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Chloramphenicol Resistance Mutation in *Escherichia coli* Which Maps in the Major Ribosomal Protein Gene Cluster

GAIL A. BAUGHMAN† AND STEPHEN R. FAHNESTOCK*

Department of Biochemistry and Biophysics, Althouse Laboratory, The Pennsylvania State University, University Park, Pennsylvania 16802

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Localized mutagenesis and selection for streptomycin resistance were utilized to isolate a chloramphenicol resistance mutation in *Escherichia coli* K-12 linked to the *strA* (*rpsL*) locus. Bacteriophage P1 transduction verified the map position of the new resistance mutation at 72 min, placing it within a dense cluster of ribosomal protein genes. The map position differs from that of known *cmlA* and *cmlB* mutations, which map at 18 and 21 min, respectively. Ribosomes prepared from chloramphenicol-resistant and -sensitive isogenic transductants were analyzed in vitro for activity in formation of *N*-formylmethionyl-puromycin, polyphenylalanine, and polylysine in the presence of inhibitory concentrations of chloramphenicol. Comparisons were also made of ^{14}C-chloramphenicol binding to 70S ribosomes and of the two-dimensional polyacrylamide gel electrophoresis pattern of ribosomal proteins from each strain. There was no detectable difference between ribosomes from sensitive and resistant strains as measured by these assays. Enzymatic modification by chloramphenicol acetyltransferase is not responsible for the observed phenotype.

The study of the action of antibiotic inhibitors of protein synthesis has contributed greatly to the understanding of the mechanism of protein synthesis (for a review, see 19) and of the role of protein synthesis in various cellular processes. The isolation and characterization of antibiotic-resistant mutants can yield valuable information concerning the mechanism of action of the antibiotic and the nature of the components on which it acts.

Chloramphenicol is believed to inhibit protein synthesis in bacteria by binding to the 50S ribosomal subunit (11, 29). Evidence from in vitro studies locates a site of action of this antibiotic at the peptidyl transferase reaction center of the ribosome, where it is believed to act by inhibiting binding of the acceptor substrate, although question remains as to the exact mechanism of this inhibition (6, 11). Previously, the only chloramphenicol resistance mutations of *Escherichia coli* known were those mapping in the 18- to 21-min region of the chromosome (*cmlA*, *cmlB*), which are believed to cause reduced cell permeability to chloramphenicol (20, 21, 24). Resistance has also been described that results from the presence of the plasmid-encoded enzyme chloramphenicol acetyl transferase, which inactivates chloramphenicol by acetylation (26). Resistance to chloramphenicol due to alteration of its site of action on the ribosome has not previously been reported.

This paper describes a new mutation which confers resistance to chloramphenicol in *E. coli* and which maps in the ribosomal protein gene cluster (72-min region of the chromosome). The mutation was obtained by localized mutagenesis of the *strA* (*rpsL*) region of the chromosome.

MATERIALS AND METHODS

Bacterial and phage strains. Wild type *E. coli* K-12 (ATCC 25404) and bacteriophage P1 (ATCC 25404B) were obtained from the American Type Culture Collection. Strains AT2472 (*aroE24 thi-1 relA1* λ^-) (28), AT700 (*aroE24 strA104* [*rpsL104*] *hisG1 argG6 thi-1*? *lacY1* or *lacZ4 malA1 mtl-2 xyl-7 gal-6 tsx-1* λ^r *supE44*?), and 2K133 (*trkA133 kdpABC5 thi-1 rha-4 lacZ82 gal-33*) (4) were obtained from Barbara Bachmann, *E. coli* Genetic Stock Center, Yale University School of Medicine. Chloramphenicol-resistant strains RE103 (*proA23 trp-30 his-51 lac-28 strA101 cmlA1*) (23, 24) and RE107 (*proA23 trp-30 his-51 lac-28 strA101 cmlB5*) (21, 23) and their parent, RE1 (*proA23 trp-30 his-51 lac-28 strA101*) (22), were the generous gift of E. C. R. Reeve, Institute of Animal Genetics, Edinburgh, Scotland. Strain X1781 (λ^-, *his-53 arg-65 lys-32 tsx-63 supE42* T3^r *xyl-14*) containing the R100 plasmid (15) was obtained from Ester Lederberg, Plasmid Reference Center, Stanford University School of Medicine. Strain NO1350 (*aroE353 thyA str^r spc^r ery^r fus^r*) was obtained from M. Nomura, University of Wisconsin.

Chemicals. Chemicals were obtained from the fol-

† Present address: Institute for Enzyme Research, University of Wisconsin, Madison, WI 53706.

lowing sources: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and chloramphenicol from Sigma Chemical Co.; streptomycin sulfate from Schwartz/Mann; puromycin from Calbiochem; ^{14}C-chloramphenicol (specific activity, 7.94 mCi/mmol) from Amersham/Searle; ^{35}S-methionine (specific activity, 466 Ci/mmol) from New England Nuclear; and $tRNA_f^{Met}$ from Boehringer Mannheim.

Media. LB medium contained (per liter): tryptone (Difco), 10 g; yeast extract (Difco), 5 g; NaCl, 5 g; 1.0 M NaOH, 2 ml; and glucose, 5 g. Minimal medium (MIN) contained (per liter): K_2HPO_4, 10.5 g; KH_2PO_4, 4.5 g; $(NH_4)_2SO_4$, 1.0 g; sodium citrate·$2H_2O$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 2.0 g; thiamine HCl, 0.5 mg; and glucose, 2 g. Solid media contained 2% agar (Difco). The concentration of chloramphenicol in LBcml medium was 10 μg/ml. LBstr medium contained streptomycin at 100 μg/ml. When amino acid supplementation was required, the L-amino acids were added at a concentration of 20 μg/ml.

Test for the Str^r Cml^r phenotype. Those cells that appeared after mutagenesis to be streptomycin resistant were replica plated onto LBcml, LBstr, and LB plates. Those which grew on LBcml, LBstr, and LB media after 24 h at 37°C were denoted as Str^r Cml^r.

Transduction procedures. Lysate preparation and P1 transduction were performed as described by Miller (13).

Test for antibiotic resistance of transductants. Transductants were tested for antibiotic resistance by growth on LB plates containing single antibiotics at the following concentrations: chloramphenicol, 10 μg/ml; streptomycin, 100 μg/ml; spectinomycin, 100 μg/ml; fusidic acid, 650 μg/ml. In the case of fusidic acid, resistance denotes the ability to produce isolated colonies in the presence of antibiotic. When Str^r was the selected marker, 5 h of growth on LB plates was allowed before application of a 3-ml soft agar overlay containing 100 μg of streptomycin per ml. Antibiotic addition was delayed to allow for expression of resistance, which requires dilution of preexisting sensitive ribosomes.

Transduction of *trkA*. The *trkA133* mutation, in the presence of the *kdpABC5* deletion, confers a growth requirement for high concentrations of potassium (4). Transductants with the *trkA*$^+$ genotype can be selected by growth in medium containing 0.1 mM K^+ (K0.1 medium [4]). Among these transductants are some that have become *kdp*$^+$, which allows growth in 0.1 mM K^+ even in the presence of *trkA133*. The *kdp*$^+$ transductants can be distinguished from *trkA*$^+$ transductants by the fact that only the former can grow on medium containing very low concentrations of K^+ (K0 medium [4]).

Ribosomes. The preparation of ribosomes (sucrose-NH_4Cl-washed) and the assay of peptidyl transferase activity by the formation of *N*-formylmethionyl-puromycin were as described by Fahnestock et al. (5).

RESULTS

Isolation of mutants. Mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine induces multiple mutations at the DNA replicating fork (7, 16). It is possible, by proper selection procedures after nitrosoguanidine treatment, to obtain a population of mutagenized cells, the replicating forks of which are at a specific region at the time of mutagenesis. The population of cells selected for a particular mutational event after nitrosoguanidine mutagenesis should be enriched for second mutations linked to the originally selected marker. Selection for streptomycin resistance after mutagenesis, with subsequent scoring for chloramphenicol resistance, could preferentially yield chloramphenicol resistance mutations closely linked to the streptomycin resistance locus. High-level streptomycin resistance in *E. coli* is due exclusively to mutation at the *strA* (*rpsL*) locus, which is part of a cluster of ribosomal protein genes located at 72 min on the genetic map. At least 27 ribosomal protein structural genes, including 15 for 50S subunit proteins, are located in this cluster (12) (Fig. 1). Mutations in this region might be expected to confer chloramphenicol resistance if alteration of one of the proteins encoded in this cluster can alter or abolish the interaction of chloramphenicol with the ribosome.

We subjected wild-type *E. coli* K-12 cells to mutagenesis as described by Miller (13), using 65 μg of nitrosoguanidine per ml at 37°C for 30 min. The mutagenized cells were washed, suspended in LB broth (5×10^9 cells/ml), and separated into 1.0-ml portions in separate tubes (each tube is equivalent to an independent selection). Each portion was diluted 30-fold with LB broth and incubated at 37°C for 3.5 h to allow expression of antibiotic resistance mutations. The resultant cultures were then concentrated by centrifugation, and 10^{10} cells were plated on LBstr selective media containing 100

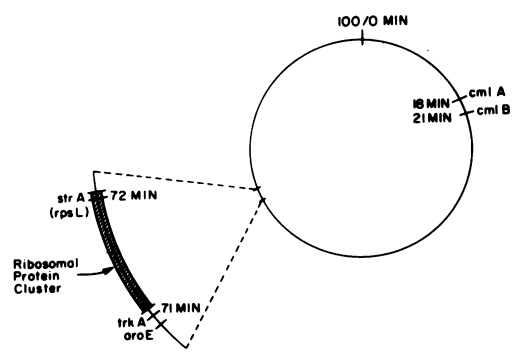

FIG. 1. *E. coli genetic map. The ribosomal protein gene cluster located at 72 min is shown as oriented to the strA and aroE genes. Known loci which determine chloramphenicol resistance (cmlA and cmlB) are physically distinct from this region (1).*

μg of streptomycin/ml. After incubation at 37°C for 36 h, the plates were replicated on LBcml (containing 10 μg of chloramphenicol/ml), LBstr, and LB plates to test for the Str^r Cml^r phenotype. The resulting Str^r Cml^r mutants were purified on LB plates for further study. Each plate yielded approximately 100 Str^r colonies, of which 5% were also Cml^r.

Mapping of the Str^r Cml^r mutations. P1 transduction was performed as described by Miller (13) to examine the linkage of the Str^r and Cml^r markers to the ribosomal cluster region of the chromosome at 72 min. P1 lysates were prepared on seven independently selected Str^r Cml^r mutants (which showed unambiguous chloramphenicol resistance) and used to infect strain AT2472, which carries the *aroE24* marker at 71 min, adjacent to the ribosomal protein cluster. Transductants were selected for ability to grow on MIN plates (Aro^+ phenotype) and subsequently scored on LBstr and LBcml media for the cotransduction of antibiotic resistance. The results of the transduction procedure for one of these mutants (K-12-N3) are shown in Table 1. The frequency of cotransduction of the Str^r (*strA*) marker with $aroE^+$ agrees with published figures (2, 24). As evidenced by the higher frequency of cotransduction of the Cml^r marker with $aroE^+$ and the rarity of $aroE^+$ Str^r Cml^s transductants, this mutation is located between the *aroE* and *strA* genes. The cotransduction frequency of Cml^r with $aroE^+$ of 81%, compared to 53% cotransduction of Str^r with $aroE^+$, allows the estimation that the Cml^r marker is 36% of the distance from *aroE* toward *strA*. This places the marker in the midst of a dense cluster of ribosomal protein genes, and suggests that the new mutation is ribosomal. Aside from *trkA* (see below), only ribosomal protein, protein synthesis elongation factor, and RNA polymerase α subunit genes have been identified in this region (12).

TABLE 1. *Cotransduction of the Str^r and Cml^r markers with aroE by bacteriophage P1*

A. Donor: K-12-N3 ($aroE^+$ Cml^r Str^r)	
Recipient: AT2472 (*aroE* Cml^s Str^s)	
Selected marker: $aroE^+$	
Segregation of nonselected markers:	
Str^r Cml^r	200 (50.6%)
Str^s Cml^r	119 (30.1%)
Str^s Cml^s	66 (16.7%)
Str^r Cml^s	10 (2.5%)
Cotransduction frequencies as calculated from above data:	
$aroE^+/Cml^r$	80.7%
$aroE^+/Str^r$	53.1%
B. Donor: AT700 (*aroE* Cml^s Str^r)	
Recipient: GB-6-R ($aroE^+$ Cml^r Str^s)	
Selected marker: Str^r	
Segregation of nonselected markers:	
aroE Cml^s	20 (28%)
$aroE^+$ Cml^s	21 (29%)
$aroE^+$ Cml^r	29 (40%)
aroE Cml^r	2 (3%)
Cotransduction frequencies as calculated from above data:	
Str^r/Cml^s	57%
Str^r/*aroE*	31%

For further studies the Cml^r marker was removed from the mutagenized background and placed into AT2472 by transduction. A chloramphenicol-resistant, streptomycin-sensitive (Str^s) transductant was chosen, along with a chloramphenicol-sensitive "isogenic" transductant, both selected for Aro^+ (GB-6-R, $aroE^+$ Str^s Cml^r; and GB-38-S, $aroE^+$ Str^s Cml^s).

To verify the gene order derived from the above results, a reciprocal transduction was performed in which the recipient was the chloramphenicol-resistant transductant GB-6-R and the donor was strain AT700 (*aroE* Str^r Cml^s). The distribution of Aro and Cml phenotypes among Str^r transductants is shown in Table 1 (experi-

(As discussed by Bachmann et al. [1] the formula of Wu [31] gives frequency of cotransduction = $[1 - d/L]^3$, where d is the distance between markers and L is the length of the transducing fragment, generally assumed to be 2 min = 84×10^3 base pairs [84 kb] [1]. For the *strA-aroE* pair, with a contransduction frequency of 0.53, the calculated d = 16 kb. The distance *strA-aroE* has been determined physically by Nomura and co-workers [12] to be approximately 39 kb. The reason for this discrepancy is unclear. The value d = 39 kb for *strA-aroE* implies that effectively L = 204 kb in this case. Assuming that the formula of Wu is valid and using L = 204 kb, the value of d for Cml^r-*aroE* is 14 kb.)

In addition to the mutant K-12-N3 characterized in Table 1, we have isolated several (approximately 20) other Cml^r mutants in this way, the chloramphenicol resistance of which also appears to map between *aroE* and *strA* and to be cotransducible with $aroE^+$ to approximately the same extent as that of K-12-N3. Transductants derived from these additional mutants, however, grow on LBcml plates only after 48 h, and grow rather weakly compared to those derived from K-12-N3. For this reason the Cml^r-$aroE^+$ cotransduction frequencies are difficult to estimate with confidence, though they are clearly between 60 and 90%. The mutant K-12-N3 was chosen for further study because its mutation yields strongly and unambiguously resistant transductants.

ment B). The gene order indicated is again *aroE-cml-strA*.

A transduction experiment involving additional antibiotic resistance markers known to map between *aroE* and *strA* is shown in Table 2. A P1 lysate was prepared on the Cml^r transductant GB-6-R, which is sensitive to spectinomycin, fusidic acid, and streptomycin, and was used to infect strain NO1350 (*aroE spcA* [*rpsE*] *fus, strA* [*rpsL*]), which is sensitive to chloramphenicol but resistant to spectinomycin, fusidic acid, and streptomycin. The distribution of antibiotic resistance markers among 100 independent, purified *aroE*$^+$ transductants is shown in Table 2. The frequencies indicate that the Cml^r marker is very closely linked to *spc* and to *fus*, too closely for the order of these three markers to be determined in this experiment. The frequencies are consistent with the following gene order: *aroE*-(*cml spcA fus*)-*strA*. The contransduction frequencies are also consistent with those in Table 1 (experiment A), though the numbers are statistically less significant because of the smaller number of transductants examined.

There is only one locus known to map between *aroE* and *strA* which is unrelated to protein (or RNA) synthesis, namely, *trkA*. Mutation in *trkA* affects transport of potassium (4). Because strain GB-6-R is *kdp*$^+$, its *trkA* genotype cannot be determined directly, since *trkA* mutations confer a requirement for elevated potassium concentrations only in the presence of a *kdp* mutation. Therefore, in order to examine the relationship between the Cml^r marker of strain GB-6-R and *trkA*, the Cml^r marker was inserted into strain

TABLE 2. *Cotransduction of antibiotic resistance markers with aroE*

Donor: GB-6-R (*aroE*$^+$ Cml^r Spc^s Fus^s Str^s)	
Recipient: NO1350 (*aroE* Cml^s Spc^r Fus^r Str^r)	
Selected marker: *aroE*$^+$	
Segregation of nonselected markers:	
Cml^s Spc^r Fus^r Str^r	27
Cml^r Spc^r Fus^r Str^r	3
Cml^r Spc^s Fus^r Str^r	2
Cml^r Spc^s Fus^s Str^r	14
Cml^r Spc^s Fus^s Str^s	47
Cml^s Spc^s Fus^s Str^s	2
Cml^s Spc^r Fus^s Str^s	2
Cml^s Spc^s Fus^r Str^r	1
Cml^s Spc^r Fus^s Str^r	1
Cml^r Spc^r Fus^s Str^r	1
Cotransduction frequencies as calculated from above data:	
aroE$^+$/Cml^r	67%
aroE$^+$/Spc^s	66%
aroE$^+$/Fus^s	67%
aroE$^+$/Str^s	51%

2K133 (*trkA133 kdpABC5*). The recipient strain 2K133 was infected with P1 phage grown on GB-6-R, and *trkA*$^+$ transductants were selected. Among 75 such *trkA*$^+$ transductants (which retained the *kdpABC5* deletion), 60 were found on subsequent scoring to be chloramphenicol resistant, and 15 were sensitive. Therefore, (i) the chloramphenicol resistance mutation of strain GB-6-R does not confer the potassium dependence of a *trkA* mutation, and (ii) because they are 20% separable in transduction the chloramphenicol resistance mutation and the *trkA133* mutation are not likely to be in the same gene. Since *trkA* is located immediately adjacent to *aroE*, on the *rpsL* side (12), the frequency of cotransduction of the Cml^r marker with *trkA* of 80% is, as expected, similar to the *aroE*/Cml^r cotransduction frequency (Table 1, experiment A).

Growth characteristics in the presence of chloramphenicol. Chloramphenicol resistance of strain GB-6-R was measured on LB-agar plates and compared to that of control strains. The resulting zones of inhibition on the confluent lawns of growth are shown in Fig. 2. Strains RE103 (*cmlA1*) and RE107 (*cmlB5*) are known chloramphenicol-resistant strains with mutations which have been mapped in the 18- to 21-min region of the *E. coli* chromosome. These results indicate that GB-6-R is more resistant to chloramphenicol on rich media plates than either of its parents (K-12, AT2472), the isogenic strain (GB-38-S), or the previously isolated chloramphenicol-resistant strains (RE103, RE107). Because this method failed to detect chloramphenicol resistance in the *cmlA* and *cmlB* strains, further tests were performed as described by Reeve (20) under conditions in which RE103 and RE107 have been shown to demonstrate resistance. The results of such tests are given in Table 3. Under these conditions, on supplemented minimal media, GB-6-R was shown to be as resistant as RE103.

GB-6-R and GB-38-S were also tested for chloramphenicol resistance in liquid media (Fig. 3). The growth rates of GB-38-S are severely inhibited at the tested concentrations of chloramphenicol as compared to GB-6-R. After 9 h, the GB-6-R cultures containing 0, 5, and 10 μg of chloramphenicol/ml showed no difference in cell density, whereas those of GB-38-S showed a definite reduction in cell density in the presence of chloramphenicol.

Ribosomal activity. From the mapping data, it appears that the chloramphenicol resistance of strain GB-6-R is likely due to a ribosomal mutation. Ribosomes prepared from the isogenic strains GB-6-R and GB-38-S were therefore tested in several in vitro assay systems for chlor-

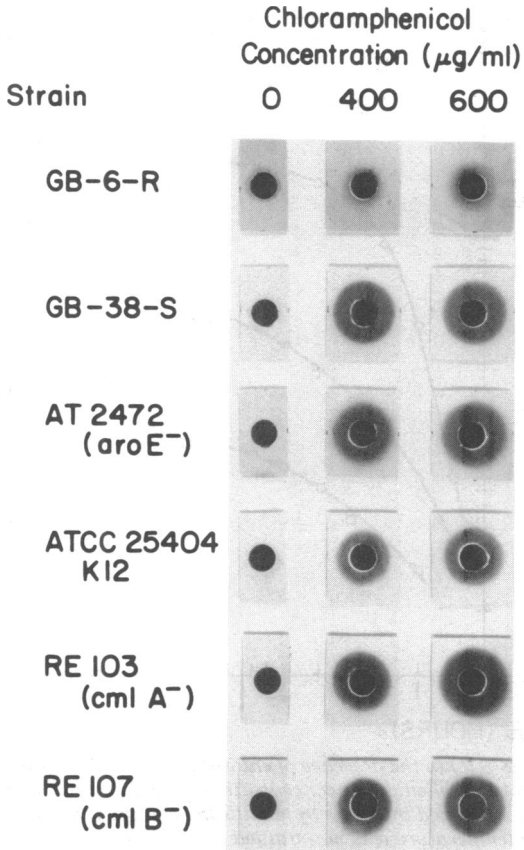

FIG. 2. *Resistance to chloramphenicol as measured by zones of growth inhibition. Soft agar overlays containing 10^7 cells were placed on LB plates. Prior to overnight incubation at 37°C, disks impregnated with chloramphenicol stock solutions containing 0, 400, and 600 μg/ml were placed on the surface of the plates.*

amphenicol inhibition or binding. The ribosome-catalyzed formation of *N*-formylmethionyl-puromycin (peptidyl transferase reaction) in the presence of inhibitory concentrations of chloramphenicol is shown in Fig. 4 for strains GB-6-R and GB-38-S. No difference in ribosomal sensitivity to chloramphenicol was observable under the conditions of this assay.

Double reciprocal plots of the rate of *N*-formylmethionyl-puromycin formation as a function of puromycin concentration were linear in the presence and absence of chloramphenicol. The apparent K_m for puromycin was 2×10^{-5} M in the absence of chloramphenicol. If chloramphenicol (at 10^{-5} to 10^{-6} M) and puromycin were added simultaneously to the assay, inhibition by chloramphenicol was competitive, with $K_I = 2 \times 10^{-6}$ to 3×10^{-6} M. Identical kinetics and inhibition were displayed by ribosomes from the sensitive (GB-38-S) and resistant (GB-6-R) strains. If chloramphenicol was added to the ribosomes before puromycin, the mode of inhibition was mixed (19; C. Coutsogeorgopoulos, Fed. Proc. **37**:1405, 1978), and again there was no apparent difference between ribosomes from GB-38-S and GB-6-R.

Studies of chloramphenicol inhibition of polyuridylic acid-directed polyphenylalanine synthesis and polyadenylic acid-directed polylysine synthesis, as well as ^{14}C-chloramphenicol binding to 50S subunits as measured by equilibrium dialysis, revealed no apparent differences between the sensitive and resistant strains. Thus, it would appear that, if a ribosomal alteration is responsible for the chloramphenicol-resistant phenotype, its effect is not being measured under these assay conditions. Furthermore, the pattern of all ribosomal proteins in two-dimensional gel electrophoresis by the method of Kaltschmidt and Wittmann (10) was identical for GB-6-R and GB-38-S.

Acetylation and permeability studies. Since the in vitro assays for ribosomal chloramphenicol sensitivity failed to detect any alteration in the mutant ribosomes, experiments were performed to compare the mechanism of resistance of GB-6-R to known nonribosomal chloramphenicol-resistant mutants of *E. coli*. Acetylation of chloramphenicol has been shown to be one mechanism responsible for chloramphenicol resistance. The enzyme responsible for this reaction, chloramphenicol acetyltransferase, is plasmid encoded (17, 27). Cell extracts of GB-6-R, GB-38-S, and X1781 (R-100) were tested for the ability to acetylate chloramphenicol as described by Shaw (26). The result is shown in Fig.

TABLE 3. *Chloramphenicol resistance as measured on supplemented MINcml plates*[a]

Strain	Minimal inhibitory concn (μg/ml)
RE1 (Cml^s)	5.0
RE103 (*cmlA*)	20.0
RE107 (*cmlB*)	7.1
GB-6-R	20.0
GB-38-S	10.0
AT2472 (*aroE*)	10.0
ATCC 25404 (K-12)	10.0

[a] Overnight cultures of cells in LB medium were diluted 100-fold (cell numbers equalized) and streaked on supplemented MINcml plates. Antibiotic concentrations were decreased in steps of a factor of $\sqrt{2}$ from 28.0 μg/ml to zero. After incubation for 48 h at 37°C, the resistance was scored as (+) or (−) growth. The lowest concentration of chloramphenicol that gave (−) growth was designated the minimal inhibitory concentration.

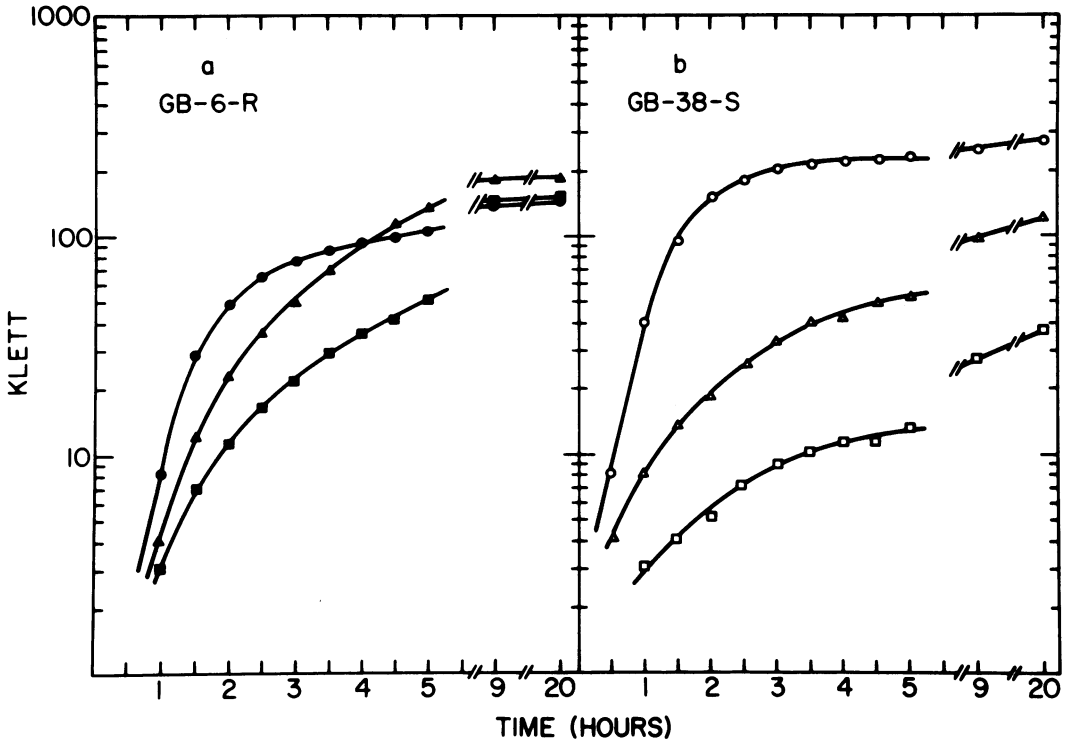

FIG. 3. *Growth curves of strains GB-6-R (a) and GB-38-S (b) in the presence of chloramphenicol. Overnight cultures were diluted 50-fold in LB media containing chloramphenicol at concentrations of 0 μg/ml* (○, ●), *5 μg/ml* (△, ▲), *and 10 μg/ml* (□, ■). *Growth was measured at timed intervals by monitoring the optical density of the culture with a Klett colorimeter (blue filter). The temperature was maintained at 37°C throughout the measurements.*

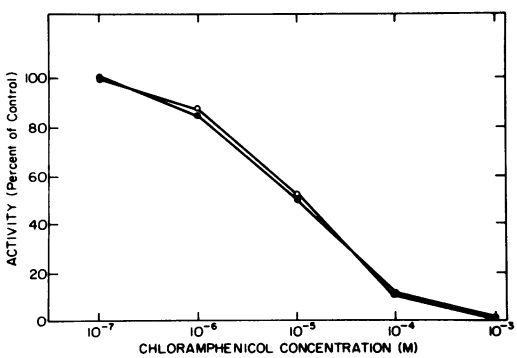

FIG. 4. *Peptidyl transferase assay in the presence of inhibitory concentrations of chloramphenicol. The ribosome-catalyzed formation of N-formylmethionyl-puromycin was measured as described by Fahnestock et al. (5) in the presence of various concentrations of chloramphenicol. The concentration of puromycin in the final assay mix was* 3.3×10^{-5} *M. Activity in the presence of chloramphenicol was represented as ethyl acetate extracted counts per minute per absorbancy unit at 260 nm of ribosomes as compared to a control (minus chloramphenicol) for each ribosome preparation.* (○) *GB-38-S;* (●) *GB-6-R.*

5. There was no increase in any detectable chloramphenicol modification in GB-6-R, compared to GB-38-S. It can be concluded from these data that strain GB-6-R does not contain the chloramphenicol acetyltransferase enzyme responsible for chloramphenicol inactivation in R-plasmid strains.

Alteration of cell membrane permeability to chloramphenicol is another mechanism proposed for chloramphenicol resistance in *E. coli* (20, 22, 30). The chloramphenicol resistance loci *cmlA* and *cmlB* which map at 18 to 21 min on the chromosome are believed to be of this type (Reeve, personal communication). Our attempts (using filtration and rapid centrifugation assays) to measure the uptake of radioactive chloramphenicol directly by strains containing these mutations have not detected differences in permeability as compared to the parental sensitive strain. Similar studies conducted to test GB-6-R cells for altered permeability have been complicated by the fact that the viable cell count of GB-6-R is consistently lower (by a factor of 2 to 10) than that of GB-38-S at equivalent optical densities (see below). However, there was no

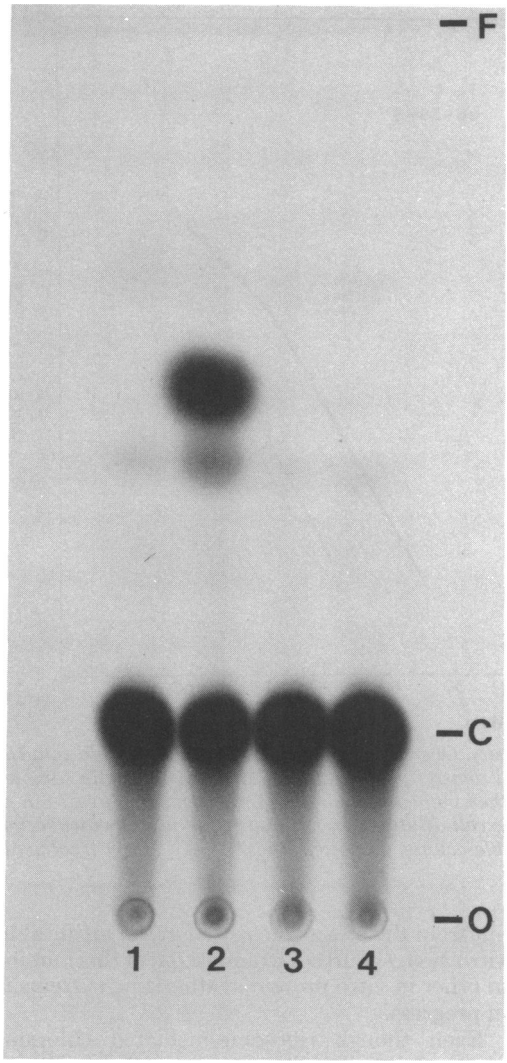

FIG. 5. *Assay for the enzyme chloramphenicol acetyl transferase in strains GB-6-R and GB-38-S. Cell extracts were prepared by ultrasonic treatment (26), and protein concentrations were measured spectrophotometrically (9). The reaction mixture consisted of 50 μmol of Tris-hydrochloride (pH 7.8), 0.1 μmol of acetyl coenzyme A, 0.05 μmol of ^{14}C-chloramphenicol (7.94 mCi/mmol), and 1.0 mg of protein from the appropriate cell extract, in a final volume of 0.5 ml. Cell extract was omitted from reaction 1. Reactions 2, 3, and 4 contained cell extracts from X1781 (R100 plasmid), GB-6-R, and GB-38-S, respectively. Incubations were conducted at 37°C for 10 min and terminated by cold ethyl acetate extraction. A sample equal to one-twentieth of the extracted material was applied to the origin of silica gel thin-layer chromatography plates, which were then developed in chloroform-methanol (95:5, vol/vol) (26). Radioactive spots were detected by autoradiography. O, origin; C, chloramphenicol; F, solvent front.*

significant difference in chloramphenicol uptake per viable cell, between GB-6-R and GB-38-S, from 30 s to 15 min after chloramphenicol addition (data not shown).

Cold lability. During the characterization of strain GB-6-R, it was observed that equivalent optical densities of GB-6-R and its isogenic partner GB-38-S contained unequal numbers of viable cells and that GB-6-R cells were substantially less motile than GB-38-S cells under microscopic examination after chilling. This relationship of cell density, viability, and temperature was investigated over a range of growth stages for each strain. Figure 6 shows that at equivalent optical densities during exponential-phase growth the viable cell counts of GB-6-R were markedly lower than those of GB-38-S, and that this difference was exaggerated upon cold treatment of the culture. The cold lability of GB-6-R was no longer observed when the culture was in stationary phase after overnight growth, but the difference in viable counts per Klett unit between GB-6-R and GB-38-S persisted. Whether this cold sensitivity is due to the same mutation responsible for chloramphenicol resistance remains to be elucidated. To our knowledge such growth phase-dependent irreversible cold lability has not been reported previously.

DISCUSSION

The nitrosoguanidine mutagenesis and streptomycin selection procedure used was designed to maximize the possibility of obtaining a chloramphenicol resistance mutation mapping in the 72-min region. Many genes located in this region are ribosomal, and therefore this method enhanced the chances of obtaining ribosome-mediated chloramphenicol resistance. The P1 transduction mapping of the Cml^r mutant obtained by this method places this new mutation within the ribosomal gene cluster.

The chloramphenicol-resistant mutant described here differs from known *cmlA* and *cmlB* mutants in two important respects. (i) It is resistant to higher concentrations of chloramphenicol in rich medium. There is an obvious difference between strain GB-6-R and the presumed permeability mutants RE103 (*cmlA*) and RE107 (*cmlB*) in the impregnated disk test in Fig. 2 and the minimal inhibitory concentration test in Table 3, taking account of differences in sensitivity of the respective "parent" strains (GB-38-S and RE1). (ii) The map positions are entirely different, Cml^r of GB-6-R mapping between 71 and 72 min, compared to 18 and 21 min for RE103 and RE107, respectively.

The cotransduction frequency of Cml^r with *aroE* of 81% is similar to reported cotransduc-

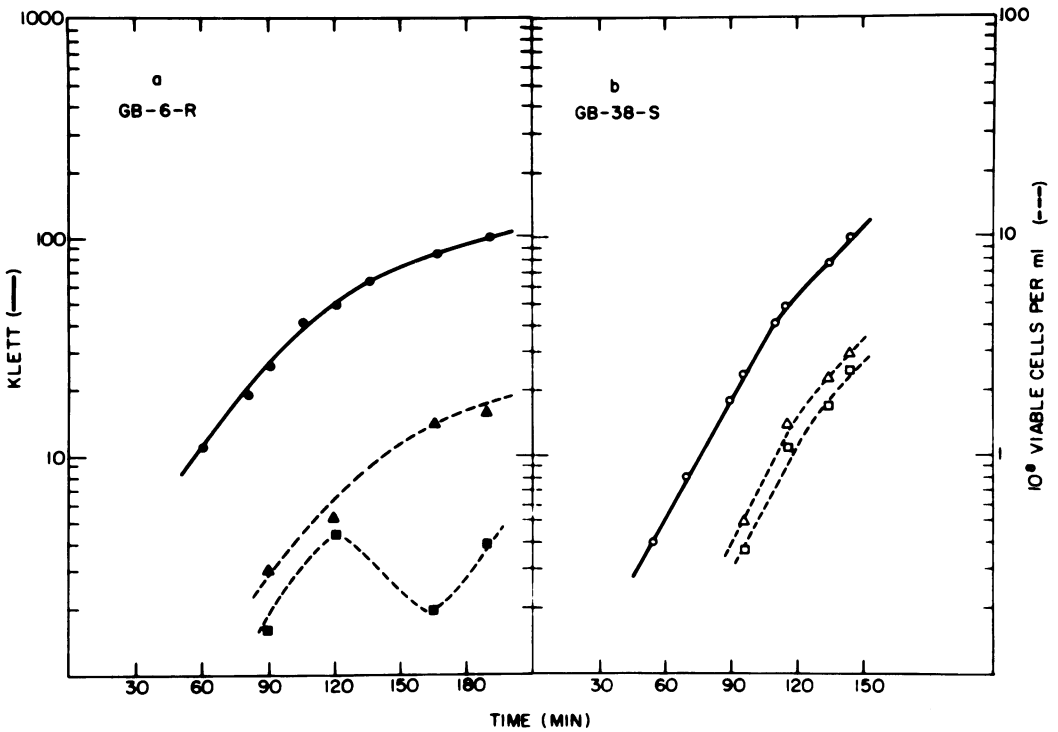

FIG. 6. *Viable-cell counts as a function of optical density. Overnight cultures were diluted 40-fold into LB media. Growth was measured by monitoring the optical density of the culture. At noted intervals samples were removed and viable-cell counts were measured either immediately (warm treatment) or after 1.0 h of incubation at 0°C (cold treatment) by plating appropriate cell dilutions on LB plates, followed by incubation at 37°C. Optical densities are plotted as solid lines; viable counts, as dotted lines.* (△, ▲) *Warm treatment;* (□, ■) *cold treatment.*

tion frequencies for the cold-sensitive Sad mutation and spectinomycin resistance (both of which are alterations in the structural gene of ribosomal protein S5) with *aroE* (frequencies are 80% [8] and 86% [25], respectively). The S5 gene (*rpsE*), located 11 kb from *aroE* (12), is very close to the proposed position of the Cml^r mutation, verifying the proposed position assignment. (Even though our Cml^r mutant is cold sensitive, we do not wish to suggest that the S5 gene is responsible.)

The unequivocal assignment of this new Cml^r mutation to a ribosomal alteration is not possible at this time. The mapping data presented suggest this assignment, as does the argument against the other known mechanisms, acetylation and impermeability, as possibilities responsible for this phenotype. The failure to demonstrate in vitro ribosomal resistance to chloramphenicol does not exclude the possibility that ribosomal alteration is responsible for resistance in vivo. It is reasonable to suppose that the mechanism by which chloramphenicol acts to inhibit protein synthesis in vivo could be different from the effects measured in the artificial in vitro assays. Further examination of this mutant in other in vitro protein-synthesizing systems is in progress.

Even though ribosome-mediated chloramphenicol resistance has not been observed previously in *E. coli*, there is no reason to suppose that such a mechanism is impossible. Osawa et al. (18) demonstrated that ribosomes from certain chloramphenicol-resistant mutants of *Bacillus subtilis* have lowered affinity for the antibiotic in vitro, and they identified altered ribosomal proteins. Indeed, one explanation for the failure to observe ribosomal chloramphenicol resistance previously in *E. coli* might be that the commonly used in vitro assays do not reflect the in vivo action of the antibiotic in *E. coli*. In vitro characterization may generally have preceded genetic mapping in previous work.

A mutation affecting the ribosome might be expected to have pleiotropic effects. For example, certain spectinomycin-resistant mutants of *E. coli*, altered in ribosomal proteins S3 and S5, display sucrose-dependent growth (3) and cold

sensitivity (14), respectively. It remains to be determined whether the cold lability of the mutant described here is due to the same mutation responsible for chloramphenicol resistance.

It is therefore concluded that, although the chloramphenicol-resistant mutant isolated in this work represents a new mutation mapping in the ribosomal gene cluster, the mechanism responsible for resistance remains to be elucidated. We propose that the locus of this mutation be assigned the symbol *cmlC* until it can be determined whether it is in fact a ribosomal protein structural gene, and that the mutation in GB-6-R be designed *cmlC1*.

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