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Chloramphenicol Resistance in *Myxococcus xanthus*

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Derivatives of *Myxococcus xanthus* FB_t resistant to chloramphenicol (25 μg/ml) arose spontaneously with a frequency of approximately 10^{-7}. One of these organisms ($FB_tCam_1^r$) was characterized. $FB_tCam_1^r$ showed a unique type of phenotypic instability. After transfer from medium containing chloramphenicol to medium lacking the drug, resistance was lost after approximately one generation. The loss resulted in a sharp drop in the total number of chloramphenicol-resistant organisms and was not due to segregation of chloramphenicol-susceptible organisms during growth. Cell-free extracts of strain $FB_tCam_1^r$ converted chloramphenicol to acetyl chloramphenicols in a fashion implicating activity of chloramphenicol acetyltransferase. This activity was lost simultaneously with the loss of chloramphenicol resistance after removal of the drug from cultures. Organisms with a similar phenotype to $FB_tCam_1^r$ could be produced at high frequencies when strain FB_t was exposed to low concentrations of chloramphenicol (2 to 5 μg/ml), to 3-acetylchloramphenicol (25 μg/ml), or to 1,3-diacetylchloramphenicol (25 μg/ml). Since strain FB_t is capable of deacetylating acetyl chloramphenicols, these effects are probably all due to low concentrations of chloramphenicol. In the presence of chloramphenicol, $FB_tCam_1^r$ produced fruiting bodies and myxospores on fruiting agar; however, glycerol-induced myxospore formation was inhibited. In the absence of the antibiotic, chloramphenicol resistance was maintained by glycerol-induced myxospores.

Myxococcus xanthus (gram-negative rod, order *Myxobacteriales*) demonstrates genetic instability with respect to several characters. Clones from liquid-grown cultures segregate into yellow and tan color types (3; Burchard, unpublished data); mutants defective in the glycerol-induced formation of myxospores (4) arise with high frequency (Burchard and Parish, unpublished data); a motility mutant (semimotile) arose with high frequency on two isolated occasions (1; Burchard, unpublished data). In this paper we report the isolation and characterization of another unstable phenotype, a chloramphenicol-resistant variant. Phenotypically similar cells can be produced by incubation in the presence of low levels of chloramphenicol or acetylated chloramphenicols. The significance of these findings for myxobacterial genetics is discussed.

MATERIALS AND METHODS

Bacteria. *M. xanthus* FB_t was derived from *M. xanthus* FB (3). *Escherichia coli* J5 K-12 F^- *met pro* lac^+ (R1-19*drd* F_{11} *fi*$^+$ *chl*^{r} *kan*^{r} *sul*^{r} *str*^{r}) (8) was a gift from S. Baumberg of the Department of Genetics, University of Leeds.

Culture conditions. *M. xanthus* FB_t was grown in CT-1 medium (3); myxospore induction was effected by adding glycerol to 0.5 M (4). CTE agar is CT-1 agar supplemented with 10^{-4} M ethylenediaminetetraacetic acid (2). Viable cells were assayed by plating on CTE agar with or without chloramphenicol (25 μg/ml).

Extraction of cells and assays of chloramphenicol and its derivatives. For assay of chloramphenicol acetyltransferase activity, bacteria were harvested by centrifugation and washed twice with 0.01 M tris(hydroxymethyl)aminomethane HCl (pH 7.6), and a suspension (approximately 10^{10} colony-forming units (CFU) per ml in tris(hydroxymethyl)aminomethane buffer as above) was disrupted by sonication (Dawe Soniprobe, 1 min, 0 C, 4 A). Cell debris was removed by centrifugation (17,000 × *g*, 15 min, 4 C), and the supernatant fraction was used as the enzyme preparation. The mixture was incubated at 37 C for 2 h in the presence of acetyl coenzyme A (CoA) and [^{14}C]chloramphenicol according to the method of Shaw (12).

Chloramphenicol and its derivatives were extracted from aqueous solutions, either from acetylase assays or from cultures of bacteria, twice with 1 volume of ethyl acetate. Ethyl acetate was removed by rotatory evaporation at 45 C and the mixture was analyzed by thin-layer chromatography (see below). In those cases in which radioactive chloramphenicol was used, the

thin-layer plates were exposed to X-ray film (Kodak) and autoradiograms were obtained. Otherwise the spots were identified under ultraviolet light. The results from autoradiograms were recorded either by scanning film blackening at 450 nm in a Unicam SP 1800 spectrophotometer fitted with a gel-scanning attachment or by scraping the silica (stationary phase) from the region of a spot (identified from the autoradiogram) off the plate and measuring the radioactivity in a Beckman LS100 liquid scintillation spectrophotometer to a standard error of 2%.

Chloramphenicol derivatives. [1-^{14}C]dichloroacetyl choramphenicol (8.8 Ci/mol) was obtained from the Radiochemical Centre, Amersham, U.K. Acetyl chloramphenicols were prepared by the following procedures.

Chloramphenicol (D(-)-threo-2,2-dichloro-*N*-[β-hydroxy-α-(hydroxymethyl)-*p*-nitrophenethyl] acetamide, molecular weight 323; Parke-Davis) was acetylated by using acetic anhydride. In naming the products, we follow the convention of Shaw (12) and refer to the β-hydroxy group as number 1 and the primary hydroxy group as 3. In pyridine solvent, the only recoverable product is 1,3-diacetyl chloramphenicol (10). Monoacetyl chloramphenicols were prepared by acetylation in aqueous solvent. For the pyridine-based method, chloramphenicol (1 mmol) dissolved in pyridine (2.0 ml) was incubated with acetic anhydride (10 mmol) for 10 min at 40 C. Water (10 ml) was added and the product was extracted with ethyl acetate (3 × 15 ml). The ethyl acetate layers were combined and dried over $MgSO_4$, and the solvent was removed at 45 C. For acetylation in aqueous solvent, chloramphenicol (1 mmol) was dissolved in 0.05 M NaOAc, pH adjusted to 6.0 with glacial acetic acid (100 ml). Acetic anhydride (100 mmol) was added and the mixture was incubated at 40 C. More acetic anhydride (100 mmol) was added after 30, 60, and 90 min. The total incubation time was 120 min. The products were extracted with ethyl acetate (3 × 50 ml), and the ethyl acetate was dried and removed as above. The products were analyzed by thin-layer chromatography on Kieselgel G (type 60; Merck) developed in a mixture of chloroform and methanol (95:5 by volume) according to the method of Shaw (12). For analytical work, the thickness of the stationary phase was 0.25 mm; for preparative work it was 1 mm. From the preparation in pyridine, 1,3-diacetyl chloramphenicol was the only detectable product. From the preparation in aqueous medium, unchanged chloramphenicol, 1-acetyl chloramphenicol (trace), 3-acetyl chloramphenicol, and 1,3-diacetyl chloramphenicol were present. The products were taken up in small volumes of ethyl acetate and streaked on a thin-layer plate. Up to 20 mg of mixed products can be fractionated in this way (15-cm streak). The plates were developed, bands corresponding to the acetylated products were scraped off and eluted with ethyl acetate, and the solvent was removed by prolonged rotatory evaporation. The samples were analyzed by thin-layer chromatography. Preparative fractionation was repeated until no detectable impurities remained. One or two preparative thin-layer fractionations are sufficient. The purified products were dissolved in ethanol and their spectra were recorded (Table 1). The literature values are taken from Shaw (12). Although preparations of 1- and 3-acetyl chloramphenicols appear not to be described in the literature, Shaw's data were based on authentic samples obtained from M. Rebstock and A. J. Glazko of Parke-Davis.

TABLE 1. *Acetylated chloramphenicols*

Antibiotic	Absorption maximum (nm)		R_f	
	Found	Literature value (12)	Found	Literature value (12)
Chloramphenicol (starting material)	274	274	0.24	0.27
Chloramphenicol (recovered from aqueous reaction)	274		0.24	
1-Acetyl chloramphenicol	268	267	0.52	0.54
3-Acetyl chloramphenicol	272	271	0.60	0.61
Diacetyl chloramphenicol (from aqueous reaction)	261	261	0.80	0.79
Diacetyl chloramphenicol (from reaction in pyridine)	261		0.80	

For experiments involving the addition of acetylated chloramphenicols to liquid cultures, the samples were dissolved in ethanol and their concentrations were calculated on the assumption that the value of $E^{1\%}_{1cm}$ at the absorbance maximum would be 298 as for chloramphenicol itself.

[^{14}C]3-acetyl chloramphenicol and [^{14}C]1,3-diacetyl chloramphenicol were obtained by following the same procedures as above, except that a very small amount of chloramphenicol (10 μCi) was used and the acetylations were performed in 1 ml of solvent, maintaining the same ratios of solvent to acetic anhydride as above. Spots of radioactive acetylated products were identified by autoradiography and extracted.

RESULTS

Chloramphenicol-resistant phenotype. *M. xanthus* FB_t grew in CT-1 containing chloramphenicol at 2 to 5 μg/ml; 10 μg/ml inhibited growth. If cultures of the organism were plated on agar containing chloramphenicol (25 μg/ml), colonies of chloramphenicol-resistant organisms were sometimes obtained. The frequency was variable and was in the range of less than 10^{-7} to 5 × 10^{-6}. It was possible to increase the frequency by exposing the cells in liquid culture to chloramphenicol or one of its derivatives (see

below). One of the spontaneous chloramphenicol-resistant isolates was $FB_tCam_1^r$.

M. xanthus $FB_tCam_1^r$ grew in liquid CT-1 medium containing chloramphenicol (25 μg/ml) at 30 C with mean generation time of 10.2 h. In the absence of chloramphenicol, the mean generation time of strain $FB_tCam_1^r$ was 5.9 h and that of strain FB_t was 5.2 h. These two values are the same within the limits of experimental error. After transfer of $FB_tCam_1^r$ cells grown in the presence of chloramphenicol to medium lacking the antibiotic, the cells continued to grow but the efficiency of plating on chloramphenicol agar showed a sharp drop after 5 h (approximately one generation) (Fig. 1). The drop reflects a fall in the absolute numbers of chloramphenicol-resistant cells, not merely the segregation of chloramphenicol-susceptible organisms. If the platings were done on agar containing 10 μg of chloramphenicol per ml, the number of colony-forming units also showed a similar decline but after a longer lag.

Chloramphenicol acetyltransferase. Chloramphenicol resistance carried by R factors in *Escherichia coli* (12) or *Staphylococcus aureus* (13) is mediated by the enzyme chloramphenicol acetyltransferase (EC 2.3.1.28). We examined cell-free extracts of *M. xanthus* $FB_tCam_1^r$ for evidence of the activity of this enzyme. The results (Fig. 2) demonstrate that extracts of the cells did contain activity characteristic of this enzyme. For comparison, Fig. 2 also demonstrates the conversion of chloramphenicol to acetylated chloramphenicols by an extract from

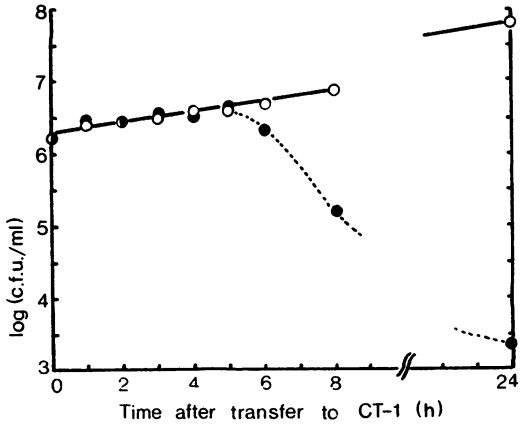

FIG. 1. *Loss of chloramphenicol resistance by M. xanthus $FB_tCam_1^r$. Bacteria were grown in the presence of chloramphenicol (25 μg/ml) to 2 × 10^8 CFU/ml and diluted to 2 × 10^6 CFU/ml in CT-1 medium. Assays were performed by plating on CTE agar (○) and CTE agar containing chloramphenicol (●).*

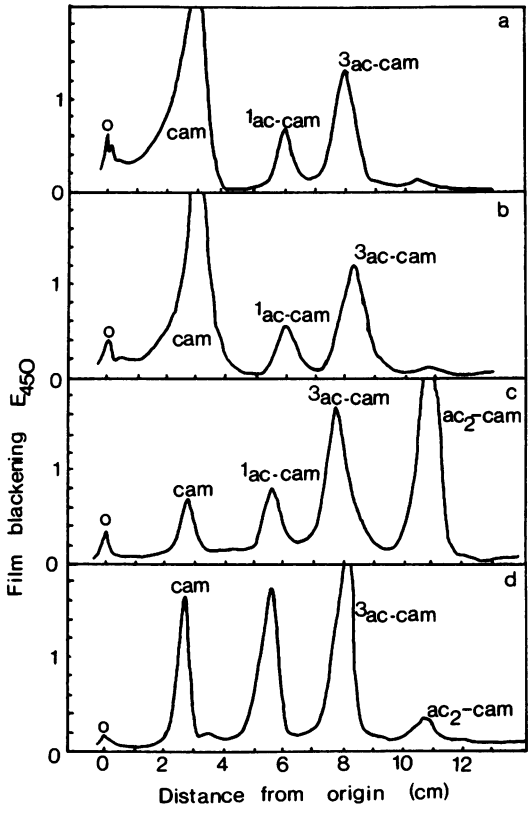

FIG. 2. *Analysis of chloramphenicol derivatives formed after incubation of ^{14}C-labeled chloramphenicol with extracts of cells as described in the text. The figure shows traces of thin-layer autoradiograms. (a) M. xanthus $FB_tCam_1^r$ extract complete incubation mixture; (b) as (a) without acetyl CoA; (c) E. coli J5 (R1-19) complete incubation mixture; (d) as (c) without acetyl CoA. Abbreviations: O, Origin; cam, chloramphenicol; 1ac-cam, 1-acetyl chloramphenicol; 3ac-cam, 3-acetyl chloramphenicol; ac_2-cam, 1,3-diacetyl chloramphenicol.*

a strain of *E. coli* R^+ *chl*^{r}, prepared by the same methods. The enzyme converts chloramphenicol into 3-acetyl chloramphenicol; 1-acetyl chloramphenicol arises as a result of a nonenzymatic rearrangement and 1,3-diacetyl chloramphenicol arises from the enzymatic acetylation of this rearranged product (12). The unfractionated extracts from the *E. coli* clearly contained some acetyl CoA, since dependence on the cofactor was only partial (Fig. 2c and d). The complete lack of dependence on acetyl CoA by the *M. xanthus* extract may have been due to an intracellular pool of the cofactor sufficient for the more limited acetylation that was observed (Fig. 2a and b). Attempts to remove endogenous acetyl CoA from the *M. xanthus* preparation by

dialysis were not feasible because of the instability of the acetyltransferase. We presume that this was due to proteolytic activity. Cell-free extracts of *M. xanthus* FB_t contained no detectable acetylase activity.

During the loss of chloramphenicol resistance that follows transfer from medium containing chloramphenicol to CT-1, the chloramphenicol acetylation activity of cell-free extracts dropped (Table 2). The loss of chloramphenicol resistance, as measured by plating, was less sharp than in Fig. 1. This presumably reflects the different method that was required in the present study, in which large concentrations of cells were removed, washed, and resuspended in CT-1; for Fig. 1, an exponentially growing culture was diluted into CT-1 medium.

Induction of chloramphenicol resistance. In chloramphenicol-resistant *S. aureus*, chloramphenicol acetyltransferase is inducible by chloramphenicol (13). We examined the effects of chloramphenicol and its acetylated derivatives upon the appearance of the chloramphenicol-resistant phenotype in *M. xanthus* FB_t (Table 3). The data demonstrate that chloramphenicol resistance can be induced in strain FB_t by exposing the cells to chloramphenicol. The highest frequencies of chloramphenicol-resistant bacteria were obtained either by prolonged exposure of the cells to sublethal concentrations of chloramphenicol or by exposure to the nontoxic, acetylated chloramphenicols. In a subsequent experiment we followed the appearance of chloramphenicol-resistant cells during incubation in medium containing 2 and 5 μg of chloramphenicol per ml (Fig. 3). After a lag, the length of which

TABLE 2. *Acetylation of chloramphenicol by extracts obtained from M. xanthus $FB_tCam_1^r$ after transfer to medium lacking chloramphenicol*[a]

Time after transfer (h)	Acetylase activity[b]	EOP on chloramphenicol agar[c]
0	12.4	1.02
4.25	0.6	1.9×10^{-1}
6.25	0.7	0.9×10^{-1}
8.00	0.2	2.7×10^{-3}

[a] Bacteria (10^8 CFU/ml) growing in CT-1 medium containing chloramphenicol were harvested by centrifugation, washed twice with CT-1 medium, and resuspended in CT-1 medium (10^8 CFU/ml). Aliquots (25 ml) were removed and used for preparation of cell-free extracts.

[b] Expressed as [(radioactivity in 3-acetyl chloramphenicol)/(radioactivity in chloramphenicol)] × 100.

[c] EOP, Efficiency of plating; expressed as (CFU/ml measured on CTE agar + chloramphenicol)/(CFU/ml measured on CTE agar).

TABLE 3. *Induction of chloramphenicol resistance in M. xanthus FB_t*[a]

Expt	Inducer[b]	Concn (μg/ml)	Time of assay (h)	Survival[c]	Frequency of cam^r phenotype among survivors[d]
1	None	0	8	1.00	6×10^{-6}
1	cam	25	8	0.03	1.4×10^{-4}
1	^{3}ac-cam	25	8	0.92	6.2×10^{-1}
1	ac_2-cam	25	8	0.65	3.2×10^{-4}
2	cam	2	48	NT	1×10^{-2}
2	cam	5	48	NT	5.5×10^{-1}

[a] *M. xanthus* FB_t was grown in CT-1 in the presence of the inducer. For experiment 1, a culture (2×10^7 CFU/ml) was divided into four. At the end of the 8-h period, the control culture had grown to 6.6×10^7 CFU/ml. For experiment 2, a light inoculum was grown (in the presence of inducer) to a final density of approximately 2×10^9 CFU/ml after the 2-day period.

[b] cam, Chloramphenicol; ^{3}ac-cam, 3-acetyl chloramphenicol; ac_2-cam, 1, 3-diacetyl chloramphenicol.

[c] Ratio of CFU per milliliter assayed on CTE agar to that of the control culture; NT, not tested.

[d] Expressed as (CFU/ml measured on CTE agar + chloramphenicol)/(CFU/ml measured on CTE agar).

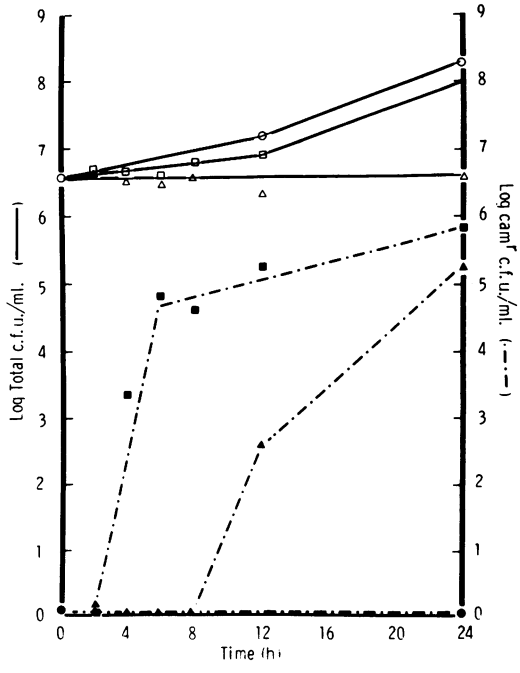

FIG. 3. *Induction of resistance to chloramphenicol (25 μg/ml) by low levels of this antibiotic. A log-phase culture of M. xanthus FB_t was diluted to 5×10^6/ml in CT-1. At time zero, chloramphenicol was added to final concentrations of 0 (○, ●), 2 (□, ■), and 5 (△, ▲) μg/ml. The cultures were incubated at 30 C. Colony-forming units on CTE ± chloramphenicol (25 μg/ml) were assayed periodically.*

depended on the concentration of the antibiotic, there was a sharp rise in cells able to form colonies on chloramphenicol agar. In the absence of the antibiotic, resistant cells did not appear. The data are inconsistent with selection of preexistent, chloramphenicol-resistant cells. Furthermore, the chloramphenicol-resistant organisms induced in this way demonstrated the same type of instability in the absence of the antibiotic that was characteristic of *M. xanthus* $FB_tCam_1^r$ (Fig. 1).

Addition of chloramphenicol or acetyl chloramphenicols to $FB_tCam_1^r$ cells during the period of loss of chloramphenicol resistance that followed transfer to medium lacking chloramphenicol resulted in a degree of rescue of the chloramphenicol-resistant phenotype (Table 4).

Since high concentrations of 3-acetyl chloramphenicol and 1,3-diacetyl chloramphenicol had effects similar to those of low concentrations of chloramphenicol in inducing resistance to the antibiotic (Tables 3 and 4), we examined the fate of these compounds in growing cultures of *M. xanthus* FB_t (Table 5). It is clear that the cells were capable of limited deacetylation of acetyl chloramphenicols. Although the data do not permit precise estimates of chloramphenicol concentrations in experiments such as those summarized in Tables 3 and 4, we presume that the inducing effects of acetyl chloramphenicols are best regarded as being due to intracellular production of low levels of chloramphenicol.

Chloramphenicol effect on myxospore formation. The effects of chloramphenicol and its derivatives upon glycerol-induced myxospore formation (4) were examined. Concentrations that induced the chloramphenicol-resistant phenotype (5 μg of chloramphenicol, 25 μg of 3-acetyl chloramphenicol, and 25 μg of 1,3-diacetyl chloramphenicol per ml) had no effect on myxospore induction in strain FB_t; 25 μg of chloramphenicol per ml inhibited differentiation.

The effects of glycerol and chloramphenicol (25 μg/ml) on *M. xanthus* $FB_tCam_1^r$ are summarized in Table 6. In the presence of these agents, most cells grew vegetatively; those few that formed myxospores did so slowly and retained chloramphenicol resistance. In the absence of chloramphenicol, resistance to the antibiotic was not lost during the formation of myxospores.

M. xanthus FB_t formed fruiting bodies on agar containing 0.02% Casitone (and other com-

TABLE 4. *Effect of inducers of chloramphenicol resistance on the stability of M. xanthus $FB_tCam_1^r$ after transfer to CT-1 medium*[a]

Time of addition of inducer (h)	Inducer[b]	Time of assay (h)	EOP[c]
	None	8	2.5×10^{-3}
0	cam	8	7.7×10^{-1}
0	^{3}ac-cam	8	2.7×10^{-2}
0	ac_2-cam	8	3.5×10^{-2}
5	cam	8	4.1×10^{-1}
5	^{3}ac-cam	8	1.1×10^{-2}
5	ac_2-cam	8	1.2×10^{-2}

[a] Bacteria were grown to 2×10^7 CFU/ml in CT-1 medium containing chloramphenicol (25 μg/ml), harvested, washed, and resuspended in CT-1 medium at the same density in the presence of the inducers.

[b] Abbreviations as in Table 3; all additions to 25 μg/ml.

[c] EOP, Efficiency of plating; ratio calculated in the same way as "frequency of Cam^r phenotype" in Table 3.

TABLE 5. *Interconversion of chloramphenicol and acetyl chloramphenicols by M. xanthus FB_t*[a]

Compound[b]	Incubation[c]	Distribution of radioactivity[d]			
		cam	^{1}ac-cam	^{3}ac-cam	ac_2-cam
cam	Control	100	0	0	0
cam	FB_t	100	0	0	0
^{3}ac-cam	Control	0	2	97	1
^{3}ac-cam	FB_t	14	4	82	0
ac_2-cam	Control	0	0	0	100
ac_2-cam	FB_t	4	1	1	94

[a] A culture in 1% (wt/vol) Casitone (10^6 CFU/ml) was divided into three aliquots (10 ml each) and incubated with ^{14}C-labeled chloramphenicol (or derivative) and incubated for 15 h.

[b] Abbreviations as in Table 3.

[c] Controls were incubations in 10-ml portions of sterile Casitone medium.

[d] Radioactivities calculated as percentage of total counts per minute in cam, ^{1}ac-cam (1-acetyl chloramphenicol), ^{3}ac-cam, and ac_2-cam.

TABLE 6. *Effect of chloramphenicol on glycerol induction of myxospores in M. xanthus $FB_tCam_1^r$*[a]

Myxospore induction medium[b]	Myxospores plated on:[b]	Myxospores (CFU/ml) after sonication[c]
CT-1-glycerol	CTE agar	2.2×10^5
	CTE-cam agar	1.2×10^5
CT-1-glycerol-cam	CTE agar	8×10^3
	CTE-cam agar	6.4×10^3

[a] Bacteria grown in CT-1 medium containing chloramphenicol (25 μg/ml) were suspended in fresh medium at a density of 2×10^5 CFU/ml.

[b] Glycerol concentration was 0.5 M; chloramphenicol (cam), 25 μg/ml.

[c] Suspensions were sonicated (see text) and plated after 9 h. The values are counts of mature myxospores in the culture.

ponents of CTE agar); chloramphenicol inhibited fruiting. *M. xanthus* $FB_tCam_1^r$ formed fruiting bodies slowly on such agar containing chloramphenicol (25 μg/ml); these fruiting bodies resembled those of FB_t and contained myxospores.

DISCUSSION

This paper demonstrates the capacity of *M. xanthus* FB_t to adapt to growth in the presence of chloramphenicol. It seems most likely that the adaptation (Fig. 3) is similar to the induction of chloramphenicol acetyltransferase in R^+ strains of *S. aureus* (13). Similarly, the loss of chloramphenicol resistance after removal of chloramphenicol and concomitant loss of acetylase activity (Fig. 1 and Table 2) imply repression of the chloramphenicol acetylase.

On the other hand, the explanation may lie in the reorganization of genetic material. Recently, "cleared lysates" of strains FB_t and $FB_tCam_1^r$ have been examined with a view to identifying plasmids. The amount of extrachromosomal deoxyribonucleic acid in strain FB_t seems variable, but a plasmid characteristic of strain $FB_tCam_1^r$ has been identified (Brown and Parish, manuscript in preparation). This aspect of *M. xanthus* should be explored, since it is possible that the genetic instability of the organism is due to the ability of genes to exist in either a chromosomal or extrachromosomal form.

The growth rate of *M. xanthus* $FB_tCam_1^r$ is slowed significantly in the presence of chloramphenicol (25 μg/ml). We presume that the acetylase activity reduces the intracellular concentration of this drug to a level that is still partially inhibitory. This correlates with the low rate of conversion to myxospores in the presence of chloramphenicol and glycerol (Table 6), since optimal growth rate is required for glycerol induction of myxospore formation (5). The data of Table 6 also demonstrate that chloramphenicol resistance is retained in those myxospores that are formed and is also retained in myxospores of strain $FB_tCam_1^r$ formed (with high frequency) in the absence of chloramphenicol. This may imply that loss of resistance requires chromosome reinitiation, since chromosome reinitiation does not occur during myxospore formation (11). The alternative explanation is that myxospore formation "freezes" the status of the vegetative cells at the time of commitment.

The presence of inducible chloramphenicol resistance in *Myxococcus* may be of value to these soil organisms, which share an ecological niche with streptomycetes and fungi capable of producing antibiotics (7, 9). Inducible antibiotic resistance may not be unique to these bacteria. The work of Garrod suggests that some strains of *S. aureus* grown on low concentrations of erythromycin become resistant to high levels of this antibiotic; this resistance is also unstable (6). It would be of interest to screen myxobacteria and other soil bacteria for inducible, unstable resistance to a variety of antibiotics.

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