Chloramphenicol resistance in *Streptomyces*: cloning and characterization of a chloramphenicol hydrolase gene from *Streptomyces venezuelae*

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(Received 4 September 1989; accepted 30 October 1989)

A 6.5 kb DNA fragment containing a chloramphenicol-resistance gene of *Streptomyces venezuelae* ISP5230 was cloned in *Streptomyces lividans* M252 using the high-copy-number plasmid vector pIJ702. The gene was located within a 2.4 kb *Kpn*I–*Sst*I fragment of the cloned DNA and encoded an enzyme (chloramphenicol hydrolase) that catalysed removal of the dichloroacetyl moiety from the antibiotic. The deacylated product, *p*-nitrophenylserinol, was metabolized to *p*-nitrobenzyl alcohol and other compounds by enzymes present in *S. lividans* M252. Examination of the genomic DNA from several sources using the cloned 6.5 kb *Sst*I fragment from *S. venezuelae* ISP5230 as a probe showed a hybridizing region in the DNA from *S. venezuelae* 13s but none in the DNA from another chloramphenicol producer, *Streptomyces phaeochromogenes* NRRLB 3559. The resistance phenotype was not expressed when the 6.5 kb *Sst*I fragment or a subfragment was subcloned behind the *lac*-promoter of plasmid pTZ18R in *Escherichia coli*.

Introduction

Chloramphenicol resistance in many Gram-positive and Gram-negative bacteria is mediated by the enzyme chloramphenicol acetyltransferase (CAT; EC 2.3.1.28) which inactivates the antibiotic by catalysing its *O*-acetylation (Shaw, 1975). However, CAT activity could not be detected in the chloramphenicol-producing actinomycete *Streptomyces venezuelae* (Shaw & Hopwood, 1976) and does not appear to have a role in protecting this organism from the toxic product of its own metabolism (Vining & Westlake, 1984). Chloramphenicol is bacteriostatic and acts primarily by inhibiting protein synthesis (Pongs, 1979). It binds to prokaryotic ribosomes, including those of *S. venezuelae*. *In vitro* protein synthesizing systems derived from chloramphenicol-producing or non-producing mycelia are equally sensitive (Malik & Vining, 1972). Non-producing cultures exposed to chloramphenicol are initially inhibited but grow after a lag that varies with the drug concentration. The bacteria are then resistant to chloramphenicol at the concentration to which they were exposed, but are returned to the sensitive state by one passage through unsupplemented medium (Vining & Westlake, 1984).

S. venezuelae possesses an intracellular enzyme that inactivates chloramphenicol by removing the dichloroacetyl substituent (Malik & Vining, 1971). Since this chloramphenicol hydrolase was found in comparable amounts in producing or non-producing cultures, it was presumed not to be responsible for the inducible characteristics of resistance in *S. venezuelae*. To account for inducibility, Malik & Vining (1970, 1972) suggested that the mycelium becomes less permeable to chloramphenicol after exposure to exogenous antibiotic; the activity of chloramphenicol hydrolase might then reduce the intracellular concentration. To clarify the mechanisms by which *S. venezuelae* is resistant to its own antibiotic, we have cloned a fragment of *S. venezuelae* genomic DNA conferring chloramphenicol resistance in a heterologous host, *Streptomyces lividans* M252. Some features of this resistance are described.

Methods

Bacterial strains and plasmids. The sources and characteristics of these are described in Table 1.

Chemicals and enzymes. Lysozyme, phage λ DNA, restriction endonucleases and T4 DNA ligase were purchased from Boehringer Mannheim or BRL. Ultrapure agarose was obtained from Bio-Rad Laboratories. Chloramphenicol, ampicillin and 1,3-diacetoxychloramphenicol were purchased from Sigma; 1,3-diacetoxychloramphenicol

Abbreviation: CAT, chloramphenicol acetyltransferase.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Source or reference
<i>Streptomyces venezuelae</i> ISP5230	Wild-type	Stuttard (1982)
<i>S. venezuelae</i> 13s	Wild-type	Ahmed & Vining (1983)
<i>Streptomyces phaeochromogenes</i> NRRLB 3559	Wild-type	Doull <i>et al.</i> (1983)
<i>Streptomyces lividans</i> 1326	Wild-type	John Innes Institute, Norwich, UK
<i>S. lividans</i> M252	Cml ^r	John Innes Institute, Norwich, UK
<i>S. lividans</i> M417	Cml ^r	Betzler <i>et al.</i> (1987)
<i>S. lividans</i> JG10	Pab ⁻	Gil & Hopwood (1983)
<i>Streptococcus lactis</i> NCDO 496	Wild-type	National Collection of Dairy Organisms, Shinfield, Reading, UK
<i>Escherichia coli</i> TG1	$\Delta(lac-pro) supE thi hsdD5/F' traD36 proA^+B^+ lacI^q lacZ\Delta M15$	Carter <i>et al.</i> (1985)
pIJ702	<i>Streptomyces</i> vector: pIJ101 replicon with <i>mel</i> and <i>tsr</i> ; insertion at <i>Sst</i> I site inactivates <i>mel</i>	Katz <i>et al.</i> (1983)
pJV3	pIJ702 carrying a 6.5 kb <i>S. venezuelae</i> ISP5230 DNA fragment containing the chloramphenicol hydrolase gene	This paper
pJV4	pJV3 with deletion in <i>mel</i> promoter and 6.5 kb insert; carries 2.4 kb DNA fragment from <i>S. venezuelae</i> ISP5230 containing the chloramphenicol hydrolase gene	This paper
pTZ18/19R	<i>E. coli</i> phagemid vector from pUC18/19; contains T7 promoter and the fl IG origin of replication	Mead & Kemper (1988)
pJV5/6	pTZ18R with a 6.5 kb DNA insert containing the chloramphenicol hydrolase gene from <i>S. venezuelae</i> ISP5230; inserts in pJV5/6 are oppositely oriented	This paper
pJV7/8	pTZ18/19R with a 2.4 kb DNA insert containing the chloramphenicol hydrolase gene from <i>S. venezuelae</i> ISP5230	This paper

was purified by TLC and recrystallization. Both *p*-nitrobenzoic acid and *p*-nitrobenzyl alcohol were purchased from Kodak. *N*-Acetyl-*p*-nitrophenylserinol was a gift from Parke-Davis. Thiostrepton was a gift from S. J. Lucania, E. R. Squibb & Sons, New Brunswick, NJ, USA.

[U-¹⁴C]Chloramphenicol was labelled biosynthetically from [U-¹⁴C]glucose (Malik & Vining, 1970); *p*-nitrophenyl[hydroxymethylene-¹⁴C]serinol was prepared by hydrolysis of [hydroxymethylene-¹⁴C]chloramphenicol (Rebstock *et al.*, 1949) purchased from Amersham.

Media and culture conditions. *Streptomyces venezuelae* ISP5230 was maintained on MYM medium as described by Doull *et al.* (1985). *Streptomyces lividans* strains were maintained on R5(-S) medium which consisted of R5 regeneration medium (Hopwood *et al.*, 1985) without sucrose. For plating *S. lividans* 3131 harbouring pIJ702, thiostrepton (30 µg ml⁻¹) was included in R5(-S) medium. Minimal medium was as described by Hopwood (1967). *Streptomyces* cultures were grown at 30 °C until sporulation (4-5 d). Spores were harvested and stored as suspensions in 20% (v/v) glycerol at -20 °C (Hopwood *et al.*, 1985).

Cultures of streptomycetes grown in liquid media were incubated at 30 °C on a rotary shaker (220 r.p.m.; 3.7 cm eccentricity). Mycelium used for protoplast formation was from cultures (25 ml per 250 ml Erlenmeyer flask) grown for 36-40 h in YEME medium (Hopwood *et al.*, 1985) supplemented with 34% (w/v) sucrose, 5 mM-MgCl₂, and 0.5% (w/v) glycine. For assessing chloramphenicol resistance, cultures (50 ml per 500 ml Erlenmeyer flask) were grown in YEME or tryptic soy (TS) medium [sucrose, 10% (w/v); tryptic soy broth, 3% (w/v); MgCl₂, 6H₂O, 1% (w/v); yeast extract, 1% (w/v); and supplemented after autoclaving with CaCl₂ to 1.5 mg ml⁻¹]. For metabolite isolation, cultures (100 ml per 500 ml Erlenmeyer flask) were grown in GNY medium (Malik & Vining 1970).

Cultures used for plasmid isolation or for vegetative inocula were initiated with spores harvested from agar plate cultures. Unless stated otherwise, vegetative inocula were grown for 48 h and added in amounts of 1% (v/v) to initiate cultures used to assess resistance, or 5% (v/v) to cultures used for metabolite isolation.

Cultures of *E. coli* on agar media were grown at 37 °C for 24 h. Shaken cultures (50 ml per 250 ml Erlenmeyer flask) used in plasmid isolation or to prepare competent cells were inoculated with 10-20 colonies and grown overnight at 37 °C in L-broth (Hopwood *et al.*, 1985). Cultures of *E. coli* TG1 harbouring pTZ18/19R were grown on L-agar or in L-broth containing 50 µg ampicillin ml⁻¹.

Plasmid and genomic DNA. Plasmid DNA from *Streptomyces* or *E. coli* was isolated by the rapid alkaline lysis method of Kieser (1984); for larger amounts the procedure was scaled up 10-fold and was followed by CsCl-ethidium bromide density gradient centrifugation. Genomic DNA was isolated and purified as described previously (Schrempf, 1982).

Restriction endonuclease digestion and ligation of DNA. Genomic DNA (10 µg) from *S. venezuelae* ISP5230 was digested with *Sst*I under conditions (Thompson *et al.*, 1982) that gave fragments predominantly 1-15 kb in size. The digest was combined with an *Sst*I digest of pIJ702 (2 µg), heated to 65 °C for 10 min, placed on ice for 5 min and diluted with 2 vols absolute ethanol. After two washes with 70% (v/v) ethanol, the precipitated DNA was dissolved in ligation buffer (20 mM-Tris/HCl pH 7.6, 10 mM-MgCl₂, 10 mM-dithioerythritol, 0.6 mM-ATP) at a DNA concentration of 40 µg ml⁻¹. The solution was incubated for 12-16 h at 14 °C with 1 unit of T4-DNA ligase, ligation being monitored by electrophoresis. The DNA was precipitated with ethanol, washed once with 70% ethanol and resuspended in TE buffer.

Protoplast transformation. Protoplasts in a small volume of P-buffer (Hopwood *et al.*, 1985) were mixed with 20 µl of ligated plasmid DNA in TE buffer (10 mM-Tris/HCl, pH 8.0, 1 mM-EDTA) and 0.5 ml 25% (w/v) polyethylene glycol 1000 in T-medium. They were immediately washed with P-buffer, recovered by centrifugation and spread (100 µl per plate) on partially dried R5 agar (Thompson *et al.*, 1982). After 18–20 h at 30 °C, regenerating colonies were overlaid with soft nutrient agar (3 ml) containing enough thiostrepton to give a plate concentration of 30 µg ml⁻¹. Transformants carrying the *tsr* gene of pIJ702 were visible within 1–2 d, and the melanin excreted by those containing intact pIJ702 was evident by the third day. At this time, all thiostrepton-resistant colonies were transferred to minimal medium supplemented with 25 or 50 µg chloramphenicol ml⁻¹.

Cells of *E. coli* TG1 were made competent and were transformed as described by Hopwood *et al.* (1985).

DNA hybridization. Genomic DNA samples were digested with *Sst*I. Fragments were precipitated with ethanol, electrophoresed in a 0.7% (w/v) agarose gel and transferred to a nitrocellulose hybridization filter. The recombinant plasmid pJV3 containing the 6.50 kb DNA insert was digested with *Sst*I, *Cl*aI and *B*amHI; since only pIJ702 DNA contains recognition sites for the latter two endonucleases, it was cleaved into fragments of 1.60, 1.70 and 2.50 kb which were readily separated from the 6.50 kb insert by electrophoresis overnight at 40 V in 0.6% (w/v) ultrapure agarose gels. The 6.5 kb DNA fragment was eluted from the gel into TBE buffer (89 mM-Tris, 89 mM-boric acid, 2 mM-EDTA); the solution was extracted with phenol/chloroform and then chloroform. The DNA was precipitated with absolute ethanol, washed with 70% ethanol and resuspended in TE buffer.

Samples of DNA were nick-translated in the presence of [α -³²P]dATP and [α -³²P]dGTP. Southern hybridization with *Sst*I-digested genomic DNA was done at 70 °C for 12 h as described by Hopwood *et al.* (1985). The membrane containing the DNA was washed four times at 70 °C for 30 min with SSC (1 × SSC is 0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0) containing 0.1% SDS. The concentration of SSC in the first two washes was 2 × and in the last two 0.2 ×. The membrane was exposed to X-ray film (Kodak X-Omat) for 5 d at -70 °C.

Analysis of cultures. Resistance to chloramphenicol and its metabolites was measured by comparing the mycelium dry weight of cultures grown in TS medium with or without the test compound at a range of concentrations. For *S. lividans* RM3 the inoculum medium was supplemented with 25 µg chloramphenicol ml⁻¹ to maintain selection for pJV3.

To identify metabolic products of chloramphenicol, cultures of *S. lividans* 1326, M252 and RM3 were supplemented with either chloramphenicol or *p*-nitrophenylserinol and harvested at 24 h intervals by centrifugation. In some cultures of *S. lividans* RM3, [U-¹⁴C]chloramphenicol or *p*-nitrophenyl[hydroxymethylene-¹⁴C]serinol was used as the supplement and cultures were grown for only 24 h. For all cultures, the supernatant solution collected by centrifugation was extracted with ethyl acetate and the extract was separated into non-acid/water (12:3:5, by vol.) for aqueous fractions. Aromatic compounds by TLC on silica gel (Sil 60 F254; Merck) using chloroform/methanol (9:1, v/v) as the solvent system for extracts, and *n*-butanol/acetic acid/water (12:3:5 by vol.) for aqueous fractions. Aromatic compounds were visualized as fluorescence-quenching zones under light of 254 nm. Chromatograms were scanned for radioactivity with a gas-flow Geiger-Mueller detector (Packard model 7200) synchronized with a strip chart recorder. The amount of radioactivity in solutions was measured with an LKB Wallac model 1215 liquid scintillation counter.

To search for possible metabolites of chloramphenicol in *E. coli* strains transformed with plasmids pJV7 and pJV8, cultures were grown for 24 h in minimal medium containing chloramphenicol (0.2 µg ml⁻¹) and isopropyl thiogalactoside. The broths were extracted with ethyl

acetate and the extracts were concentrated and examined by TLC on silica gel with chloroform/methanol (9:1, v/v) and ethyl acetate/acetic acid/water (14:7:1, by vol.) as solvent systems. Authentic samples of chloramphenicol, *p*-nitrophenylserinol and *N*-acetyl-*p*-nitrophenylserinol were included as reference compounds.

Isolation of metabolites. Cultures of *S. lividans* 1326 and RM3 that had been supplemented with the test compound were centrifuged and the pelleted mycelium was washed with water; the pellet was then leached with acetone. The supernatant solution from the culture was extracted with ethyl acetate successively at pH 8.5, 5.0 and 2.5. The acetone and ethyl acetate extracts were each evaporated, redissolved in 250 µl ethyl acetate and chromatographed on preparative layers of silica gel using chloroform/methanol (9:1, v/v) as solvent. Fluorescence-quenching zones were scraped from plates; the adsorbed substances were eluted with acetone and the solutions were evaporated.

In the non-acidic (pH 8.5) extract from *S. lividans* RM3 cultures supplemented with [U-¹⁴C]chloramphenicol (3 µg ml⁻¹; 25.6 µCi mmol⁻¹), the residue from a zone with the same mobility (*R*_F 0.72) as *p*-nitrobenzyl alcohol contained 0.89 nCi of radioactivity. Admixture with unlabelled *p*-nitrobenzyl alcohol (25 mg) and three recrystallizations from water gave specific activities of 5.5, 6.5 and 6.1 nCi mmol⁻¹. A second radioactive zone in the non-acidic extract matched *N*-acetyl-*p*-nitrophenylserinol (*R*_F 0.34) in mobility. The eluate (1.3 nCi) was mixed with unlabelled *N*-acetyl-*p*-nitrophenylserinol (25 mg). In successive recrystallizations from water, its specific activity remained constant at 13.2 nCi mmol⁻¹. (1 Ci = 37 GBq.)

Metabolites with *R*_F values of 0.34 and 0.72 detected by TLC analysis of non-acidic extracts from *S. lividans* M252 cultures supplemented with *p*-nitrophenylserinol, and from *S. lividans* RM3 cultures supplemented with *p*-nitrophenyl[hydroxymethylene-¹⁴C]serinol (200 µg ml⁻¹; 1.06 µCi mmol⁻¹), were separated by preparative TLC, eluted and crystallized from water. The substances at *R*_F 0.34 from each source were obtained as needles, m.p. 129 °C, indistinguishable in melting point, infrared and ¹H-nuclear magnetic resonance (NMR) spectra from authentic *N*-acetyl-*p*-nitrophenylserinol (Doull *et al.*, 1985). The specific activity of the product from *S. lividans* RM3 was 1.01 µCi mmol⁻¹. The substances at *R*_F 0.72 were obtained as needles, m.p. 92 °C, unchanged on admixture with authentic *p*-nitrobenzyl alcohol. Its identity was confirmed by comparison of infrared and ¹H-NMR spectra with those of *p*-nitrobenzyl alcohol.

In the non-acidic extract from *S. lividans* 1326 cultures supplemented with chloramphenicol, a zone at *R*_F 0.92 corresponded in mobility to 1,3-diacetoxychloramphenicol. When the product eluted from this zone was recrystallized from water, it yielded colourless needles, m.p. 141 °C alone or after mixing with authentic 1,3-diacetoxychloramphenicol. The ¹H-NMR spectrum, recorded in deuterochloroform at 300 MHz on a Bruker model MSL-300 spectrometer with tetramethylsilane as the internal reference, contained signals at δ (p.p.m.) 2.11 (s, 3H, acetoxy CH₃), 2.19 (s, 3H, acetoxy CH₃), 4.06 (q, 1H, H-1a', *J*_{1a',1b} 6.1 Hz, *J*_{1a',2} 5.6 Hz), 4.19 (q, 1H, H-1b', *J*_{1b',1a} 6.1 Hz, *J*_{1b',2} 5.6 Hz), 4.61 (q, 1H, H-2', *J*_{2',1a} 5.6 Hz, *J*_{2',1b} 5.6 Hz), 4.82 (bs, 1H, NH), 5.86 (s, 1H, CHCl₂), 6.07 (d, 1H, H-3', *J*_{3',2} 5.7 Hz), 7.89 (AA'BB', 4H, H-2, H-3, H-5, H-6, consisting of doublets at 7.53 and 8.24 p.p.m., *J*_{2,3} = *J*_{5,6} = 8.7 Hz). The data were consistent with the assigned structure and matched those obtained with the authentic sample.

Results

Sensitivity of S. venezuelae and S. lividans strains to chloramphenicol

The minimum inhibitory concentration (MIC) of chloramphenicol for *S. venezuelae* ISP5230 grown on MYM medium was 200 µg ml⁻¹. *S. lividans* strains 1326

Fig. 1. Restriction map of plasmid pJV3 linearized with *Bam*HI; sizes were determined by comparison with *Hind*III- and *Pst*I-digested λ phage DNA. The position of restriction sites in the 6.5 kb insert was determined by single and multiple digestions with the restriction enzymes indicated in the diagram. The position of sites in the vector region is as given by Hopwood *et al.* (1985).

and JG10 had an MIC of 20 $\mu\text{g ml}^{-1}$ but strains M417 and M252, both of which had been selected for sensitivity to chloramphenicol (Betzler *et al.*, 1987; Schottel *et al.*, 1981), failed to grow at drug concentrations above 3 and 6 $\mu\text{g ml}^{-1}$, respectively.

Isolation and characterization of plasmid pJV3

After complete digestion with *Sst*I, genomic DNA from *S. venezuelae* ISP5230 contained fragments of predominantly 1–15 kb. These were ligated to *Sst*I-cleaved pIJ702 and the ligation mixture was used to transform protoplasts of *S. lividans* M252. Regeneration of the protoplasts under selective conditions yielded semi-confluent lawns of thiostrepton-resistant transformants. When these were replica-plated on minimal medium supplemented with 25 or 50 μg chloramphenicol ml^{-1} , three colonies from approximately 10000 melanin-negative transformants grew rapidly at both drug concentrations.

All three resistant transformants contained recombinant plasmids. Two of the plasmids, with inserts of 0.7 and 1.7 kb, failed to confer chloramphenicol resistance when they were isolated and reintroduced into *S. lividans* M252. The third plasmid, from transformant RM3, contained a 6.5 kb insert. When this plasmid (pJV3) was used to transform *S. lividans* M252, all of the 16 thiostrepton-resistant colonies tested were also chloramphenicol resistant. With a second host, *S. lividans* M417, all 12 colonies selected for resistance to 50 μg thiostrepton ml^{-1} after transformation with pJV3 were resistant to 12.5 μg chloramphenicol ml^{-1} .

Plasmid DNA from *S. lividans* RM3 was extracted by the rapid alkaline procedure of Kieser (1984) and was digested with restriction enzymes, both singly and in combination. Comparison of the fragments by agarose gel electrophoresis with those obtained from pIJ702 gave the restriction map shown in Fig. 1. To locate the region

Fig. 2. Derivation of recombinant plasmids. 1. pJV3 was digested with *Kpn*I and the ligated mixture was used to transform *S. lividans* M252. From the transformants, a clone containing pJV4 was isolated; this had undergone a 4.1 kb deletion from the insert and carried the *cml*^r determinant in a 2.4 kb *Kpn*I–*Sst*I segment of DNA. 2. pJV5 was formed by digesting both pJV3 and pTZ18R with *Sst*I, ligating the mixture and using it to transform *E. coli* TG1; screening of the transformants yielded a clone containing pJV5. 3. pJV5 was digested with *Kpn*I, ligated and used to transform *E. coli*; among the transformants was one in which the resident plasmid had undergone a 4.1 kb deletion to yield pJV7. 4. Digestion of pJV7 with *Kpn*I and *Sst*I gave a 2.4 kb fragment; this was ligated to pTZ19R digested with the same enzymes and the mixture used to transform *E. coli* TG1. From the transformants, a clone containing pJV8 was isolated. The diagram is not drawn to scale. A, *Apa*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; S, *Sst*I; X, *Xmn*I.

within the cloned fragment that conferred chloramphenicol resistance, pJV3 was digested with *Kpn*I. Ligation of the products with T4-DNA ligase gave DNA that transformed *S. lividans* M252 to thiostrepton resistance. All these transformants grew on a medium containing 12.5 μg chloramphenicol ml^{-1} . Plasmid extraction afforded pJV4 in which a 5.2 kb fragment of pJV3 had been deleted (Fig. 2). The deleted region consisted of a 4.1 kb *Sst*I–*Kpn*I fragment derived from the insert, along with a 1.1 kb *Kpn*I–*Sst*I fragment from pIJ702. This

Fig. 3. Agarose gel electrophoresis (a) of restriction endonuclease digests of genomic DNA samples and Southern hybridization (b) with a ^{32}P -labelled 6.5 kb *Sst*I DNA fragment from *S. venezuelae* ISP5230 under stringent conditions (70 °C). The samples are: lane A, λ ; B, *S. lividans* JG10; C, *S. phaeochromogenes*; D, *S. venezuelae* 13s; E, *S. lividans* M417; F, *S. lividans* RM3; G, *S. lactis*; H, *S. lividans* M252; I, *S. venezuelae* ISP5230; J, λ . λ DNA was digested with *Hind*III; all other samples were digested with *Sst*I.

pIJ702 fragment is believed to carry the promoter region of the *mel* gene (Hopwood *et al.*, 1985). Retransformation of *S. lividans* M252 with pJV4 gave thiostrepton-resistant colonies that were also chloramphenicol resistant.

The 6.5 kb fragment excised from pJV3 was shotgun-cloned into an *Sst*I site within the polylinker sequence downstream of the *lac*-promoter in pTZ18R to give two recombinant plasmids, pJV5 and pJV6. From single and double digestions of pJV5 and pJV6 with *Sst*I, *Kpn*I and *Pst*I, and electrophoretic measurement of fragment sizes against a *Hind*III-digested λ DNA reference (data not shown), it was concluded that the 6.5 kb insert was present in opposite orientations in the two plasmids. In cultures grown with or without isopropyl thiogalactoside as an inducer, neither plasmid conferred chloramphenicol resistance on *E. coli* TG1. Subcloning the 2.4 kb *Kpn*I–*Sst*I *S. venezuelae* DNA fragment from pJV4 in the polylinker regions of pTZ18R and pTZ19R (vectors with

the same polylinker sequence in reverse orientations to the *lac* promoter) gave the recombinant plasmids pJV7 and pJV8, respectively. These also failed to confer chloramphenicol resistance on *E. coli* transformants.

To ascertain whether the chloramphenicol hydrolase gene was expressed in the *E. coli* transformants without conferring resistance to the antibiotic, cultures were grown in a sub-inhibitory concentration of chloramphenicol and extracts were examined by TLC. Whereas unchanged antibiotic could be readily located, no substances corresponding in R_f value with *p*-nitrophenylserinol or *N*-acetyl-*p*-nitrophenylserinol were detected, even when *lac* expression was induced with isopropyl thiogalactoside.

Southern hybridization

To confirm that the 6.5 kb insert of pJV3 hybridized to a region in the genomic DNA of *S. venezuelae* ISP5230,

Table 2. Effect of chloramphenicol on the growth of *S. lividans* strains M252 and RM3

Cultures were harvested at 48 h and 96 h: the mycelium was collected by filtration and dried to constant weight.

Chloramphenicol ($\mu\text{g ml}^{-1}$)	Growth (g l^{-1})			
	48 h		96 h	
	M252	RM3	M252	RM3
Nil	3.7	4.4	3.5	3.6
15	0.18	2.6	0.06	3.7
30	0.15	1.3	0.02	4.1
45	0.16	0.51	0.17	4.2

and also to determine whether the nucleotide sequence was present in the genome of related organisms, insert DNA was isolated by electroelution and labelled with ^{32}P by nick-translation. Samples of the total DNA extracted from *S. venezuelae* strains ISP5230 and 13s, *S. phaeochromogenes* NRRLB 3559, *S. lividans* strains JG10, M252, M417 and RM3, and from *Streptococcus lactis* were digested with *Sst*I and electrophoresed through a 0.7% agarose gel along with *Hind*III-digested λ DNA as size markers. The DNA fragments in the gel were transferred to a nitrocellulose filter, immobilized and exposed under hybridizing conditions to the ^{32}P -labelled 6.5 kb insert from pJV3. Strong hybridization was observed to 6.5 kb fragments in the DNA from *S. venezuelae* ISP5230, *S. venezuelae* 13s and *S. lividans* RM3 (Fig. 3). The DNA from ISP5230 also showed a second hybridizing fragment of 8 kb. No hybridization was detected with DNA from the other organisms.

Effect of chloramphenicol on growth

Cultures of RM3 and M252 in TS medium without antibiotic grew at the same rate and achieved a similar maximum yield after 2 d. However, *S. lividans* M252 was markedly inhibited by $6 \mu\text{g chloramphenicol ml}^{-1}$ and little growth occurred at $15 \mu\text{g ml}^{-1}$. In contrast, cultures of *S. lividans* RM3 containing chloramphenicol supplements of from 15 to $45 \mu\text{g ml}^{-1}$ showed an approximately linear decrease in growth after 2 d incubation (Table 2). By the fourth day, all cultures had accumulated similar amounts of biomass.

Chloramphenicol metabolism by *S. lividans* RM3

To determine whether the antibiotic was metabolized, cultures of *S. lividans* RM3 were grown in a medium supplemented with [$\text{U-}^{14}\text{C}$]chloramphenicol. Of the total radioactivity administered, 19% became associated with the mycelium; less than half of this was recovered by

acetone extraction and examination of the extract by TLC and scanning for radioactivity showed the label to be distributed among numerous unidentified components. The combined ethyl acetate extracts of the culture supernatant accounted for a further 20% of the radioactivity initially added; the remainder was associated with non-extractable metabolites in the culture supernatant fraction.

When an extract from the culture supernatant at pH 7.3 was fractionated by TLC and scanned for radioactivity, two radioactive metabolites were detected. These were identified by co-crystallization with authentic material to constant specific activity as *p*-nitrobenzyl alcohol and *N*-acetyl-*p*-nitrophenylserinol (Fig. 4). Examination by TLC of the metabolites extracted at pH 2.5 gave a radioactive peak that co-migrated with *p*-nitrobenzoic acid. The amount of radioactivity eluted from the zone was too small to allow further characterization.

Chloramphenicol metabolism by *S. lividans* M252

Examination by TLC of ethyl acetate extracts from cultures of *S. lividans* M252 that had been supplemented after 24 h growth with a sublethal concentration ($2 \mu\text{g ml}^{-1}$) of chloramphenicol showed that the antibiotic was slowly metabolized. The main product was an aromatic compound with an R_F value matching that of 1,3-diacetoxychloramphenicol (Fig. 4). The same metabolite was formed in the more drug-tolerant parent strain, *S. lividans* 1326, when chloramphenicol was added to cultures at $15 \mu\text{g ml}^{-1}$. The substance isolated from these cultures was confirmed from its spectroscopic properties to be 1,3-diacetoxychloramphenicol.

Metabolism of *p*-nitrophenylserinol by *S. lividans*

N-Acetyl-*p*-nitrophenylserinol and *p*-nitrophenylserinol added to 24 h cultures of *S. lividans* RM3 at concentrations of 100 and $200 \mu\text{g ml}^{-1}$, respectively, did not affect the subsequent growth of the organism. In contrast, growth of *S. lividans* M252 was inhibited by these concentrations, and in the case of the acetyl derivative, by as little as $20 \mu\text{g ml}^{-1}$. When added at $15 \mu\text{g ml}^{-1}$, *p*-nitrophenylserinol was metabolized in cultures of *S. lividans* M252 to substances that were isolated and identified spectroscopically as *N*-acetyl-*p*-nitrophenylserinol and *p*-nitrobenzyl alcohol.

In cultures of *S. lividans* RM3 to which $190 \mu\text{g p-nitrophenyl[hydroxymethylene-}^{14}\text{C]serinol ml}^{-1}$ was added at inoculation, only 14% of the radioactivity in the culture 24 h later was in the non-acidic ethyl acetate extract. TLC of this fraction and scanning for radioactivity showed that the ^{14}C was present in only one

Fig. 4. Pathways by which chloramphenicol (compound 2) is inactivated: formation of 1,3-diacetoxychloramphenicol (1), catalysed by chloramphenicol acetyltransferase (CAT), was found in *S. lividans* 1326 and M252; the route via *p*-nitrophenylserinol (3), catalysed by chloramphenicol hydrolase, is postulated to occur in *S. venezuelae* and in *S. lividans* RM3 which accumulate *N*-acetyl-*p*-nitrophenylserinol (4), *p*-nitrobenzoic acid (7) and *p*-nitrobenzyl alcohol (9). *p*-Nitrophenylserine (5) and *p*-nitrobenzaldehyde (6) have been identified in *S. venezuelae* and *p*-aminobenzoic acid (8) has been isolated from *S. lividans* RM3 (N. P. Ranade, unpublished).

metabolite, the R_F of which (0.34) coincided with that of *N*-acetyl-*p*-nitrophenylserinol. An unlabelled product at R_F 0.72 co-chromatographed with *p*-nitrobenzyl alcohol but no unmetabolized *p*-nitrophenylserinol could be detected. *N*-Acetyl-*p*-nitrophenylserinol and *p*-nitrobenzyl alcohol were isolated and identified spectroscopically as above.

Discussion

Since a 5.2 kb segment lying between a *Kpn*I site in the vector and the single *Kpn*I site in the 6.5 kb DNA insert in pJV3 could be removed without altering the chloramphenicol-resistance phenotype conferred by the plasmid,

the *cmI^r* determinant is present on the remaining 2.4 kb of insert DNA. Besides the deleted 4.1 kb portion of the original insert, pJV4 also lacks a 1.1 kb *Kpn*I-*Sst*I fragment of the pIJ702 vector. The latter DNA encodes all of the upstream portions of the *melC1* gene (Lee *et al.*, 1988). Thus the possibility that the *cmI^r* determinant in pJV3 might be expressed from *melC1* promoter sequences is absent for pJV4. Although the opportunity for read-through from an upstream plasmid promoter remains, the results suggest that the 2.4 kb insert in pJV4 contains a promoter for *cmI^r*.

The absence of increased chloramphenicol resistance when the 6.5 kb insert from pJV3 was introduced into the polylinker site of pTZ18R and, in both orientations with respect to the upstream *lac* promoter, subcloned in

E. coli TG1, is probably due to the resistance gene not being expressed. In cultures of the *E. coli* transformants, sublethal chloramphenicol supplements remained unchanged and the products expected from their metabolism could not be detected. The lack of expression from the *lac* promoter in the vector is presumably due to an inappropriate reading frame or the presence of an intervening termination sequence. Absence of expression from the indigenous *cmI*^r promoter is consistent with the observation (Bibb *et al.*, 1985) that many *Streptomyces* promoters are not recognized by the DNA transcribing system in *E. coli*.

Cultures of *S. lividans* RM3 metabolized [U-¹⁴C]chloramphenicol to radioactive *N*-acetyl-*p*-nitrophenylserinol and *p*-nitrobenzyl alcohol. A small amount of a substance chromatographically similar to *p*-nitrobenzoic acid was detected as well but could not be conclusively identified. The absence of *p*-nitrophenylserinol is explained by the rapid conversion of this compound to *N*-acetyl-*p*-nitrophenylserinol, demonstrated by supplementing cultures with *p*-nitrophenyl[hydroxymethylene-¹⁴C]serinol. The *p*-nitrobenzyl alcohol also accumulated in these cultures was unlabelled, as anticipated from the location of the label in the supplement. In *S. venezuelae* 13s, labelled *p*-nitrophenylserinol as well as *p*-nitrobenzoic acid have been identified in cultures after the addition of [U-¹⁴C]chloramphenicol (Malik & Vining, 1970) but the products found were otherwise identical to those found in *S. lividans* RM3. The difference in amounts of *p*-nitrobenzoic acid may be due to a more active reduction of this compound to *p*-aminobenzoic acid demonstrated in *S. lividans* (N. P. Ranade, unpublished). The overall results suggest that the antibiotic is inactivated by a similar pathway in the two organisms (Fig. 4).

No conversion of chloramphenicol to these products could be detected in *S. lividans* strains not transformed with pJV3 or pJV4. Instead, *S. lividans* 1326 and M252 modified the antibiotic by *O*-acetylation. The inferred presence of CAT activity supports observations of weak CAT activity in strains M252 (Gil & Hopwood, 1985) and 1326 (W. Dittrich & H. Schrempf, unpublished), and would seem to conflict with earlier reports of its absence from strain 1326 (Shaw & Hopwood, 1976; Gil & Hopwood, 1985). However, *O*-acetylation of chloramphenicol by this strain depends on the composition of the growth medium and the age of cultures (N. P. Ranade, unpublished) and it is possible that CAT activity is catabolite-repressed under some conditions. The results suggest that *S. lividans* strains M252 and 1326 tolerate chloramphenicol at concentrations up to 6 µg ml⁻¹ and 20 µg ml⁻¹, respectively, because of inactivation of the antibiotic mediated by CAT. Consistent with this, and with the absence of chloramphenicol hydrolase activity in these strains, is the absence of hybridization between

the 6.5 kb DNA insert of pJV3 and genomic DNA from *S. lividans* M252 and JG10 (a *pab* mutant of strain 1326 which exhibits wild-type resistance to chloramphenicol). Whether chloramphenicol resistance in 1326 is due primarily to CAT activity is nevertheless uncertain since W. Dittrich & H. Schrempf (unpublished) did not observe increased activity in cell extracts from hyper-resistant derivatives of this strain.

The absence of chloramphenicol degradation products in *S. lividans* M252 and their formation in *S. lividans* RM3 suggests that the resident *S. venezuelae* DNA of pJV3 confers resistance to the antibiotic by inactivation. The presence of metabolites derived from *p*-nitrophenylserinol and devoid of the dichloroacetyl substituent of chloramphenicol implicates chloramphenicol hydrolase (Malik & Vining, 1971) as the initial enzyme catalysing the reactions. Since *S. lividans* M252 metabolized *p*-nitrophenylserinol (but not chloramphenicol) to *N*-acetyl-*p*-nitrophenylserinol and *p*-nitrobenzyl alcohol, introduction of the DNA coding for chloramphenicol hydrolase from *S. venezuelae* would be sufficient to enable this strain to generate the range of metabolic products observed. Removal from pJV3 of a 4.1 kb segment of the DNA insert without affecting the chloramphenicol resistance phenotype conferred on *S. lividans* M252 by the plasmid is consistent with the assumption that the cloned fragment contains only the determinant for chloramphenicol hydrolase. Of potential interest is the observation that *N*-acetyl-*p*-nitrophenylserinol exhibits antibiotic activity (Suzuki *et al.*, 1972). Accumulation of this product, especially in the absence of competing degradative reactions, might cause growth inhibition and mask phenotypic expression of resistance.

The results of the Southern hybridization experiment indicated that the 6.5 kb fragment from *S. venezuelae* ISP5230 had strong sequence similarity to a comparably sized DNA fragment from *S. venezuelae* 13s. The 8 kb hybridizing fragment in the *S. venezuelae* ISP5230 digest is probably due to incomplete digestion. Because *S. phaeochromogenes* NRRLB 3559, like *S. venezuelae* ISP5230 and 13s, produces chloramphenicol (Doull *et al.*, 1983), the lack of hybridization between the probe and its genomic DNA was surprising. It may be that *S. phaeochromogenes* possesses a chloramphenicol hydrolase gene with sufficient sequence similarity to be detected under less stringent conditions for hybridization and washing than those used, which were relatively severe. It is also possible that an *S. phaeochromogenes* gene possesses numerous *Sst*I sites and was digested into small fragments not recognized in the hybridization. However, it is not known for certain whether the mechanism by which *S. phaeochromogenes* acquires resistance to its own antibiotic is of the same type as in *S. venezuelae*.

This research was supported by the Natural Sciences and Engineering Research Council of Canada. We thank C. Stuttard, J. L. Doull, J. M. Wright, D. A. Aidoo, D. A. Hopwood, J. A. Gil and E. Katz for gifts of cultures. We also thank J. A. Walter, Atlantic Research Laboratory, National Research Council of Canada, for the nuclear magnetic resonance spectra.

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