JOURNAL OF BACTERIOLOGY, October 1973, p. 74-83

Vol. 116, No. 1
Printed in U.S.A.

Mutation to Erythromycin Dependence in *Escherichia coli* K-12

P. FREDERICK SPARLING AND ELEANOR BLACKMAN

Departments of Medicine and Bacteriology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514

Received for publication 22 June 1973

A nitrosoguanidine-induced mutant of *Escherichia coli* K-12 strain JC12 was absolutely dependent on erythromycin or related macrolide antibiotics for growth. The only other drugs which permitted growth (lincomycin and chloramphenicol) are, like the macrolides, inhibitors of the 50*S* ribosome. The order of relative effectiveness of these drugs was macrolides > lincomycin > chloramphenicol. Rates of growth with all drugs were concentration dependent. Erythromycin starvation was followed by normal rates of increase in cell mass and macromolecular synthesis for approximately one mass-doubling time, after which macromolecular synthesis abruptly ceased and cell lysis and death occurred. The dependent mutant gave rise spontaneously to revertants to independence with very high frequency (10^{-4}). The gene (*mac*) for macrolide dependence is located near minute 25 on the *E. coli* chromosome; it does not result in increased resistance to these drugs. A separate gene for erythromycin resistance (*eryA*) is located in the cluster of ribosomal structural genes near *spc*, close to minute 63. Dependence on macrolides was most clearly evident in strains carrying mutations at both *eryA* and *mac*.

There are relatively few described mutations which specifically alter 50*S* ribosomal subunit structure and function. The best characterized of these are mutants to increased resistance to the macrolide antibiotic erythromycin (Ery). Erythromycin is known to bind to the 50*S* ribosome (8, 16, 30), although its binding site is apparently shared with the chemically unrelated drugs chloramphenicol (Cml) and lincomycin (Lin) (8). Mutation at the *eryA* (Ery resistance) locus maps near *spc* at minute 63, in the cluster of ribosomal protein genes on the *Escherichia coli* chromosome (6, 24). Osawa's group has shown that the *eryA* gene product is ribosomal protein 50-8 (7, 19, 20, 24), and that alteration of this protein is associated with decreased binding of Ery and Cml (20, 25), increased resistance to several macrolides (26), and other changes in 50*S* ribosome function (20, 26). Other Ery-resistant (Ery^R) mutants map near minute 11 on the *E. coli* chromosome (1) but are less well characterized biochemically.

We report here a novel mutant of *E. coli* which is absolutely dependent on the macrolide antibiotics or other antibiotics which act on the 50*S* ribosome (Cml, Lin). Mutations of the 30*S* ribosome to drug dependence have been extensively studied (4, 9, 10), but similar mutants dependent on drugs acting on the 50*S* ribosome have not been reported. The erythromycin-dependent mutant is shown to be a double mutant, with alteration of a gene close to or identical with *eryA* as well as alteration of a second gene, *mac*. The latter determines the drug-dependent phenotype.

MATERIALS AND METHODS

Strains used (all *E. coli* K-12) are listed in Table 1. A schematic map of the *E. coli* chromosome with position of relevant markers and points of origin and direction of transfer of Hfr strains is shown in Fig. 1.

Media and growth. L broth was as described by Lennox (15). All broth cultures contained 0.2% glucose. Enriched agar medium was identical to L broth excepting addition of 1.5% agar (wt/vol) (L plates). Minimal medium A was described by Davis and Mingioli (5). Amino acids were added to 50 μg/ml, purines to 2×10^{-4} M, carbohydrates to 0.2% (wt/vol), thiamine to 1 μg/ml, and antibiotics as stated. All incubations were at 37 C.

Kinetics of growth of the Ery-dependent mutant were measured in L broth as follows. An overnight culture containing 100 to 200 μg of Ery per ml was washed by centrifugation before being suspended in fresh L broth without added drug; this was used to inoculate identical 10-ml L-broth cultures containing various concentrations of drug in 125-ml baffled side-arm flasks. Growth was measured as change in

TABLE 1. *Strains used*

Strain	Source	Description
JC12	G. Jacoby	Hfr; *met, purC, gal, lac,* (λ^-)
χ408	G. Jacoby	Hfr; *thi, proA*
HfrC	G. Jacoby	Hfr; *thi, met*
KL19	CGSC[a]	Hfr; *thi-1*, λ^-
KL25	CGSC	Hfr; *sup*
KL208	CGSC	Hfr; *rel-1*, λ^-
KL96	CGSC	Hfr; *thi-1, rel-1*, λ^-
MX129	F. Bastarrachea	F^-; *argG, aroE, metB, thr, lac, gal, tsx, his*
χ478	CGSC	F^-; *leu, proC, purE, trp, metE, lacZ, lysA, str*
FS141	NGN[b] from JC12	As JC12 but *eryA7* (Ery resistant) and *mac-1* (macrolide dependent)
FS179	Spontaneous Ery-independent revertant from FS141	As FS141 but *rev-1*
FS186	Spontaneous Ery-independent revertant from FS141	As FS141 but *rev-2*
FS187	Spontaneous Ery-independent revertant from FS141	As FS141 but *rev-3*
FS191	Spontaneous Ery-independent revertant from FS141	As FS141 but *rev-4*
FS224	NGN from χ478	As χ478 but *his-2*
FS246	Spontaneous from FS179	As FS179 but *spc15*
FS285	Spontaneous from FS141	As FS141 but *spc18*
FS287	$AroE^+$ $PurC^+$ recombinant from FS141 × MX129	As MX129, but *eryA7, mac-1*, $aroE^+$
FS289	$AroE^+$ $PurC^+$ recombinant from FS141 × MX129	As MX129, but *eryA7*, $aroE^+$
FS296	Spontaneous from FS224	As FS224 but *nal-2*
FS298	Thr^+ $PurC^+$ recombinant from FS141 × MX129	As MX129, but *mac-1*, thr^+, $argG^+$
FS299	Trp^+ Nal^R recombinant from FS141 × FS296	As FS296 but trp^+, *mac-1*

[a] CGSC, Coli Genetic Stock Center, Yale.
[b] NGN, nitrosoguanidine mutagenesis.

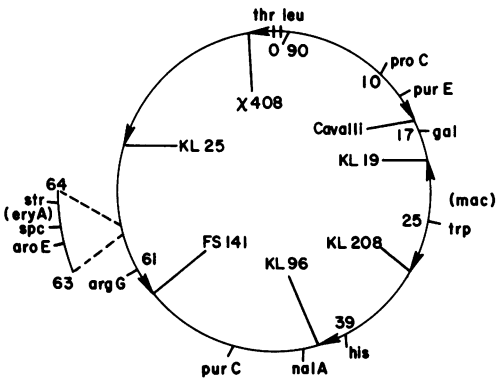


FIG. 1. *Location of eryA and approximate location of mac on the E. coli map. Origins and orientation of transfer of relevant Hfr strains are indicated by arrow heads. Genetic map and symbols from Taylor (27).*

optical density at 540 nm during incubation in a gyratory bath.

Macromolecular synthesis following Ery starvation was measured in A medium supplemented with 0.1% (wt/vol) Casamino Acids. ^{3}H-leucine was added to a final concentration of 0.20 μCi per 10 μg per ml, and ^{14}C-uracil was added to a final concentration of 0.02 μCi per 10 μg per ml at least two mass doublings before transfer of washed cells to the same medium with or without added Ery (100 μg/ml). Samples (1.0 ml) were removed at intervals to 1.0 ml of ice-cold 10% trichloracetic acid, and the precipitate collected by filtration onto membrane filters (HA; Millipore Corp.) was counted in a liquid scintillation counter. Correction was made for 20% spillover of ^{14}C counts into the ^{3}H window.

Conjugation and transduction were generally as previously described (23). Phage $P1_{kc}$ was used for all transductions. Matings between Ery-sensitive and Ery-dependent strains were in L broth containing 50 μg of Ery per ml. At the conclusion of most matings, the frequency of Ery-independent revertants from the Ery-dependent parent was checked as follows. Suitable dilutions from the mating mixture were plated onto minimal medium selective for the dependent strain, containing either no Ery (only independent revertants grow) or 100 μg of Ery per ml (dependent and independent cells grown). Most matings were for 60 to 120 min, after which they were interrupted by vigorous agitation before plating. Recombinants were selected, purified, and scored on minimal medium containing just enough Ery (25 to 50 μg/ml) to allow full growth of both Ery-sensitive (Ery^s) and Ery-dependent recombinants. Dependence was scored as

inability to grow on L plates without added drug. Ery resistance was scored on L agar containing doubling dilutions of Ery. The MIC (minimum-inhibiting concentration) of Ery for Ery^S strains was 100 to 200 μg/ml and was 400 to 800 μg/ml for Ery^R strains.

Mutagenesis. Nitrosoguanidine (NGN) mutagenesis was as previously described (23). Spontaneous nalidixic acid-resistant (*nal*) mutants were selected on L plates containing 50 μg of Nal per ml, and spectinomycin-resistant (*spc*) mutants were selected on plates containing 500 μg of Spc per ml.

Chemicals. Erythromycin, lincomycin, and spectinomycin were gifts from The Upjohn Co. Oleandomycin (Ole) was a gift from Pfizer Co., Inc., and kasugamycin (Ksg) was a gift from Bristol Laboratories, Inc. Chloramphenicol was from Parke, Davis, and Co. Streptomycin was from Eli Lilly and Co. ^{3}H-leucine and ^{14}C-uracil were from New England Nuclear Corp. N-methyl-N′-nitrosoguanidine was from Aldrich Chemical Co., Inc. Other chemicals were of the highest grade commercially available.

RESULTS

Isolation of Ery-dependent mutant. One of 131 nitrosoguanidine-induced mutants of *E. coli* K-12 strain JC12 selected for resistance to 800 μg of Ery per ml grew only on medium containing at least 50 μg of Ery per ml; it was not inhibited by 800 μg of Ery per ml. It thus was phenotypically both Ery resistant and Ery dependent. Since the gene for dependence was subsequently found to be nonallelic with the *eryA* locus for Ery^R, the dependent phenotype and genotype were given the symbols Mac^D and *mac*, respectively.

Effect of various drugs. The activity of Ery and other compounds as stimulators of growth of the dependent mutant (FS141) was tested by an agar diffusion method. An overnight culture of FS141 in L broth plus 200 μg of Ery per ml was washed by centrifugation before it was spread onto L agar plates without added drug. A center well was cut in the agar and 0.1 ml of one of several drugs in concentrations of 0.1 to 25.0 mg/ml was placed in the center well. Plates were examined for a concentric ring of growth occurring at a variable distance from the central well after 20 to 44 h of incubation at 37 C. Stimulation was observed by erythromycin and oleandomycin and, to a lesser degree, by lincomycin and chloramphenicol. Kasugamycin had a weak but definite growth-stimulatory effect. No other drugs were effective, including streptomycin, spectinomycin, paromomycin, neomycin, and 5 to 50% ethyl alcohol. The distance from the center well at which growth occurred varied inversely with the concentration of drug in the well. When over 10^7 colony-forming units of FS141 were spread onto the plates, sufficient revertants to erythromycin independence grew to obscure the results.

Kinetics of growth. The quantitative effects of various drugs on growth of FS141 were examined by suspending washed cells in L broth containing various concentrations of drug and following optical density with incubation at 37 C in a gyratory shaker bath. The effects of starvation for Ery were not evident until starved cells had grown for 90 to 150 min (1.2 to 2.2 mass doublings), at which time cell lysis became evident (Fig. 2). Concentrations of 25 μg/ml or less of Ery had almost no growth-stimulating effect, but dose-related increases in rate of growth were observed between 50 and 100 μg/ml. No further stimulation was achieved by concentrations up to 400 μg of Ery per ml, and concentrations greater than 400 μg/ml slowed or totally inhibited growth. Similar dose-related increases in rate of growth were observed with lincomycin and chloramphenicol (Fig. 3), although maximal rates of growth were slower than with Ery. Addition of any of these drugs to a culture 90 or more after Ery starvation failed to restore growth, suggesting that an irreversible process was triggered by drug deprivation. The doubling time of FS141 under optimal conditions was 70 to 80 min, as compared to 25 to 30 min for the *ery*$^+$ parent strain JC12 grown under similar conditions except omission of Ery.

Inhibition of rate of growth was generally observed if Cml or Lin was added to cultures growing optimally in 100 μg of Ery per ml (Fig. 3). Contrariwise, addition of Cml or Lin to cultures growing at less than peak rates in the presence of Ery regularly resulted in faster growth (not shown).

The effects of these and other drugs are summarized in Table 2. The only other drug tested which stimulated growth as much as erythromycin was oleandomycin, which is a closely related member of the macrolide group of antibiotics (17). Maximal stimulation by oleandomycin required molar concentrations almost fivefold higher than needed with erythromycin, however. Rates of growth with lincomycin and chloramphenicol were on the average only 70 and 38% of those observed with Ery, and maximal stimulation by these drugs required concentrations almost 10-fold greater than Ery. The only stimulation of growth by a drug which is not known to act on the 50*S* subunit was by Ksg, which is well documented to exert its primary effect on the 30*S* ribosomal subunit (11, 22). Stimulation by Ksg was variable but always minimal. Addition of other antibiotics which act on the 30*S* subunit (strep-

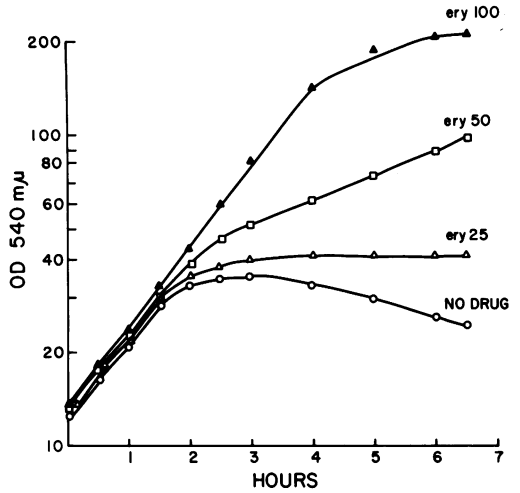


FIG. 2. *Growth in L broth of eryA7 mac-1 strain FS141 related to concentration of erythromycin (μg/ml). Ery, erythromycin. Cells grown overnight in L broth plus 100 μg of Ery per ml were washed and suspended in the same medium containing various concentrations of Ery at time zero.*

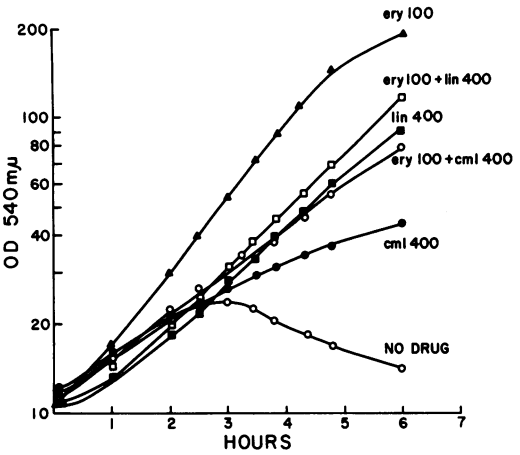


FIG. 3. *Inhibition of growth of eryA7 mac-1 strain FS141 growing at maximal rate in L broth containing 100 μg of erythromycin per ml by addition of lincomycin (400 μg/ml) or chloramphenicol (400 μg/ml). Comparison is made with growth in presence of lincomycin (400 μg/ml) or chloramphenicol (400 μg/ml) alone. Conditions as in Fig. 2.*

tomycin, spectinomycin) to an Ery-starved culture resulted in accelerated rates of cell lysis.

Macromolecular synthesis. The effects of Ery starvation on protein and RNA systhesis are shown in Fig. 4. The most dramatic effect was abrupt cessation of ribonucleic acid (RNA) synthesis approximately 90 min after cells of an Ery-stimulated culture were suspended in medium without Ery. Protein synthesis slowed at the same time, but there was continued slow net incorporation of leucine for at least 4 h after Ery starvation. At no time was any stimulation of RNA synthesis observed.

Viability after Ery starvation. Samples of an Ery-starved culture were taken at intervals for determination of viability (Fig. 5). Normal growth was observed for 60 to 90 min after Ery starvation, but after 4 h of starvation 90 to 99% of the dependent cells were nonviable. Onset of cell death coincided closely with cell lysis (decreased optical density, Fig. 2) and termination of macromolecular synthesis (Fig. 4). Killing as a result of Ery starvation appeared to plateau after 5 to 6 h, but this was due to selection of frequent revertants to erythromycin independence (Mac^{IND}), which grew equally well with or without Ery, and became dominant in the Ery-starved culture after 6 to 12 h (Fig. 4).

Samples of supernatant fluid from cultures of the Ery-starved strain were taken after cell lysis and death had occurred, for purposes of testing for presence of lytic phage which might have been induced by Ery starvation. No evidence of phage active in spot tests was found by using FS141, JC12, and other strands of *E. coli* K-12 and *Shigella* as indicators.

Variable phenotype of FS141. Growth of FS141 was influenced markedly by which drug

TABLE 2. *Relative effect of various drugs on stimulation of growth of FS141*

Drug	Generation time (Ery)/generation time (other drug)[a]
Erythromycin (1.3×10^{-4} M; 100 μg/ml)	1.0
Oleandomycin (5.8×10^{-4} M; 400 μg/ml)	1.0
Lincomycin (10^{-3} M; 400 μg/ml)	0.7
Chloramphenicol (1.2×10^{-3} M; 400 μg/ml)	0.38
Kasugamycin (1.1×10^{-3} M; 400 g/ml)	<0.2
Tetracycline	0
Streptomycin	0
Spectinomycin	0
Paromomycin	0
(Ethyl alcohol)	0

[a] Maximal rates of growth of FS141 in 10 ml of L broth in 125-ml flasks incubated at 37 C in a gyratory shaker. Relative activity of each drug at its most effective concentration compared to erythromycin. 0 indicates no growth (tetracycline) or lysis (streptomycin, spectinomycin, paromomycin, ethanol). Means of 3 to 10 experiments.

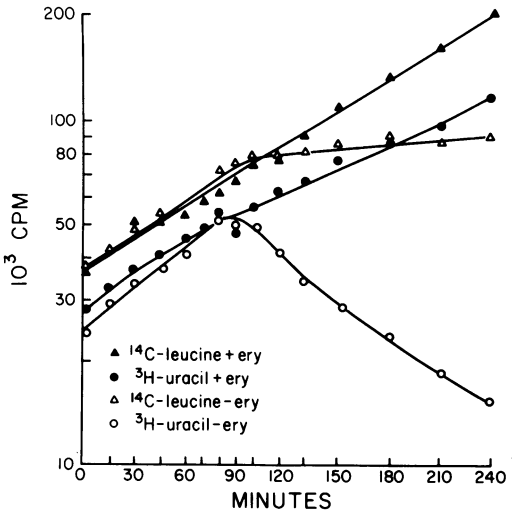


FIG. 4. *Effect of starvation for erythromycin on macromolecular synthesis of eryA7 mac-1 strain FS141. Cells grown in 100 μg of erythromycin per ml for three mass doublings in labeling medium (Materials and Methods) were washed and resuspended at time zero in the same medium with or without erythromycin (100 μg/ml).*

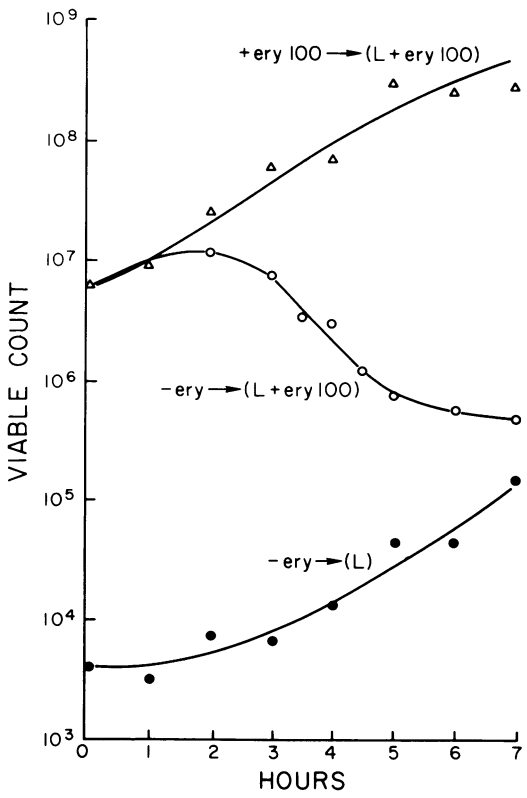


FIG. 5. *Effect of starvation for erythromycin on viability of eryA7 mac-1 strain FS141. Conditions as in Fig. 2. Symbols: Δ, viable counts from control flask containing 100 μg of Ery per ml plated onto L agar with 100 μg of Ery per ml; O, viable counts from flask without Ery plated onto L agar with 100 μg of Ery per ml; ●, viable counts from flask without Ery plated onto L agar without Ery.*

was used to stimulate growth before transfer to broth containing either lincomycin or erythromycin (Table 3). Mass-doubling time was 70 min in broth containing 100 μg of Ery per ml when prior exposure was to Ery, but was 131 min when prior exposure was to Lin. Prior exposure to oleandomycin (Ole) resulted in normal rates of growth when transferred to Ery but no growth (lysis) when transferred to Lin. The slow growth of FS141 when transferred from Lin to Ery was maintained without change for at least three mass doublings (not shown).

Erythromycin-independent mutants. Strain FS141 gave rise with high frequency (10^{-3} to 2×10^{-5}) to revertants to erythromycin independence. These remained resistant to 400 to 800 μg of Ery per ml but were no longer dependent on Ery for growth (Table 4). Some were still stimulated somewhat by added Ery (FS179, FS191), whereas others were nearly totally indifferent to Ery (FS187) or were inhibited by Ery (FS186). The phenotypic response to Ery of several Mac^{IND} strains dependent on whether they had been previously exposed to Ery; thus, although FS191 was able to grow slowly without drug, addition of Ery for several mass doublings not only increased the rate of growth but also rendered the cells phenotypically Mac^{D} upon transfer to medium without drug. Another revertant to Mac^{IND} (FS186) was

TABLE 3. *Effect of prior growth in various drugs on phenotype FS141*

Prior growth[a] (μg/ml)	Generation time (min) when transferred to L broth containing (in μg/ml)	
	Ery (100)	Lin (400)
Erythromycin (100)	70	100
Lincomycin (400)	131	184
Oleandomycin (400)	72	Lysis

[a] Cells were grown overnight in L broth containing either erythromycin, lincomycin, or oleandomycin and were washed before resuspension in L broth with erythromycin, lincomycin, or no drug. Generation time is time for one mass doubling (determined by optical density at 540 nm) after at least two mass doublings in the fresh medium.

partially inhibited by added Ery unless exposed to Ery for several mass doublings, in which case it was indifferent to the presence of Ery.

Mapping of mac. Considerable difficulty was encountered in attempts to map the locus (*mac*) for erythromycin dependence. Matings of the erythromycin-dependent donor FS141 with erythromycin-sensitive strain JC411, with selection for ArgG$^+$ PurC$^+$, Leu$^+$ PurC$^+$ recombinants on plates containing variable concentrations of Ery did not yield more than 2% which were Ery dependent, regardless of the markers selected. These difficulties are unexplained but were overcome by using other recipient strains. Conjugation was made between strain FS141 and recipient MX129, with selection on plates containing just enough Ery for all classes of recombinant to grow; when selection was for the proximal donor marker *aroE*$^+$, over 80% of recombinants also acquired the donor erythromycin-resistant phenotype, but none was dependent on erythromycin (Table 5). However, when selection was made for the more distal donor marker *thr*$^+$, 5.4 to 13.6% of recombinants were Mac^D, and many of these were not Ery^R. This suggested that dependence and resistance to erythromycin were not allelic forms of the same gene, but rather that dependence was determined by a separate locus (*mac*).

The location of *mac* was more closely approximated by a second mating by using recipient strain FS296 (Table 6). The results clearly indicated proximity of *mac* to *trp*, since over 50% of Trp$^+$ Nal^R recombinants were Mac^D, whereas all other classes of recombinants had much lower frequencies of acquisition of the unselected donor marker *mac*.

The low frequency of Mac^D (18%) and Trp$^+$ (29%) among His$^+$ Nal^R recombinants suggested genetic interference. Because of possible bias against expression of *mac* under the conditions used, caution is indicated in strict interpretation of the data in Table 6. With this in mind, the relative frequency of the crossover class Trp$^+$ Mac$^+$ (13) as opposed to Trp$^-$ Mac^D (5) among His$^+$ Nal^R recombinants suggested a gene order *purE...gal...mac...trp*.

Confirmation of the approximate chromosomal position of *mac* was obtained by mating several *leu*$^+$ *purE*$^+$ *his*$^+$ *mac*$^+$ *nal*^{s} Hfr strains with different points of origin and directions of transfer (Fig. 1) with *leu*$^-$ *purE*$^-$ *his*$^-$ *mac-1* *nal*^{r} recipient strain FS299. Selection was for Leu$^+$ Nal^R, PurE$^+$ Nal^R, or His$^+$ Nal^R recombinants on plates containing enough Ery (25 μg/ml) to allow growth of both Mac^D and Mac$^+$ organisms; these were scored for acquisition of the donor *mac*$^+$ allele. When Hfr C was the donor strain, none of 160 Leu$^+$ Nal^R recombinants was Mac$^+$, but when the donor was either KL25 or χ408, approximately 5 to 10% of Leu$^+$ Nal^R recombinants were Mac$^+$. When the donor strain was either KL208 or KL96, close to 50% of Leu$^+$ Nal^R or PurE$^+$ Nal^R recombinants acquired the donor Mac$^+$ character, and approximately 50% of His$^+$ Nal^R recombinants from KL19 × FS299 acquired the donor Mac$^+$ phenotype. These results placed *mac* between the points of origin of KL208 and KL19, or between minutes 29 and 21.

TABLE 4. *Erythromycin-independent revertants from FS141: effect of prior growth in Ery on phenotype*

Strain[a]	Previous drug exposure[b] (μg/ml)	Generation time (min) when transferred to L broth containing	
		No drug	Ery (200 μg/ml)
JC12	None	30	345
FS141	Ery (100)	Lysis	70
FS179	None	84	68
	Ery (100)	114	80
FS186	None	86	140
	Ery (100)	86	79
FS187	None	92	102
	Ery (100)	92	90
FS191	None	195	110
	Ery (100)	>400	72

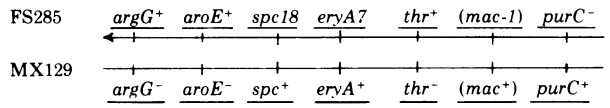
[a] FS179, FS186, FS187, and FS191 are spontaneous revertants to drug independence selected on drug-free plates from an Ery-dependent strain FS141.

[b] Grown overnight in L broth containing either no drug or Ery (100 μg/ml) before being washed and resuspended in similar medium with or without added Ery. Generation times were measured after at least two mass doublings in fresh medium.

Preliminary experiments were undertaken to determine whether reversion from Mac^D to Mac^{IND} was due to an unlinked suppressor mutation. Conjugation between FS246 (a spontaneous revertant from Mac^D to Mac^{IND} which also is Spc^R) and MX129 failed to produce any Mac^D recombinants, even though all recombinants were selected on plates containing sufficient Ery to allow growth of Mac^D strains (Table 7). The data are insufficient to detect a suppressor mutation close to *mac* but do reasonably exclude existence of such a suppressor in the cluster of ribosomal structural genes near *spc*.

Mapping eryA7. The chromosomal location of *eryA7* can be determined from the data in Tables 5 and 7. When selection was made for the distal donor marker Thr$^+$ after conjugation of an AroE$^+$ Spc^R Ery^R Thr$^+$ donor and an Aroe$^-$ Spc^S Ery^S Thr$^-$ recipient, the crossover

TABLE 5. *Demonstration of separate loci for erythromycin resistance and erythromycin (macrolide) dependence*

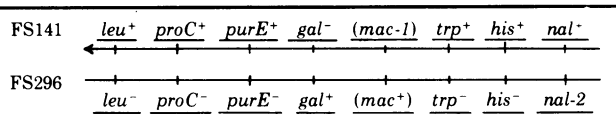


Selected[a] markers	No.	Unselected markers (%)					
		Arg+	Aro+	Spc^R	Ery^R	Thr+	Mac^D
AroE+ PurC+	172	69.8	100	91.3	89.0	6.4	0
Thr+ PurC+[b]	167	57.5	58.7	59.3	61.7	100	5.4

[a] Selected on minimal plates containing 50 μg of Ery per ml, which concentration allows growth of Ery^S as well as Mac^D recombinants.

[b] Among Thr+ PurC+ recombinants, there were five Ery^R Spc^S but no Ery^S Spc^R.

TABLE 6. *Locus for erythromycin dependence linked to trp*

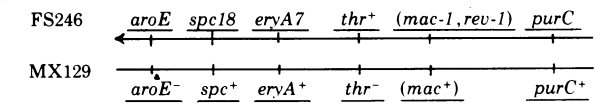


Selected recombinants[a]	No.	Percent receiving unselected donor						
		Leu	Pro	Pur	Gal	Mac^D	Trp	His
Leu+ Nal^R	75	100	37.4	22.6	8	0	0	0
ProC+ Nal^R	74	41.9	100	62.1	18.9	1.4	2.7	0
PurE+ Nal^R	73	38.4	57.5	100	42.4	2.7	0	0
Trp+ Nal^R	83	25.3	24.1	45.8	36.2	53.1	100	6.1
His+ Nal^R[b]	72	40.3	36.2	40.3	57.8	18.1	29.2	100

[a] All recombinants were selected, purified, and scored on plates containing 50 μg of Ery per ml, which permits growth of both Ery+ and Mac^D recombinants as per Materials and Methods.

[b] Among His+ Nal^R recombinants, 13 were Trp+ Mac+ but only five were Trp- Mac^D.

TABLE 7. *Locus for erythromycin independence closely linked to locus for erythromycin dependence*



Selected markers[a]	No.	Unselected markers (%)				
		Aro	Spc^R	Ery^R	Thr+	Mac^D
Aro+ PurC+	195	100	79.5	77.4	12.3	0
Ery^R PurC+	187	90.4	94.6	100	9.6	0
Thr+ PurC+[b]	465	56.8	55.1	58.3	100	0

[a] Aro+ PurC+ and Thr+ PurC+ were selected in presence of 50 μg of Ery per ml; Ery^R PurC+ were selected with 200 μg of Ery per ml.

[b] Among Thr+ PurC+ recombinants, there were 20 Ery^R Spc^S but only five Ery^S Spc^R.

class Ery^R Spc^S was considerably more frequent than Ery^S Spc^R. When selection was for the proximal donor marker AroE+, more recombinants were Spc^R than were Ery^R. In the transductional cross Pl_{kc} (246) × MX129, 126 of 160 AroE+ transductants were Spc^R (78.8%), but only 121 of 160 were Ery^R (75.6%). Difficulties in direct selection of Ery^R or Spc^R transductants prevented more accurate mapping by reciprocal three-factor transductions. These data are consistent and suggest a gene order *aroE*...*spc*...-*eryA7*, whereas experiments reported previously

by Dekio (6) with other Ery^R strains demonstrated a gene order *aroE...eryA...spc*. It cannot be stated whether the discrepancy is due to two closely adjacent *ery* loci, or to error in the mapping of a single *eryA* locus. The latter is not unlikely considering the extensive interactions among ribosomal mutations with resultant difficulties in accurate genetic analysis (2, 24). Indeed, we frequently observed strong genetic interference when *eryA7* was introduced into streptomycin-resistant (*str^r*) recipients, and therefore no ordering of *eryA7* relative to *str* was attempted.

Separate effects of eryA and mac. It was possible to study the effect of *eryA* and *mac* separately by comparison of strain MX129 (*eryA$^+$ mac$^+$*) with its derivatives obtained after mating with FS141: FS289 (*eryA7 mac$^+$*), FS298 (*eryA$^+$ mac-1*), and FS287 (*eryA7 mac-1*). FS289 and FS287 (both *eryA7*) were two- to fourfold more resistant to Ery and Ole than MX129 and FS298 (both *eryA$^+$*), but were not resistant to Lin or Cml. FS287 (*eryA7 mac-1*) was also drug dependent; the order of relative drug effectiveness was Ole > Ery > Lin; Cml had no stimulatory effect. (The relative effectiveness of these drugs was therefore slightly different than in FS141.) FS298 (*eryA$^+$ mac-1*) was dependent on Ole, Ery, or Lin but was not resistant to any drug except slightly to Ole. The maximal doubling time of *eryA$^+$ mac-1* strain FS298 in broth plus Ery was 200 min, as compared with approximately 90 min for *eryA7 mac-1* strain FS287. Reversion frequencies to Mac^{IND} of *eryA$^+$ mac-1* strain FS298 after overnight growth in broth were often as high as 1%, but were approximately 10^{-4} for *eryA7 mac-1* strain FS287. Thus, *mac* determined macrolide-lincomycin dependence, but the dependent phenotype was more stable and growth was faster in strains carrying both *eryA7* and *mac*.

DISCUSSION

These experiments describe for the first time a mutant of *E. coli* which is dependent on antibiotics (erythromycin, oleandomycin, lincomycin, and chloramphenicol) which act on the 50*S* ribosomal subunit.

Analogous mutants which are dependent on inhibitors of the 30*S* ribosome (streptomycin and paromomycin) have been extensively studied (9, 10). Mutations to Str^D are allelic with the locus for Str^S and Str^R (10), and the structural effect of mutations at this locus has been shown to be alteration of 30*S* protein S12 (28). The physiology and genetics of Str mutations were recently reviewed by Davies and Nomura (4).

The locus for dependence on erythromycin or related inhibitors of the 50*S* ribosome (*mac*, near minute 25) was not found to be allelic with the well-characterized locus for ribosomal resistance to erythromycin (*eryA*, near minute 63). Other loci for increased sensitivity or resistance to erythromycin and lincomycin have been described by Apirion; most of these mapped near minute 11 on the *E. coli* chromosome (1) and therefore are quite unlikely to be related to the *mac* locus described here. It is possible that *mac* is related to Apirion's lincomycin-resistant (*lin*) mutant, although the latter was only mapped very roughly between minutes 10 and 40 (1).

The apparent rarity of Mac^D mutants may be due to the fact that *mac* resulted in dependence but not resistance to Ery and related drugs. Moreover, strains bearing mutation only at *mac* are apparently at great selective disadvantage because of their extremely slow growth, even in the presence of Ery. Strains carrying mutations at both *mac* and *eryA* grew much more readily in the presence of Ery, were more resistant to Ery, and were less likely to revert to macrolide independence. It therefore is not surprising that the Mac^D strain FS141 was found to be mutant at both *eryA* and *mac*.

We have not shown in these experiments that *mac* affects the ribosome. However, it seems likely that the gene product of *mac* does affect the ribosome, presumably the 50*S* subunit, since growth of Mac^D strain FS141 was stimulated almost exclusively by drugs which inhibit the 50*S* ribosome (21), and the relative order of effectiveness of these drugs (Ery, Ole > Lin > Cml; Table 2) coincides with the apparent affinities of these drugs for binding to a shared site on the 50*S* subunit (8, 29). The simplest conclusion, therefore, is that *mac* alters the ribosome in such a manner that binding of Ery or related drugs is required for ribosomal activity.

There is excellent documentation both genetically and biochemically that alterations of the structural gene for protein 50–8 (in Osawa's terminology) by *eryA* mutations (19) results in change in the binding site for the macrolides, with resultant decreased binding of ^{14}C-erythromycin (20) and ^{14}C-chloramphenicol (25), and cross-resistance between the macrolides (26). If the gene product of *mac* affects this binding site, it must therefore either modify protein 50–8 or else it affects a separate ribosomal structural component which is part of (or functionally related to) the macrolide binding site. The latter seems quite plausible, considering the evidence for a structurally complex

macrolide binding site: the biochemical effects of Ery, Lin, and Cml on protein synthesis are quite distinct (3, 12, 14, 18, 21); the binding of these drugs to the 50*S* ribosome can be differentially affected by agents such as puromycin or ethanol (8); and Mao and Putterman have demonstrated the functional importance of both protein and 23*S* RNA in *E. coli* for binding of erythromycin (16).

The altered phenotypic behavior of FS141 (*mac eryA*) (Table 3) after prior exposure to either Ery, Ole, or Lin might be due to specific conformational changes induced in the ribosome by binding of these drugs. A similar hypothesis might also explain the changes in phenotype of the Mac^{IND} revertants of FS141 after growth in Ery (Table 4). There is considerable evidence that *ery* mutations result in conformational changes in the 50*S* ribosome (20) and that binding of Ery and related drugs is dependent on the conformation of the ribosome (20, 30). Similar changes in the phenotype of 30*S* ribosomal mutants to drug dependence as a function of prior growth in different drugs were described by Gorini et al. (9).

Nonribosomal mechanisms for Mac^{D} are theoretically possible. For instance, *mac* could primarily affect a ribosomal cofactor or a metabolic process (e.g., cation transport) essential to ribosomal activity; a ribosome secondarily altered in this manner might be restored to activity by binding of Ery. Moreover, the failure to observe increased net RNA synthesis after Ery starvation is somewhat surprising if one assumes that Ery starvation of a Mac^{D} ribosome would be functionally equivalent to adding Ery to an Ery^{S} ribosome, since the latter characteristically results in temporary stimulation of RNA synthesis (13).

Studies of the structure and function of ribosomes from several of these strains will be reported in a separate communication.

ACKNOWLEDGMENTS

Thanks are expressed to Y. Ikeya and R. Zimmermann for useful discussions.

This work was supported by a Public Health Service grant (5-RO1-AI09574) from the National Institute of Allergy and Infectious Diseases and by a Public Health Service Research Career Development Award to P.F.S.

LITERATURE CITED

1. Apirion, D. 1967. Three genes that affect *Escherichia coli* ribosomes. J. Mol. Biol. **30**:255-275.
2. Apirion, D., S. L. Phillips, and D. Schlessinger. 1969. Approaches to the genetics of *Escherichia coli* ribosomes. Cold Spring Harbor Symp. Quant. Biol. **34**:117-128.
3. Cundliffe, E., and K. McQuillen. 1967. Bacterial protein synthesis. The effect of antibiotics. J. Mol. Biol. **30**:137-146.
4. Davies, J., and M. Nomura. 1972. The genetics of bacterial ribosomes. Annu. Rev. Genet. **6**:203-234.
5. Davis, B. D., and E. S. Mingioli. 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B12. J. Bacteriol. **60**:17-28.
6. Dekio, S. 1971. Genetic studies of ribosomal proteins VII. Mapping of several ribosomal protein components by transduction experiments between *Shigella dysenteriae* and *Escherichia coli*, and between different strains of *Escherichia coli*. Mol. Gen. Genet. **113**:20-30.
7. Dekio, S., R. Takata, S. Osawa, K. Tanaka, and M. Tamaki. 1970. Genetic studies of ribosomal proteins in *Escherichia coli*. IV. Pattern of alteration of ribosomal protein components in mutants resistant to spectinomycin or erythromycin in different strains of *Escherichia coli*. Mol. Gen. Genet. **107**:39-49.
8. Fernandez-Munoz, R., R. E. Monro, R. Torres-Pinedo, and D. Vazquez. 1971. Substrate- and antibiotic-binding sites at the peptidyl-transferase centre of *Escherichia coli* ribosomes. Studies on the chloramphenicol, lincomycin, and erythromycin sites. Eur. J. Biochem. **23**:185-193.
9. Gorini, L., R. Rosset, and R. A. Zimmermann. 1967. Phenotypic masking and streptomycin dependence. Science **157**:1314-1317.
10. Hashimoto, K. 1960. Streptomycin resistance in *Escherichia coli* analyzed by transduction. Genetics **45**:49-62.
11. Helser, T. L., J. E. Davies, and J. E. Dahlberg. 1972. Mechanism of kasugamycin resistance in *Escherichia coli*. Nature N. Biol. **233**:12-14.
12. Igarishi, K., H. Ishitsuka, and A. Kaji. 1969. Comparative studies on the mechanism of action of lincomycin, streptomycin, and erythromycin. Biochem. Biophys. Res. Commun. **37**:499-504.
13. Imamoto, F. 1973. Diversity of regulation of genetic transcription. I. Effect of antibiotics which inhibit the process of translation on RNA metabolism in *Escherichia coli*. J. Mol. Biol. **74**:113-136.
14. Ishitsuka, H., and A. Kaji. 1970. Release of tRNA from ribosomes by a factor other than G factor. Proc. Nat. Acad. Sci. U.S.A. **66**:168-173.
15. Lennox, E. S. 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190-206.
16. Mao, J. C.-H., and M. Putterman. 1969. The intermolecular complex of erythromycin and the ribosome. J. Mol. Biol. **44**:347-361.
17. Mao, J. C.-H., and R. G. Wiegand. 1968. Mode of action of macrolides. Biochim. Biophys. Acta **157**:404-413.
18. Monro, R. E., and D. Vazquez. 1967. Ribosome-catalyzed peptidyl transfer: effect of some inhibitors of protein synthesis. J. Mol. Biol. **28**:161-165.
19. Otaka, E., T. Itoh, S. Osawa, K. Tanaka, and M. Tamaki. 1972. Peptide analyses of a protein component, 50-8, of 50S ribosomal subunit from erythromycin resistant mutants of *Escherichia coli* and *Escherichia freundii*. Mol. Gen. Genet. **114**:14-22.
20. Otaka, E., H. Teraoko, M. Tamaki, K. Tanaka, and S. Osawa. 1970. Ribosomes from erythromycin-resistant mutants of *Escherichia coli* Q13. J. Mol. Biol. **48**:499-510.
21. Pestka, S. 1971. Inhibitors of ribosome functions. Annu. Rev. Microbiol. **25**:487-562.
22. Sparling, P. F. 1970. Kasugamycin resistance: a 30S ribosomal mutation with unusual map location in *E. coli*. Science **167**:56-57.
23. Sparling, P. F., Y. Ikeya, and D. Elliot. 1973. Two genetic loci for resistance to kasugamycin in *Escherichia coli*. J. Bacteriol. **113**:704-710.
24. Takata, R., S. Osawa, K. Tanaka, H. Teraoka, and M. Tamaki. 1970. Genetic studies of the ribosomal proteins of *Escherichia coli*. V. Mapping of erythromycin

resistance mutations which lead to alteration of a 50S ribosomal protein component. Mol. Gen. Genet. **109**:123-130.
25. Tanaka, K., M. Tamaki, R. Takata, and S. Osawa. 1972. Low affinity of chloramphenicol of erythromycin resistant *Escherichia coli* ribosomes having an altered protein component. Biochem. Biophys. Res. Commun. **46**:1979-1983.
26. Tanaka, K., H. Teraoka, M. Tamaki, R. Takata, and S. Osawa. 1972. Phenotypes represented by a mutational change in a 50S ribosomal component, 50-8, in *Escherichia coli*. Mol. Gen. Genet. **114**:9-13.
27. Taylor, A. L. 1970. Current linkage map of *Escherichia coli*. Bacteriol. Rev. **34**:155-175.
28. Traub, P., and M. Nomura. 1968. Streptomycin resistance mutation in *Escherichia coli*: altered ribosomal protein. Science **160**:198-199.
29. Vazquez, D., and R. E. Monro. 1967. Effects of some inhibitors of protein synthesis on the binding of aminoacyl tRNA to ribosomal subunits. Biochim. Biophys. Acta **142**:155-173.
30. Vogel, Z., T. Vogel, A. Zamir, and D. Elson. 1971. Correlation between the peptidyl transferase activity of the 50S ribosomal subunit and the ability of the subunit to interact with antibiotics. J. Mol. Biol. **60**:339-346.