 detected homologous sequences in total DNA preparations of *S. coelicolor* A3(2) but not in *S. lividans* 66. In addition to this, SLP1 plasmids could be constructed in

vitro by digesting and religating total DNA from S. coelicolor A3(2) and transforming S. lividans 66 with the mixture. Mutations mapping to the S. coelicolor A3(2) chromosome abolished the ability of SLP1 plasmids to elicit lethal zygosis and it was concluded that the SLP1 plasmids were excised from the S. coelicolor chromosome during mating with S. lividans. The SLP1 plasmids acted as fertility factors.

Pocking has frequently been correlated with the transfer of fertility plasmids in Streptomyces but this is not always the case. Plasmid pJV1 from Streptomyces phaeochromogenes NRRLB-3559 is transferred at high frequency from plasmid<sup>+</sup> to plasmid<sup>-</sup> strains, and this transfer is attended by a lethal zygosis reaction. However, pJV1 apparently does not promote chromosomal recombination (Mosher, 1984).

Whether lethal zygosis is always associated with plasmid transfer also remains to be determined. Two self-transmissible plasmids tentatively identified in S. lividans 66 by their lethal zygosis reactions could not be isolated (Hopwood et al., 1983). When protoplasts of S. lividans 66 were regenerated (a procedure shown to eliminate plasmids, Hopwood et al., 1981) numerous isolates were obtained on which the parental strain could elicit lethal zygosis. Two types of inhibition zones were attributed to two different plasmids, SLP2 and SLP3. As predicted, putative SLP2<sup>-</sup> strains produced lethal zygosis on SLP3<sup>-</sup> strains while SLP3<sup>-</sup> strains produced inhibition zones on SLP2<sup>-</sup> strains. Putative SLP2<sup>-</sup> SLP3<sup>-</sup> strains were also isolated. These were sensitive to inhibition by both SLP3<sup>-</sup> and SLP2<sup>-</sup> strains but did not elicit a lethal zygosis reaction. SLP2 acted as a sex factor but SLP3 did not promote recombination.

In several cases thus far examined, plasmids associated with a

lethal zygotis reaction appear to encode genes associated with the Ltz phenotype. Kicsor et al. (1982) found that insertion into or deletion of a segment of the fertility plasmid pIJ101 either abolished or reduced the lethal zygotis reaction. Similarly, Kobayashi et al. (1984) found that insertion of a resistance gene into a BclI site of plasmid pTA4001 abolished the Ltz phenotype. Clearly further study is required to explain this phenomenon.

I. Strains

Streptomyces venezuelae ISP5230 (ATCC10712) was originally received from E. Wellington (Stuttard, 1982). Streptomyces venezuelae strains NRRLB-902 and NRRL2277, Streptomyces phaeochromogenes strains NRRLB-3559 and NRRLB-2119, and Streptomyces omiyaensis NRRLB-1587 were received from the Northern Regional Research Laboratories, Peoria, Illinois. Streptomyces venezuelae PDO4828 and PDO5080 were provided by Parke, Davis and Co., Detroit, Michigan. Streptomyces lividans strains 3141 (erroneously sent instead of strain 3041) and 3041, containing plasmids pIJ701 (Kieser et al., 1983) and pIJ303 (Katz et al., 1983), respectively, were from the culture collection of the John Innes Institute, Norwich, U.K. In the following study, unless otherwise noted, S. venezuelae refers to strain ISP5230 and S. phaeochromogenes or SP1 to strain NRRLB-3559. Strain HP-1 is a single colony isolate of S. venezuelae ISP5230 which produced up to  $110 \text{ ug. mL}^{-1}$  of chloramphenicol on glucose-isoleucine medium.

Mutant S. venezuelae strains VS113, VS160, VS191, VS192, and VS194 were received from C. Stuttard, as were strains VS99, VS130, VS141, VS143, VS144, VS146, VS153 and VS163, which had all been obtained by mutagenizing spores of  $\text{Cml}^-$  mutants with long-wavelength ultraviolet light in the presence of 8-methoxypsoralen and selecting auxotrophic derivatives. Strain VS206 was derived as a spontaneous streptomycin resistant mutant by plating VS191 spores on MYM medium containing streptomycin sulfate ( $100 \text{ ug. mL}^{-1}$ ). Strain EB116 was obtained from SP1 by ethidium bromide treatment.

## II. Chemicals

Bacto-agar, bacto-peptone, casamino acid, yeast extract, nutrient broth and malt extract were purchased from Difco Laboratories, Detroit, Michigan; lysozyme (grade I), BamHI and p-aminophenylalanine were from Sigma Chemical Co., St. Louis, Missouri; ribonuclease A and pronase E were from Boehringer Mannheim Canada, Dorval, Québec. Chloramphenicol and D-threo-1-p-nitrophenyl-2-acetamido-1,3-propanediol were gifts from Parke, Davis and Co.. 2(S)-Dichloroacetamido-3-(p-acetamidophenyl)propane-1-ol (Wat et al., 1971), N-acetyl-p-nitrophenylalanine, p-nitrophenylalanine, p-aminophenyl[carboxyl-<sup>14</sup>C]alanine, p-aminophenyl[carboxyl-<sup>14</sup>C]serine (McGrath et al., 1968) and N-dichloroacetyl-p-nitrophenylalanine (synthesized by K. Paramasigamani in this laboratory) were supplied by L.C. Vining.

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 hydrochloric acid. It was purified by preparative thin-layer chromatography and crystallization from dichloroethane to give colorless needles, m.p. 108-110°C; R<sub>f</sub> (thin-layer chromatography, system A), 0.24. The <sup>1</sup>H-nuclear magnetic resonance (NMR) spectrum, recorded in chloroform-d at 80 MHz on a Varian model FT-80 spectrometer with tetramethylsilane as the internal reference, showed signals at  $\delta$  1.04 (t, 3H, CH<sub>3</sub>, J<sub>CH<sub>3</sub>,CH<sub>2</sub></sub> 7.6 Hz), 2.15 (q, 2H, CH<sub>2</sub>, J<sub>CH<sub>2</sub>,CH<sub>3</sub></sub> 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<sub>2',3'</sub> 3.3 Hz), 6.15 (bd, 1H, NH, J<sub>NH,2'</sub> 7.6 Hz), 7.88 (AA'BB', 4H, H-2, H-3, H-5, H-6,  $\Delta\nu_{AB}$  51.1 Hz,  $N = \underline{J}_{AB} + \underline{J}_{AB}' =$

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  $-10^{\circ}\text{C}$  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 colorless needles, m.p.  $130-131^{\circ}\text{C}$ ; Rf (thin-layer chromatography, system A), 0.30. The  $^1\text{H-NMR}$  spectrum, recorded as above, showed signals at  $\delta$  1.01 (d, 3H,  $\text{CH}_3$ ,  $J_{\text{CH}_3, \text{H}}$  6.9 Hz), 1.06 (d, 3H,  $\text{CH}_3$ ,  $J_{\text{CH}_3, \text{H}}$  6.9 Hz), 1.62 (bs, 1H, OH), 2.27 (qn, 1H,  $\text{CH}$ ,  $J_{\text{H}, \text{CH}_3}$  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).

### III. Media

All media were heat-sterilized in an autoclave at  $121^{\circ}\text{C}$  and 15 pounds per square inch for 20-25 min.

Complex (GNY) liquid medium contained:

Glycerol	20 mL
Nutrient broth	8.0 g
Yeast extract	3.0 g
Dipotassium hydrogen phosphate	5.0 g
Distilled water	1000 mL

Complex (MYM) media contained:

Maltose	4.0 g
Yeast extract	4.0 g
Malt extract	10.0 g
Agar	20.0 g
Distilled water	1000 mL

Chloramphenicol-production (glucose-isoleucine) medium (Chatterjee

et al., 1983) contained:

Glucose	30 g
Magnesium sulfate heptahydrate	0.2 g
Potassium dihydrogen phosphate	4.5 g
Dipotassium hydrogen phosphate	10.5 g
Isoleucine	7.5 g
Sodium chloride	90 mg
Calcium chloride	90 mg
Ferrous sulfate heptahydrate	9.0 mg
Zinc sulfate heptahydrate	4.0 mg
Cupric sulfate pentahydrate	0.18 mg
Boric acid	26 µg
Ammonium molybdate tetrahydrate	17 µg
Manganese sulfate tetrahydrate	27 µg
Distilled water	1000 mL

To determine which of eight amino acids supported the best chloramphenicol production, cultures of S. venezuelae and S. phaeochromogenes were grown in the production medium modified by substituting each of the following amino acids in turn for isoleucine: proline (6.6 g), DL-serine (6.0 g), leucine (7.5 g), alanine (5.1 g), glycine (4.3 g), threonine (6.8 g), and valine (6.7 g).

Minimal medium (MM) (Hopwood, 1967), used to test for prototrophy and, supplemented with various growth factors, for auxotrophic requirements, consisted of:

Maltose (instead of glucose)	10.0 g
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Asparagine	0.5 g
Dipotassium hydrogen phosphate	0.5 g
Magnesium sulfate heptahydrate	0.2 g
Ferrous sulfate heptahydrate	9.0 mg
Agar	15.0 g
Distilled water	1000 mL

Growth factors were sterilized by filtration and were added to autoclaved minimal medium as required. The concentrations were as follows:

Amino acids,  $56 \mu\text{g}\cdot\text{mL}^{-1}$ , except histidine,  $75 \mu\text{g}\cdot\text{mL}^{-1}$ , nucleic acid bases,  $11.25 \mu\text{g}\cdot\text{mL}^{-1}$ , vitamins  $0.75 \mu\text{g}\cdot\text{mL}^{-1}$ . Streptomycin sulfate was added to a final concentration of  $50 \mu\text{g}\cdot\text{mL}^{-1}$ .

Defined GGC medium in which S. phaeochromogenes was grown for plasmid isolation (Okanishi, 1980) contained:

Glycerol	4.0 mL
Glycine	4.0 g
Casamino acid	4.0 g
Magnesium sulfate heptahydrate	1.0 g
Potassium dihydrogen phosphate	2.0 g
Disodium hydrogen phosphate	3.1 g
Trace element solution	4.0 mL
Distilled water	1000 mL

The trace element solution consisted of:

Zinc chloride	40 mg
Ferric chloride	120 mg
Cupric chloride dihydrate	10 mg
Manganous chloride tetrahydrate	10 mg
Boric acid	1.6 mg
Ammonium molybdate tetrahydrate	10 mg
Distilled water	1000 mL

This trace element solution was also used in medium R2, R3 and P:

Medium S (Okanishi et al., 1974) in which S. phaeochromogenes and S. venezuelae strains were grown to prepare protoplasts consisted of:

Glucose	10.0 g
Peptone	4.0 g
Yeast extract	4.0 g
Magnesium sulfate heptahydrate	0.5 g
Potassium dihydrogen phosphate	2.0 g
Dipotassium hydrogen phosphate	4.0 g
Distilled water	1000 mL

After the solution had been autoclaved, glycine was added to a final concentration of 3% for S. phaeochromogenes and 1% for S. venezuelae cultures.

Protoplast regeneration (R2) medium (Okanishi et al., 1974), on which protoplasts of S. phaeochromogenes strains were plated, consisted of:

Sucrose	103 g
Glucose	10.0 g
Casamino acid	0.1 g
Potassium sulfate	0.25 g
Magnesium chloride hexahydrate	10.12 g
Agar	22.0 g
Distilled water	800 mL

After the solution had been autoclaved the following sterile solutions were added to each 800 mL:

Trace element solution	2 mL
Potassium dihydrogen phosphate (0.5%)	10 mL
Calcium chloride dihydrate (3.68%)	80.2 mL
Proline (20%)	15 mL
N-tris-(Hydroxymethyl)methyl-2-aminoethane sulphonate (0.25 M, pH 7.2)	100 mL
Sodium hydroxide (1 N)	5 mL

Protoplast regeneration (R3) medium (modified from Shirahama et al., 1981), on which protoplasts of S. venezuelae strains were plated, consisted of:

Sucrose	100 g
Maltose	10.0 g
Peptone	4.0 g
Magnesium chloride hexahydrate	8.0 g
Agar	22 g
Distilled water	700 mL

After autoclaving, the following sterile solutions were added to each 700 mL:

Potassium chloride (5.0%)	10 mL
Calcium chloride dihydrate (2.0%)	100 mL
Dipotassium hydrogen phosphate (2.0%)	10 mL
N-tris-(Hydroxymethyl)methyl-2-aminoethane sulfonate (0.25 M, pH 7.2)	100 mL

Medium P (Okanishi et al., 1974), used to prepare protoplasts of S. phaeochromogenes and S. venezuelae strains, consisted of:

Sucrose	103 g
Potassium sulfate	0.25 g
Trace element solution	2 mL
Magnesium chloride hexahydrate	2.03 g
Distilled water	800 mL

After autoclaving the following sterile solutions were added:

Potassium dihydrogen phosphate (0.5%)	10 mL
Calcium chloride dihydrate (3.68%)	80.2 mL
N-tris-(Hydroxymethyl)methyl-2-aminoethane sulfonate (0.25 M, pH 7.2)	100 mL

#### IV. Culture Conditions

##### a. Maintenance

Spores of S. venezuelae, its mutant strains, and S. phaeochromogenes were preserved as 20% aqueous glycerol suspensions at -20°C. Micrococcus luteus was maintained on GNY agar slants stored at 5°C.

b. Glycerol spore suspensions

Sterile water was added to the surface of well-sporulated cultures growing on MYM agar plates. The surface was scraped with a sterile loop to dislodge the spores. The fluid was then removed, filtered through sterile cotton wool to remove mycelial fragments and centrifuged. The supernatant fluid was decanted and the spore pellet was resuspended in 20% (v/v) aqueous glycerol.

c. Vegetative inocula

Vegetative mycelial suspensions used as culture inocula were prepared by transferring a loopful of spores from a stock spore suspension in glycerol to 50 mL of GNY medium in a 250-mL Erlenmeyer flask. Cultures were grown for 48 h at 27°C on a shaker with a platform rotating at 220 rpm describing an arc of 18 cm.

d. Chloramphenicol production in liquid culture

A 1% (v/v) vegetative inoculum was aseptically added to 50-mL portions of chloramphenicol-production medium in 500-mL Erlenmeyer flasks and incubated with shaking for 7 d at 27°C. When large volumes of culture fluids were required to isolate chloramphenicol-pathway intermediates or shunt products from mutant strains, vegetative inocula (1%) were added to 500 mL of chloramphenicol-production medium in 2-L Erlenmeyer flasks which were incubated at 27°C on a rotary shaker for 7 d.

e. Cultures for plasmid isolation (*Streptomyces phaeochromogenes*)

A 1% inoculum was added to 100 mL of GGC medium in a 500-mL Erlenmeyer flask and the culture was incubated overnight with shaking at

27°C.

f. Cultures of Streptomyces phaeochromogenes and Streptomyces venezuelae strains for protoplast formation

Mycelium of S. phaeochromogenes or strain EB116 to be used for protoplast formation was obtained by transferring a loopful of spores to 25 mL of medium S (3% glycine) and incubating for 5 d with shaking at 27°C. Mycelium of S. venezuelae strains to be used for protoplast formation was prepared by transferring a loopful of spores to 25 mL of medium S (1% glycine) and incubating the culture for 24 h with shaking at 27°C.

g. Growth

Growth was estimated as increase in optical density at 640 nm ( $OD_{640}$ ) of samples having a 1.2-cm light path.

V. Assays

a. p-Aminophenylalanine  $\beta$ -hydroxylase

The reaction mixture for p-aminophenylalanine  $\beta$ -hydroxylase contained the following in a 3.0-mL final volume:

p-Aminophenyl[carboxyl- $^{14}$ C]alanine (2.7 mM)	1 mL
Phosphate buffer (0.1 M)	1 mL
Cell extract	1 mL

The mixture was incubated with shaking at 27°C for 30 min. To prepare cell extracts, mycelium from a 3-d culture grown in chloramphenicol-production medium was collected and washed twice with 0.1 M KCl, then resuspended in 4 volumes of lysis buffer (10 mM potassium phosphate, pH 7.0, 7 mM mercaptoethanol and 1 mM disodium EDTA). After cooling to 4°C, the cells were disrupted with a sonic oscillator at intensity 7,

using six 30-s bursts. The lysate was cleared by centrifugation.

b. Chemical assays

i. Aromatic amino- or nitro-compounds

Aromatic amino- or nitro- compounds were assayed according to a modification of the procedure of Levine and Fischbach (1951). Sodium hydroxide (1 mL of a 0.3 M solution) and a fresh solution of sodium dithionite (1 mL containing 25 mg) were added to 1 mL of culture supernatant fluid. After mixing, the reactants were kept at room temperature for 15 min, then treated successively with 0.5 mL of 5% (w/v) sodium nitrite, 0.1 mL of concentrated hydrochloric acid and, after 5 min with 0.1 mL of a 50% (w/v)-aqueous solution of urea. After 10 min, 2.5 mL of a 2% (w/v) solution of sulphamic acid in sodium dihydrogen phosphate buffer (2.2 M) was added and the solution was allowed to stand for 5 min before mixing with N-1-napthylethylenediamine dihydrochloride (1 mL of a 0.2% (w/v) solution). After 15 min, absorbance at 550 nm was measured and compared with a standard curve prepared using authentic chloramphenicol.

To assay for aromatic amino compounds following acid hydrolysis of culture broths, the above procedure was followed except that 3.0 M sodium hydroxide (1 mL) was added as the first step and the addition of sodium dithionite was omitted.

ii. p-Aminopheny[carboxyl-<sup>14</sup>C]serine

Sodium hydroxide (200  $\mu$ L of a 1 M solution) and saturated sodium metaperiodate (500  $\mu$ L) were added to a 3-mL enzyme incubation mixture. After vortexing, the mixture was allowed to stand for 1 min, then 500 mg of activated charcoal was added and the mixture was filtered through a

medium porosity sintered glass filter. A 250- $\mu$ L portion of the filtrate was added to 15 mL of scintillation fluid (2,5-diphenyloxazole (4 g) in a mixture of toluene (600 mL) and ethanol (378 mL)). Radioactivity was measured with a liquid scintillation spectrometer (model 1215, LKB-Wallac, Helsinki).

c. Bioassay for chloramphenicol

Spores from the colonies to be tested were spread in patches on 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 *M. luteus*. The overlaid plates were incubated overnight and inhibition zones were examined.

VI. Plasmid Detection, Isolation and Elimination

a. Buffers

TES buffer (Okanishi, 1980) used in the large-scale isolation of plasmid DNA from *S. phaeochromogenes* contained tris-(hydroxymethyl) aminomethane (Tris)-hydrochloride, disodium ethylenediamine tetraacetic acid (EDTA), and sodium chloride, each at a 25 mM concentration. The pH was adjusted to 7.4.

Digestion buffer (Malik, 1979) in which DNA was digested with Bam HI contained:

Tris-hydrochloric acid, pH 7.4	10 mM
Magnesium chloride	5 mM
Dithiothreitol	1 mM
Sodium chloride	50 mM

Tris-acetate buffer, used both for making agarose gels and as a

running buffer for electrophoresis, consisted of:

Tris-base	40 mM
Sodium acetate	5 mM
Disodium EDTA	1 mM

The pH was adjusted to 7.8.

b. Plasmid detection

Spores of Streptomyces strains were spread in patches on MYM agar and incubated overnight at 27°C. A loopful of mycelium from each patch was then examined for the presence of plasmid DNA using the rapid alkaline extraction procedure of Birnboim and Doly (1979).

c. Large scale isolation of plasmid DNA

Plasmid DNA was isolated by centrifugation in cesium chloride-ethidium bromide of sodium lauryl sulfate-cleared lysates using a procedure based on that of Okanishi et al. (1980). Cells (1-2 g wet weight) from an overnight culture in GGC medium were washed with TES buffer, resuspended in 30 mL of 0.1 N ammonium hydroxide containing 10 mM disodium EDTA, and incubated at 37°C for 20 min. The cells were then washed and resuspended in 38 mL of double-strength TES buffer. Lysozyme (1 mL of a 40 mg.mL<sup>-1</sup> solution) and RNase (0.5 mL of a 5 mg.mL<sup>-1</sup> solution) were added and the mixture was incubated for 1 hr at 37°C. Sodium lauryl sulfate (6 mL of a 10% (w/v) solution) was added and the mixture was incubated another 20 min to obtain a cleared lysate. Sodium chloride (5 M) was added to a final concentration of 1 M and the mixture was kept in an ice bath for 2.5 h, then centrifugated for 15 min at 0°C and 1200xg. The supernatant fluid was mixed with pronase E (incubated for 1 h at 37°C in advance) at a final concentration of 100 ug.mL<sup>-1</sup> and the solution was held at 37°C for 20 min. Polyethylene glycol 6000 (40% w/v) was added to a final concentration of 10% and kept at 5°C for 16-18 h. It

was then centrifuged at 2000xg for 20 min and the sedimented material redissolved in 2.5 mL of TES buffer and dialyzed against 1 L of TES buffer for 3-4 h.

After dialysis, the DNA solution was diluted to 10.6 mL with TES buffer. Ethidium bromide (0.2 mL of a 10 mg.mL<sup>-1</sup> solution) and cesium chloride (10.4 g) were added to give a final volume of 13.5 mL. The solution was centrifuged at 100,000xg and 17°C for 24 h, after which the plasmid band was removed and the plasmid-containing solution was extracted with isoamyl alcohol to remove the ethidium bromide. The solution was then dialyzed for 24 h against a buffer containing 1 mM Tris-hydrochloride, pH 7.0, and 0.01 mM disodium EDTA, and freeze dried.

d. Agarose electrophoresis

Samples of digested DNA were loaded on to horizontal slab gels of 0.7% (w/v) agarose in Tris-acetate buffer and subjected to electrophoresis for 16 h at 15 mA using Tris-acetate running buffer.

e. Staining of agarose gels

After electrophoresis the gels were stained by soaking in ethidium bromide solution (0.1 µg.mL<sup>-1</sup>) for 15 min in the dark, then destained in an excess of water for 30 min. The DNA fragments were located by viewing under ultraviolet light of 300-nm wavelength.

f. Photography of agarose gels

Gels were photographed using Polaroid type 55 Positive/Negative 4x5 Land film, with a Polaroid M-P4 Land camera.

g. Ethidium bromide treatment of *Streptomyces phaeochromogenes*

Spores of *S. phaeochromogenes* were inoculated into 50 mL of GNY medium containing either 10  $\mu\text{g.mL}^{-1}$ , 15  $\mu\text{g.mL}^{-1}$  or 20  $\mu\text{g.mL}^{-1}$  of ethidium bromide and the cultures were grown for 2-3 d. Samples were removed, vortexed vigorously to break up mycelial fragments, diluted, and plated on MYM agar. Single colonies were screened for plasmid using the rapid alkaline extraction technique (Birnboim and Doly, 1979).

VII. Probing of Total Cellular DNA with  $^{32}\text{P}$ -Labeled pJV1.a. Buffers

SSC (20x) consisted of:

Sodium chloride	3 M
Sodium citrate, pH 7.0	0.3 M

Neutralization buffer consisted of:

Tris-hydrochloride, pH 7.5	1 M
Sodium chloride	0.6 M

Prehybridization buffer consisted of:

Sodium chloride	0.75 M
Sodium citrate, pH 7.0	75 mM
Sodium phosphate, pH 7.0	0.1 M
Polyvinylpyrrolidone 360	0.02%
Bovine serum albumin	0.02%
Ficoll	0.02%
Salmon sperm DNA	0.005%
EDTA	10 mM
Sarkosyl	0.5%

Nick translation buffer consisted of:

Tris-hydrochloride, pH 7.5	50 mM
Magnesium chloride	5 mM
Dithiothreitol	1 mM
dGTP	1 $\mu\text{M}$
dCTP	1 $\mu\text{M}$
dTTP	1 $\mu\text{M}$

b. Isolation of total cellular DNA

A small quantity of mycelium from patches of S. phaeochromogenes strains grown overnight on MYM agar was added to 475  $\mu\text{L}$  of TES buffer. Lysozyme (25  $\mu\text{L}$  of a 50  $\text{mg}\cdot\text{mL}^{-1}$  solution in TES buffer) was added and the mixture incubated for 1 h at 37°C. Sarkosyl (25  $\mu\text{L}$  of a 10  $\text{mg}\cdot\text{mL}^{-1}$  solution) was added and the resulting lysate treated sequentially with RNase A (5  $\mu\text{L}$  of a 10  $\text{mg}\cdot\text{mL}^{-1}$  solution, 1 h) and pronase (5  $\mu\text{L}$  of a 0.02  $\text{mg}\cdot\text{mL}^{-1}$  solution, 1 h). It was then extracted twice with phenol. The aqueous phase was reextracted twice with chloroform and the DNA was precipitated with 2 volumes of cold ethanol.

c. Nick translation

Nick translation buffer (50  $\mu\text{L}$  of double-strength buffer) was added to approximately 1  $\mu\text{g}$  of pJV1 DNA, prepared according to the large-scale isolation procedure, in 45  $\mu\text{L}$  of 10  $\text{mM}$  Tris, pH 7.5.  $^{32}\text{P}$ -labeled dATP (3  $\mu\text{L}$  of a 15  $\mu\text{Ci}\cdot\mu\text{L}^{-1}$  solution), DNase I (1  $\mu\text{L}$  of a 0.5- $\mu\text{g}\cdot\text{mL}^{-1}$  solution), and DNA polymerase I (2-4 units) were added and the mixture was incubated at 37°C for 35 min. It was then incubated at 100°C for 5 min and stored at -15°C until required.

d. Hybridization

BamHI digests of total cellular DNA and pJV1 DNA were electrophoresed in a 0.7% agarose gel, then irradiated for 90 min. using a 30W germicidal lamp at a distance of 30 cm. The DNA was denatured by soaking the gel in 500 mL of denaturant (0.2 M sodium hydroxide and 0.6 M sodium chloride) for 40 min. The gel was neutralized by soaking in 500 mL of neutralization buffer and the DNA was transferred to a nitrocellulose filter using the method of Southern (1975). After

drying, the filter was soaked in 10 mL of prehybridization solution for 24 h at 65°C, then 5 mL of the solution was removed and the labeled pJV1 probe was added. The filter was hybridized at 65°C for 24 h, then washed twice for 30 min with 4x SSC buffer, once for 60 min with 2x SSC buffer and once for 30 min successively with 1x SSC, 0.3x SSC and 0.1x SSC buffer. The filter was then dried overnight and exposed for 20 h to Kodak X-Omat XAR5 film at -70°C.

#### VIII. Transformation of Protoplasts

##### a. Formation of Protoplasts

Protoplasts were prepared by the method of Hopwood and Wright (1978). Mycelium from 5-d cultures of S. phaeochromogenes or of strain EB116 in medium S was washed twice with 20-mL volumes of 0.3 M sucrose and suspended in 1.5 mL of a lytic mixture which consisted of lysozyme (10 mg) in 10 mL of medium P, sterilized by passing through a 0.45 $\mu$ m filter. The mycelium suspension was incubated at 37°C for up to 2 h. Medium P (4 mL) was then added and the mixture was drawn repeatedly through the orifice of a 10-mL pipette and finally filtered through sterile cotton wool. The protoplasts were diluted with medium P and plated on R2 medium. The proportion of unprotoplasted colony-forming units in the mixture was estimated by making parallel dilutions in sterile distilled water and plating on R2 medium.

Protoplasts of S. venezuelae and strains VS160, VS144, VS130, and VS143 were prepared as above with the exception that mycelium from a 24 h culture in medium S containing 1% glycine was used. Protoplasts were regenerated on R3 medium.

b. Transformation

i. DNA isolation

Plasmids pIJ701, pIJ702 and pIJ303 were isolated from cultures of S. lividans grown overnight in liquid MYM medium. The method of Birnboim and Doly (1979) was used with the following modification: after the first ethanol precipitation, DNA pellets from 4 tubes were combined and resuspended in 400  $\mu$ L of TES buffer. The solution was extracted once with an equal volume of phenol saturated with TES buffer. Plasmid DNA was precipitated twice with ethanol and resuspended in 50-100  $\mu$ L of medium P.

ii. Transformation

Approximately  $10^9$  protoplasts in 100-200  $\mu$ L of medium P were combined with 1  $\mu$ g of plasmid DNA in 50-100  $\mu$ L of medium P and 0.5 mL of a 20% solution of polyethylene glycol 1000. The mixture was left at room temperature for 1 min then 0.5 mL of a 10% solution of polyethylene glycol 1000 was added. The mixture was left for 3 min. Medium P (4 mL) was then added and the suspension centrifuged at a low speed. The resulting pellet was resuspended in a small volume of medium P then plated on medium R3. The resulting lawn of growth was allowed to sporulate. The spores were harvested and approximately  $10^8$  spores plated on MYM medium containing 200  $\mu$ g/mL of thiostrepton.

IX. Isolation and characterization of chloramphenicol nonproducing mutants of Streptomyces venezuelae

a. Mutagenesis

i. Mutagenesis with ultraviolet light in the presence of 8-methoxysporalen

Spores were suspended in 0.015 M phosphate buffer, pH 7.0,

containing 8-methoxy-psoralen ( $0.1 \text{ mg.mL}^{-1}$ ) for 30 min. The spore suspension was then exposed to long wavelength (366 nm) ultraviolet light ( $7.5 \text{ J.m}^{-2}$ ) from a Blak-Ray 13-100A ultraviolet lamp (Ultra-Violet Products, Inc., San Gabriel, California) for 15 min. The spore suspension was serially diluted and plated on MYM agar. This procedure gave about 1% survival of colony-forming units when viabilities were compared before and after irradiation.

ii. Mutagenesis with short-wavelength ultraviolet light.

Spore suspensions in  $0.1 \text{ M}$  phosphate buffer, pH 7.0, were irradiated for 4 min at  $0.8 \text{ J.m}^{-2}$  using a 254 nm germicidal lamp (Ultra-Violet Products, Inc., San Gabriel, California). The irradiated suspension was serially diluted and plated on (i) MYM agar or (ii) MYM agar containing caffeine ( $500 \text{ } \mu\text{g.mL}^{-1}$ ).

iii. Mutagenesis with ethyl methanesulfonate (EMS)

To a suspension of spores in  $0.1 \text{ M}$  phosphate buffer, EMS (final concentration  $70 \text{ mg.mL}^{-1}$ ) was added and the mixture was shaken for 2 h at  $27^\circ\text{C}$ . Spores were collected on a membrane filter, washed with 250 mL of phosphate buffer and resuspended in a small volume of the buffer. Finally, they were plated to obtain isolated colonies on MYM agar.

b. Isolation of chloramphenicol-nonproducing mutants

Colonies that grew from mutagenized spores were allowed to sporulate, streaked in patches on MYM agar and tested for chloramphenicol production using the bioassay procedure. Inocula from patches that did not inhibit the indicator bacteria 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 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. Those giving positive tests were extracted with ethyl acetate, initially at neutral pH and again after acidification to pH 2.0. The extracts were concentrated separately and examined by thin-layer chromatography.

c. Compounds A, B, and C from mutant Cm1-2

The supernatant fluid (2 L) from cultures of mutant Cm1-2 grown in chloramphenicol-production medium was extracted with three 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 (1.4x15 cm). The column was developed with successive 40-mL portions of chloroform-ethyl acetate mixtures in the ratios (9:1, v/v), (4:1, v/v), (1:1, v/v), ethyl acetate, ethyl acetate-acetone mixtures in the ratios (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 thin-layer chromatography (system A) showed three aromatic nitro-compounds A, B, and C. Fractions containing each 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. The structures of compounds A and B were examined using  $^1\text{H-NMR}$  spectroscopy.

d. 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 (2.5x30 cm) of Dowex 50x8 ( $H^+$ ) 50-100 mesh (Bio-Rad Laboratories, Richmond, California) 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 (1.5x20 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. The residues were examined by thin-layer chromatography using systems C and D. The residue from Cml-4 was examined using  $^1H$ -NMR spectroscopy.

e. 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 thin-layer chromatography in system C.

f. Hydrolysis of culture supernatant fluids

Concentrated hydrochloric acid (0.3 mL) was added to 10-mL test tubes containing clarified broths (2 mL) from cultures of each mutant grown in chloramphenicol-production medium. The test 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. The validity of the method for measuring N-acylated aromatic amines in culture supernatant fluids was established with known amounts of 2-dichloroacetamido-3-(p-acetoamidophenyl)propan-1-ol.

g. Feeding with p-aminophenylalanine

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.

h. Crossfeeding

To observe crossfeeding 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 earlier to detect antibiotic production. To test for crossfeeding 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 production colorimetrically.

i. 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 (Macherey-Nagel and

Co., Duren); 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) and sprayed in turn with aqueous stannous chloride and with a solution of p-dimethylaminobenz-aldehyde in 2 M hydrochloric acid. Zones containing aromatic nitro- or amino-compounds became yellow. For preparative chromatograms, 20x20-cm plates of silica gel F254 (0.2 mm thickness; Machery-Nagel and Co., Duren) 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 (Analtech Inc., Newark, Delaware). The chromatograms were developed in 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).

j. <sup>1</sup>H-Nuclear magnetic resonance spectroscopy

Spectra were recorded using a Varian model FT-80 nuclear magnetic resonance spectrometer with tetramethylsilane in chloroform-d as the external reference.

X. Chloramphenicol Resistance in Strain HP-1 and Nonproducing Mutants

To test for chloramphenicol resistance at the beginning of the growth phase, 500-mL flasks containing chloramphenicol-production medium (50 mL) with and without chloramphenicol at a final concentration of 150  $\mu\text{g}\cdot\text{mL}^{-1}$  received 1% inocula from 48-h GNY cultures of strains HP-1 or Cml-9. Cultures were grown for 6 d and the  $\text{OD}_{640}$  of each culture was measured periodically. To test for chloramphenicol resistance in 4 d-cultures of strains HP-1 and each Cml mutant in chloramphenicol-

production medium, a sterile aqueous solution of chloramphenicol was added to a final concentration of  $150 \mu\text{g}\cdot\text{mL}^{-1}$ . Control cultures received no chloramphenicol. After 12-13 d, 1% inocula were transferred from chloramphenicol-supplemented cultures of the Cml mutants to fresh flasks of production medium with and without chloramphenicol. These cultures were grown for 7 d. To test for reversion of the mutants to the chloramphenicol-producing phenotype, clarified broths of the unsupplemented cultures were then tested for the presence of the antibiotic using the colorimetric procedure. In the case of strain HP-1, 1% inocula from 7-d chloramphenicol supplemented cultures were transferred to fresh production medium with and without chloramphenicol as well as to GNY medium (25 mL in a 125-mL flask). After 24 h the GNY culture was tested for chloramphenicol sensitivity by inoculation into production medium with and without chloramphenicol.

Reversibility of chloramphenicol resistance in mutants Cml-1, Cml-8, Cml-10 and Cml-12 was examined by transferring 1% inocula from resistant cultures grown for 7 d in the absence of chloramphenicol to flasks of production medium with and without chloramphenicol. The cultures were grown for 7 d.

## XI. Genetic Mapping in *Streptomyces venezuelae*

### a. Crosses between differentially-marked strains

Crosses were performed by the procedure of Hopwood (1967). Spores of parental strains were mixed and spread on MYM agar and allowed to grow until sporulation occurred. Progeny spores were harvested, diluted and plated on selective media. This consisted of minimal medium supple-

mented with appropriate growth factors, chosen to allow growth of recombinant types but not the parental strains. Patches of parental strains were replicated on to each selective medium as controls. After sporulation, recombinant colonies were picked from the initial selective media and patched on agar media of the same composition. After sporulation these patches were replica plated to diagnostic media. Alternatively, the selective plates on which recombinants first arose were sometimes replicated directly to diagnostic media.

b. Mapping the chloramphenicol locus

Recombinant progeny from crosses between chloramphenicol-producing and nonproducing mutants were picked from diagnostic plates and restreaked on MYM agar to obtain single colonies. After sporulation, these colonies were again replica plated to diagnostic media to confirm their phenotypes. Single colonies were patched on to MYM agar and tested for chloramphenicol production using the bioassay procedure.

## RESULTS

I. Growth and Chloramphenicol Production on Various Amino Acids

To select a suitable chloramphenicol-production medium, eight amino acids were tested as nitrogen sources for their ability to support synthesis of the antibiotic in cultures of S. phaeochromogenes and S. venezuelae. Chloramphenicol production closely paralleled the growth on each amino acid. However, lower concentrations of chloramphenicol were accumulated by cultures growing on those amino acids such as alanine or glycine that supported rapid growth than on amino acids such as isoleucine or leucine that supported very slow growth (Table 1). Overall, isoleucine was the best nitrogen source for obtaining maximum chloramphenicol production.

II. Screening of Chloramphenicol-producing Strains for Plasmid DNA

Eight chloramphenicol-producing strains were examined for the presence of ccc DNA using the rapid alkaline-extraction technique. Of these eight, five were strains of S. venezuelae (ATCC10712, NRRLB-902, NRRL2277, PDO4828 and PDO5080), two were strains of S. phaeochromogenes (NRRLB-2119 and NRRLB-3559) and one was a strain of S. omiyaensis (NRRLB-1587). Only in S. phaeochromogenes NRRLB-3559 could ccc DNA be detected. Density-gradient centrifugation of a cleared lysate of this strain yielded plasmid DNA with corresponding electrophoretic mobility on agarose gels. The plasmid has been designated pJV1. It has a molecular weight of 10.8 kb and contains three cutting sites for the restriction enzyme BamHI, four for SmaI and one site for EcoRI. Details of the characterization of pJV1, have been reported (Doull et

Table 1. Effect of nitrogen sources on growth and chloramphenicol production in cultures of *S. venezuelae* and *S. phaeochromogenes*\*

Nitrogen Source	<i>S. venezuelae</i>		<i>S. phaeochromogenes</i>	
	$t_{max}^{**}$ (days)	$y_{max}^{**}$ ( $\mu\text{g}\cdot\text{mL}^{-1}$ )	$t_{max}^{**}$ (days)	$y_{max}^{**}$ ( $\mu\text{g}\cdot\text{mL}^{-1}$ )
Isoleucine	7	63	7	57
Leucine	7	76	7	38
Valine	6	48	6	64
Proline	5	56	5	27
Threonine	5	23	5	33
Serine	5	36	5	32
Glycine	5	48	5	27
Alanine	5	27	3	22

\*Cultures were grown for 7 days in chloramphenicol-production medium with each amino acid as the sole nitrogen source. Samples were taken each day and the  $\text{OD}_{640}$  and chloramphenicol titres measured.

\*\* $t_{max}$  = day on which cultures reached their maximum  $\text{OD}_{640}$ ;  
 $y_{max}$  = maximum concentration of chloramphenicol accumulated by the culture.

al., 1983). No plasmid DNA could be detected after density-gradient centrifugation of cleared lysates of S. venezuelae ATCC10712.

### III. Elimination of pJV1

To determine whether pJV1 had a role in chloramphenicol biosynthesis, attempts were made to eliminate it from cultures of S. phaeochromogenes. For this purpose, protoplast formation and regeneration was selected as the method of choice, since it has been reported to eliminate plasmid DNA from up to 25% of regenerant colonies of S. coelicolor A3(2) (Hopwood, 1981). Furthermore, it was thought that protoplast formation and regeneration would be less likely to promote DNA rearrangements or mutations than traditional curing agents such as the acridines and ethidium bromide (Shrempf and Goebel, 1979).

When mycelium of S. phaeochromogenes was treated to form protoplasts and plated on regeneration medium, approximately 70% of the resultant colonies were derived from osmotically fragile units. Of sixty such colonies screened for the presence of plasmid DNA using the rapid alkaline-extraction technique, all harboured ccc DNA identical in electrophoretic mobility to pJV1.

In a further attempt to eliminate pJV1, S. phaeochromogenes cultures were grown in GNY medium in the presence of ethidium bromide at concentrations of 10, 15 and 20  $\mu\text{g.mL}^{-1}$ . Sixty single colony isolates obtained from these cultures all contained plasmid DNA of the same mobility in agarose gels as pJV1. However, one of the isolates, EB116, which was obtained from a culture grown for 3'd in the presence of 10  $\mu\text{g.mL}^{-1}$  ethidium bromide, consistently yielded less plasmid DNA than did

the parental strain (Figure 3). Single-colony isolates of strain EB116 all yielded relatively small amounts of pJV1 DNA. Protoplasts of EB116 were prepared and regenerated and the resulting colonies again tested for the presence of plasmid DNA. One out of 25 colonies screened contained no detectable plasmid DNA (Figure 4).

The possibility that pJV1 had become integrated into the chromosome of this apparently pJV1<sup>-</sup> strain was tested by Southern hybridization. Total DNA was digested with BamHI, electrophoresed, transferred to a nitrocellulose filter and exposed to a <sup>32</sup>P-labeled probe of pJV1. The probe did not hybridize with DNA from the pJV1<sup>-</sup> strain; it did hybridize with fragments from the wild-type strain. (Figure 5)

#### IV. Chloramphenicol Biosynthesis in Strains SP1, SP2 and EB116.

Streptomyces phaeochromogenes (henceforth referred to as SP1), its plasmid-less derivative (SP2) and the ethidium bromide-treated strain EB116, were grown for 6 d in chloramphenicol-production medium. No significant differences were found when growth and chloramphenicol broth titres were compared. (Figure 6) Comparisons of sporulation and of sensitivity to tetracycline, ampicillin, and streptomycin between SP1 and SP2 also failed to show any appreciable differences. (Dough et al., 1983). Also, examination of SP1 and SP2 for restriction endonuclease SphI activity showed it to be present in both cultures.

#### V. Isolation of Chloramphenicol-nonproducing Mutants

To determine whether genetic markers for chloramphenicol production and chromosomally-located genes for primary pathways are linked, chlor-

Figure 3. Plasmid DNA isolated from Streptomyces phaeochromogenes using the rapid alkaline-extraction technique. Lanes 1 and 4 - strain SP1; lanes 2 and 3 - strain EB116. Band a represents the open circular, band b the linear and band c the covalently closed circular (ccc) form of the plasmid.

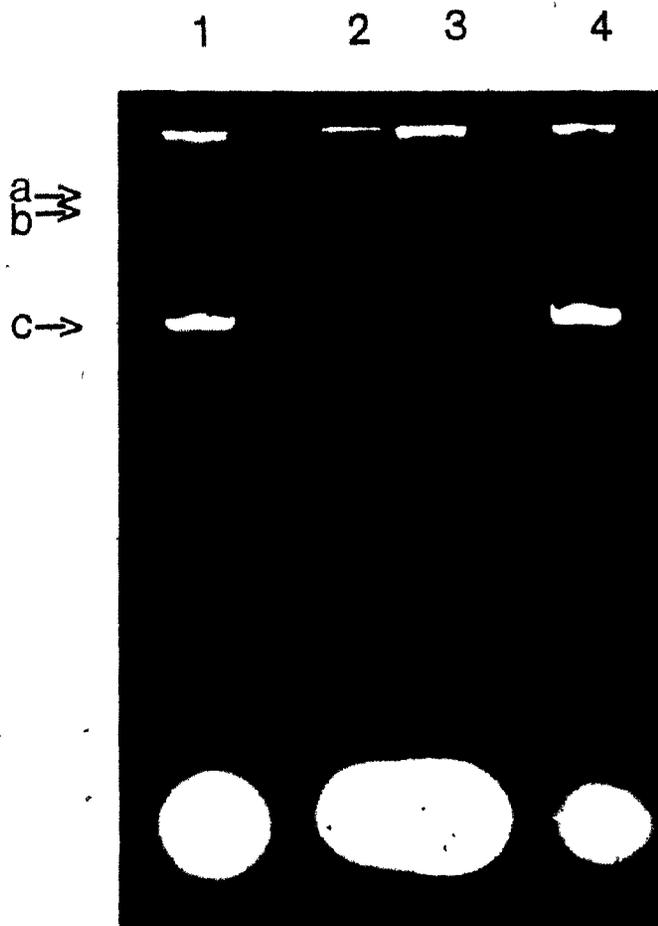


Figure 4. Rapid alkaline-extraction of strain EB116 and the pJV1-  
cured strain, SP2. Lanes 1 and 4 - strain EB116; lanes 2  
and 3 - strain SP2.

1 2 3 4

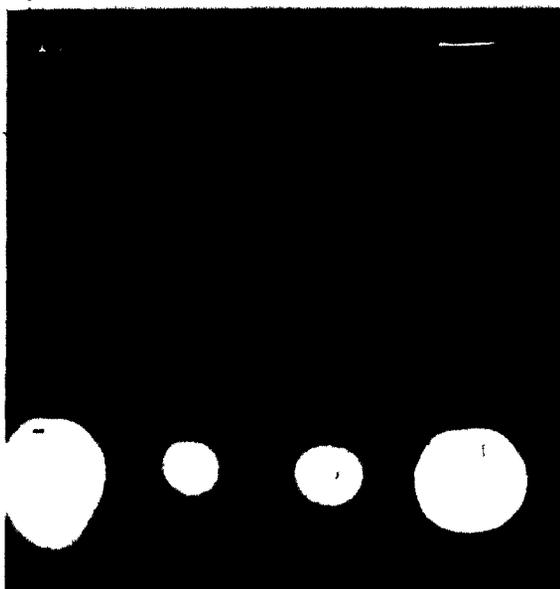


Figure 5. Total DNA from strains SP1 and SP2 probed with  $^{32}\text{P}$ -labeled pJV1 DNA. Left - agarose gel showing BamHI digests of: pJV1 DNA (lane 1), total DNA from SP1 (lane 2) and total DNA from SP2 (lane 3). DNA from this gel was transferred to a nitrocellulose filter. Right - autoradiograph of nitrocellulose filter probed with  $^{32}\text{P}$ -labeled pJV1 DNA: lane 1 - pJV1 DNA, lane 2 - SP1 total DNA, lane 3 - SP2 total DNA.

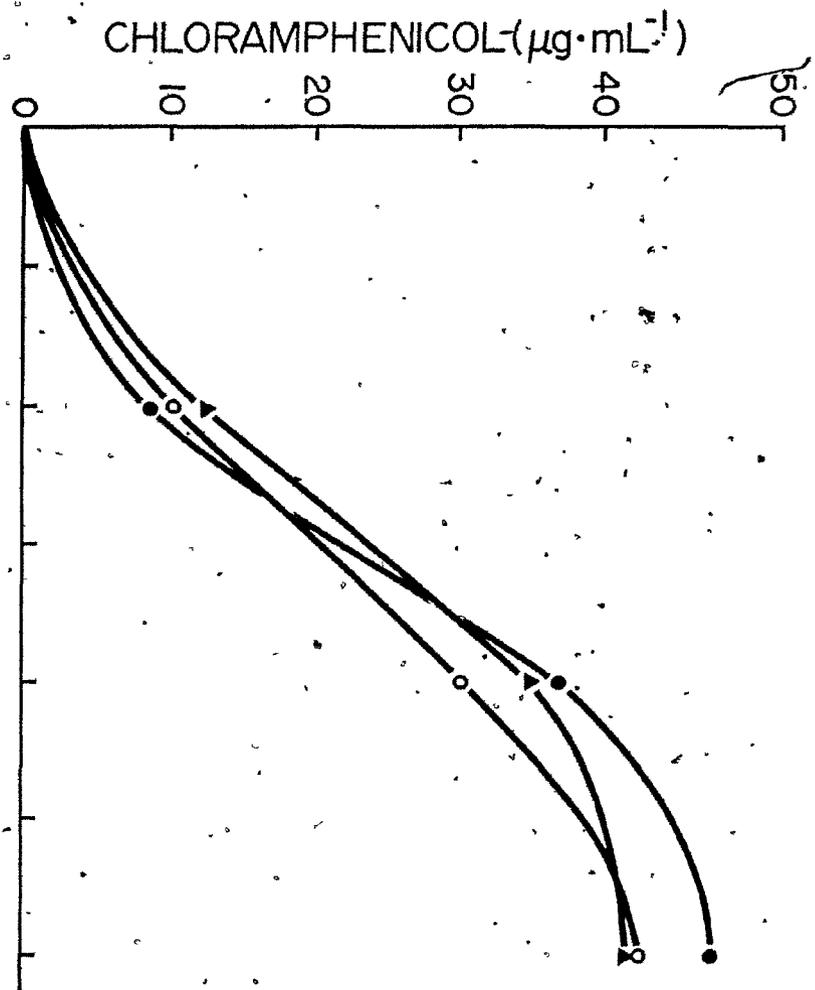
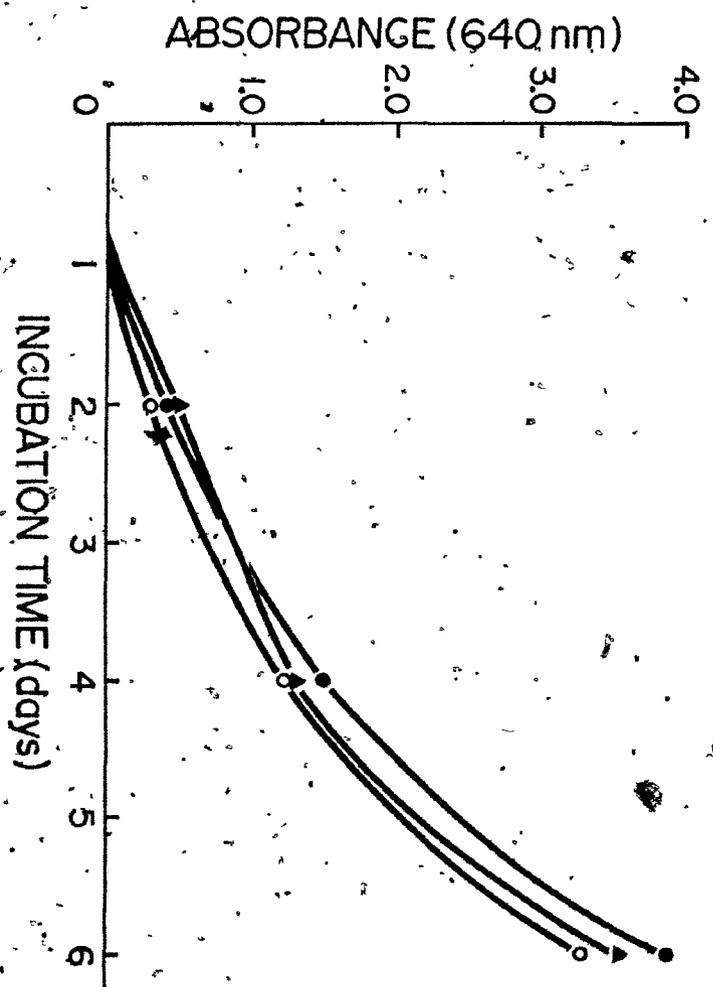
1 2 3



1 2 3



Figure 6. Growth and chloramphenicol biosynthesis in Streptomyces  
phaeochromogenes strains SP1, SP2 and EB116. Top panel -  
chloramphenicol production in glucose-isoleucine medium;  
bottom panel - growth (OD<sub>640</sub>) in glucose-isoleucine medium.  
●—● strain SP1 (pJV1<sup>+</sup>); ▲—▲ strain EB116 (plasmid copy  
number mutant); ○—○ strain SP2 (pJV1<sup>-</sup>).



amphenicol-nonproducing strains were isolated for use in mapping studies. All nonproducing strains were derived from strain HP-1, a prototrophic single-colony isolate of S. venezuelae selected for its ability to produce up to  $110 \mu\text{g}\cdot\text{mL}^{-1}$  of chloramphenicol on glucose-isoleucine medium. Strain HP-1 was mutagenized by four different procedures: in two of these, spores were irradiated with short-wavelength ultraviolet light; this was followed by plating on either (i) MYM agar or (ii) MYM agar containing caffeine ( $500 \mu\text{g}\cdot\text{mL}^{-1}$ ). In the third procedure, spores were irradiated with long-wavelength ultraviolet light in the presence of 8-methoxypsoralen; in the fourth they were treated with ethyl methanesulfonate (EMS). Approximately 25,000 colonies arising from the mutagenized spores were screened for chloramphenicol production using the bioassay technique. Twelve mutants showing absolutely no inhibition of M. luteus were isolated. Nonproducing strains were isolated at a rate of approximately  $3/4500$  (0.07%) from the short-wavelength ultraviolet irradiation treatment in the absence of caffeine,  $1/3000$  (0.03%) from the short-wavelength ultraviolet irradiation and caffeine treatment,  $5/9000$  (0.06%) from mutagenesis by long-wavelength ultraviolet irradiation in the presence of 8-methoxypsoralen, and  $3/8000$  (0.04%) after EMS mutagenesis. Table 2 gives the mutagenic treatment by which each mutant was derived. Leaky mutants, producing small amounts of chloramphenicol, were obtained at a rate of approximately 0.3% for each mutagenic treatment.

#### VI. Mutant characterization

All twelve mutants were still prototrophic and grew well on minimal agar, thus lesions in the shikimic acid pathway common to the biosyn-

Table 2. Aromatic amino- or nitro-compounds in, and bioactivity of, clarified broths from chloramphenicol-nonproducing strains of *S. venezuelae*

Mutant	Mutagen*	Colorimetric Assay** ( $\mu\text{g}\cdot\text{mL}^{-1}$ )	Bioassay**
Cml-1	EMS	3	-
Cml-2	EMS	32	+
Cml-3	EMS	<2	-
Cml-4	UVSC	70-100	-
Cml-5	UVS	56	-
Cml-6	*MOP-UVL	<2	-
Cml-7	MOP-UVL	<2	-
Cml-8	UVS	17	-
Cml-9	UVS	3.5	-
Cml-10	MOP-UVL	4.5	-
Cml-11	MOP-UVL	3.5	-
Cml-12	MOP-UVL	<2	-

\* The mutagens were:

EMS - ethyl methanesulfonate

UVS - short-wavelength ultraviolet light

UVSC - short-wavelength ultraviolet light in the presence of caffeine

MOP-UVL - long-wavelength ultraviolet light in the presence of 8-methoxypsoralen.

\*\*Each mutant was grown for 7 d in chloramphenicol-production medium and the clarified broth was examined for chloramphenicol pathway intermediates using the colorimetric assay. Absorbance at  $\text{OD}_{550}$  nm of the chromogen formed in the colorimetric assay was compared to a standard curve prepared using chloramphenicol. Culture fluids were then extracted with ethyl acetate and the extracts were concentrated. Absorbent paper assay disks soaked with the concentrated extracts were tested for bioactivity against *M. luteus*.

thesis of aromatic amino acids and chloramphenicol can be ruled out. The specific lesions in the chloramphenicol biosynthetic pathway responsible for the absence of antibiotic activity were investigated by growing each mutant in chloramphenicol-production medium for 7 d and examining the culture fluids for chloramphenicol pathway intermediates. Cultures of eight mutants showed no reaction for aromatic amino- or nitro-compounds when the clarified fluids were analysed colorimetrically (Table 2). In addition, concentrates of the ethyl acetate extracts of these culture fluids gave no zones that reacted with *p*-dimethylaminobenzaldehyde when they were chromatographed on thin layers of silica gel. Absorbent paper assay disks soaked with the concentrated extracts failed to show antibiotic activity against *M. luteus*.

a. Characterization of Cml-1 and Cml-12

To determine whether any of the eight strains that did not excrete chloramphenicol or its precursors was blocked in the conversion of chorismic acid to *p*-aminophenylalanine (the aromatization step that initiates chloramphenicol biosynthesis, (Figure 2)) each mutant was grown for 7 d in chloramphenicol-production medium containing 1 mM *p*-aminophenylalanine. Two of the strains, Cml-1 and Cml-12, then produced an aromatic nitro compound (Table 3) which was extractable into ethyl acetate and which had the same R<sub>f</sub> value as that of authentic chloramphenicol on a thin-layer chromatogram in system A. These two strains are therefore presumed to be deficient in arylamine synthetase activity.

b. Hydrolysis of culture supernatant fluids

The possibility was considered that some of the six strains (Cml-3,-6,-7,-9,-10,-11) that did not excrete chloramphenicol nor any of its

Table 3. Chloramphenicol production by blocked mutants in cultures supplemented with p-amino-L-phenylalanine

Mutant	Colorimetric Assay ( $\mu\text{g}\cdot\text{mL}^{-1}$ )*
Cm1-1	77
Cm1-3	<1
Cm1-6	<1
Cm1-7	4.5
Cm1-9	<2
Cm1-10	<2
Cm1-11	<1
Cm1-12	57

\*Each mutant was grown for 7 d in chloramphenicol-production medium containing 1 mM p-amino-L-phenylalanine. Clarified broths were extracted with ethyl acetate and the extract assayed colorimetrically for aromatic amino- or nitro-compounds. The absorbance of the chromogen at 550 nm was compared to a standard curve prepared using chloramphenicol.

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, was isolated by Wat et al. (1971) and identified as 2-dichloroacetamido-3-(*p*-acetamidophenyl)propan-1-ol. Bioassays showed that it 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 in the six mutants (Table 4). Mixing the culture filtrate from Cml-12 with 2-dichloroacetamido-3-(*p*-acetamidophenyl)propan-1-ol before hydrolysis did not alter the colorimetric yield of aromatic amine from the latter compound.

#### c. Characterization of Cml-3 and Cml-10

To determine whether any of the mutant strains Cml-3, Cml-6, Cml-7, Cml-9, Cml-10 or Cml-11 were blocked at a step where an intermediate was accumulated but not excreted, ethanol extracts of washed cells were examined by thin-layer chromatography. Cells from two mutant strains, Cml-3 and Cml-10, contained small amounts of an arylamine with an *R<sub>f</sub>* value similar to that of authentic *p*-aminophenylalanine. Accumulation of this compound and its failure to promote chloramphenicol production when added as a medium supplement suggests a pathway lesion after the arylamine synthetase step. Cell extracts of the four remaining strains did not contain any aromatic amino- or nitro-compounds.

Table 4. Effect of acid hydrolysis on aromatic amine content of solutions of 2-dichloroacetamido-3-(p-acetamidophenyl)propan-1-ol (DAPP) and clarified broths from cultures of blocked mutants\*

Sample	Before hydrolysis	After hydrolysis	Increase
DAPP	0		
3.125 $\mu\text{g.mL}^{-1}$ in $\text{H}_2\text{O}$	0	0.02	0.02
12.5 $\mu\text{g.mL}^{-1}$ in $\text{H}_2\text{O}$	0	0.06	0.06
25 $\mu\text{g.mL}^{-1}$ in $\text{H}_2\text{O}$	0	0.20	0.20
50 $\mu\text{g.mL}^{-1}$ in $\text{H}_2\text{O}$	0	0.34	0.34
12.5 $\mu\text{g.mL}^{-1}$ in Cml-2 broth	0.01	0.09	0.08
50 $\mu\text{g.mL}^{-1}$ in Cml-2 broth	0.01	0.33	0.32
Cml-1	0.06	0.09	0.03
Cml-2	0.19	0.16	-
Cml-3	0.02	0.05	0.03
Cml-4	1.38	1.38	-
Cml-5	0.55	0.69	0.14
Cml-6	0.03	0.07	0.04
Cml-7	0.02	0.06	0.04
Cml-8	0.05	0.10	0.05
Cml-9	0.04	0.07	0.03
Cml-10	0.07	0.12	0.05
Cml-11	0.02	0.03	0.01
Cml-12	0.01	0.03	0.02

\*Concentrated HCl (0.3 mL) was added to samples (2 mL) and the mixtures were heated at  $100^\circ\text{C}$  for 4 h; 1-mL portions were assayed for aromatic amines colorimetrically. Solutions of DAPP served as controls. Aromatic amine content is expressed as increase in absorbance at 550 nm.

d. Characterization of Cml-2

Blocked mutant Cml-2 gave a positive response when culture fluids were examined colorimetrically for aromatic nitro- or amino-compounds (Table 2). Disks permeated with a concentrated ethyl acetate extract of clarified broth from a Cml-2 culture showed very weak inhibition of M. luteus. Thin-layer chromatography of the extracts showed three zones that reacted with p-dimethylaminobenzaldehyde only after treatment with stannous chloride, and had mobilities different from chloramphenicol. These aromatic nitro compounds were isolated by silica-gel column chromatography of the extracts. Compound A was eluted with a 1:1 mixture of ethyl acetate and chloroform, compound B with ethyl acetate, and compound C with a 9:1 mixture of ethyl acetate and acetone.

The structures of compounds A and B were established from their chromatographic behaviour (Table 5) and <sup>1</sup>H-NMR spectra, which corresponded to those of authentic specimens of D-threo-1-p-nitrophenyl-2-isobutyramido-1,3-propanediol and D-threo-1-p-nitrophenyl-2-propionamido-1,3-propanediol, respectively. Compound C was identical chromatographically with D-threo-1-p-nitrophenyl-2-acetamido-1,3-propanediol (Table 5) but the size and purity of the sample were insufficient for NMR spectral identification. Since it accumulates these three non-chlorinated chloramphenicol analogues, mutant Cml-2 appears to be blocked in the reaction that chlorinates the N-acyl group of chloramphenicol (Figure 2).

Culture fluids of Cml-2 also contained an aromatic amine that could not be extracted into ethyl acetate, even after acidification of the culture fluid to pH 2.0. This compound was isolated by chromatography on Dowex 50. It was retained on the hydrogen form of the cation

Table 5. Thin-layer chromatographic comparison of compounds A, B, and C from Cml-2 with corynecins I, II, and III.

Compound	System A* Rf	System B** Rf
Compound C	0.17	0.60
<u>D-threo-1-p-Nitrophenyl-</u> <u>2-acetamido-1,3-propanediol</u>	0.18	0.60
Compound B	0.24	-
<u>D-threo-1-p-Nitrophenyl-</u> <u>2-propionamido-1,3-propanediol</u>	0.24	-
Compound A	0.31	-
<u>D-threo-1-p-Nitrophenyl-</u> <u>2-isobutyramido-1,3-propanediol</u>	0.30	-

\*Silica gel plates developed in chloroform-methanol (9:1).

\*\*Silica gel plates developed in butanol-acetic acid-water (12:3:5)

exchange resin and eluted with 2 M ammonium hydroxide. Thin-layer chromatography of the product showed it to have an Rf value identical to that of authentic p-aminophenylalanine.

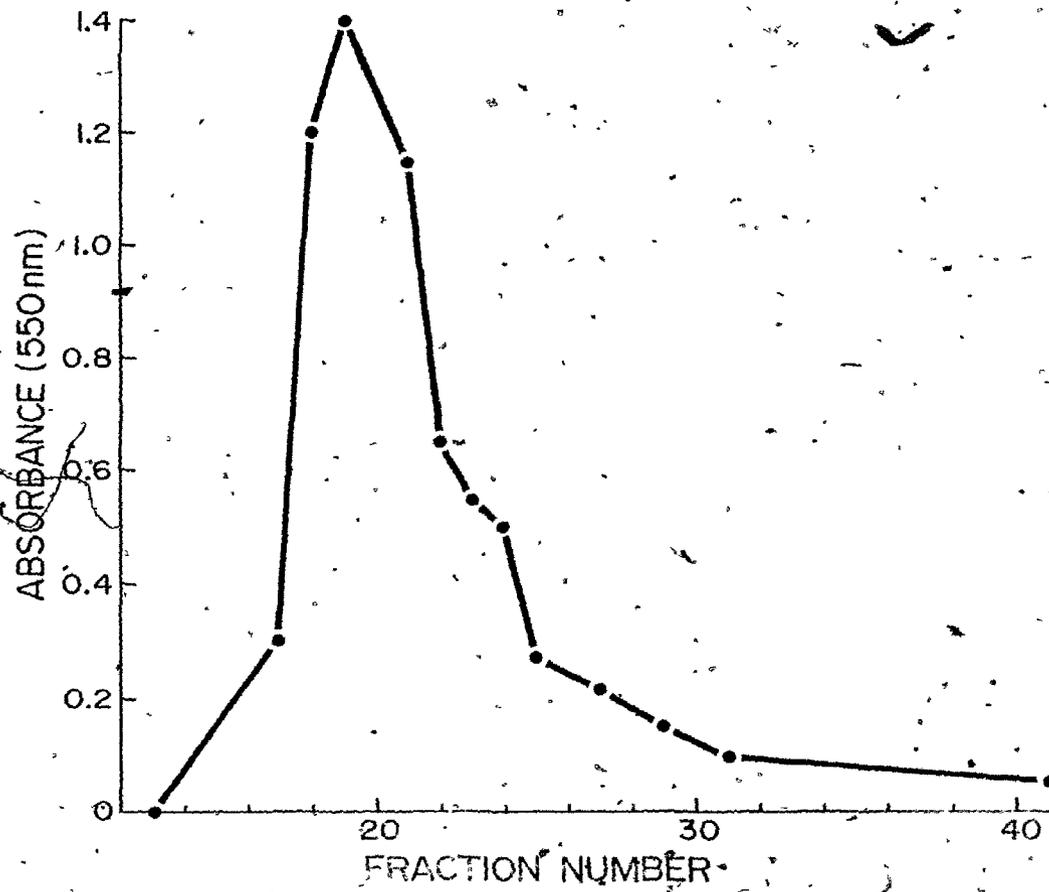
e. Characterization of Cml-4, Cml-5 and Cml-8

Culture filtrates of Cml-4, Cml-5 and Cml-8 also contained a polar arylamine (compound D) that could not be extracted into ethyl acetate, even after acidification. In each case the compound could be eluted in a single peak with 2 M ammonium hydroxide from a column of Dowex 50 (H<sup>+</sup>).

Figure 7 gives the elution profile for compound D from culture fluids of Cml-4. Positive fractions were pooled and purified further by chromatography on a column of Dowex 50 in the pyridinium form. Stepwise elution with 0.1 M and 0.5 M pyridine gave fractions that exhibited a single response peak when tested colorimetrically for arylamines. Arylamine-positive fractions were pooled and lyophilized. Compound D isolated in this way from culture fluids of Cml-4 was identified from its NMR spectrum and chromatographic comparison with an authentic sample as p-aminophenylalanine. Its <sup>1</sup>H-NMR spectrum, recorded in deuteriated water at 80 MHz with tetramethylsilane in chloroform-d as the external reference, contained signals matching those given by p-aminophenylalanine at  $\delta$  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).

The products isolated from Cml-5 and Cml-8 were of insufficient size and purity for spectral identification. However, their chromatographic properties coincided with those of authentic p-aminophenyl-

Figure 7. Elution profile for compound D from culture fluids of Cml-4. The supernatant fluid from a 750-ml culture was applied to a column (2.5 x 30 cm) of Dowex 50x8 (H<sup>+</sup>) 50-100 mesh. Amino acids were eluted with 2 M ammonium hydroxide. Fractions (10 ml) were collected and assayed for aromatic amines using the colorimetric procedure.



alanine; Rf values for compound D were 0.23 and 0.27 in systems C and D, respectively. Rf values for authentic p-aminophenylalanine were 0.24 and 0.28 in systems C and D, respectively. No p-aminophenylserine (Rf value = 0.19 in system C) could be detected in the culture fluids of these mutants. Thus they are probably blocked in the hydroxylation of p-aminophenylalanine to p-aminophenylserine (Figure 2).

Broths of mutants Cml-4 and Cml-5 adjusted to pH 2.0 after the initial extraction with ethyl acetate and reextracted under acidic conditions yielded an aromatic nitro compound (compound E). The thin-layer chromatographic properties of compound E were compared with those of three potential metabolites, N-dichloroacetyl-p-nitrophenylalanine, N-acetyl-p-nitrophenylalanine and p-nitrophenylalanine, using systems B and C (Table 6). Compound E was tentatively identified as N-acetyl-p-nitrophenylalanine. Attempts to isolate this aromatic nitro compound from broths of Cml-4 to allow for more rigorous identification failed due to the small amounts accumulated by the mutant.

Clarified broths of Cml-5 showed an increase in aromatic amine content after acid hydrolysis (Table 4), suggesting that an acylated aromatic amine was also present. Examination of broth extracts by thin-layer chromatography (system A) failed to reveal any fluorescence-quenching zone corresponding in mobility to that given by a sample of 2(S)-dichloroacetamido-3-(p-acetamidophenyl)propan-1-ol. It was concluded that the masked aromatic amine accumulated by Cml-5 might be p-N-acetylaminophenylalanine.

Table 6. Thin-layer chromatographic comparison of compound E  
from mutants Cml-4 and Cml-5 with standard aromatic  
nitro-compounds

Compound	System B* Rf.	System C** Rf
p-Nitrophenylalanine	0.37	0.53
N-Acetyl-p-nitrophenylalanine	0.55	0.51 "
Compound E	0.55	0.50
N-Dichloroacetyl-p-nitrophenylalanine	0.66	0.69
Chloramphenicol	0.73	

\*Silica gel plates developed in n-butanol-acetic acid-water  
(12:3:5)

\*\*Cellulose plates developed in n-butanol-ethanol-4M ammonium  
hydroxide (8:1:3)

## VII. Cosynthesis

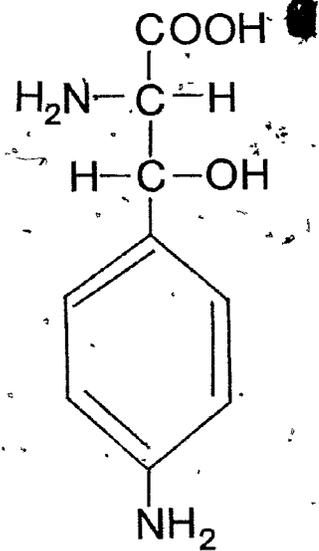
Attempts were made to detect cosynthesis of chloramphenicol by growing mixtures of mutants in all possible pairwise combinations on MYM agar or in chloramphenicol-production medium. In agreement with the preceding findings, mutants Cml-4 and Cml-5 (which accumulated p-aminophenylalanine) promoted chloramphenicol synthesis when grown with mutants Cml-1 and Cml-12 (blocked in the conversion of chorismic acid to p-aminophenylalanine). No other instances of cosynthesis were observed.

## VIII. p-Aminophenylalanine $\beta$ -Hydroxylase

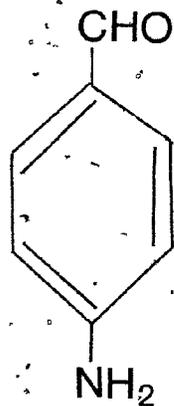
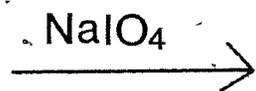
### a. Development of an assay

One of the early steps in the chloramphenicol biosynthetic pathway is hydroxylation at the  $\beta$ -carbon of p-aminophenylalanine to form p-aminophenylserine (Westlake and Vining, 1969). To detect enzyme activity catalysing the reaction in cell extracts of S. venezuelae, a method for distinguishing substrate from product was required. Since these differ only slightly in their chromatographic properties, differences in chemical reactivity due to the hydroxyl group at the  $\beta$ -carbon of p-aminophenylserine were exploited. The presence of a vicinal amino alcohol renders this compound susceptible to oxidation with sodium metaperiodate. If p-aminophenylalanine labeled at the carboxyl carbon were converted to p-aminophenylserine, periodate oxidation of the mixture should yield radiolabeled glyoxylic acid derived only from the product (Figure 8). To estimate the amount present, the reaction mixture could then be filtered through activated charcoal which would adsorb unconverted p-aminophenylalanine while allowing the [ $^{14}\text{C}$ ]gly-

Figure 8. Periodate oxidation of p-aminophenylserine.

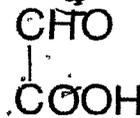


p-Aminophenyl-serine



p-Aminobenzaldehyde

+



Glyoxylic Acid

+



oxylic acid to pass through. The amount of p-aminophenylalanine converted to p-aminophenylserine should thus be represented by the amount of radioactivity recovered in the charcoal filtrate.

In preliminary experiments using a reaction mixture containing periodic acid, approximately 30% of the initial radioactivity in p-aminophenyl[carboxyl- $^{14}\text{C}$ ]serine was present in the filtrate recovered after charcoal treatment. Radioisotope yields decreased with extended reaction times, suggesting that overoxidation of [ $^{14}\text{C}$ ]glyoxylic acid was responsible, at least in part, for the low recoveries. In addition, an appreciable and time-dependent amount of radioactivity appeared in the charcoal filtrate when p-aminophenyl[carboxyl- $^{14}\text{C}$ ]alanine was treated under these conditions, indicating that the intended enzyme substrate was slowly oxidized by periodic acid in acidic reaction mixtures.

Better discrimination was obtained by mixing a solution of p-aminophenyl[carboxyl- $^{14}\text{C}$ ]serine in 0.1 M phosphate buffer, pH 7.0, with a saturated solution of sodium metaperiodate (final pH of 7.5) and, after 1 min, filtering the solutions through activated charcoal. The filtrate then contained 73% of the total initial radioactivity of the sample. When the reaction was carried out at final pH values of 2.5 and 5.5, only 15% and 37%, respectively, of the initial radioactivity of the sample was recovered in the filtrate, demonstrating the pH dependence of the reaction. A control solution to which sodium metaperiodate was not added gave a filtrate with only 10% of the total initial radioactivity. A second control in which saturated sodium metaperiodate solution was mixed with a  $0.38 \text{ mg}\cdot\text{mL}^{-1}$  solution of p-aminophenyl[carboxyl- $^{14}\text{C}$ ]alanine and the solution was filtered after 1 min through charcoal, gave a filtrate containing only 5.5% of the

initial radioactivity in the sample. These results confirmed that under suitable conditions, *p*-aminophenylserine is rapidly oxidized by periodate whereas *p*-aminophenylalanine reacts very slowly.

b. Hydroxylation by cell extracts

*p*-Aminophenyl[carboxyl- $^{14}\text{C}$ ]alanine was added to incubation mixtures containing 100  $\mu\text{L}$ , 500  $\mu\text{L}$ , and 1000  $\mu\text{L}$  of cell extract of *S. venezuelae*. After incubation, sodium meta-periodate was added and the mixtures were filtered through charcoal. Increased amounts of cell extract in the incubation mixture gave an increase in filterable radioactivity (Table 7). Controls receiving boiled cell extracts gave significantly less filterable radioactivity. The results indicate that cell extracts of *S. venezuelae* contain an enzyme that converts *p*-aminophenylalanine to a product, probably *p*-aminophenylserine, which is susceptible to periodate oxidation.

IX. Chloramphenicol Resistance in Strain HP-1 and the Cml Mutants

Cultures of strain HP-1 growing in chloramphenicol-production medium were sensitive to chloramphenicol early in the growth phase before chloramphenicol production began, but became resistant with the production of endogenous antibiotic; addition of chloramphenicol to a final concentration of 150  $\mu\text{g}\cdot\text{mL}^{-1}$  at the beginning of growth extended the lag phase for an additional 24 h whereas addition after 4 d had no effect (Figure 9 and 10).

Cultures of mutant Cml-9 showed greater sensitivity to chloramphenicol compared to those of strain HP-1. Addition of the antibiotic at the beginning of growth extended the lag phase for 2 d (Figure 9).

Table 7. p-Aminophenylalanine  $\beta$ -hydroxylase activity (dpm·mL<sup>-1</sup>)  
in cell extracts of *S. venezuelae*\*

Cell extract added ( $\mu$ L)	Charcoal filtrate		
	Cell extracts (dpm·mL <sup>-1</sup> )	Boiled extracts (dpm·mL <sup>-1</sup> )	Net activity**
100	378	366	14
500	472	346	151
1000	638	530	130

\*Various amounts of cell extract, or boiled-extract were mixed with p-aminophenyl[carboxyl-<sup>14</sup>C]alanine solution (1 mL) and 0.1 M phosphate buffer, pH 7.0 (1 mL), and the volume was adjusted to 3 mL with water. The mixture was shaken at 27°C for 30 min and samples were analyzed as described in Materials and Methods.

\*\* Net activity as dpm per mL of incubation mixture.

Figure 9. Effect of chloramphenicol on Streptomyces venezuelae strains at the beginning of growth in chloramphenicol production medium. Top panel - strain Cm1-9; bottom panel, - strain HP-1.

● — ● control cultures receiving no chloramphenicol supplement; ▲ — ▲ cultures receiving chloramphenicol supplement to a final concentration of  $150 \mu\text{g}\cdot\text{mL}^{-1}$ .

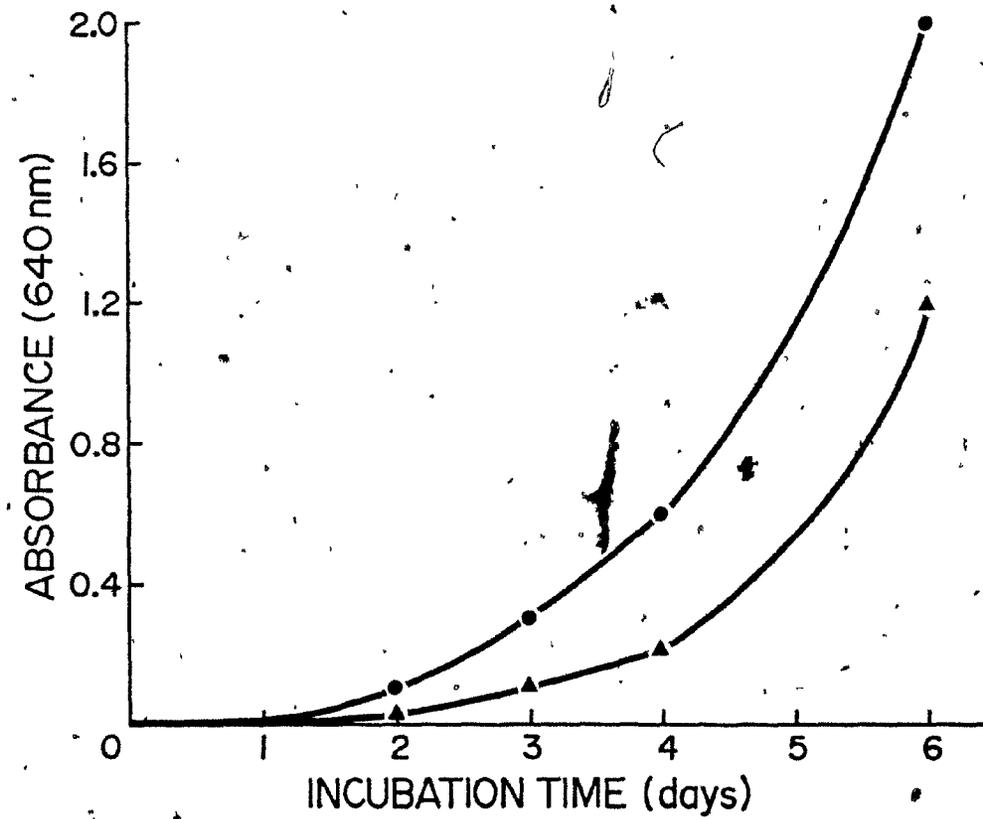
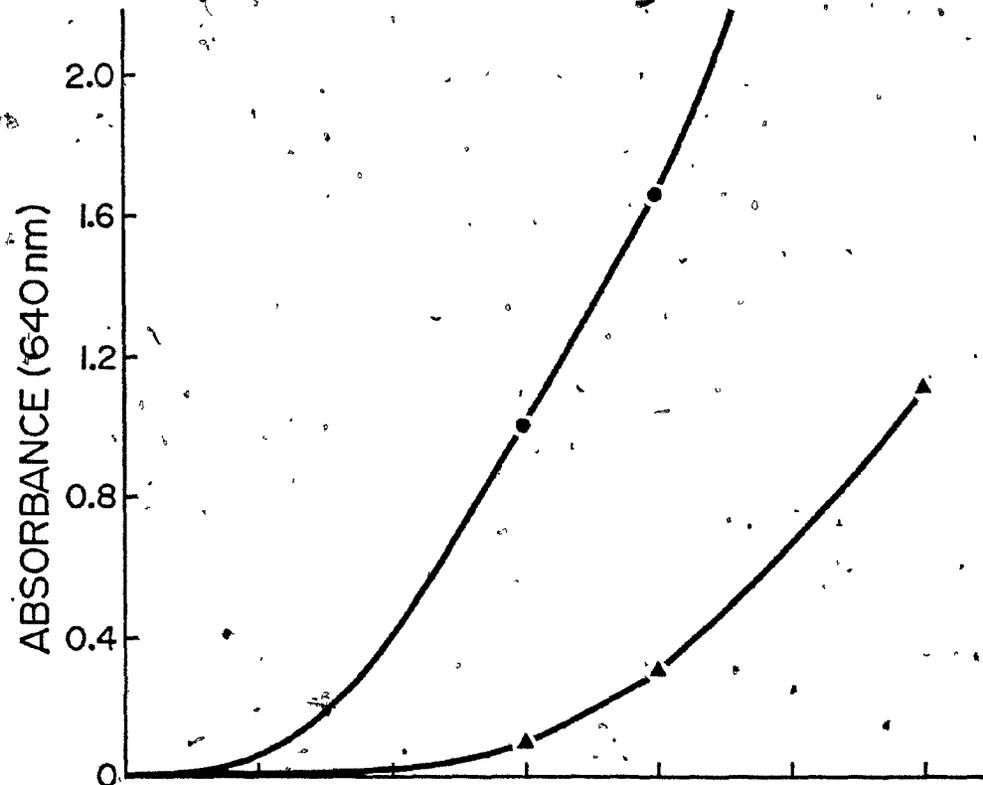
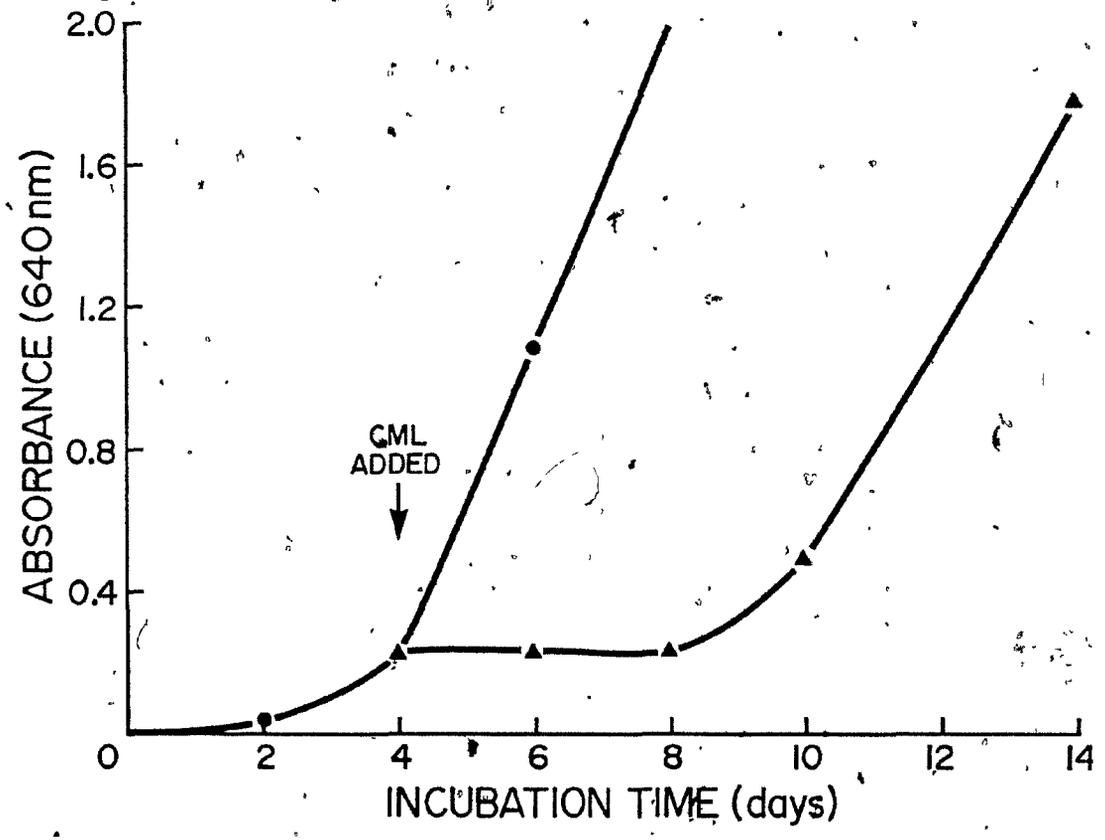
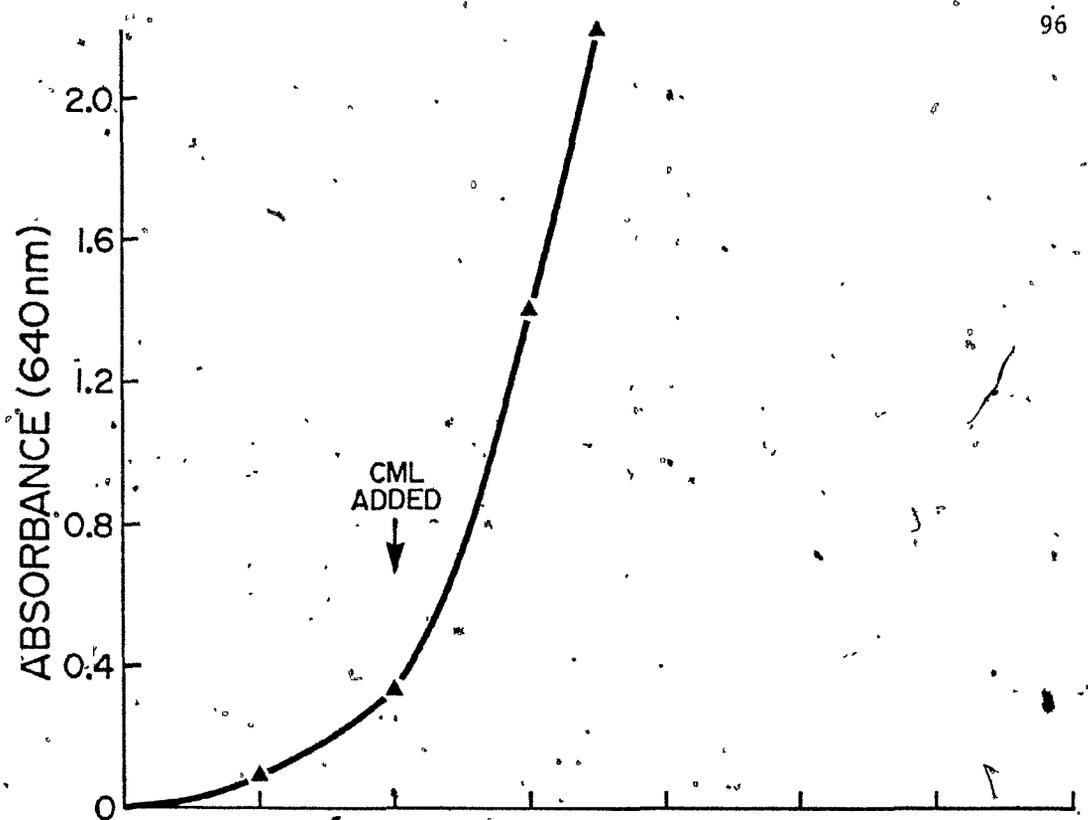


Figure 10. Effect of chloramphenicol on growth of Streptomyces venezuelae in chloramphenicol production medium. Top panel - strain HP-1; bottom panel - composite of results for all Cml mutants. ●—● control cultures receiving no chloramphenicol supplement; ▲—▲ cultures receiving chloramphenicol supplement to a final concentration of  $150 \mu\text{g mL}^{-1}$ . In the case of strain HP-1, growth curves for cultures with and without chloramphenicol were superimposable.



Furthermore, addition of chloramphenicol to cultures of each nonproducing mutant after 4 d of growth (at which time strain HP-1 had become resistant) temporarily arrested growth for 3-4 d (see appendix). All 12 Cml mutants were sensitive to chloramphenicol. Figure 10 gives a generalized growth curve demonstrating the effect of chloramphenicol addition on the growth of nonproducing strains.

When mycelium from cultures of mutants Cml-1, Cml-2, Cml-3, Cml-8, Cml-9, Cml-10 and Cml-12 grown for 12-13 d in the presence of chloramphenicol was used to inoculate flasks of production medium with and without chloramphenicol, the chloramphenicol-supplemented cultures lagged only slightly behind the unsupplemented cultures (Figure 11 and appendix). Broths of the unsupplemented cultures were tested for the presence of chloramphenicol using the colorimetric method to ascertain whether these "resistant" mutants had reverted to become chloramphenicol producers. Only with Cml-12 was chloramphenicol detected in the broth and it was present only at the very low concentration of  $6 \mu\text{g}\cdot\text{mL}^{-1}$ .

Resistant cultures of Cml-1, Cml-8, Cml-9, Cml-10 and Cml-12 were tested for reversibility of the chloramphenicol-resistant phenotype by growing them for 7 d in the absence of chloramphenicol and then using them to inoculate chloramphenicol-supplemented medium. These cultures retained their antibiotic resistance (Figure 11 and appendix). On the other hand, resistant cultures of strain HP-1 grown for 24 h in the absence of chloramphenicol in a medium (GNY) supporting only low levels of antibiotic synthesis returned to a sensitive state. (Figure 12).

Figure 11. Effect of chloramphenicol on growth of resistant Cm1 mutants.

Top panel - composite figure: mutants transferred to medium with and without chloramphenicol after growth for 1 week in the absence of chloramphenicol; bottom panel - composite figure: mutants transferred directly from chloramphenicol-containing medium to medium with the same concentration of chloramphenicol and to medium without chloramphenicol. ● — ● control cultures receiving no chloramphenicol supplement; ▲ — ▲ cultures receiving chloramphenicol supplement to a final concentration of  $150 \mu\text{g}\cdot\text{mL}^{-1}$ . All curves show growth in chloramphenicol production medium.

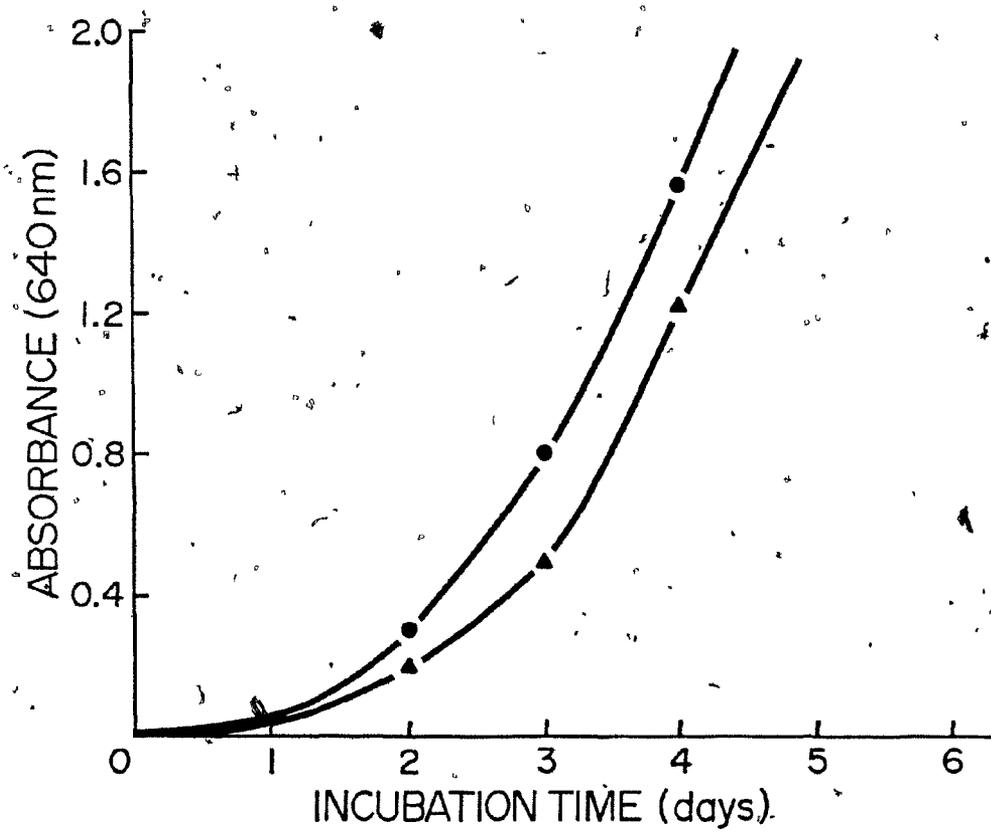
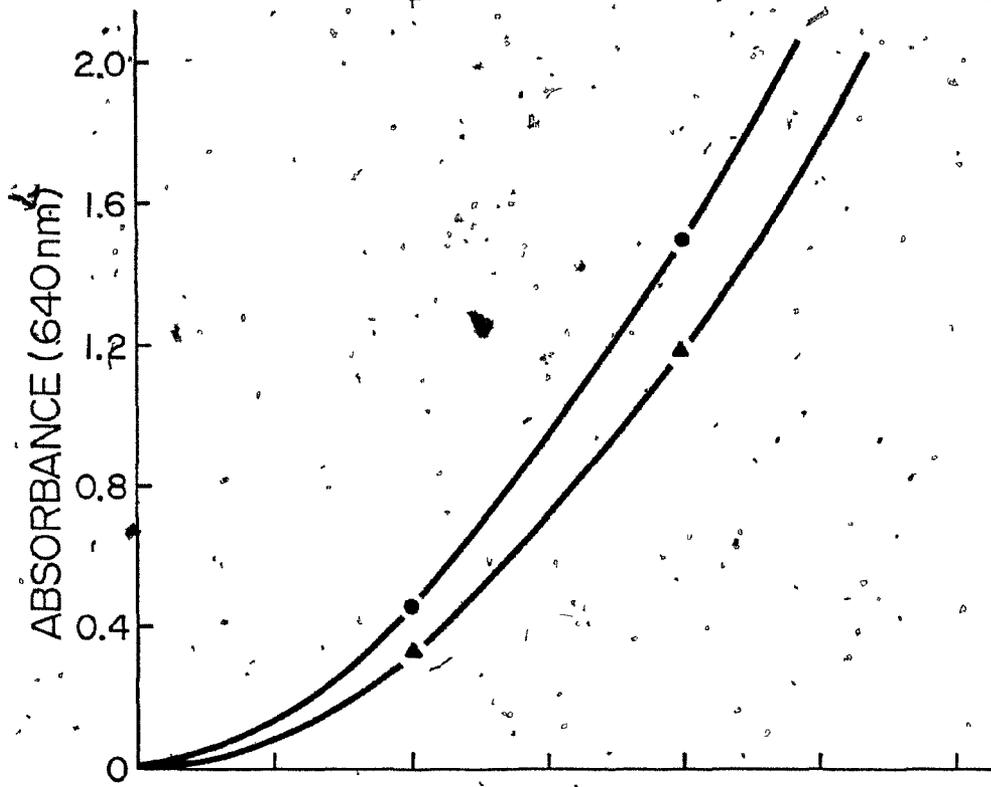
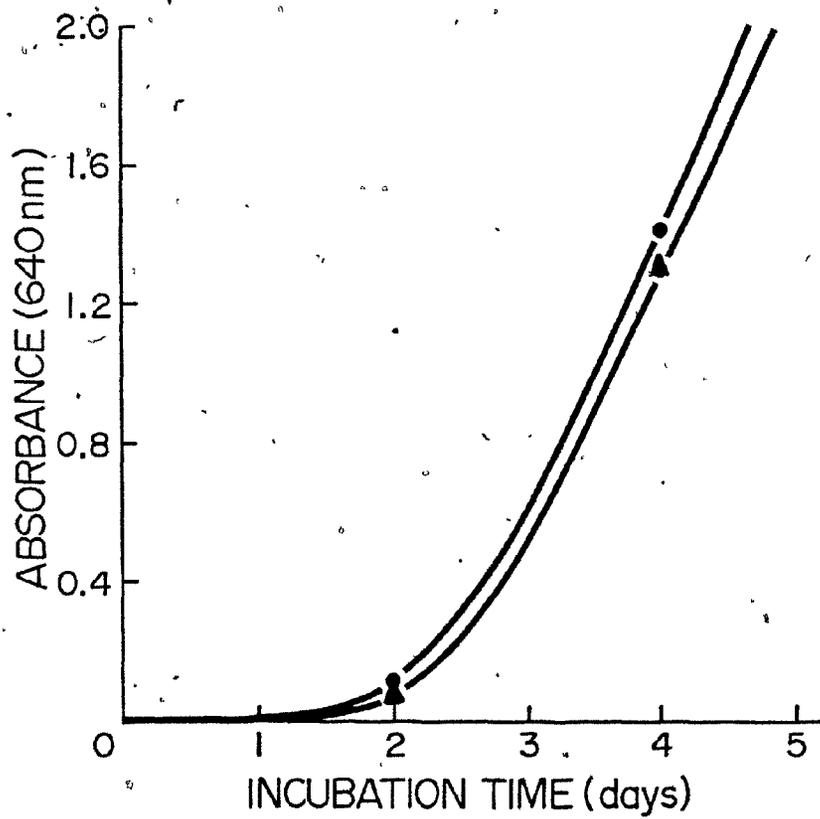
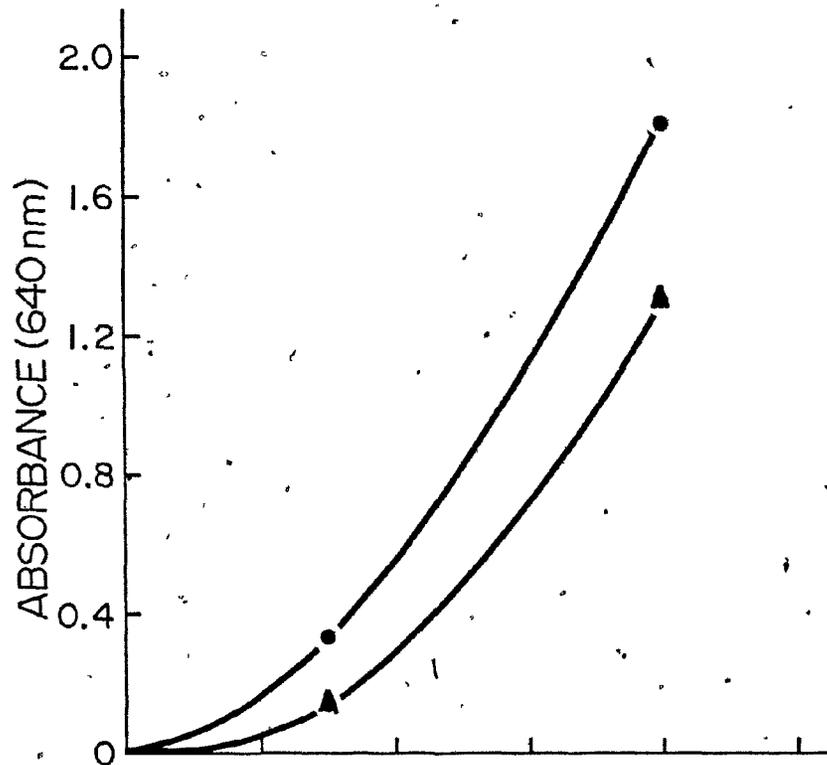


Figure 12. Loss of chloramphenicol resistance in strain HP-1 during growth in a medium lacking chloramphenicol. Top panel - culture transferred to a medium with and without chloramphenicol after growth for 24 h in GNY medium; bottom panel - culture transferred directly from chloramphenicol-containing medium to medium with and without the same concentration of chloramphenicol. ●—● control cultures receiving no chloramphenicol supplement; ▲—▲ cultures receiving chloramphenicol supplement to a final concentration of  $150 \mu\text{g}\cdot\text{mL}^{-1}$ . All curves show growth in chloramphenicol production medium.



X. Fertility in *Streptomyces venezuelae*

Crosses in which each of the auxotrophically marked Cml mutants: VS146 (nic-6, cml-7), VS163 (hom-5, cml-4), VS130 (cys-13, cml-4) and VS99 (his-9, cml-4) was mated with the triply auxotrophic chloramphenicol producer, VS160 (ura-1, thr-1, trp-1, cml<sup>+</sup>) gave threonine-independent progeny at a frequency of only 1 per  $10^7$  parental c.f.u. or less. This was a frequency not significantly higher than that for reversion to wild-type of the nic-6, hom-5, cys-13, his-9 and thr-1 markers.

A very low frequency of recombination was not limited to crosses involving the Cml mutants and VS160. Crosses between strain VS113 (his-6, ade-10, cml<sup>+</sup>) and the Cml derivatives VS144 (met-15, cml-7) and VS143 (met-14, cml-7) yielded prototrophic, histidine-requiring and adenine-requiring recombinants at a frequency of only 1 per  $10^6$  c.f.u. of the minority parent. The frequency of reversion to wild type was 4 per  $10^7$  c.f.u. for ade-10, 6 per  $10^8$  c.f.u. for his-6, 2 per  $10^7$  c.f.u. for met-15 and 1 per  $10^7$  c.f.u. for met-14.

In an effort to acquire fertile strains for use in mapping, attempts were made to transform protoplasts of mutants VS130, VS143, and VS144 with the *S. lividans* derived fertility plasmid pIJ303. These attempts were unsuccessful. However, a derivative of VS160 that displayed increased fertility in some crosses was obtained following transformation with another *S. lividans* derived plasmid, pIJ701. Recombination frequencies using this strain varied markedly in different crosses and were often unrepeatabile; therefore this strain was abandoned for use in mapping studies.

Fertile strains (VS191 and VS192) of *S. venezuelae* were obtained following protoplast formation and regeneration of VS113 (Stuttard,

personal communication). VS113 elicited lethal zygotis on both of these strains and VS191 and VS192 elicited this reaction on each other. A third fertile strain, VS194 was derived from VS191 by protoplast formation and regeneration. VS113, VS191, VS192, and VS194 produced chloramphenicol at concentrations of approximately 170, 130, 130 and 120  $\mu\text{g}\cdot\text{mL}^{-1}$  after 7 d in chloramphenicol-production medium. In crosses with auxotrophic derivatives of Cml mutants, VS191, VS192 and VS194 all yielded recombinants at frequencies ranging from 1 per  $10^4$  to 1 per  $10^3$  c.f.u. of the minority parent.

#### XL. Genetic Mapping

Four crosses were initially carried out using strain VS206 (his-6 ade-10 strA6) and singly auxotrophic derivatives of three different Cml mutants. For each cross the relative locations of the three auxotrophic markers and the streptomycin resistance marker were determined by the procedure of Hopwood (1972). The Cml marker was not considered in this initial procedure. Spores from a mixed culture of mutant strains were plated on to four selective media. The phenotypes of recombinants that appeared on each medium were determined by replica plating to diagnostic media, as demonstrated below for cross I. In this manner, nine different phenotypes reflecting the genotypes of recombinant progeny could be detected, including two reciprocal pairs. As Hopwood (1972) pointed out, subsequent analysis depends upon the data satisfying two internal checks: i) in several instances, the frequency of a particular recombinant class (c.f.u. per unit volume of the spore suspension at a standard dilution) should be estimated on more than one medium. To exclude the possibility of bias in the frequency with which a parti-

cular class is recovered under different selective conditions, these estimates must be similar; ii) In cases where both members of a pair of complementary genotypes can be recovered, both members of the pair should have approximately the same frequency, indicating a statistically equal contribution of genetic material by the two parents. The cross is then said to be balanced. In all crosses using VS206 as one parent, this was achieved by mixing VS206 spores with spores of the other parental strain in a ratio of approximately 20:1. Mixing of spores in a 1:1 ratio resulted in unbalanced outputs of progeny spores.

Consistently unbalanced crosses could reflect polarized gene transfer with one parent functioning predominantly as a DNA donor and the other as the recipient, as in F- mediated conjugation in E. coli.

a. Cross I: VS143(met-14 cml-7) x VS206(his-6 ade-10 strA 6)

Spores from the mixed culture were initially plated on the following media, each of which was countersselective for the parental spores:

1. MM + adenine
2. MM + methionine, adenine, and streptomycin
3. MM + methionine, histidine, and streptomycin
4. MM + histidine

Medium 1 selected Met<sup>+</sup> and His<sup>+</sup> phenotypes and allowed growth of the following four phenotypes of recombinant progeny:

	Ade	His	Met	Str
(a)	+	+	+	+
(b)	-	+	+	+
(c)	+	+	+	-
(d)	-	+	+	-

Note:  $\text{Str}^+$  indicates sensitivity to streptomycin,  $\text{Str}^-$  indicates resistance.

To distinguish between progeny differing in the unselected markers ade and str, colonies on medium 1 were replicated to the following media:

<u>Medium</u>	<u>Phenotype selected</u>
MM	(a), (c)
MM + streptomycin	(c)
MM + adenine and streptomycin	(c), (d)
MM + adenine	(a), (b), (c), (d)

Medium 2 selected for  $\text{His}^+$  and  $\text{Str}^-$  phenotypes and allowed growth of the following four types of recombinant progeny:

	Ade	Met	His	Str
(a)	+	+	+	-
(b)	-	+	+	-
(c)	-	-	+	-
(d)	+	-	+	-

To distinguish between progeny differing in the unselected markers ade and met, colonies were replicated to the following media:

<u>Medium</u>	<u>Phenotype Selected</u>
MM + streptomycin	(a)
MM + adenine and streptomycin	(b), (a)
MM + methionine and streptomycin	(a), (d)
MM + adenine, methionine and streptomycin	(a), (b), (c), (d)

Medium 3 selected for the  $\text{Ade}^+$  and  $\text{Str}^-$  phenotypes and allowed growth of the following four types of recombinant progeny:

	Ade	Met	His	Str
(a)	+	+	+	-
(b)	+	+	-	-
(c)	+	-	-	-
(d)	+	-	+	-

To distinguish between progeny differing in the unselected markers met and his, colonies were replicated to the following media:

<u>Medium</u>	<u>Phenotype Selected</u>
MM + streptomycin	(a)
MM + histidine and streptomycin	(a), (b)
MM + methionine and streptomycin	(a), (d)
MM + histidine, streptomycin and methionine	(a), (b), (c), (d)

Medium 4 selected for Met<sup>+</sup> and Ade<sup>+</sup> phenotypes and allowed growth of the following four types of recombinant progeny:

	Ade	Met	His	Str
(a)	+	+	+	-
(b)	+	+	-	-
(c)	+	+	-	+
(d)	+	+	+	+

To distinguish between progeny differing in the unselected markers his and str, colonies were replicated to the following media:

<u>Medium</u>	<u>Phenotype Selected</u>
MM	(d), (a)
MM + histidine + streptomycin	(a), (b)
MM + streptomycin	(a)
MM + histidine	(a), (b), (c), (d)

Frequencies for each inferred genotypic class of recombinants from cross I are given in Table 8. As can be seen from this table, the

Table 8. Analysis of a four-factor cross (Cross I: met-14 cml-7 X his-6 ade-10 strA6)\*

Genotypes of selectable progeny	Selective media supplements								Average frequency of complementary genotypes
	ade		met, ade str		met, his str		his		
	A	B	A	B	A	B	A	B	
+ + + +	2	8					20	11	10
+ + + <u>str</u>	9	34	8	25	24	24	51	30	28
<u>met</u> + + <u>str</u>			11	35	31	31			33
+ <u>his</u> + <u>str</u>					8	8	4	2	5
+ + <u>ade str</u>	58	224	83	261					242
+ <u>his</u> + +							0	0	>0
<u>met</u> + <u>ade str</u>			0	0					
+ + <u>ade</u> +	7	27							>17
<u>met his</u> + <u>str</u>					7	7			
Sample size:	76		102		70		75		
Total recombinants per plate:	293		321		70		43		

Relative recombination frequencies in each interval \*\*

	<u>met-ade</u>	<u>ade-his</u>	<u>met-his</u>	<u>his-str</u>	<u>ade-str</u>	<u>met-str</u>
	10	5	10	28	28	10
	28	242	28	33	33	33
	5	0	242	242	5	0
	0	17	17	0	17	17
Total	43	264	297	303	83	60

\*A sample of recombinants from each selective medium was classified into the four possible putative genotypes. The frequencies are shown in column A. Column B gives adjusted frequencies as a proportion of the total recombinants per plate. The adjusted frequencies averaged to give the frequency of each complementary pair of recombinants per unit volume of spore suspension are shown in the far right-hand column.

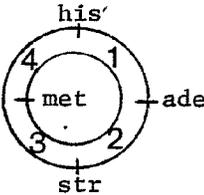
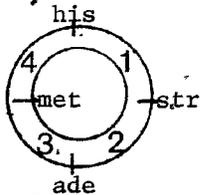
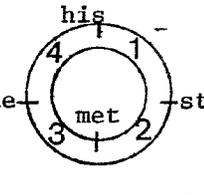
\*\*Relative recombination frequencies in each interval were obtained by summing the average frequencies for each recombinant class produced by a crossover in that interval.

above-mentioned internal checks were satisfied. The frequencies of each genotypic class could therefore be used to calculate the relative frequencies of recombination between each pair of markers in the cross. Pairs that showed the highest crossover frequencies were considered to be the farthest apart while those that showed low crossover frequencies were probably closer together. The results from Table 8 suggest that the pairs met-14 and ade-10 are closer together and likewise met-14 and strA6 are close together while met-14 and his-6 are far apart and strA6 and his-6 are, far apart.

The next step in the analysis was assignment of the four loci to relative positions on the map. The chromosome was assumed to be circular. The four loci could therefore be arranged in three different sequences, ignoring mirror images. These three arrangements and the crossovers required to produce the nine genotypes are shown in Table 9. Models in which genotypes requiring quadruple crossovers were not the rarest class were discarded. The data are most consistent with model 3.

A further analysis of linkage relationships was made by examining the segregation of each pair of unselected markers when arranged in 2X2 tables. A chi-square test can be applied to verify the null hypothesis: that there are no associations among members of each pair of markers when distributed to recombinant progeny. At the 0.05 significance level, the null hypothesis is accepted if  $\chi^2 \leq 3.841$ , and rejected if  $\chi^2 > 3.841$ . P gives the probability that the difference between the observed and expected values is due to chance. Since only one degree of freedom exists in analysing these 2X2 tables, the Yates correction factor was used (Stansfield, 1969). Hopwood has pointed out that when two unselected markers are adjacent, that is, lie on the same arc of a

Table 9. Comparison of genotype frequencies with the crossover regions required by the three possible marker models (cross I: met-14 cml-7 x his-6 ade-10 strA6)\*

Medium	Genotype	Frequency	Crossovers			Indicated Model
			1	2	3	
						
MM	+ + + +	2	3,4	3,4	2,3	3
+ ade	+ + + <u>str</u>	9	2,4	1,2,3,4	1,3	
	+ + <u>ade</u> <u>str</u>	58	1,4	1,4	1,4	
	+ + <u>ade</u> +	7	1,2,3,4,	2,4	2,4	
MM	+ + + <u>str</u>	8	2,4	1,2,3,4	1,3	1 or 3
+ ade	<u>met</u> + + <u>str</u>	11	2,3	1,2	1,2	
,met	+ + <u>ade</u> <u>str</u>	83	1,4	1,4	1,4	
str	<u>met</u> + <u>ade</u> <u>str</u>	0	1,3	1,3	1,2,3,4	
MM	+ + + <u>str</u>	24	2,4	1,2,3,4	1,3	3
+ his	<u>met</u> + + <u>str</u>	31	2,3	1,2	1,2	
met	+ <u>his</u> + <u>str</u>	8	1,2	2,3	3,4	
str	<u>met</u> + <u>his</u> <u>str</u>	7	1,2,3,4,	2,4	2,4	
MM	+ + + +	20	3,4	3,4	2,3	1 or 3
+ his	+ + + <u>str</u>	51	2,4	1,2,3,4,	1,3	
	+ <u>his</u> + <u>str</u>	4	1,2	2,3	3,4	
	+ <u>his</u> + +	0	1,3	1,3	1,2,3,4,	

\*Each model gives a possible arrangement for the four markers. Listed under each model are the crossovers required to form each genotype. Models in which genotypes requiring quadruple crossovers were not the rarest class were discarded.

circular chromosome between two selected markers, they will demonstrate dependent segregation. When the unselected markers are separated by a marker and are therefore nonadjacent, they will demonstrate independent segregation.

Analysis of the segregation of unselected alleles on each medium for cross I is given in Table 10 and suggests that:

ade-10 and strA6 are nonadjacent

met-14 and ade-10 are adjacent

met-14 and his-6 are nonadjacent

his-6 and strA6 are apparently nonadjacent

Again, the data are most consistent with model 3.

After ordering the auxotrophic and streptomycin resistance markers, the segregation of the cml-7 marker in cross I was analysed by examining chloramphenicol production in approximately 100 progeny. The results are given in Table 11. These data indicated that cml-7 was located between his-6 and strA6. The assignment of cml-7 to this arc did not require that any of the recombinants be formed by quadruple crossovers.

No infectious transfer of the  $Cml^+$  phenotype to the non-producing parent was observed.

Three additional crosses were performed and the results analysed as above.

b. Cross II: VS146 (nic-6 cml-7) X VS206 (his-6 ade-10 strA6)

Table 12 gives the frequencies for the recombinant classes in cross II. From the relative recombination frequencies it can be seen that ade-10 and strA6 are more closely linked than any other pairs of

Table 10. Segregation of unselected alleles (cross I)\*

(met-14 cml-7) X (his-6 ade-10 strA6)

Medium 1 (MM + ade)

Medium 2 (MM + ade met and str)

Selected alleles: his<sup>+</sup>/met<sup>+</sup>

Selected alleles: his<sup>+</sup>/str

	<u>ade<sup>+</sup></u>	<u>ade<sup>-</sup></u>
<u>str</u>	9	58
<u>str<sup>+</sup></u>	2	7

	<u>met<sup>+</sup></u>	<u>met<sup>-</sup></u>
<u>ade<sup>+</sup></u>	8	11
<u>ade<sup>-</sup></u>	83	0

Chi-square = 0.57  
0.50 < P < .30

Chi-square = 45.23  
P < .001

Medium 3 (MM + met his and str)

Medium 4 (MM + his)

Selected alleles: ade<sup>+</sup>/str

Selected alleles: met<sup>+</sup>ade<sup>+</sup>

	<u>met<sup>+</sup></u>	<u>met<sup>-</sup></u>
<u>his<sup>+</sup></u>	24	31
<u>his<sup>-</sup></u>	8	7

	<u>his<sup>+</sup></u>	<u>his<sup>-</sup></u>
<u>str</u>	51	4
<u>str<sup>+</sup></u>	20	0

Chi-square = 0.13  
0.80 < P < .70

Chi-square = 0.043  
0.70 < P < 0.50

Conclusions:

1. ade-10 and strA6 are nonadjacent
2. met-14 and ade-10 are adjacent
3. met-14 and his-6 are nonadjacent
4. his-6 and strA6 are apparently nonadjacent

\*Values for each genotype frequency on each medium are taken from column A in Table 8. Probability values were obtained from Stansfield, 1969.

Table 11. Chloramphenicol production in progeny from cross I

(met-14 cml-7) X (his-6 ade-10 strA6)

Genotype	Number screened	Chloramphenicol producers*	
		Number	%
+ <u>his</u> + <u>str</u>	10	10	100
<u>met</u> + + <u>str</u>	21	1	5
+ + + <u>str</u>	25	0	0
+ + <u>ade str</u>	27	4	15
+ + <u>ade</u> +	2	0	0
<u>met</u> + + +**	36	0	0

\*A sample of progeny from the cross was screened for chloramphenicol production using the bioassay.

\*\*Parental Type

Table 12. Analysis of a four-factor cross (Cross II: nic-6 cml-7 X his-6 ade-10 strA6)\*

Genotypes <sup>a</sup> of selectable progeny	Selective media supplements						Average frequency of complementary - genotypes	
	ade		his		nic,ade str			
	A	B	A	B	A	B	A	B
+ + + +	196	196	165	165				181
+ + + <u>str</u>	0	0	0	0	0	0	0	0
<u>nic</u> + + <u>str</u>					67	67	89	37
+ <u>his</u> + <u>str</u>			25	25			49	20
+ + <u>ade</u> <u>str</u>	4	4			7	7		6
+ <u>his</u> + +			310	310				>210
<u>nic</u> + <u>ade</u> <u>str</u>					109	109		
+ + <u>ade</u> +	0	0						>0
<u>nic</u> <u>his</u> + <u>str</u>							1	0
sample size:	200		500		183		139	
total recombinants per plate:		200		500		183		57

Relative recombination frequencies in each interval \*\*

<u>nic-ade</u>	<u>ade-his</u>	<u>nic-his</u>	<u>his-str</u>	<u>ade-str</u>	<u>nic-str</u>
181	23	181	0	0	181
0	6	0	52	52	52
23	210	6	6	23	210
210	0	0	210	0	0
Totals	414	239	187	268	75
					443

\* See Table 8.

\*\* See Table 8.

markers. The data from Table 13 suggest that model 2 is the most likely relative order of markers. This is consistent with the segregational analysis presented in Table 14, which suggests that:

- ade-10 and strA6 are adjacent
- his-6 and strA6 are adjacent
- nic-6 and ade-10 are apparently nonadjacent
- nic-6 and his-6 are adjacent

Table 15 gives an analysis of chloramphenicol production in progeny from cross II. The data again suggest that cml-7 is located in the arc between his-6 and strA6.

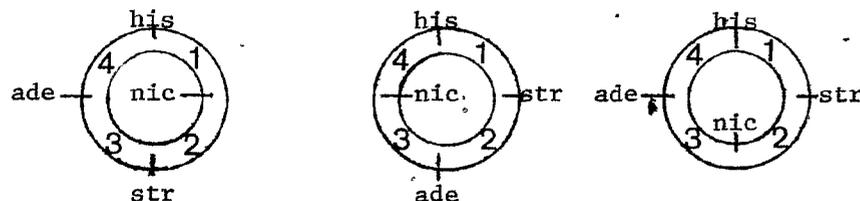
c. Cross III: VS141(lysA7 cml-1) X VS206(his-6 ade-10 strA6)

Table 16 gives the frequencies for the recombinant classes in cross III. From the relative recombination frequencies it can be seen that ade-10 is close to strA6 and lysA7 is close to strA6 while lysA7 and his-6 and are further apart, as also are his-6 and strA6. The data from Table 17 suggest that model 1 is the most likely relative order of markers. This is consistent with the segregational analysis presented in Table 18, which suggests that:

- ade-10 and strA6 are adjacent
- his-6 and strA6 are nonadjacent
- ade-10 and lysA7 are nonadjacent
- his-6 and lysA7 are apparently nonadjacent

Table 19 gives an analysis of chloramphenicol production in progeny from cross III. The data suggest that cml-1 is located in the arc between his-6 and lysA7.

Table 13. Comparison of the genotype frequencies with the cross-over regions required by the three possible chromosomal marker models (Cross II: nic-6 cml-7 X his-6 ade-10 strA6)\*



Medium	Genotype	Frequency	Crossovers			Indicated Model
			1	2	3	
MM	+ + + +	196	1,2	3,4	2,3	1,2,3
+ ade	+ + + <u>str</u>	0	1,3	1,2,3,4,	1,3	
	+ + <u>ade</u> <u>str</u>	4	1,4	1,4	1,4	
	+ + <u>ade</u> +	0	1,2,3,4	2,4	2,4	
MM	+ + + +	165	1,2	3,4	2,3	1 or 2
+ his	+ + + <u>str</u>	0	1,3	1,2,3,4	1,3	
	+ <u>his</u> + <u>str</u>	25	3,4	2,3	3,4	
	+ <u>his</u> + +	310	2,4	1,3	1,2,3,4	
MM	+ + + <u>str</u>	0	1,3	1,2,3,4	1,3	1 or 2
+ nic	<u>nic</u> + + <u>str</u>	67	2,3	1,2	1,2	
ade	+ + <u>ade</u> <u>str</u>	7	1,4	1,4	1,4	
str	<u>nic</u> + <u>ade</u> <u>str</u>	109	2,4	1,3	1,2,3,4	
MM	+ + + <u>str</u>	0	1,3	1,2,3,4	1,3	2 or 3
+ nic	<u>nic</u> + + <u>str</u>	89	2,3	1,2	1,2	
his	+ <u>his</u> + <u>str</u>	49	3,4	2,3	3,4	
str	<u>nic</u> <u>his</u> + <u>str</u>	1	1,2,3,4	2,4	2,4	

\* See Table 9.

Table 14. Segregation of unselected alleles (cross II)\*

(nic-6 cml-7) X (his-6 ade-10 strA6)

Medium 1 (MM + ade)Medium 2 (MM + his)Selected alleles: his<sup>+</sup>/nic<sup>+</sup>Selected alleles: ade<sup>+</sup>/nic<sup>+</sup>

	<u>ade<sup>+</sup></u>	<u>ade<sup>-</sup></u>
<u>str</u>	0	4
<u>str<sup>+</sup></u>	196	0

Chi-square = 198.1  
P<.001

	<u>his<sup>+</sup></u>	<u>his<sup>-</sup></u>
<u>str</u>	0	25
<u>str<sup>+</sup></u>	165	310

Chi-square = 11.43  
P<.001Medium 3 (MM + nic ade str)Medium 4 (MM + nic his str)Selected alleles: his<sup>+</sup>/strSelected alleles: ade<sup>+</sup>/str

	<u>nic<sup>+</sup></u>	<u>nic<sup>-</sup></u>
<u>ade<sup>+</sup></u>	0	67
<u>ade<sup>-</sup></u>	7	109

Chi-square = 2.72  
P=.10

	<u>nic<sup>+</sup></u>	<u>nic<sup>-</sup></u>
<u>his<sup>+</sup></u>	0	89
<u>his<sup>-</sup></u>	49	1

Chi-square = 102.15  
P<.001

## Conclusions:

1. ade-10 and strA6 are adjacent
2. his-6 and strA6 are adjacent
3. nic-6 and ade-10 are apparently nonadjacent
4. nic-6 and his-6 are adjacent

\* See Table 8 for procedure. Values for each genotype frequency are taken from column A in Table 12.

Table 15.  
Chloramphenicol production in progeny from cross II\*

(nic-6 cm1-7) X (his-6 ade-10 strA6)

Genotype	Number screened	Chloramphenicol producers	
		Number	%
+ + + +	28	0	0
<u>nic</u> + + <u>str</u>	19	0	0
<u>nic</u> + <u>ade</u> <u>str</u>	21	4	19
+ <u>his</u> + <u>str</u>	12	12	100
+ <u>his</u> + +	5	1	20
<u>nic</u> + + +**	76	0	0

\*See Table 11.

\*\*Parental type

Table 16. Analysis of a four-factor cross (Cross III: lysA7 cml-1 X his-6 ade-10 strA6)\*

Genotypes of selectable progeny	Selective media supplements						lys, his str		Average frequency of complementary genotypes
	ade		his		lys,ade str				
	A	B	A	B	A	B	A	B	
+ + + +	5	5	68	7					6
+ + + <u>str</u>	1	1	19	2	1	1	23	2	2
<u>lys</u> + + <u>str</u>					1	1	29	3	2
+ <u>his</u> + <u>str</u>			1	1			4	1	1
+ + <u>ade</u> <u>str</u>	47	47			38	38			43
+ <u>his</u> + +			7	1					>3
<u>lys</u> + <u>ade</u> <u>str</u>					4	4			
+ + <u>ade</u> +	0	0							>0
<u>lys</u> <u>his</u> + <u>str</u>							0	0	
Sample Size:	53		95		44		56		
Total Recombinants per plate:		53		10		44		5	

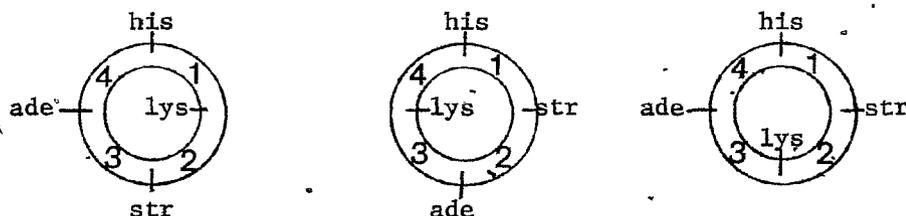
Relative Recombination Frequencies in Each Interval\*\*

	<u>lys-ade</u>	<u>ade-his</u>	<u>lys-his</u>	<u>his-str</u>	<u>ade-str</u>	<u>lys-str</u>
	6	0	6	2	2	2
	2	43	2	2	2	2
	0	3	43	43	0	3
	3	0	3	3	0	0
Totals	11	46	54	50	4	7

\*See Table 8.

\*\*See Table 8.

Table 17. Comparison of the genotype frequencies with the crossover regions required by the three possible chromosomal marker models (Cross III: lysA7 cml-1 X his-6 ade-10 strA6) \*



Medium	Genotype	Frequency	Crossovers			Indicated Model
			1	2	3	
MM + ade	+ + + +	5	1,2	3,4	2,3	1 or 3
	+ + + <u>str</u>	1	1,3	1,2,3,4	1,3	
	+ + <u>ade</u> <u>str</u>	47	1,4	1,4	1,4	
	+ + <u>ade</u> +	0	1,2,3,4	2,4	2,4	
MM + his	+ + + +	68	1,2	3,4	2,3	1
	+ + + <u>str</u>	19	1,3	1,2,3,4	1,3	
	+ <u>his</u> + <u>str</u>	1	3,4	2,3	3,4	
	+ <u>his</u> + +	7	2,4	1,3	1,2,3,4	
MM + lys ade str	+ + + <u>str</u>	1	1,3	1,2,3,4	1,3	1
	<u>lys</u> + + <u>str</u>	1	2,3	1,2	1,2	
	+ + <u>ade</u> <u>str</u>	38	1,4	1,4	1;	
	<u>lys</u> + <u>ade</u> <u>str</u>	4	2,4	1,3	1,2,3,4	
MM + lys his str	+ + + <u>str</u>	23	1,3	1,2,3,4	1,3	1 or 3
	<u>lys</u> + + <u>str</u>	29	2,3	1,2	1,2	
	+ <u>his</u> + <u>str</u>	4	3,4	2,3	3,4	
	<u>lys</u> <u>his</u> + <u>str</u>	0	1,2,3,4	2,4	2,4	

\*See Table 9.

Table 18. Segregation of unselected alleles (cross III) \*

(lysA7 cml-1) X (his-6 ade-10 strA6)

Medium 1 (MM + ade)

Medium 2 (MM + his)

Selected alleles: his<sup>+</sup>/lys<sup>+</sup>

Selected alleles: ade<sup>+</sup>/lys<sup>+</sup>

	<u>ade</u> <sup>+</sup>	<u>ade</u> <sup>-</sup>
<u>str</u>	1	47
<u>str</u> <sup>+</sup>	5	0

	<u>his</u> <sup>+</sup>	<u>his</u> <sup>-</sup>
<u>str</u>	19	1
<u>str</u> <sup>+</sup>	68	7

Chi-square = 33.76  
P < .001

Chi-square = .027  
0.70 < P < 0.50

Medium 3 (MM + lys ade str)

Medium 4 (MM + his lys str)

Selected alleles: his<sup>+</sup>/str

Selected alleles: ade<sup>+</sup>/str

	<u>ade</u> <sup>+</sup>	<u>ade</u> <sup>-</sup>
<u>lys</u> <sup>+</sup>	1	38
<u>lys</u> <sup>-</sup>	1	4

	<u>his</u> <sup>+</sup>	<u>his</u> <sup>-</sup>
<u>lys</u> <sup>+</sup>	23	4
<u>lys</u> <sup>-</sup>	29	0

Chi-square = 0.385  
0.70 < P < 0.50

Chi-square = 2.66  
0.20 < P < 0.10

Conclusions:

1. ade-10 and strA6 are adjacent
2. his-6 and strA6 are nonadjacent
3. ade-10 and lysA7 are nonadjacent
4. his-6 and lysA7 are nonadjacent

\* See Table 10 for procedure. Values for each genotype frequency on each medium are taken from column A in Table 16.

Table 19. Chloramphenicol production in progeny from cross III\*

(lysA7 cml-1) X (his-6 ade-10 strA6)

Genotype	Number screened	Chloramphenicol producers	
		Number	%
+ + + +	15	0	0
+ + + <u>str</u>	36	2	6
+ + <u>ade str</u>	34	12	35
+ <u>his</u> + <u>str</u>	5	5	100
<u>lys</u> + + +**	30	0	0

\*See Table 11.

\*\*Parental type

d. Cross IV: VS153(trp-3 cml-5) X VS206(his-6 ade-10 strA6)

Table 20 gives the frequencies for the recombinant classes of cross IV. From the relative frequencies it can be seen that trp-3 and his-6 are very closely linked. Because there is very little crossing over between these two markers the data are ambiguous (Table 21) and consistent with models 2 or 3. Chi-square analysis was applicable to only one pair of markers, trp-3 and his-6, and confirmed that these are closely linked. (Table 22). Table 23 gives an analysis of chloramphenicol production in progeny from cross IV. The data suggest that cml-5 is located in the arc between his-6 and strA6. A summary of the results of the previous four crosses is presented in Figure 13.

e. Crosses between prototrophic Cml mutants and VS206.

Since auxotrophic derivatives of the remaining Cml mutants were not available, these prototrophs were crossed with VS206 and the following progeny were isolated:

- a) + + str
- b) + ade str
- c) his + str

Approximately 100 progeny from each cross were tested for chloramphenicol production. The results are given in Table 24. In all crosses 100% of the his + str progeny were chloramphenicol producers. This finding suggests that the wild type alleles of all the cml mutations represented in these crosses are located in the arc between his-6 and strA6, that is, opposite rather than adjacent to the ade-10 region.

Table 20. Analysis of a four-factor cross (Cross IV: trp-3 cml-5 X his-6 ade-10 strA6)\*

Genotypes of selectable progeny	Selective media supplements								Average frequency of complementary genotypes
	ade		his		his, trp str		ade, trp str		
	A	B	A	B	A	B	A	B	
+ + + +	0	0	0	0					0
+ + + <u>str</u>	0	0	0	0	0	0	0	0	0
<u>trp</u> + + <u>str</u>					92	25	43	23	24
+ <u>his</u> + <u>str</u>			1	1	1	0			1
+ + <u>ade</u> <u>str</u>	2	2					0	0	1
+ <u>his</u> + +			66	66					>46
<u>trp</u> + <u>ade</u> <u>str</u>							50	26	
+ + <u>ade</u> +	0	0							>0
<u>trp</u> <u>his</u> + <u>str</u>					0	0			
Sample Size:	2		67		93		93		
Total Recombinants per Plate:	2		67		25		49		

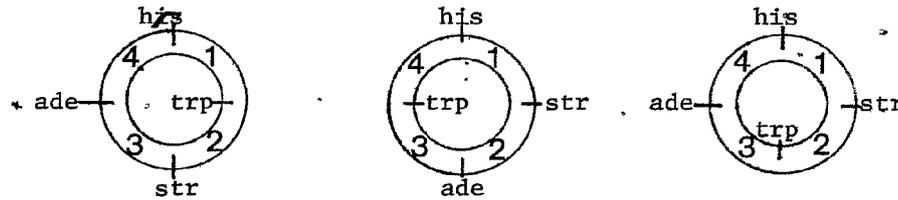
Relative Recombination Frequencies in Each Interval \* \*

<u>trp-ade</u>	<u>ade-his</u>	<u>trp-his</u>	<u>his-str</u>	<u>trp-str</u>	<u>ade-str</u>
0	1	0	0	0	0
0	1	0	24	43	24
1	46	1	1	46	1
46	0	0	46	0	0
Total	47	48	1	71	89

\*See Table 8.

\*\*See Table 8.

Table 21. Comparison of the genotype frequencies with the crossover regions required by the three possible chromosomal marker models (Cross IV: trp-3 cml-5 X his-6 ade-10 strA6)\*



Medium	Genotype	Frequency	Crossovers			Indicated Model
			1	2	3	
MM + ade	+ + + +	0	1,2	3,4	2,3	1,2,3
	+ + + <u>str</u>	0	1,3	1,2,3,4	1,3	
	+ + <u>ade</u> <u>str</u>	2	1,4	1,4	1,4	
	+ + <u>ade</u> +	0	1,2,3,4	2,4	2,4	
MM + his	+ + + +	0	1,2	3,4	2,3	1 or 2
	+ + + <u>str</u>	0	1,3	1,2,3,4	1,3	
	+ <u>his</u> + <u>str</u>	1	3,4	2,3	3,4	
	+ <u>his</u> + +	66	2,4	1,3	1,2,3,4	
MM + trp ade str	+ + + <u>str</u>	0	1,3	1,2,3,4	1,3	1 or 2
	<u>trp</u> + + <u>str</u>	43	2,3	1,2	1,2	
	+ + <u>ade</u> <u>str</u>	0	1,4	1,4	1,4	
	<u>trp</u> + <u>ade</u> <u>str</u>	50	2,4	1,3	1,2,3,4	
MM + trp his str	+ + + <u>str</u>	0	1,3	1,2,3,4	1,3	1, 2 or 3
	<u>trp</u> + + <u>str</u>	92	2,3	1,2	1,2	
	+ <u>his</u> + <u>str</u>	1	3,4	2,3	3,4	
	<u>trp</u> <u>his</u> + <u>str</u>	0	1,2,3,4	2,4	2,4	

\*See Table 9.

Table 22. Segregation of Unselected Alleles (Cross IV) \*

(trp-3 cml-5) X (his-6 ade-10 strA6)

Medium 1 (MM + ade)Medium 2 (MM + his)Selected alleles: his<sup>+</sup>/trp<sup>+</sup>Selected alleles: ade<sup>+</sup>/trp<sup>+</sup>

	<u>ade<sup>+</sup></u>	<u>ade<sup>-</sup></u>
<u>str<sup>-</sup></u>	0	2
<u>str<sup>+</sup></u>	0	0

	<u>his<sup>+</sup></u>	<u>his<sup>-</sup></u>
<u>str<sup>-</sup></u>	0	1
<u>str<sup>+</sup></u>	0	66

Chi-square cannot be calculated

Chi-square cannot be calculated

Medium 3 (MM + his trp str)Medium 4 (MM + ade trp and str)Selected alleles: ade<sup>+</sup>/str<sup>+</sup>Selected alleles: his<sup>+</sup>/str<sup>+</sup>

	<u>his<sup>+</sup></u>	<u>his<sup>-</sup></u>
<u>trp<sup>+</sup></u>	0	1
<u>trp<sup>-</sup></u>	92	0

	<u>ade<sup>+</sup></u>	<u>ade<sup>-</sup></u>
<u>trp<sup>+</sup></u>	0	0
<u>trp<sup>-</sup></u>	43	50

Chi-square = 24.43  
P < .001

Chi-square cannot be calculated

Conclusion:

1. his-6 and trp-3 are adjacent

\*See Table 10. Values for each genotype frequency are taken from column A in Table 20.

Table 23. Chloramphenicol production in progeny from cross IV\*

(trp-3 cml-5) X (his-6 ade-10 strA6)

Genotype	Number screened	Chloramphenicol Number	production <sup>**</sup> %
<u>trp</u> + + <u>str</u>	31	3	10
+ <u>his</u> + <u>str</u>	24	24	100
+ <u>his</u> + +	9	2	22
<u>trp</u> + <u>ade</u> <u>str</u>	10	0	0
<u>Lrp</u> + + + **	46	0	0

\*See Table 11.

\*\*Parental type

Figure 13. Relative order of auxotrophic and cml markers from crosses I-IV. cml-1 lies in the arc between his-6 and lysA7 and cml-5 and cml-7 lie in the arc between his-6 and strA6. Due to the ambiguity of the data concerning its location the marker trp-3 has not been included.

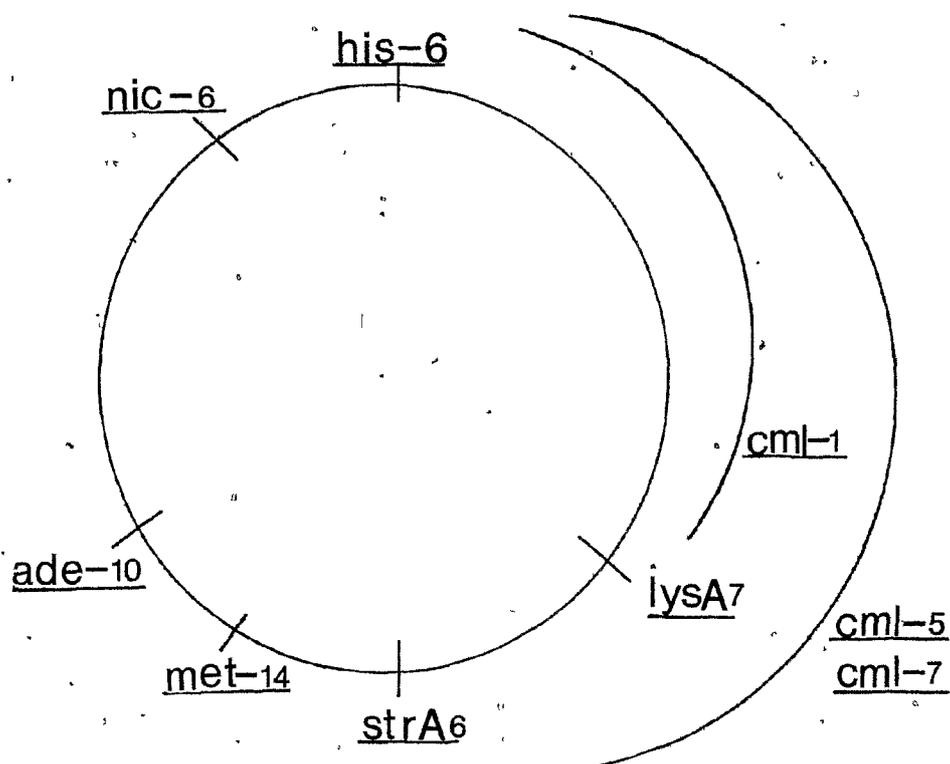


Table 24. Chloramphenicol production in progeny from crosses between Cml mutants and VS206\*

Cml mutant	Progeny genotypes					
	+ + + str		+ his + str		+ + ade str	
	Number tested	Cml <sup>+</sup>	Number tested	Cml <sup>+</sup>	Number tested	Cml <sup>+</sup>
Cml-2	48	2	8	8	31	9
Cml-3	57	0	7	7	31	2
Cml-6	47	1	21	21	29	12
Cml-8	48	1	13	13	27	10
Cml-9	20	0	3	3	20	7
Cml-11	39	0	13	13	20	11
Cml-12	43	3	19	19	11	7

\* A sample of progeny from the three recombinant classes indicated was screened for chloramphenicol production. The number of colonies of each genotype screened and the number of these that were chloramphenicol producers is listed for each genotype. In the far left-hand column the Cml mutant acting as the parent in each cross is listed.

## DISCUSSION

I. Growth and Chloramphenicol Production on Various Amino Acids

When individual amino acids were used as the sole source of nitrogen for growth of S. phaeochromogenes and S. venezuelae, chloramphenicol titres in cultures growing on amino acids that supported rapid growth were lower than in those that supported slow growth. Similar results were obtained with cultures of S. venezuelae strain 13s (Westlake et al., 1968; Shapiro and Vining, 1983). These observations indicate a possible link between chloramphenicol production and primary metabolic pathways that control nitrogen utilization. The similar relationship between growth and chloramphenicol production in S. venezuelae and S. phaeochromogenes cultures with each amino acid suggests that the same regulatory mechanisms function in both organisms. Control of antibiotic biosynthesis through nitrogen metabolite regulation appears to be common among streptomycetes and has been reported in the biosynthesis of nourseothricin in Streptomyces noursei (Gräfe et al., 1977) cephalosporin in Streptomyces clavuligerus (Aharonowitz and Demain, 1979), and tylosin in S. fradiae (Omura et al., 1984).

II. Screening of Chloramphenicol-producing Streptomycetes for Plasmid DNA

Of eight chloramphenicol-producing streptomycetes screened using the rapid alkaline-extraction technique, only S. phaeochromogenes NRRLB-3559 was found to contain ccc DNA. This may be an underestimate of the frequency with which plasmids occur as the technique may not have revealed large plasmids. However, a similarly low (2/21) frequency of

plasmid-containing strains was reported by Omura et al. (1981) in a sample of macrolide-producing streptomycetes examined using a technique that allowed very large plasmids to be isolated. Also consistent with this low frequency are the findings of Daniel and Tiraby (1983) who found only 21 plasmid-containing strains in 120 different streptomycetes. Moreover, the proportion of such strains among antibiotic producers was no higher than the proportion among nonproducers, leading to the conclusion that plasmid-borne genes are not necessarily involved in antibiotic biosynthesis. Slightly higher proportions of plasmid-containing strains were found in screening studies by Kirby et al. (1982) and Okanishi et al. (1980); however, both groups concluded that plasmids are not ubiquitous in Streptomyces.

Although genetic studies (Akagawa et al., 1979) had suggested possible plasmid involvement in chloramphenicol production in S. venezuelae ISP5230, no plasmid DNA could be isolated from the S. venezuelae strain used in the present studies, although it is derived from the same parental stock. Only the rapid-alkaline extraction and density-gradient centrifugation techniques were used, but the application of several other plasmid isolation techniques to a strain of S. venezuelae ISP5230 received from A. Seino, Kaken Chemical Co., as strain KCCS-0526 also failed to reveal ccc DNA (Z. Ahmed, personal communication). Okanishi et al. (1980) and Kirby et al. (1982) have recently reported fruitless attempts to isolate plasmid from this strain. It seems that, if S. venezuelae ISP5230 harbours a plasmid, it possesses unusual characteristics, i.e. it may be very large or possibly even linear (Hayakawa et al., 1979) so that its isolation is not possible by standard techniques.

III. Elimination of pJV1

Neither ethidium bromide treatment nor protoplast formation and regeneration alone proved effective in eliminating pJV1 from cultures of S. phaeochromogenes. Instead, a combination of both procedures was used; ethidium bromide treatment generated a strain, EB116, carrying a putative low-copy-number plasmid. Subsequently a plasmid-free derivative, strain SP2, was obtained after subjecting strain EB116 to protoplast formation and regeneration. That strain SP2 was indeed pJV1<sup>-</sup> and did not harbour the plasmid in an integrated state was demonstrated by probing total DNA of strain SP2 with <sup>32</sup>P-labeled pJV1; no evidence of hybridization was seen, even after X-ray film was exposed to the nitrocellulose filter for 5 d.

Although the copy number of pJV1 was not determined in this study, the large amount of plasmid DNA extractable from S. phaeochromogenes mycelium suggests that the plasmid multiplicity is very high. This may explain why pJV1 was difficult to eliminate from the wild type strain whereas it could be eliminated from strain EB116 by protoplast formation and regeneration with relative ease. Hopwood (1981) considered the possible involvement of plasmid copy number in determining the effectiveness of curing by protoplasting. He reported that two low copy number plasmids, SCP1 and SCP2 could be eliminated using this technique while SCP2\* could not. This he attributed to the higher copy number of SCP2\*. However, in their work with Staphylococcus aureus, Novick et al. (1980) found that the curing effect of protoplast formation and regeneration was not related to copy number as all of the curable plasmids they observed had greater copy numbers than the noncurable ones. They

suggested that plasmids from the former group may be associated with the cell envelope while those from the latter might be associated with the chromosome. Since the mechanism by which plasmids are eliminated from protoplast regenerants remains obscure and may differ in the mycelial Streptomyces from that operating in unicellular gram-positive bacteria, the potential conflict in these observations cannot be resolved without further study. The possibility that pJV1 is associated differently with cellular components of strain EB116 compared with strain SP1, making it easier to eliminate, seems unlikely but has not been excluded.

The nature of the defect in strain EB116 has not been investigated in the present study; however, the observation that pJV1 can be reintroduced into a genetically marked derivative of strain SP2 in mixed cultures with strain SP1 and is reestablished at an apparently high copy number (R. Mosher, personal communication) is consistent with strain EB116 having an altered plasmid (pJV101) rather than a chromosomal mutation affecting plasmid maintenance. Any such plasmid alteration could not involve large changes in the amount of DNA as plasmid DNA from strain EB116 has the same electrophoretic mobility as that from strain SP1.

#### IV. Chloramphenicol Production in Strains SP1, SP2, and EB116

To obtain the pJV1-cured strain it was necessary to expose SP1 to ethidium bromide before protoplast formation and regeneration. Treatment with ethidium bromide has been known to produce DNA rearrangements and deletions in streptomycetes (Schrempf, 1983). Even the process of protoplast formation and regeneration, once regarded as a gentle and "safe" method of plasmid elimination, has been reported to cause pheno-

typic alterations which cannot be attributed solely to plasmid loss (Furumai et al., 1982; Ikeda et al., 1983). It is possible, therefore, that strains SP2 and EB116 have undergone different chromosomal changes.

Despite this possibility no differences were found between strains SP1, SP2 and EB116 when growth and chloramphenicol production levels in glucose-isoleucine medium were compared. This strongly suggests that pJV1 is not involved in chloramphenicol biosynthesis. A similar finding was reported by Ahmed and Vining (1983): elimination of plasmid pUC3 from *S. venezuelae* strain 13s had no effect on chloramphenicol production. These results do not exclude the possibility that an as yet unidentified plasmid may be involved in antibiotic production in these two species.

## V. Isolation of Chloramphenicol-nonproducing Strains

### a. Mutagenesis

Of the four mutagenic treatments employed, long-wavelength ultraviolet irradiation in the presence of 8-methoxypsoralen and short-wavelength ultraviolet irradiation in the absence of caffeine yielded the highest frequencies of nonproducing mutants from strain HP-1. Contrary to previous results with *S. venezuelae* strain 13s (Francis et al., 1975), posttreatment with caffeine did not increase the mutagenic effect of short-wavelength radiation. However, slight differences in method as well as strain differences may account for the discrepancy.

The mutagenic methods used were chosen to maximize the number of chloramphenicol nonproducers likely to bear only single point mutations.

Nitrosoguanidine, which is an efficient mutagen in Streptomyces (Delić et al., 1970) was not used since it produces clustered multiple mutations in bacteria such as E. coli (Cerdeña-Olmedo et al., 1968). Such mutations would hinder subsequent genetic analysis.

b. Mutant characterization

i. Cml-1 and Cml-12

Of the twelve mutants isolated, Cml-1 and Cml-2 produced chloramphenicol when grown in production medium supplemented with p-aminophenylalanine and were therefore blocked in the initial stage of chloramphenicol biosynthesis - the conversion of chorismic acid to p-aminophenylalanine (Jones and Vining, 1976). The possibility that either of these mutants was blocked in the synthesis of chorismic acid can be ruled out because both strains were prototrophic and thus could produce chorismate-derived amino acids and cofactors. The conclusion that Cml-1 was blocked in this initial step is strengthened by the finding that no arylamine synthetase activity could be found in cell extracts whereas activity was detected in extracts of the parental strain, HP-1, prepared under similar conditions (R. Bhatnagar, personal communication). Cml-12 has not yet been tested for arylamine synthetase activity. Furthermore chloramphenicol is produced when Cml-4 and Cml-5 (which excrete p-aminophenylalanine) are grown in mixed cultures with either Cml-1 or Cml-12. The weak cosynthesis observed with another p-aminophenylalanine excretor, Cml-8, is attributable to the smaller amount released by this mutant.

Jones and Vining (1976) and Jones et al. (1978) have suggested that the conversion of chorismic acid to p-aminophenylalanine proceeds in at

least two steps, namely aromatization producing p-aminophenylpyruvic acid followed by transamination to yield p-aminophenylalanine. This proposal was consistent with the discovery in cultures of S. venezuelae strain 13s of an aminotransferase that accepts p-aminophenylalanine as a substrate (Jones et al., 1978). It is not known at which step mutants Cml-1 and Cml-12 are blocked and the instability of p-aminophenylpyruvic acid prevents its use in feeding studies to locate the lesion, but it would be instructive to examine cell extracts for aminotransferase activity.

ii. Cml-2

Mutant Cml-2 accumulated three aromatic nitro-compounds identified as: D-threo-1-p-nitrophenyl-2-isobutyramido-1,3-propanediol, D-threo-1-p-nitrophenyl-2-propionamido-1,3-propanediol and D-threo-1-p-nitrophenyl-2-acetamido-1,3-propanediol.

These same three derivatives of p-nitrophenylserinol are produced by S. venezuelae in halide-free medium (Smith, 1958) and by Corynebacterium hydrocarboclastus (Shirahata et al., 1972). Trace amounts of these compounds have also been reported to accompany chloramphenicol under conditions of antibiotic synthesis in S. venezuelae (Stratton and Rebstock, 1963). The finding that concentrated broths of Cml-2 gave weak inhibition of M. luteus is consistent with the findings of Suzuki et al. (1972) that these compounds possess antibiotic activity.

Since it produces three nonchlorinated chloramphenicol analogues, Cml-2 appears to be blocked in the reaction that chlorinates the N-acyl group of chloramphenicol. Simonsen et al. (1978) proposed a model for the dichloroacylation reaction in which N-malonyl-p-aminophenylserine is the substrate for a chloroperoxidase enzyme. The finding that neither

N-malonyl-p-aminophenylserine nor its derivatives were accumulated by Cml-2 suggests that the acyl substituent might be chlorinated before attachment to the phenylpropanoid intermediate. In the absence of the halogenated acylation substrate; a low specificity transferase catalyzing the acylation reaction may accept less preferred substrates such as acetyl, propionyl and isobutyryl coenzyme A.

iii. Mutants Cml-4, Cml-5 and Cml-8

Three mutants, Cml-4, Cml-5 and Cml-8 accumulated a polar aromatic amino-compound that could be isolated using cation-exchange chromatography. This compound was identified from its <sup>1</sup>H-NMR spectrum and by chromatographic comparison with an authentic sample as p-aminophenylalanine. It is a direct precursor of chloramphenicol (Siddiqueullah et al., 1967) and has been detected in the mycelium of producing cultures (McGrath et al., 1968). It has also been detected in culture broths of Cml-2, the producing strain HP-1 (data not shown), and corynecin-producing cultures of Corynebacterium hydrocarboclastus (Nakano et al., 1976).

No p-aminophenylserine was detected in the culture broths of mutants Cml-4, -5 and -8. It is likely therefore that they are blocked in the conversion of p-aminophenylalanine to p-aminophenylserine. Consistent with this suggestion is the finding that no p-aminophenylalanine β-hydroxylase activity could be detected in Cml-4 (R. Bhatnagar, personal communication). Mutants Cml-5 and Cml-8 have not yet been tested for this activity. A further confirmation that these mutants are blocked in the hydroxylation reaction would be the demonstration that they produce chloramphenicol when supplied with p-aminophenylserine.

Unfortunately, this compound was not available for feeding studies.

Akagawa et al. (1979) reported the isolation of a mutant of S. venezuelae ISP5320 which excreted deoxychloramphenicol and was putatively blocked in this same  $\beta$ -hydroxylation reaction. The mutant accumulated only one tenth as much p-aminophenylalanine as the producing strain from which it was derived and cell extracts showed only about one tenth of the arylamine synthetase activity. By contrast, mutant Cml-4 had slightly elevated arylamine synthetase activity compared with that found in HP-1 (R. Bhatnagar, personal communication) and neither Cml-4, -5 nor -8 accumulated deoxychloramphenicol. No explanation for these differences can be offered at this time.

Culture broths of Cml-4 and Cml-5 also contained an aromatic nitro compound which could be extracted from acidified broths and was tentatively identified by its chromatographic properties as N-acetyl-p-nitrophenylalanine. This compound accumulates in small amounts and is presumably derived from p-aminophenylalanine by the activity of enzymes catalyzing the acylation and oxidation reactions normally found in the pathway. That these enzymes should accept the nonhydroxylated substrate is illustrative of the low substrate specificity of many enzymes involved in secondary metabolism (Martin and Demain, 1980). The apparent activity of these enzymes diminishes the possibility that Cml-4 and Cml-5 have blocks further on in the pathway indirectly responsible for p-aminophenylalanine accumulation.

Extracts of Cml-5 showed an increase in aromatic amine content following acid hydrolysis, suggesting that an acylated aromatic amine is present. Such a compound; 2-dichloroacetamido-3-(p-acetamidophenyl) propan-1-ol, has been isolated from chloramphenicol producing cultures to

which p-nitrophenylserinol had been added (Wat et al., 1971). The latter compound, it was suggested, inhibited the hydroxylation of p-aminophenylalanine to p-aminophenylserine. The p-aminophenylalanine thus accumulated was modified by dichloroacetylation and reduction but underwent acetylation instead of oxidation at the p-amino group. 2-Dichloroacetamido-3-(p-acetamidophenyl)propan-1-ol could not be detected in culture broths of Cml-5 and it was concluded that the masked aromatic amine accumulated might be p-acetamidophenylalanine or N-acetyl-p-acetamidophenylalanine (or both).

iv. Mutants Cml-3 and Cml-10

Mutants Cml-3 and Cml-10 accumulated p-aminophenylalanine intracellularly whereas no such intracellular accumulation could be detected in the p-aminophenylalanine excretors, Cml-5 and Cml-8, using the same technique. This observation raises the possibility that mutants Cml-3 and Cml-10 are somehow defective in excretion of chloramphenicol intermediates.

v. Mutants Cml-6, -7, -9, and -11

Four mutant strains (Cml-6, -7, -9 and -11) did not accumulate aromatic amino- or nitro-compounds either extracellularly or intracellularly, nor were any "masked" aromatic amino-compounds formed. They also failed to produce chloramphenicol when fed p-aminophenylalanine. Several possibilities might explain this phenotype; i) the mutants have suffered multisite mutations. This seems improbable because simultaneous acquisition of two or more independent mutations would be a relatively rare event unlikely to account for the high proportion of "nonexcretors" found. ii) they are defective in the synthesis of p-

aminophenylpyruvic acid which might act as an inducer for the synthesis of other chloramphenicol biosynthetic enzymes. This possibility could not be tested since the potential inducer is too unstable to be synthesized. iii) the mutants accumulate enzyme-bound intermediates, as is the case with some of the peptide antibiotics (Kurylo-Borowska, 1975; Kurahashi, 1974). iv) they accumulate intermediates which act as powerful feedback effectors and turn off earlier steps in the pathway. v) they are regulatory mutants. This last suggestion raises the possibility that these mutants are blocked in the production of a bioregulator such as A-factor, a substance which is excreted by S. griseus and S. bikiniensis and is essential for streptomycin production, streptomycin resistance, and spore formation in these species (Hara and Beppu, 1982a,b). However, the observation that none of the mutants in this group (Cm1-6, -7, -9 and -11) produced chloramphenicol when grown in any pairwise combinations with other mutants, including those that accumulated p-aminophenylalanine or corynecins and are thus unlikely to be lacking a bioregulator, tends to exclude this suggestion.

The failure to obtain mutants accumulating intermediates other than p-aminophenylalanine and the corynecins might be due to the low specificity of additional enzymes in the pathway. Mutants of Streptomyces aureofaciens blocked in early steps in the tetracycline pathway produced congeners which retained a high level of antibiotic activity (Vaněk et al., 1971). A similar series of events in S. venezuelae might yield mutant strains that would have escaped attention since the initial screening was for absence of antibiotic activity. Again, a further possibility might be that some mutants accumulate enzyme-bound intermediates.

## VI . p-Aminophenylalanine $\beta$ -Hydroxylase

Cell extracts of S. venezuelae converted p-aminophenylalanine to a product, probably p-aminophenylserine, more susceptible to periodate oxidation. Although conclusions from this preliminary evidence are necessarily tentative, this activity is expected to be associated with the chloramphenicol pathway enzyme responsible for  $\beta$ -hydroxylation of p-aminophenylalanine (Figure 2 ). The conclusion is strengthened by the finding that cell extracts of Cml-4, a mutant thought to lack such an enzyme, did not convert p-aminophenylalanine to a periodate-oxidizable product (R. Bhatnagar, personal communication).

## VII. Chloramphenicol Resistance in Strain HP-1 and the Cml Mutants

Cultures of strain HP-1 were sensitive to chloramphenicol early in the growth phase before production of endogenous antibiotic began, but became resistant after the onset of antibiotic biosynthesis. Resistant cultures of strain HP-1 grown in the absence of chloramphenicol returned to a more sensitive state. Thus, chloramphenicol tolerance in strain HP-1 resembles that in another chloramphenicol producer, S. venezuelae strain 13s (Malik and Vining, 1972).

All 12 Cml mutants were sensitive to chloramphenicol added at a stage of growth in which the producer, strain HP-1, had become tolerant. Furthermore, Cml-9 was more sensitive than strain HP-1 to chloramphenicol added at the beginning of growth. This is in agreement with Akagawa et al. (1979) who reported that low and nonproducing mutants of S. venezuelae ISP5230 were more sensitive to chloramphenicol than the

chloramphenicol-producing strains from which they were derived. When chloramphenicol-supplemented Cml<sup>-</sup> cultures eventually grew, the cells were then chloramphenicol resistant. However, mycelium transferred into fresh chloramphenicol-containing medium lagged slightly behind unsupplemented cultures in growth. This lag may be due to heterogeneity of the cultures with respect to resistance. Only in one of the Cml mutants tested was chloramphenicol resistance accompanied by reversion to a chloramphenicol-producing phenotype.

A surprising finding was that resistant Cml cultures grown for 7 d in the absence of chloramphenicol did not return to a sensitive state as did the parent HP-1 strain and as also reported for S. venezuelae strain 13s (Malik and Vining, 1972). This implies that the mechanism of resistance in the Cml mutants is different from that in the producing strain. Malik and Vining (1972) suggested that chloramphenicol tolerance in S. venezuelae strain 13s is established through the reversible development of a permeability barrier which excludes antibiotic from the site of protein synthesis. It is possible that this mechanism is inextricably linked to chloramphenicol biosynthesis and that, in its absence, Cml mutants under selective pressure have acquired resistance by a different mechanism, possibly through modification of the 50s ribosomal subunit. Ribosomal modification through the action of specific methylases has been shown to be the mechanism by which the erythromycin producer, Streptomyces erythraeus (Skinner and Cundliffe, 1982) and the thiostrepton producer Streptomyces azureus (Cundliffe, 1978) protect themselves from the toxicity of products that they produce. As an alternative to target-site modification, the Cml mutants may have acquired changes altering the reversibility of the resistance mechanism normally found in

producing strains. Mutations causing constitutive rather than inducible resistance in producing strains to the macrolide, lincosamide and streptogramin type antibiotics have been described (Weisblum et al., 1982). Clearly, these suggestions require further study and might be explored by examining the susceptibility of ribosomes from resistant Cm1 mutants to the inhibitory effects of chloramphenicol, as well as by examining uptake of the antibiotic.

#### VIII. Fertility in *Streptomyces venezuelae*

Recombinants were obtained at very low frequencies in crosses between Cm1 mutants and the auxotrophic chloramphenicol-producing strains, VS160 and VS113. However, fertile derivatives of *S. venezuelae* (strains VS191 and VS192) were obtained following protoplast formation and regeneration of strain VS113 (C. Stuttard and K. Wong, personal communication). In spot-patch tests on MYM agar, spots of strain VS113 elicited inhibition of growth (lethal zygosis) of both VS191 and VS192, and these latter strains elicited the reaction in each other. A third fertile strain, VS194, was derived from VS191 after protoplast formation and regeneration. Both VS191 and VS192 as well as strain VS113 elicited lethal zygosis in this strain. Subsequent tests showed that the protoplasting treatment was probably irrelevant in the generation of lethal zygosis-sensitive strains from strain VS113 as these could be isolated with the same frequency from spores of unprotoplasted single colony isolates (C. Stuttard and K. Wong, personal communication).

Since lethal zygosis has often been shown to accompany plasmid transfer (Murakami et al., 1983; Ohnuki et al., 1983), it is possible that strains VS191, VS192 and VS194 have lost fertility plasmids which

are as yet unidentified. The increased fertility of these strains in crosses with other derivatives of S. venezuelae, including the Cml mutants which are presumably fertility plasmid<sup>+</sup>, might be due to increased recombination in plasmid<sup>-</sup> x plasmid<sup>+</sup> crosses compared with plasmid<sup>+</sup> x plasmid<sup>+</sup> crosses. This would resemble the situation with fertility plasmids SRP1 in S. rimosus (Friend et al., 1978) and SCP2 in S. coelicolor A3(2) (Bibb et al., 1977).

Any explanation for the increased fertility of strains VS191, VS192 and VS194 can be only tentative at this time as very little is known about conjugational fertility in Streptomyces. In several other bacterial and fungal systems there is considerable evidence for soluble factors that influence genetic exchange (Gooday, 1974 and Goldfarb et al., 1973). In Streptococcus faecalis, for example, plasmidless recipient strains excrete pheromones (Dunny et al., 1979). Conjugative plasmids in donor strains prevent the formation of endogenous pheromones but allow these strains to respond to exogenous pheromone by forming aggregating clumps of cells. Plasmid and chromosomal gene transfer is thus facilitated. In several instances, plasmids appear to be involved in fertility in Streptomyces (Bibb et al., 1977, 1981; Hopwood et al., 1983; Kieser et al., 1982). Indeed, the fertility of some strains has been increased by introducing plasmids from other species (Kieser et al., 1982; Hopwood, 1984). Whether plasmids are essential for all gene exchange in Streptomyces and if so what role they play is yet unknown. Their possible involvement in the production of substances or structures that promote cell contact or fusion leading to gene exchange, or their involvement in chromosomal gene mobilization or recombination is an interesting area of further study.

## IX. Mapping the cml Genes

All of the cml mutations isolated (except Cml-4 and Cml-10 which have not yet been included in crosses) mapped to the chromosome of S. venezuelae in the arc between his-6 and strA6, opposite the ade-10 region. Furthermore, when colonies arising from unselected spores harvested from crosses between Cml<sup>+</sup> and Cml<sup>-</sup> parents were tested for chloramphenicol production, none of those bearing the auxotrophic markers of the nonproducing parent in each cross were Cml<sup>+</sup>; This suggested that there was no independent transfer of chloramphenicol genes in the absence of chromosomal marker transfer. From the results of this study it appears that at least three of the chloramphenicol biosynthetic genes - those coding for arylamine synthetase, p-aminophenylalanine  $\beta$ -hydroxylase, and the enzyme responsible for chlorination - are located on the chromosome in the same arc. Mutations in strains that did not accumulate identifiable intermediates, and in strains that accumulated p-aminophenylalanine intracellularly, also mapped to this region. In so far as elements regulating chloramphenicol biosynthesis are represented by the former group, these all appear to be located in the same chromosomal region.

Akagawa et al. (1979) reported that five chloramphenicol pathway mutations, one causing production of deoxychloramphenicol, and four others which were for the most part uncharacterized, mapped to the S. venezuelae chromosome between met and ilv markers. Assuming a similarity between the S. venezuelae and S. coelicolor A3(2) maps, the met marker was thought to correspond to metA in S. coelicolor and the

ilv marker to ilvB (Akagawa et al., 1975). If as we predict, the his-6, ade-10 and strA6 markers used in this present study correspond to a lesion in the hisA, B, C, F, G, I cluster, adeA or C and strA in S. coelicolor A3(2), then our Cml mutations and those mapped by Akagawa et al. would be located in the same arc.

The three low-producing strains isolated by Akagawa et al. (1979) after treatment with "curing" agents carried mutations that showed no linkage with chromosomal markers, and crosses between such strains failed to yield recombinants that were high producers. These low producers were presumed therefore to have lost a plasmid with a regulatory role in increasing chloramphenicol production levels. Several results presented in this study raise doubts about this suggestion of plasmid involvement: i) plasmid DNA was not detected in the majority of chloramphenicol-producing strains; ii) pJV1, the only plasmid isolated from the eight producers screened, does not appear to have any role in chloramphenicol production; iii) mapping studies using blocked mutants failed to provide any evidence of extrachromosomal genes involved in chloramphenicol production; iv) no physical evidence of a plasmid in S. venezuelae strain HP-1 could be obtained. It has been suggested (C. Stuttard, personal communication) that the lethal zygosis phenomenon seen when VS113 is spotted on lawns of the fertile strains, VS191, VS192, and VS194 could result from absence of one or more fertility plasmids in these latter strains. If this is so, such plasmids apparently have no role in chloramphenicol production as all three derivatives of VS113 produce better than 100  $\mu\text{g.mL}^{-1}$  of chloramphenicol.

Although the mechanisms causing it remain to be fully explained it is becoming increasingly obvious that the instability of such features

as antibiotic production in Streptomyces cannot usually be attributed solely to plasmid loss (Komatsu et al., 1981; Ikeda et al., 1981b; Nojiri et al., 1980 and Yi-guang and Davies, 1981). Schrempf (1982), for example, demonstrated that although a large plasmid could be eliminated from strains of Streptomyces reticuli with acridine orange or ethidium bromide and variants producing no aerial mycelium, spores, antibiotic substances nor melanin were thus obtained, these variants also contained amplified nucleotide sequences within their chromosomal DNA. The number and size of the amplifications varied from strain to strain. It was assumed that the alterations in secondary metabolism were due to changes within both the chromosomal and plasmid DNA s of S. reticuli. Similarly amplified chromosomal sequences have been found to accompany spontaneous phenotypic changes (chloramphenicol sensitivity and a requirement for arginine) in S. lividans 66 (Altenbuchner, 1984) and chromosomal deletions have been shown to accompany alterations in melanin production in S. glaucescens (Hintermann et al., 1981). Future research may well reveal that mechanisms such as DNA rearrangement are the principal cause of the generally-observed instability of secondary metabolism in Streptomyces.

## SUMMARY and CONCLUSIONS

Of eight chloramphenicol-producing streptomycetes screened for the presence of ccc DNA, only S. phaeochromogenes NRRLB-3559 was found to harbour a plasmid. This plasmid, pJV1, could be eliminated using a combination of two techniques: ethidium bromide treatment and protoplast formation and regeneration. The elimination of pJV1 had no effect on chloramphenicol production in S. phaeochromogenes. It appears that plasmid DNA is not found in most chloramphenicol producers and that pJV1 in particular plays no role in the production of this antibiotic.

Twelve mutants of S. venezuelae ISP5230 blocked in the production of chloramphenicol were isolated following treatment of spores with EMS, long-wavelength ultraviolet light in the presence of 8-methoxypsoralen, or short-wavelength ultraviolet light followed by plating on a medium with and without caffeine. One of these mutants accumulates D-threo-1-p-nitrophenyl-2-isobutyramido-1,3-propanediol, D-threo-1-p-nitrophenyl-2-propionamido-1,3-propanediol, and D-threo-1-p-nitrophenyl-2-acetamido-1,3-propanediol and is presumably blocked in the reaction that chlorinates the substituted  $\alpha$ -amino group. Three others accumulated p-aminophenylalanine and are probably blocked in the hydroxylation reaction that converts this compound to p-aminophenylserine. Two other mutants produced chloramphenicol when fed p-aminophenylalanine and are probably blocked in the conversion of chorismic acid to p-aminophenylalanine. The above findings were consistent with the overall pathway suggested for chloramphenicol biosynthesis but the isolation of a mutant accumulating nonhalogenated acyl analogues of the antibiotic suggests

that the acyl substituent is chlorinated before attachment to the phenylpropanoid intermediate. In the absence of the halogenated acylation substrate, a low-specificity acyl transferase catalyzing the acylation reaction accepts less preferred substrates such as acetyl-, propionyl-, and isobutyryl- coenzyme A.

Two mutants accumulated p-aminophenylalanine intracellularly but not in culture broths, raising the possibility that they are defective in excretion of chloramphenicol intermediates. The final four mutant strains did not accumulate aromatic amino- or nitro- compounds either extracellularly or intracellularly, nor did they produce any "masked" aromatic amino compounds. The possibility exists therefore that some or all of these are regulatory mutants.

Cell extracts of S. venezuelae converted p-aminophenylalanine to a product, probably p-aminophenylserine, more susceptible to periodate oxidation. Although conclusions at this point are tentative, this activity is expected to be associated with the chloramphenicol pathway enzyme responsible for  $\beta$ -hydroxylation of p-aminophenylalanine.

All of the chloramphenicol-nonproducing mutants isolated were more sensitive to chloramphenicol than the wild-type strain but became resistant after exposure to the antibiotic. Unlike that in the wild-type, this resistance was not reversible. The nonproducing mutants may thus have acquired resistance by a different mechanism.

All of the cml mutations included in crosses mapped to the chromosome of S. venezuelae in the arc between the markers his-6 and strA6 opposite to the ade-10 region. From the results of this study, it appears that at least three of the chloramphenicol biosynthetic genes,

i.e. those coding for p-aminophenylalanine  $\beta$ -hydroxylase, arylamine synthetase, and the enzyme responsible for chlorination, are located on the chromosome and in the same arc.

Table 25. Effect of chloramphenicol supplement on growth ( $OD_{640}$ ) of nonproducing mutants of *S. venezuelae*

Culture	Supplement*	Age of culture (days)					
		2	4	6	8	10	12
Cml-1	+		0.36	0.40	0.60	1.00	1.40
	-		0.34	1.64	3.20		
Cml-2	+		0.80	0.82	0.96	1.32	
	-		0.50	1.62	2.40		
Cml-3	+		0.46	0.50	0.54	0.80	1.26
Cml-4	+		0.40	0.50	0.50	0.76	1.20
	-		0.50	1.50	2.40		
Cml-5	+	0.06	0.50	0.66	0.80		
	-	0.06	0.40	1.64	2.60		
Cml-6	+	0.10	0.48	0.60	0.64	1.00	
	-	0.14	0.60	1.74			
Cml-7	+	0.20	0.66	0.74	1.00	1.64	
	-	0.24	0.80	1.44	2.64		
Cml-8	+	0.08	0.46	0.32	0.56		
	-	0.06	0.46	1.32	2.60		
Cml-9	+	0.04	0.60	0.80	0.90	1.08	1.94
	-	0.04	0.70	2.40	3.00		
Cml-10	+	0.04	0.46	0.44	0.44	0.45	0.70
	-	0.04	0.40	1.36	2.48		
Cml-11	+	0.20	0.54	0.60	0.60	0.90	
	-	0.08	0.40	0.96	3.00		
Cml-12	+	0.10	0.40	0.46	0.63	1.08	1.74
	-	0.10	0.55	1.50	2.60		

\* Chloramphenicol ( $150 \mu\text{g}\cdot\text{mL}^{-1}$  final concentration) was added where indicated after 4 d growth in glucose-isoleucine medium.

Table 26. Effect of chloramphenicol on growth ( $OD_{640}$ ) of chloramphenicol-resistant nonproducing mutants of *S. venezuelae*\*

Culture	Supplement	Age of culture (days)		
		2	4	6
Cml-1	+	0.12	1.16	2.10
	-	0.20	1.30	2.24
Cml-2	+	0.24	0.80	3.60
	-	0.24	1.60	2.10
Cml-3	+	0.16	0.48	1.40
	-	0.20	0.90	2.40
Cml-8	+	0.20	0.80	1.60
	-	0.32	1.10	1.90
Cml-9	+	0.40	1.20	1.80
	-	0.70	1.80	2.70
Cml-10	+	0.20	1.86	3.00
	-	0.34	2.70	3.00
Cml-12	+	0.24	0.60	2.85
	-	0.26	0.70	2.85

\* Inocula from cultures grown in the presence of chloramphenicol ( $150 \mu\text{g}\cdot\text{mL}^{-1}$ ) for 8-9 d were transferred to fresh glucose-isoleucine medium with and without chloramphenicol ( $150 \mu\text{g}\cdot\text{mL}^{-1}$ ).

Table 27. Effect of chloramphenicol on growth ( $OD_{640}$ ) of chloramphenicol-resistant nonproducing mutants of *S. venezuelae* grown for one week in the absence of chloramphenicol\*

Culture	Supplement	Age of culture (days)		
		2	4	6
Cml-1	+	0.10	1.00	2.25
	-	0.30	1.70	2.85
Cml-8	+	0.34	1.20	1.70
	-	0.44	1.54	
Cml-9	+	0.14	2.00	
	-	0.20	3.80	
Cml-10	+	0.20	1.80	1.95
	-	0.54	2.10	1.95
Cml-12	+	1.08	2.80	
	-	1.30	3.20	

\* Chloramphenicol-resistant cultures were grown for 1 week in glucose-isoleucine medium in the absence of chloramphenicol. Inocula were then transferred from these cultures to fresh glucose-isoleucine medium with and without chloramphenicol supplement ( $150 \mu\text{g}\cdot\text{mL}^{-1}$ ).

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