DISTRIBUTION OF CHLORAMPHENICOL ACETYLTRANSFERASE AND CHLORAMPHENICOL-3-ACETATE ESTERASE AMONG *STREPTOMYCES* AND *CORYNEBACTERIUM*

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Chloramphenicol-3-acetate esterase activity was detected in cell-free extracts of strains of *Streptomyces venezuelae, Streptomyces* sp. and *Streptosporangium viridogriseum* var. *kofuense* which produced chloramphenicol and also *Corynebacterium hydrocarboclastus* which produced chloramphenicol analogs (corynecins).

None of the cell-free extracts of chloramphenicol- or corynecin-producing strains possessed chloramphenicol acetyltransferase activity under conditions which avoided the influence of the esterase activity.

Among 20 strains examined that did not produce chloramphenicol, chloramphenicol acetyltransferase was detected in cell-free extracts of one strain of *Streptomyces coelicolor* Müller and one strain of *S. fradiae* ISP5063.

Plasmids are believed to be involved in the biosyntheses of a number of antibiotics[1~3] and resistance to chloramphenicol and aminoglycosidic antibiotics is due to specific acetyl, phosphate or adenylate transferases which are determined by plasmids[4]. However, it is not certain whether there is a relationship between the origin of enzymes involved in the biosynthesis of antibiotics and the origin of the enzymes involved in resistance mechanisms. In order to test this relationship, we have studied the distribution of chloramphenicol acetyltransferase in various species of *Streptomyces* and strains of *Corynebacterium hydrocarboclastus*. The latter produce corynecins[5] which are structurally related to chloramphenicol. As shown in this paper, chloramphenicol-3-acetate esterase activity was widely distributed in streptomyces and, under conditions which avoided the influence of this esterase, chloramphenicol acetyltransferase activity could not be detected in cell-free extracts of strains which produced chloramphenicol or corynecins.

Materials and Methods

Strains

Strains used in this experiment are listed in Table 1.

Culture Media

(1) Bacto nutrient broth was purchased from Difco Laboratories.

(2) IS5Y medium contained the following's ingredients per liter: glycerol, 10 g; asparagine, 1 g; K_2HPO_4, 1 g and Bacto yeast extract, 2 g.

(3) CF8YP medium contained per liter: sucrose, 70 g; $(NH_4)_2SO_4$, 5 g; $MgSO_4 \cdot 7H_2O$, 1 g; KCl, 8 g; KH_2PO_4, 0.25 g; $FeSO_4 \cdot 7H_2O$, 40 mg; $ZnSO_4 \cdot 7H_2O$, 3 mg; thiamine-HCl, 10 mg; inositol, 40 mg; Bacto yeast extract, 2 g; and peptone, 2 g; pH was adjusted to 7.2 with 1 N NaOH.

(4) GSL-Y medium as described by MALIK and VINING[6] for production of chloramphenicol

col contained per liter: glycerol, 10 g; DL-serine, 3 g; 60 % sodium lactate, 28 ml; and Bacto yeast extract, 0.2 g.

These media were solidified with 2 % Bacto agar (purchased from Difco Laboratories), if necessary.

Determination of Resistance to Chloramphenicol

Organisms grown in shake-cultures in IS5Y medium were spread on agar plates of the same medium containing 5 or 50 μg/ml of chloramphenicol. After various periods of incubation at 30°C, the growth on each plate was compared with the control which was grown in the absence of chloramphenicol.

Preparation of Cell-free Extract

Cells were harvested during exponential growth phase in Bacto nutrient broth or at various periods of growth in GSL-Y and CF8YP media, washed once with 50 mM Tris-HCl pH 7.8, and disrupted by grinding with sea sand. The resulting suspension was centrifuged at 30,000 $\times$ g for 30 minutes. The supernatant was used as the cell-free extract.

Assay of Chloramphenicol Acetyltransferase

(1) Acetylation of chloramphenicol by growing cells: Cells were grown to exponential phase in 2 ml of Bacto nutrient broth at 30°C with shaking, and then chloramphenicol (100 μg) was added. After further incubation for 18 hours at 30°C, the incubation mixture was centrifuged and the supernatant liquid was extracted with ethyl acetate (2 ml). The residual potency of the antibiotic in the extract was determined by a paper disc method using *Bacillus subtilis* PCI219 as indicator. Production of chloramphenicol-3-acetate, 1-acetate or 1, 3-diacetate was analyzed by subjecting the extract to silica gel thin-layer chromatography as described below.

(2) Acetylation of chloramphenicol by cell-free extracts:

(a) Spectrophotometric assay: The composition of the reaction mixture which contained chloramphenicol, acetyl-CoA and dithiobisnitrobenzoic acid is described in Table 2, and the spectrophotometric assay described by SHAW[7] was used to determine the amount of CoA produced.

(b) Radioisotope assay: The reaction mixture contained 0.1 M Tris-HCl buffer (pH 6.8), 0.2 mM acetyl-CoA, 0.06 M KCl, 0.1 mM nonlabeled chloramphenicol, ^{14}C-chloramphenicol (0.2 μCi/ml) and the crude extract (86~490 μg protein/ml) of the strain. After 30 minutes at 30°C, the mixture (100 μl) was extracted with ethyl acetate (500 μl). The extract was dried and redissolved in ethanol (50 μl). Ten μl were applied to Silica gel G plates (Merck) together with carrier chloramphenicol and chloramphenicol-3-acetate. The chromatogram was developed with a mixture of $CHCl_3$ and methanol (97:3). Chloramphenicol and its acetates were detected by exposing the plates to an ultraviolet light and the radioactivity of each spot was measured by liquid scintillation counting. The liquid scintillation fluid was prepared by adding 5 g PPO (2, 5-diphenyloxazole) and 0.3 g dimethyl-POPOP (1, 4-bis [2-(4-methyl-5-phenyloxazolyl)]-benzene) to 1 liter of toluene].

Measurement of Chloramphenicol-3-acetate Esterase Activity.

Chloramphenicol-3-acetate was prepared by acetylation of chloramphenicol using the strain *Escherichia coli* ML4079 which produces chloramphenicol acetyltransferase. The supernatant (300 ml) of the culture of *E. coli* ML4079 grown in Bacto nutrient broth containing 100 μg/ml of chloramphenicol was extracted with ethyl acetate (300 ml). The extract was dried over anhydrous sodium sulfate and then concentrated *in vacuo*. The crude chloramphenicol inactivated product thus obtained was purified by silica gel column chromatography; chloramphenicol-3-acetate was eluted from the column with chloroform.

The reaction mixture (1 ml) contained the following: 0.1 M Tris-HCl buffer (pH 6.8), 0.06 M KCl, 0.1 mM chloramphenicol-3-acetate and the crude cell-free extract (10 μl). After 30 minutes at 30°C, the chloramphenicol produced was determined by bioassay with *B. subtilis* PCI219 and thin-layer chromatography.

Results

Chloramphenicol Inactivation by Growing Cultures

As shown in Table 1, a strain of *Streptomyces coelicolor* Müller and a strain of *Streptomyces fradiae* ISP5063 inactivated chloramphenicol present in the growing culture. The transformation products were similar to those reported by ARGOUDELIS *et al.*[11] and the main product was indistinguishable by thin-layer chromatography from chloramphenicol-1-acetate or chloramphenicol-3-acetate.

On the other hand, the chloramphenicol remained intact when added to cultures of *S. venezuelae*, *S.* sp. MI7633 and *Streptosporangium viridogriseum* var. *kofuense* which produced chloramphenicol, and *Corynebacterium hydrocarboclastus* KY8826 and KY8834 which produced

Table 1. Chloramphenicol acetyltransferase activity, chloramphenicol-3-acetate esterase activity and chloramphenicol sensitivity of the strains investigated

Strain	Antibiotic produced	MIC** μg/ml	Esterase	Chloramphenicol acetyltransferase
Streptomyces venezuelae ATCC15068	Chloramphenicol	5~50	+	−
S. venezuelae ISP5230-SVM1[8]	Chloramphenicol	>50	+	−
S. venezuelae ISP5230-SVM4[8]	None	>50	+	−
〃 IFO3365	None	>50	+	−
〃 NIHJ213	None	>50	+	−
〃 ATCC14584	Anthelvencin	5~50	+	−
〃 NRRL2718	Lemacidin	5~50	+	−
〃 ATCC15439	Methymycin	5~50	+	−
S. griseus ISP5236	Streptomycin	5~50	−	−
S. kasugaensis M338-M1	Kasugamycin	>50	+	−
S. fradiae ISP5063	Neomycin	5~50	+	+
S. roseochromogenes ISP5463	Streptothricin	5~50	−	−
S. lavendulae ISP5069		5~50	+	−
S. coelicolor KY621*	None	>50	+	−
S. coelicolor Müller	None	5~50	−	+
S. sp. MI7633*	Chloramphenicol	>50	+	−
Streptosporangium viridogriseum var. *kofuense*	Chloramphenicol	5~50	+	−
Nocardia asteroides IFO3384	None	5~50	−	−
Corynebacterium hydrocarboclastus KY8826*	Corynecins	>50	+	−
〃 KY8834*	Corynecins	>50	+	−
〃 KY4309*	None	5~50	+	−
〃 KY4336*	None	5~50	+	−
E. coli K12	None	<5	−	−
〃 K12/R5	None	>50	−	+
〃 K12W677	None	<5	−	−
〃 K12W677/JR66	None	>50	−	+
〃 ML4079	None	>50	−	+

* These strains were obtained from Kyowa Hakko Kogyo Co., Ltd.

** MIC (Minimum inhibitory concentration of chloramphenicol) was examined by testing the growth on nutrient agar containing 5 or 50 μg of chloramphenicol.

chloramphenicol analogs (corynecins).

Sensitivity of Chloramphenicol

As shown in Table 1, there was no correlation between resistance to chloramphenicol and chloramphenicol acetylation activity in streptomyces strains tested in this experiment. *S. coelicolor* Müller and *S. fradiae* ISP5063 which did not grow on an agar containing 50 μg/ml of chloramphenicol, inactivated chloramphenicol added to the culture at a concentration of 50 μg/ml. There was also no relation between resistance to chloramphenicol and production of this antibiotic. Some strains which produced chloramphenicol did not grow on an agar containing 50 μg/ml chloramphenicol per/ml, while some chloramphenicol non-producing strains could grow on this plate.

Chloramphenicol-3-acetate Esterase Activity in *Streptomyces* and *Corynebacterium*

In studies on biosynthesis of aminoglycoside antibiotics it has been suggested that antibiotics might be formed from inactivated compounds by hydrolytic reaction at the final stage of biosynthesis[8,9]. Therefore, we examined the activity of chloramphenicol-3-acetate esterase in streptomyces and corynebacterium. As shown in Table 1, all strains of *Corynebacterium hydrocarboclastus* and almost all streptomyces strains possessed strong chloramphenicol-3-acetate esterase activity. Therefore, as described below, we examined the activity of chloramphenicol acetyltransferase under conditions which avoided the influence of this esterase.

Table 2. Spectrophotometric assay of chloramphenicol acetyltransferase in crude cell-free extracts.

Strain	mμmole/min/mg protein**
E. coli K12W677	—
E. coli K12W677/JR66	1,550
E. coli K12	—
E. coli K12/R5	560
E. coli ML4079	1,060
E. coli ML4079+*C. hydrocarboclastus* KY8834	1,010
C. hydrocarboclastus	—
〃 (induction*)	—
S. coelicolor Müller	—
S. fradiae ISP5063	10
S. venezuelae ATCC15068	—
〃 (induction*)	—
S. venezuelae SVM-1	—
〃 (induction*)	—

* Cells were grown in a medium with 0.02 mM chloramphenicol.

** The reaction was carried out at 30°C in a Gilford recording spectrophotometer with thermostatic control. Each cuvette contained: 0.1 M Tris-HCl (pH 6.8); 0.1 mM acetylCoA, 5,5′-dithiobis-2-nitrobenzoic acid (0.4 mg/ml) and the crude cell-free extract (86~490 μg protein) in a final volume of 1.0 ml. The reaction was initiated by addition of chloramphenicol (0.1 mM).

Table 3. Inhibitors of chloramphenicol-3-acetate esterase.

Inhibitor	Concentration	Inhibition (%)
p-Nitrophenylacetate	1×10^{-3} M	0
〃	1×10^{-4} M	0
Bis-*p*-nitrophenylphosphate	1×10^{-3} M	1
〃	1×10^{-4} M	0
Isoamylalcohol	1×10^{-3} M	100
〃	5×10^{-4} M	44
NaF	1×10^{-3} M	0
〃	1×10^{-4} M	0
Acetylsalicilate	2.5×10^{-2} M	100
〃	1.2×10^{-2} M	100
〃	6×10^{-3} M	63
〃	3×10^{-3} M	40
〃	1×10^{-3} M	20
Ethylacetate	10%	100
〃	5%	28

Spectrophotometric Assay of Chloramphenicol Acetyltransferase

When chloramphenicol acetyltransferase catalyzes the acetylation of chloramphenicol in the presence of acetyl-CoA, CoA is liberated. The release of SH- of CoA is detected by reaction with 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to form a mixed disulfide with an increase in absorbance at 412 mμ due to the thionitrobenzoic acid liberated. By this method, it is possible to detect chloramphenicol acetyltransferase activity without the influence of chloramphenicol-3-acetate esterase. As shown in Table 2, chloramphenicol acetyltransferase of *E. coli* ML4079 was not inhibited by addition of an extract from *C. hydrocarboclastus* containing the esterase. In this test, which avoided the influence of the esterase, none of cell-free extracts from strains producing chloramphenicol or corynecins catalyzed the chloramphenicol dependent liberation of CoASH.

However, the mycelial extract of *S. coelicolor* which produced chloramphenicol-3-acetate in shake culture did not show positive activity in the spectrophotometric method described above. Therefore, we had to develop another method to detect chloramphenicol acetyltransferase in the presence of the esterase.

Table 4. Determination of chloramphenicol acetyltransferase by measuring ^{14}C-chloramphenicol acetylation in the presence of esterase inhibitors.

Strain	^{14}C-Chloramphenicol-3-acetate formed, dpm	
	None	Acetyl salicilate
E. coli K12W677	—	—
E. coli K12W677/JR66	2619	2482
S. griseus ISP5236	—	—
S. kasugaensis M338-M1	—	—
S. fradiae ISP5063	—	2638
S. roseochromogenes ISP5463	—	—
S. lavendulae ISP5069	—	—
S. coelicolor KY621	—	—
S. coelicolor Müller	672	602
S. venezuelae ATCC15068	—	—
〃 SVM-1	—	—
〃 SVM-4	—	—
S. sp. M17633	—	—
N. asteroides IFO3384	—	—
C. hydrocarboclastus		
KY8826	—	—
〃 KY8834	—	—
〃 KY4336	—	—

* The reaction mixtures (100 μl) with the crude cell-free extract (10 μl) were incubated for 30 min at 30°C and extracted with ethylacetate (50 μl). The extracts (250 μl) were concentrated to dryness and dissolved in methanol (50 μl). Thereafter, 10 μl was applied on silica gel plate. Other conditions were described in Method.

Inhibition of Chloramphenicol-3-acetate Esterase

A number of compounds which have been reported to inhibit various hydrolases including esterases, were tested for their effect on chloramphenicol-3-acetate esterase. The esterase activity in the cell-free extract of *Corynebacterium hydrocarboclastus* was assayed with 0.1 mM chloramphenicol-3-acetate. As shown in Table 3, organophosphates did not show a significant inhibition, whereas the addition of acetylesters, such as acetylsalicilate and ethyl acetate at high concentration (1×10^{-2} M) inhibited completely the hydrolysis of chloramphenicol-3-acetate.

Chloramphenicol Acetyltransferase Activity Measured in the Presence of Esterase Inhibitors

Table 4 indicates that acetylesters at concentrations which inhibited chloramphenicol-3-acetate esterase did not affect the chloramphenicol acetyltransferase activity of *E. coli* and *S. coelicolor* Müller. This method made it possible to detect weak acetyltransferase activity; for instance 2~3 % of the activity

shown by *S. coelicolor* Müller. Even by this more sensitive detection method, none of the strains that produce chloramphenicol or corynecins showed the presence of the acetyltransferase.

Chloramphenicol Acetyltransferase in Cells of Different Culture Ages

It has been suggested that some modified aminoglycosides may be precursors of the aminoglycoside antibiotics in *Streptomyces* and that the activity of modification enzymes changes during cultivation of the bacteria[10]. Therefore, chloramphenicol acetyltransferase activity was tested at various periods in the culture of strains producing chloramphenicol or corynecin. The cell-free extracts of chloramphenicol- or corynecin-producing strains did not show any activity that catalyzed the acetylation of chloramphenicol during cultivation in the production medium (Table 5). Moreover, the cell-free extracts thus tested were not capable of acetylating chloramphenicol under different reaction conditions (temperature of 6~60°C or KCl concentration of 0~1.0 M; data are not shown).

The chloramphenicol acetyltransferase activity of *S. coelicolor* Müller was detected in cell-free extracts prepared from all cultures under the conditions examined.

Table 5. Chloramphenicol acetyltransferase (CAT) in cells of different culture ages in chloramphenicol or corynecin-producing medium.

Medium	Time (hr)	*S. venezuelae* ATCC15068		*S.* sp. M17633		*C. hydrocarboclastus* KY8834		*S. coelicolor* Müller	
		Antibiotic* mm	CAT** dpm	Antibiotic mm	CAT dpm	Antibiotic mm	CAT dpm	Antibiotic mm	CAT dpm
CF8	12	130	—	—	—	—	—	—	610
	36	142	—	104	—	—	—	—	757
	60	155	—	127	—	125	—	—	397
	86	120	—	120	—	190	—	—	624
GSL	24	165	—	—	—	—	—	—	524
	72	120	—	90	—	—	—	—	372

* The antibiotic activity of culture supernatant was assayed by paper disc method against *S. subtilis* PCI219.

** CAT was assayed as described in Table 4.

Discussion

As first shown in this report, the esterase of chloramphenicol-3-acetate is widely distributed in streptomyces. Therefore, in order to detect chloramphenicol acetyltransferase in streptomyces cells, it is necessary to use a method which excludes the influence of this enzyme. We have shown that the spectrophotometric assay of CoA liberated from acetyl-CoA after reaction with chloramphenicol, or the radioisotopic method measuring ^{14}C-chloramphenicol-3-acetate produced in the presence of an esterase inhibitor can be applied for this purpose. Even by these methods, no chloramphenicol acetyltransferase activity was detected in organisms producing chloramphenicol or its analogs (corynecin) under any conditions.

On the other hand, the acetyltransferase was detected in two chloramphenicol-nonproducing strains, *S. fradiae* and *S. coelicolor* among 20 streptomyces strains tested. These results are consistent with observations made by other authors on strains of *S. coelicolor*[11], *S. griseus*[12], *S. acrimycini*[13] and *Flavobacterium* sp.[14], which did not produce chloramphenicol and have been reported to acetylate chloramphenicol.

In general, an antibiotic-producing strain is resistant to its own product and it has been suggested that antibiotic-modifying enzymes originated from streptomyces. The hypothesis that plasmids for resistance to an antibiotic and for its biosynthesis might have the same origin is attractive, because it would explain the appearance of resistant strains after wide use of that antibiotic. However, as described in this paper, chloramphenicol acetyltransferase is not involved in biosynthesis of this antibiotic or corynecin. In addition, there is no relationship between resistance to chloramphenicol and its production in *Streptomyces*.

The immunoassay method using an antibody to an enzyme involved in resistance is useful for the detection of the enzyme in strains producing chloramphenicol. However, as already reported, chloramphenicol acetyltransferases from different species or different group of R-factor were distinguished immunologically[15]. Therefore this method could not be used for the present study.

Although chloramphenicol acetyltransferase could not be detected in strains producing chloramphenicol or its analogs, we cannot eliminate the possibility that the genetic materials for biosynthesis and resistance originated from the same DNA and that they followed different evolutionary paths thereafter.

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