Chloramphenicol Acetylation in *Streptomyces*

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SUMMARY

Twenty-one strains of actinomycetes were screened for the presence of chloramphenicol acetyltransferase, the enzyme responsible for chloramphenicol resistance in many species of bacteria. Only five strains, belonging to three species, yielded mycelial lysates which catalysed the formation of chloramphenicol acetates in the presence of acetyl-coenzyme A: *Streptomyces coelicolor* Müller, *S. acrimycini*, and *S. griseus*. A mutant of *S. acrimycini* selected for an increase in resistance to chloramphenicol had a higher specific activity for chloramphenicol acetyltransferase than that found in the parental strain; the enzyme was not inducible in the mutant, the parental strain, or any other strain tested. Chloramphenicol was not acetylated by lysates of a strain of *S. venezuelae*, the organism known to produce it.

INTRODUCTION

Chloramphenicol resistance in numerous species of bacteria is associated with inactivation of the antibiotic by enzymic acetylation (Shaw, 1974). The presence of the enzyme responsible for the resistant phenotype, chloramphenicol acetyltransferase, was originally noted in isolates of *Escherichia coli* and related enteric bacteria which carried R factors (Shaw, 1967; Suzuki & Okamoto, 1967) and in staphylococci harbouring plasmids for chloramphenicol resistance (Shaw & Brodsky, 1968). More recently, the acetylating enzyme has been described in *Proteus* spp., *Agrobacterium tumefaciens*, *Streptococcus faecalis*, *Diplococcus pneumoniae* (reviewed by Shaw, 1974), and *Myxococcus* spp. (Burchard & Parish, 1975).

In most cases examined it has been easier to ascribe the mechanism of chloramphenicol resistance to enzymic acetylation than rigorously to define its genetic determination. Circumstantial evidence for plasmid linkage has been found for the chloramphenicol acetyltransferase gene in some of the organisms noted above.

The notion that the commonly found plasmid-linked genes for the acetylating enzymes may have arisen by mobilization of a chromosomal precursor in *Proteus* or some other species has been an attractive one. The observation of Argoudelis & Coats (1971) that a member of the genus *Streptomyces* was capable of acetylating chloramphenicol suggested that a survey of other members of the genus might be informative. Organisms potentially capable of antibiotic synthesis might, *a priori*, be expected to carry out antibiotic-specific enzyme transformations, a point emphasized by Benveniste & Davies (1973). The present study was designed to examine a number of species of actinomycetes for the presence of chloramphenicol acetyltransferase, to correlate their rates of acetylation with their sensitivity to chloramphenicol, and to acquire preliminary information on the nature of the enzyme in actinomycetes.

Table 1. *Actinomycete strains tested for chloramphenicol acetylation by the chromatographic method*

John Innes stock no.	Description	Origin/remarks	Chloramphenicol acetylation
104	<i>Streptomyces coelicolor</i> *	<i>his</i> , <i>str-R</i> , SCP1 ⁺ derivative of A3(2)	—
2159	<i>S. coelicolor</i> Müller*	UC5240 of Argoudelis & Coats (1971)	+++
1157	<i>S. coelicolor</i> Müller*	Kutzner 1122	++
1201	<i>S. acrimycini</i>	IPV1610	+++
1326	<i>S. lividans</i>	66 of Lomovskaya <i>et al.</i> (1972)	—
1472	<i>S. glaucescens</i>	ETH22794	—
1459	<i>S. rimosus</i>	NRRL2234	—
2246	<i>S. rimosus</i>		—
2248	<i>S. rimosus</i>	18274 of Pfizer Ltd., Sandwich, Kent	—
2207	<i>S. roseochromogenes</i>	CUB520	—
2209	<i>S. viridochromogenes</i>	CUB530	—
2210	<i>S. viridochromogenes</i>	CUB539 (ATCC14920)	—
2155	<i>S. venezuelae</i>	At-40 (Okanishi)	—
2222	<i>S. venezuelae</i> var. <i>spiralis</i>	CUB528	—
2204	<i>S. erythrochromogenes</i>	CUB486	—
2211	<i>S. griseus</i>	CUB609	+
2212	<i>S. griseus</i>	CUB625	+++
1158	<i>Streptomyces</i> sp. (unidentified)	.	—
1159	<i>Streptomyces</i> sp. (unidentified)	.	—
1195	<i>Streptomyces</i> sp. (unidentified)	.	—
1276	<i>Nocardia mediterranei</i>	ATCC13685	—

* Kutzner & Waksman (1959) found that strains earlier classified as *S. coelicolor* fell into two classes and proposed a reclassification of one class as *S. violaceoruber*; 1157 and 2159 are strains for which the name *S. coelicolor* was retained, while A3(2), represented here by its derivative 104, is a strain of the *S. violaceoruber* type. Strain A3(2) has been extensively studied genetically (Hopwood, *et al.*, 1973).

CUB, Collection of the University of Bradford (T. Cross); ETH, Eidgenössische Technische Hochschule, Zurich; IPV, Istituto Patologia Vegetale, Milan. AT, Department of Antibiotics, National Institute of Health, Tokyo.

METHODS

Organisms and media. The strains of actinomycetes screened for the presence of chloramphenicol acetyltransferase are listed in Table 1. For the preparation of extracts, organisms were grown by the inoculation of spore suspensions into 200 or 500 ml of the following liquid medium in 2000 ml Erlenmeyer flasks, followed by orbital shaking at 30 °C for 40 to 48 h. The medium contained (g l⁻¹): Difco Casamino acids, 24; glycine, 5; glucose, 5; MgCl₂, 10.25. Vitamins were included by the addition of 1 ml of a stock solution containing (g/100 ml): riboflavine, 0.1; nicotinamide, 0.1; *p*-aminobenzoic acid, 0.01; pyridoxine-HCl, 0.05; thiamine-HCl, 0.05; biotin, 0.02. The major components of the medium were chosen to facilitate subsequent lysis of the mycelium by lysozyme; vitamins were probably unimportant for the purposes of the present study.

Preparation of cell-free extracts. The mycelium was collected by centrifugation, washed with 0.05 M-Tris-HCl, pH 7.6, and resuspended in 5 to 10 ml of the same buffer. Lysozyme was added at a final concentration of 2.5 mg ml⁻¹ and the suspension incubated at 30 °C for 30 to 60 min, depending on the strain and rapidity of lysis. Approximately 5 to 10 µg of deoxyribonuclease were added and, after incubating for several more minutes, the preparation was sonicated briefly and frozen. After thawing, each 5 to 10 ml of culture extract was clarified by centrifugation at 18000 *g* for 30 min, after which the supernatant was taken for dialysis against 1 l of 0.02 M-Tris-HCl, pH 7.8 at 4 °C for 16 h. Protein was determined by the method of Lowry *et al.* (1951).

Assay of chloramphenicol acetyltransferase. Acetylation of chloramphenicol was detected chromatographically following the incubation of dialysed cell-free extracts with [^{14}C]-chloramphenicol and acetyl-CoA as described by Shaw (1975). For the present experiments the incubation mixtures contained the following, at the final concentrations indicated, in a total volume of 0.15 ml: crude extract (protein), 130 to 330 $\mu\text{g ml}^{-1}$; acetyl-CoA, 1 mM; [^{14}C]chloramphenicol (The Radiochemical Centre, Amersham, Buckinghamshire; specific activity 8.8 mCi/mmol), 0.1 mM; Tris-HCl buffer, pH 7.8, 50 mM. After 20 min at 37 °C, the assay mixture was extracted twice with 1 ml ethyl acetate, the extracts were pooled and dried, and the samples were then taken up in a few drops of 95 % (v/v) ethanol and spotted at the origin of silica gel thin-layer sheets for ascending chromatography in chloroform-methanol (97.5:2.5, v/v). Autoradiographs were used to locate the radioactive substrate and products. Quantification of enzyme activity was achieved by cutting out the chromatogram areas containing radioactive substrate and product and placing them directly in scintillation vials for counting. The specific activity of chloramphenicol acetyltransferase was then determined from a knowledge of the total radioactivity and concentration of chloramphenicol present in the incubation, the fraction of [^{14}C]chloramphenicol converted to the 3-acetate, and the protein concentration of the extract.

Each experiment with one or more strains of actinomycetes was accompanied by parallel incubations containing either no culture lysate or a cell-free extract of *E. coli* strain K12 carrying R factor 222 (same as R100; see Nisioka, Mitani & Clowes, 1970) which mediates the synthesis of chloramphenicol acetyltransferase. The occurrence of chloramphenicol acetylation was scored by comparison of the autoradiographs of each actinomycete incubation with the appropriate controls. Culture lysates showing evidence compatible with chloramphenicol acetylation were retested by examination of the specificity of the reaction as described by Shaw (1975). Since chloramphenicol acetyltransferase is specific for the biologically active D-threo isomer of the antibiotic, each presumptive positive was checked by incubating the lysate in question with [^{14}C]acetyl-CoA and non-radioactive chloramphenicol of either the D-threo or L-erythro configuration. All such incubations yielded autoradiographs showing acetylation of only the D-threo stereoisomer. Because of the low level of chloramphenicol acetyltransferase activity in the streptomyces species examined, it was not possible to use the more convenient spectrophotometric assay for the enzyme (Shaw, 1975) when crude wild-type culture lysates were examined. The latter method was used only for the measurement of chloramphenicol acetylation in a mutant with an increased level of activity (see Results). The correlation between the results of the radioactive chromatographic assay and those obtained with the spectrophotometric procedure was excellent.

Determination of chloramphenicol resistance. A glucose-asparagine minimal medium (Hopwood, 1967) in liquid or agar gel form and supplemented with growth factors was used for the determination of antibiotic resistance. L-Arginine, L-cysteine, DL-homoserine, L-leucine, L-phenylalanine and L-proline were added at 500 mg l^{-1} ; L-histidine was included at 750 mg l^{-1} . Uracil and adenine were present at 100 mg l^{-1} and nicotinamide was present at 7 mg l^{-1} . Spore suspensions were inoculated into liquid media or spread on agar plates containing concentrations of chloramphenicol over the range from 5 to 200 $\mu\text{g ml}^{-1}$. After varying periods of incubation at 30 °C, each culture was compared with controls incubated in the absence of antibiotic. The minimum concentration required for complete inhibition of growth in liquid medium was in each case approximately twice that observed for the same organism on solid medium.

Tests for destruction of chloramphenicol. Spores were inoculated into 30 ml amounts of liquid minimal medium in 250 ml Erlenmeyer flasks with 10 or 15 μg chloramphenicol/ml,

and incubated at 30 °C in a shaking water bath. After various periods, 2 ml samples were withdrawn, Millipore filtered and stored at 4 °C. When all samples had been collected, a few drops of a stationary-phase culture of *E. coli*, sensitive to inhibition by chloramphenicol at a concentration of approximately 4 µg ml⁻¹, were added. Visible turbidity after incubation at 37 °C for 8 h indicated destruction of chloramphenicol.

Attempts to induce increases in chloramphenicol resistance and enzymic acetylation. Samples of spore suspensions were inoculated and incubated with shaking, as described above, in the presence or absence of 15 µg chloramphenicol/ml. After 24 h incubation the mycelium was centrifuged and samples were plated on agar containing various concentrations of chloramphenicol. Samples were also taken for the preparation of lysates and assay of chloramphenicol acetylation by either the radioactive method (strains 2159 and 1201) or the spectrophotometric method (strain 2236).

Selection of a chloramphenicol-resistant mutant of Streptomyces acrimycini. Strain 1201 was mutagenized under the conditions described by DeliĆ, Hopwood & Friend (1970). A spore suspension was incubated for 60 min at 30 °C in a solution containing *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (1 mg ml⁻¹) and 0.05 M-Tris-maleate buffer, pH 9. The spores were collected by centrifugation and resuspended in 30 ml of liquid medium in a 250 ml Erlenmeyer flask and incubated at 30 °C on a gyratory shaker for 9 h to allow mutation expression. The very young mycelium was centrifuged, resuspended in a small volume of water and samples were spread on plates containing 5, 10, 15 or 20 µg chloramphenicol/ml. After incubation at 30 °C for several days, near-confluent growth occurred on chloramphenicol concentrations up to 15 µg ml⁻¹. There was considerable background growth at 20 µg ml⁻¹, but six discrete colonies were recognized on three plates and streaked on a fresh plate containing 20 µg chloramphenicol/ml, together with a control patch of parent strain 1201. After incubation the plate was replica-plated on to solid medium containing 20, 40 or 60 µg chloramphenicol/ml. The control patch and five of the possible mutant cultures grew at 20 µg ml⁻¹ but not at 40 µg ml⁻¹, while one culture (designated 2236) grew at 40 µg ml⁻¹ but not at 60 µg ml⁻¹. This increased resistance of 2236 compared with 1201 was reproducible. Two attempts were made to increase the resistance of 2236 by a further round of mutagenesis by NTG or by exposure to ultraviolet radiation, but no mutant resistant to higher chloramphenicol concentrations was isolated.

Fig. 1. Autoradiographic demonstration of chloramphenicol acetylation by lysates of streptomyces. (a) The formation of radioactive products [chloramphenicol 1-acetate (CM-1-Ac), chloramphenicol 3-acetate (CM-3-Ac) and chloramphenicol 1,3-diacetate (CM(Ac)₂)] formed after the incubation of [¹⁴C]chloramphenicol (CM) with acetyl-CoA and lysates of the following: (1) *S. coelicolor* Müller (strain 2159); (2) *S. coelicolor* A3(2)(104); (3) *S. lividans* (1326); (4) *S. glaucescens* (1472); (5) *S. acrimycini* (1201) and (6) *E. coli* K12 carrying an R factor 222 conferring chloramphenicol resistance. The formation of chloramphenicol diacetate in (6) was due to the higher specific activity of chloramphenicol acetyltransferase in R⁺ strains of *E. coli* as compared with the two acetyltransferase containing streptomyces strains represented by (1) and (5). (b) The results of an identical experiment using lysates of the following: (7) *Nocardia mediterranei* (1276); (8) *S. rimosus* (1459); (9) *S. roseochromogenes* (2207); (10) *S. viridochromogenes* (2209); (11) *S. viridochromogenes* (2210); (12) *S. venezuelae* (2222); (13) *S. erythrophromogenes* (2204); (14) *S. griseus* (2211); (15) *S. griseus* (2212); (16) *S. rimosus* (2248) and (17) *S. rimosus* (2246). In (b) note the strong positive reaction in (15); all the other reactions were negative except for a very weak positive reaction in (14) revealed only in an over-exposed autoradiograph (not shown). Similar experiments with the three unidentified strains of streptomyces, numbers 1158, 1159, 1195 (chromatograms not shown) showed no evidence of chloramphenicol acetylation, while *S. coelicolor* Müller strain 1157 showed a weak acetylation reaction. See Table 1 for further details of the strains.

Fig. 1 (a) and (b). For legend see facing page.

Table 2. *Levels of chloramphenicol resistance and chloramphenicol acetyltransferase in, and destruction of chloramphenicol by, certain of the strains in Table 1*

John Innes stock no.	Strain	M.i.c.* for chloramphenicol ($\mu\text{g/ml}$)	Destruction of antibiotic activity	Chloramphenicol acetylation	Chloramphenicol acetyltransferase†
2159	<i>Streptomyces coelicolor</i> Müller	5	Yes	Yes	0.6
104	<i>S. coelicolor</i> A3(2)	20	No	No	—
1201	<i>S. acrimycini</i>	10	Yes	Yes	1.0
2236	<i>S. acrimycini</i> (chloramphenicol-resistant mutant of 1201)	40	Yes	Yes	32.0
2212	<i>S. griseus</i>	NT	NT	Yes	0.8
1326	<i>S. lividans</i>	10	NT	No	—
1472	<i>S. glaucescens</i>	10	NT	No	—
2155	<i>S. venezuelae</i>	> 100, < 200	NT	No	—

NT, Not tested.

* Minimum inhibitory concentration in agar.

† Specific activity of chloramphenicol acetyltransferase in crude mycelial lysates; units are nmol chloramphenicol acetylated/min per mg protein.

RESULTS

Occurrence of the chloramphenicol acetylation phenotype

The strains of actinomycetes tested for evidence of chloramphenicol acetylation, and the results, are listed in Table 1. The strain 2159 of *Streptomyces coelicolor* Müller originally described by Argoudelis & Coats (1971) as being capable of chloramphenicol acetylation was included in this group, together with most of those strains in which genetic studies have been carried out, since it was hoped to identify at least one genetically characterized strain possessing chloramphenicol acetyltransferase. Such strains were *S. coelicolor* A3(2)/(104), *S. lividans* (1326), *S. glaucescens* (1472), *S. rimosus* (1459) and *Nocardia mediterranei* (1276). The group was enlarged by the addition of a number of other strains chosen by essentially arbitrary criteria. See the footnote to Table 1 for a note on the taxonomy of '*S. coelicolor*' strains.

Qualitative tests for the formation of the expected mono-acetylated products, chloramphenicol 3-acetate and chloramphenicol 1-acetate, showed that the occurrence of the chloramphenicol acetylation phenotype was rarer than its absence (Fig. 1). A clear positive reaction was given by the 2159 (*S. coelicolor* Müller) strain and an approximately equal activity by strains 1201 (*S. acrimycini*) and 2212 (*S. griseus*). A second strain (1157) classified as *S. coelicolor* Müller gave a rather weaker positive reaction, while a second *S. griseus* strain (2211) was so weakly positive that the chloramphenicol mono-acetates could be detected only on over-exposed autoradiographs. Thus, of 21 independent natural isolates of actinomycetes, only five strains, belonging to three species, were found to acetylate chloramphenicol. Disappointingly, none of the genetically studied strains was positive.

Chloramphenicol inactivation and the resistance phenotype

The data in Table 2 suggest that the presence of the acetylating enzyme is not the only factor contributing to the level of sensitivity of streptomyces strains to chloramphenicol. A case in point is *Streptomyces venezuelae*, the chloramphenicol-producing organism, which, although resistant or indifferent to a concentration in excess of $100 \mu\text{g ml}^{-1}$, showed no evidence of acetylation. However, the three species shown to inactivate chloramphenicol

contained chloramphenicol acetyltransferase in their mycelial lysates. Selection for an isolate of *S. acrimycini* with increased resistance to chloramphenicol (approximately 2- to 4-fold) yielded strain 2236 which had an enzyme specific activity more than 30-fold higher than that of the parental organism (strain 1201).

Tests for enzyme induction were made with *S. coelicolor* Müller (strain 2159) and with both the parent (strain 1201) and chloramphenicol-resistant mutant (strain 2236) of *S. acrimycini*. The level of enzyme in mycelial lysates was not found to be influenced by the presence of chloramphenicol at the time of inoculation or by further additions of the antibiotic during growth or immediately before harvest (secondary additions tested only in *S. acrimycini*). *Streptomyces coelicolor* Müller (strain 2159) was also examined for evidence of induction of an increased expression of resistance to chloramphenicol following exposure to the antibiotic. The minimal inhibitory concentration for chloramphenicol in agar was the same for mycelium grown from medium containing 15 µg antibiotic/ml as it was for that obtained after growth in the absence of the drug.

DISCUSSION

The findings of the present study confirm the report of Argoudelis & Coats (1971) that *S. coelicolor* Müller is capable of acetylating chloramphenicol. The earlier study was performed with growing cultures, and the products included the 3-propionyl and 3-butyryl (or iso-butyryl) esters of chloramphenicol in addition to the predominant 1-acetyl and 3-acetyl derivatives. Similar results were obtained in the present investigation when undialysed extracts of *S. coelicolor* Müller, *S. acrimycini*, and *S. griseus* were tested for acetylation activity using [¹⁴C]chloramphenicol and acetyl-CoA with the chromatographic assay. Autoradiographs of the products revealed not only the expected acetate esters but also small amounts of three radioactive products with the chromatographic properties of the propionyl and butyryl mono-esters and the di-propionyl ester of chloramphenicol. Taken together with earlier studies on chloramphenicol acetylation in *E. coli* (Shaw, 1975), it seems likely that the acetyltransferase activity of streptomyces strains is not specific for the acetyl thioester of CoA and that crude undialysed extracts contain both the necessary short-chain fatty acids and a transesterification system to synthesize the propionyl- and butyryl-CoA.

Only five of 21 isolates of actinomycetes tested were capable of enzymic chloramphenicol acetylation. This finding is compatible with the notion that the acetylation phenotype is mediated by a plasmid-linked gene, although a chromosomal location is by no means excluded. Further studies are needed on this point, including a correlation of the acetylation phenotype with the presence or absence of plasmid DNA. The isolation of a *S. acrimycini* mutant (strain 2236) which has an increased level of both chloramphenicol resistance and acetylating activity compared with the parental strain 1201 should be a useful step towards such studies.

Since the genes for enzymes which inactivate chloramphenicol and the aminoglycoside family of antibiotics are extra-chromosomal in naturally-occurring isolates of enteric bacteria and staphylococci, there has been a tendency to look to other genera for their possible origin. Benveniste & Davies (1973) called attention to the ubiquity of aminoglycoside-inactivating enzymes in actinomycetes and pointed out that these enzymes have certain properties in common with those which are plasmid-mediated in clinically-important organisms. The streptomycin phosphorylating enzyme in the producing organism *S. griseus* (Walker & Skorvaga, 1973) might suggest that antibiotic-modifying enzymes specific for the synthesized antibiotic would be generally present in the producing species. The absence of

evidence for chloramphenicol acetylation in *S. venezuelae*, which is highly resistant to its antibiotic product, suggests that there may be no general rule and that *S. venezuelae* may use an alternative mechanism to circumvent auto-inhibition. Experiments reviewed by Vining, Malik & Westlake (1968) suggest that *S. venezuelae* is indeed capable of modifying chloramphenicol, but it is by no means clear that enzymic inactivation is the sole mechanism preventing the inhibition of host ribosomal protein synthesis by chloramphenicol.

Current efforts are being directed at a characterization of the purified enzyme from *S. acrimycini* to compare it with variants found in other genera, and studies are also in progress to define the genetic determination of the acetylation phenotype.

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