MECHANISM OF CHLORAMPHENICOL RESISTANCE IN *BACILLUS BADIUS* 211

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Six strains of chloramphenicol (CM)-resistant endospore-forming bacteria, which can grow in the presence of 100 μg/ml of CM, were isolated and identified as *Bacillus badius*. Mechanism of CM-resistance in one of the isolated strains, *Bacillus badius* 211, was investigated. No inactivation of CM was demonstrated when the strain was grown in nutrient broth containing 100 μg/ml of CM, as evidenced by paper-disc bioassay of CM in the growth medium. In accordance with this result, no CM acetylation activity was demonstrated either with the intact cells or with the crude extracts of the CM-resistant strain. Poly U- and Poly A-directed polyphenylalanine and polylysine syntheses by S-30 preparations of both CM-resistant and CM-sensitive strains of *Bacillus badius* were almost equally inhibited by CM. From these results, the mechanism of CM resistance in *Bacillus badius* 211 seems to be due to other unknown mechanism.

The mechanism of chloramphenicol (CM)-resistance in R-factor bearing *Escherichia coli* and other enteric bacteria, as well as in plasmid bearing *Staphylococcus aureus* has been shown to be inactivation of CM by CM-acetyltransferase.[1~8] Other mechanisms of CM resistance, such as decreased membrane permeability of CM, have been postulated both in *E. coli* and in *Pseudomonas*.[9,10] On the other hand, there has been no report to our knowledge, of CM-resistant *Bacillus* found in nature. We have isolated several strains of endospore forming and CM-resistant bacteria from soil and studied the mechanism of CM resistance in these bacteria.

Materials and Methods

Bacterial strains

Bacillus badius Nos. 17, 28, 37, 43, 211, and Q, which were isolated as CM-resistant bacteria from soil, were used. *Bacillus badius* 11059, which was kindly supplied from the Institute of Applied Microbiology, University of Tokyo, was used as a CM-sensitive strain. A CM-sensitive strain of *E. coli* W 2252 was used for the bioassay of CM and R-factor bearing *E. coli* W 2252 R was also used as a CM-resistant strain.

Media and growth conditions

The organisms were usually grown at 30°C with shaking in nutrient broth (pH 7.2) containing 1% Polypeptone (Daigo Eiyo Kagaku Co., Ltd., Osaka), 1% meat extract (Kyokuto Seiyaku Co., Ltd., Tokyo) and 0.5% NaCl. Nutrient agar medium was prepared by adding 1.5% agar to the nutrient broth.

Chemicals

Chloramphenicol (CM) was a product of the Sankyo Co., Ltd., Tokyo. D-*Threo* (dichloroacetyl-1-^{14}C) chloramphenicol was purchased from the Radiochemical Centre, Amersham, England. The antibiotics thiophenicol, leucomycin, lincomycin, erythromycin, thiopeptin and mikamycin B were

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Isolation of endospore-forming CM-resistant bacteria

Samples taken from the soil surface and from a depth of about 10 cm underground were suspended in 6 ml of distilled water and heated at 80°C for 30 minutes to select spore-forming bacteria. Then, the suspensions were diluted, plated out on nutrient agar containing 50 μg/ml of CM, and incubated at 37°C for 4 days. The colonies formed on the plates were isolated and purified on nutrient agar with and without 50 μg/ml of CM.

Determination of minimal inhibitory concentration of various antibiotics

Overnight cultures of isolated bacteria were inoculated into 6 ml of fresh nutrient broth containing various concentrations of antibiotics, adjusting the O.D. at 660 nm to about 0.1, and the cultures were incubated at 37°C with shaking for 10 hours. Bacterial growth was measured by O.D. determinations at 660 nm using a Hitachi photoelectric colorimeter (FPW–4) and growth inhibitory concentrations of various drugs were determined.

Quantitative analysis of chloramphenicol concentration in the growth medium

The concentration of CM in the medium during bacterial growth was measured by paper-disc bioassay using *E. coli* W 2252 as a test strain. Six milliliters of overnight culture of *B. badius* 211 were inoculated into 100 ml of fresh nutrient broth containing 100 μg/ml of CM and incubated at 37°C with shaking for 40 hours. Five milliliters of the culture were taken at various incubation time periods and heated at 95°C for 3 minutes. After the cells were removed by centrifugation at 12,000$\times g$ for 5 minutes, the concentration of CM in the supernatant was determined by paper-disc bioassay.

Radioisotope assay of chloramphenicol acetylating activity *in vivo* and *in vitro*

In vivo assay: Overnight culture in nutrient broth was inoculated into 6 ml of fresh nutrient broth containing about 0.32 nmole (600,000 dpm) of ^{14}C-chloramphenicol; the O.D. was adjusted at 660 nm to 0.2. The cells were grown at 37°C with shaking until the optical density of the culture reached 1.0. The cells were then removed by centrifugation at 12,000$\times g$ for 5 minutes, and to the supernatant an equal volume of cold ethyl acetate was added. The mixture was agitated by means of a Vortex mixer for 1 minute, and the upper layer was removed after clarification by low speed centrifugation. The extraction procedure was repeated once more. The combined ethyl acetate fractions were pooled and concentrated using an evaporator (Model TC–8 Taiyō Kagaku Co., Ltd.,) under reduced pressure. The residue was then dissolved in 20 μl of ethyl acetate and the solution spotted on Tōyō Filter paper No. 51 A. The paper was developed with benzene – methanol – water (98: 2: 2, upper phase) for about 3 hours. ^{14}C-Chloramphenicol and its acetylated products were located by radioautography, using a Fuji X-ray film. Areas of the paper corresponding to radioactive compounds were cut out, placed in vials and counted in a liquid scintillation spectrometer (Packard Tri-Carb Model 3385) using a scintillation fluid composed of 4 g of 2.5-diphenyloxazole in 1 liter of toluene.

In vitro assay: Enzymatically formed acetyl derivatives of CM were assayed according to the method described previously[11] with slight modification. The assay was performed on a reaction mixture which contained 10 μmoles of Tris-HCl buffer (pH 7.8), 8 μmoles of acetyl CoA, and 3.0 nmoles (5.3×10^4 dpm) of ^{14}C-chloramphenicol in a total volume of 40 μl. The reaction was initiated by adding 40 μl of enzyme preparation (70~80 μg protein), and the mixture was incubated at 37°C for 30 minutes. The reaction was terminated by heating at 95°C for 10 minutes. The reaction mixture was extracted twice with 100 μl of cold ethyl acetate. Separation of acetylated CM from CM by paper chromatography and counting of radioactivity were carried out in the same way as in the *in vivo* assay.

Preparation of cell-free extracts for assay of CM-acetylating activity

The cells were grown in nutrient broth to an O.D. of *ca.* 0.8, harvested by centrifugation, and washed twice with 0.02 M Tris-HCl buffer (pH 7.8) containing 0.06 M KCl, 0.01 M $MgSO_4$ and 0.006 M 2-mercaptoethanol. The washed cells were finally suspended in 10 volumes of the same buffer and

sonicated at 10 KHz for 10 minutes using a sonic oscillator (Kubota Seisakujo Co., Ltd., Tokyo). The supernatant obtained by centrifugation of the sonicate at 24,000×*g* for 30 minutes was dialyzed for 24 hours at 4°C against 20 volumes of the same buffer with 3 changes of the buffer. The cell-free extracts were stored at −80°C until used.

Preparation of S–30 fraction

Bacteria were grown at 37°C with shaking in nutrient broth enriched with 0.2% yeast extract. The cells in their logarithmic growth phase (O.D. at 660 nm 0.4~0.6) were harvested by centrifugation and washed twice with cold buffer (0.01 M Tris-HCl buffer pH 7.8, 0.01 M magnesium acetate, 0.06 M KCl and 0.006 M mercaptoethanol). The cells were finally suspended in 3 volumes of the same buffer, and disrupted in a French pressure cell (Model 5615, Ohtake Seisakujo Co., Ltd., Tokyo) at 400~500 kg/cm^2 and 0°C. DNase was added to the cell homogenates at the concentration of 20 μg/ml, and the mixture centrifuged three times: 15,000×*g* for 10 minutes, 20,000×*g* for 20 minutes and 30,000×*g* for 30 minutes. The pellets were discarded after each centrifugation. The final supernatant (S–30) was dialyzed for 24 hours at 4°C against 30 volumes of the same buffer and stored frozen at −80°C.

Conditions for amino acid incorporation

Reaction mixtures contained the following components: Tris-HCl pH 8.0, 40 μmoles; magnesium acetate, 10 μmoles; NH_4Cl, 40 μmoles; GTP (Na-salt), 0.2 μmoles; ATP (Na-salt), 0.8 μmoles; phosphoenolypyruvate (K-salt), 4.0 μmoles; mercaptoethanol, 40 μmoles; spermidine, 0.8 μmoles; ^{3}H-phenylalanine or ^{3}H-lysine (0.5 μCi), 1.5 nmoles; pyruvate kinase, 10 μg; t-RNA, 600 μg; polyU or polyA, 50 μg; and S–30 preparation, 200 μl (3.0~4.0 mg protein); total volume: 1 ml. The reaction mixture containing all the components except the S–30 preparation was preincubated at 37°C for 5 minutes. The reaction was started by adding 200 μl of S–30 preparation. The mixture was incubated for 30 minutes and the reaction terminated by the addition of 1 ml of 10% trichloroacetic acid (TCA). The precipitates were washed twice with 2 ml of 5% TCA, heated at 90°C for 15 minutes in 2 ml of 5% TCA, washed successively with 95% ethanol, ethanol-ether (1: 1), and ether. The washed precipitates were dried at 30°C, then solubilized in 1 ml of 5% sodium dodecyl sulfate solution at 37°C. Radioactivity of the sample was counted in the liquid scintillation spectrometer using a toluene-Nonione scintillation fluid, from which POPOP was omitted.[12]

Results

Isolation and Identification of CM-resistant Bacteria

Six strains of endospore-forming, CM-resistant bacteria (strains 17, 28, 37, 43, 211 and Q) were isolated from soil sampled in the Miyagi Prefecture. These bacteria were resistant to more than 50 μg/ml of CM. Taxonomical characteristics of these bacteria are reported in Table 1; all six strains were identified as *Bacillus badius*. One of these strains, *Bacillus badius* 211, was mainly used in the following experiments.

Minimal Inhibitory Concentrations of Various Antibiotics for Isolated Bacteria

Minimal inhibitory concentrations of various antibiotics including CM were determined for strains 211 and 11059 (sensitive control). Results are shown in Table 2. In this experiment, antibiotics, known as inhibitors of protein synthesis in bacteria, were used. *B. badius* 211 was resistant to more than 100 μg/ml of CM and thiophenicol. This strain was sensitive to streptomycin (SM), kanamycin (KM), and tetracycline (TC) at concentrations lower than 5 μg/ml; these antibiotics are inhibitors of the 30-S subunit of the ribosome.[13] On the other hand, strain 211 was resistant to some of the antibiotics which are inhibitors of the 50-S subunit, such as spiramycin, erythromycin, lincomycin and mikamycin B.[13] This strain, however, was sensitive to low concentrations of leucomycin and thiopeptin. CM-sensitive *B. badius* 11059 was sensitive to all antibiotics at con-

Table 1. Taxonomical characteristics of isolated CM-resistant bacteria

Rods: Width, μm	0.5~0.7
Length, μm	1.5~3.0
Spore: Shape	Elliptical
Position	Central
Distension of sporangium	None
Acid from: Glucose	−
Xylose	−
Mannose	−
Arabinose	−
Gas from: Glucose	−
Xylose	−
Mannose	−
Acetoin from glucose	−
Hydrolysis of starch	+
Deamination of phenylalanine	−
Growth in 7% NaCl	+
Reduction of NO_3^- to NO_2^-	−
Uric acid utilization	−
G+C content of DNA, %	44

Isolated strains were designated 17, 28, 37, 43, 211 and Q. Position of spore and distention of sporangium were observed by phase contrast microscopy. G+C content of DNA was determined by melting temperature.

Table 2. Minimal inhibitory concentrations of various antibiotics against *B. badius* 211 and *B. badius* 11059

Antibiotics	μg/ml	
	B. badius 211	*B. badius* 11059
Chloramphenicol	100	10
Streptomycin	5	5
Kanamycin	5	5
Tetracycline	5	5
Thiophenicol	100	10
Leucomycin	5	5
Spiramycin	100	10
Erythromycin	100	10
Lincomycin	100	25
Thiopeptin	5	5
Mikamycin B	50	5

Cells were incubated at 37°C for 10 hours in nutrient broth containing various concentrations of drugs and growth inhibitory concentrations were determined.

centrations lower than 10 μg/ml (except lincomycin).

Absence of CM Inactivation in CM-resistant *Bacillus badius* 211

Inactivation of CM in CM-resistant *B. badius* 211 during growth was examined by paper-disc bioassay. The results are shown in Fig. 1. No significant change in the concentration of CM was observed for 36 hours of incubation. R factor-bearing *E. coli, Pseudomonas* and plasmid-bearing *Staphylococcus* are known to inactivate CM by chloramphenicol acetyltransferase. To examine CM-acetylating activity in CM-resistant *B. badius* 211, radioisotope assay was carried out using both intact cells (*in vivo* assay) and crude extracts (*in vitro* assay). Since CM-acetylating activity may be inducible in CM-resistant *B. badius* 211, the organism was grown in nutrient broth with and without 20 μg/ml of CM. In the case of the CM-sensitive strain, *B. badius* 11059, the cells were grown in nutrient broth with and without 2 μg/ml of CM. When the reaction mixture was analyzed by paper chromatography, acetylated compounds were well separated from CM. After autoradiography, radioactivities of both acetylated and unchanged CM were counted. As shown in Table 3, approximately 90% of added CM were converted to acetylated CM, when the cells of *E. coli* W 2252 R were incubated with radioactive CM, while essentially no change of CM was observed with the cells of *B. badius* 211. The same results were observed with cell-free extracts. No significant CM-acetylating activity was observed with the crude extracts from CM-resistant *B. badius* 211 and CM-sensitive *B. badius* 11059. As shown in Table 4, CM-acetylating activity was only detected in *E. coli* W 2252 R.

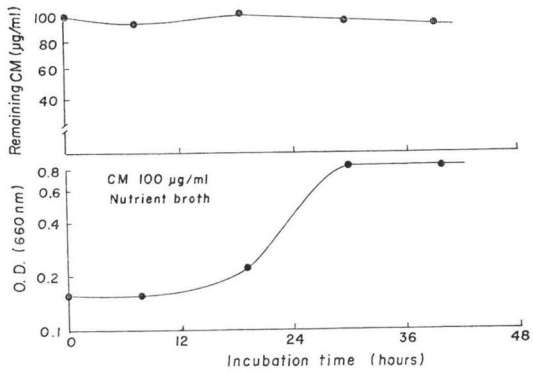
Effect of CM on Polyphenylalanine and Polylysine Syntheses by S-30 Preparation

Effect of CM on polyphenylalanine and polylysine syntheses by S-30 preparation was investigated. Fig. 2 shows the effect of CM on incorporation of ^{3}H-phenylalanine (A) and ^{3}H-lysine (B) into

polypeptide, using S–30 preparations of both *B. badius* 211 and *B. badius* 11059. Both syntheses were similarly affected in CM-sensitive and CM-resistant strains. As reported previously,[14] inhibition of polylysine synthesis by CM is more marked than that of polyphenylalanine synthesis.

Fig. 1. Change of chloramphenicol (CM) concentration during growth of CM-resistant *B. badius* 211.

Cells of *B. badius* 211 were grown in nutrient broth containing 100 μg/ml of CM for 40 hours. Five milliliters of the culture were taken at various incubation periods and the concentration of CM was analyzed by bioassay.



Discussion

Two major types of mechanism of CM resistance in bacteria have been reported. One is inactivation of CM by CM acetyltransferase in R-factor-bearing enteric bacteria and plasmid-bearing *Staphylococcus*,[1,3~6] the other is decreased permeability of bacteria to CM[9,10]. In our isolated *B. badius* 211, neither inactivation nor acetylation of CM were demonstrated by either bioassay or radioisotope assay. When we examined the effect of CM on polyphenylalanine and polylysine syntheses using S–30 preparations of both CM-sensitive and CM-resistant strains of *B. badius*, only a slight difference in CM sensitivity was found between these two strains. There is a possibility that the resistant strain of *B. badius* excreted antagonists of CM into the medium and therefore was resistant to CM. This possibility, however, can be ruled out because the sensitive strain of *B. badius* could not grow in nutrient broth containing 50 μg/ml of CM in which the resistant strain of *B. badius* had been pregrown for more than 20 hours. From these results, it is concluded that the mechanism of CM resistance in *B. badius* 211 is neither inactivation of CM nor the presence of CM-resistant protein synthesis, but is due to an unknown factor, probably the impossibility of CM to reach the target site. Although we have examined the uptakes of ^{14}C-chloramphenicol by the cells of both sensitive and resistant strains, we have not yet obtained reliable data, probably due to technical difficulty. As shown in Table 1, this resistant strain of *B. badius* was also resistant to various antibiotics, such as erythromycin, spiramycin,

Table 3. CM-acetylating activities of CM-resistant *E. coli* W 2252 and *B. badius* 211

Strain	Radioactivity (dpm)	
	CM	Acetylated CM
B. badius 211		
Pregrown with CM	54890	170
Pregrown without CM	53240	260
E. coli W 2252 R		
Pregrown with CM	8540	49130
Pregrown without CM	8640	53430
Minus cells	40910	280

Cells of *B. badius* 211 and *E. coli* W 2252 R were grown in nutrient broth containing radioactive CM for 5~7 hours to reach an O.D. of 1.0. Then, the supernatant obtained by centrifugation was extracted with ethyl acetate, and analyzed for the formation of acetylated chloramphenicol.

Table 4. CM-acetylating activities of various strains of bacteria

Strain	Radioactivity (dpm)	
	CM	Acetylated CM
E. coli W 2252 R	43800	4580
B. badius 211		
Pregrown with CM	42620	80
Pregrown without CM	27930	80
B. badius 11059		
Pregrown with CM	40410	60
Pregrown without CM	31490	80

Crude extracts were prepared from the cells of *E. coli* W 2252 R (non-induced), *B. badius* 211 and *B. badius* 11059 (induced and non-induced). CM-acetylating activities of these crude extracts were assayed.

Fig. 2. (A) Effect of chloramphenicol on incorporation of ^{3}H-phenylalanine into polypeptide by S–30 preparation.

S–30 preparations were prepared from the cells of both *B. badius* 11059 (CM-sensitive strain) and *B. badius* 211 (CM-resistant strain) and the effects of CM on polyphenylalanine syntheses by these S–30 preparations were examined.

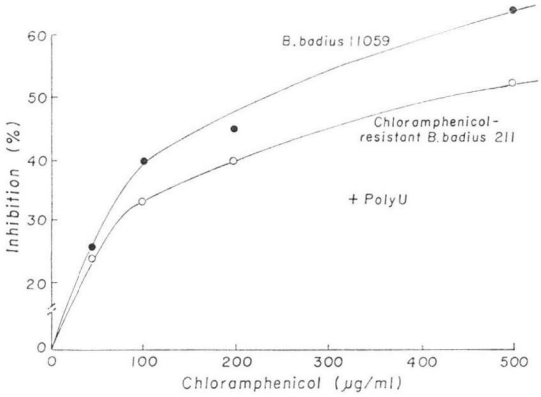
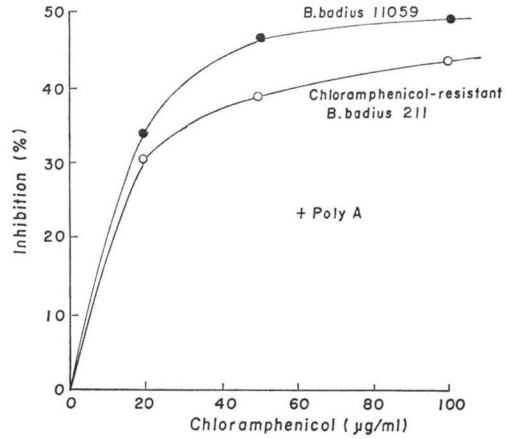


Fig. 2. (B) Effect of chloramphenicol on incorporation of ^{3}H-lysine into polypeptide by S–30 preparation.

S–30 preparations were prepared from the cells of both *B. badius* 11059 (CM-sensitive strain) and *B. badius* 211 (CM-resistant strain) and the effects of CM on polylysine syntheses by these S–30 preparations were examined.



lincomycin and mikamycin. We have obtained a partially CM-sensitive mutant from CM-resistant parent strain, *B. badius* 211, by nitrosoguanidine treatment. This strain, however, retained a similar level of erythromycin resistance to that of the parent strain. Therefore, the factor determining erythromycin resistance seems to be different from that of CM resistance in this parent strain. Inactivation of erythromycin has not been demonstrated in this resistant strain.

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