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Contribution of Two Different Mechanisms to Erythromycin Resistance in *Escherichia coli*

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***Escherichia coli* BM2570 was resistant to high levels of erythromycin by two different mechanisms. The two genes conferring resistance to erythromycin in BM2570 were carried by a 150-kilobase self-transferable plasmid, pIP1527, and were cloned separately in *E. coli*. A single polypeptide with an M_r of 27,000 was encoded by the gene *ermA* and conferred high-level resistance to macrolide, lincosamide, and streptogramin B-type antibiotics by a mechanism other than drug inactivation. This resistance phenotype, not previously reported for a clinical isolate of enterobacteria, was probably due to modification of the ribosomes. The gene *ereB* encoded an enzyme with an M_r of 51,000 which inactivated erythromycin and oleandomycin. The two different mechanisms specified by *ermA* and *ereB* contributed in more than an additive fashion to the high level of resistance to erythromycin conferred by plasmid pIP1527 to *E. coli* BM2570.**

Macrolide, lincosamide, and streptogramin (MLS) antibiotics are mainly active against gram-positive bacteria. Acquired resistance to MLS antibiotics in these bacteria involves generally the N^6-dimethylation of a specific adenine residue in 23S rRNA (34). By contrast, enterobacteria, like most gram-negative microorganisms, are naturally resistant to low levels of MLS, which precludes the therapeutic use of MLS in systemic infections. Cell impermeability is probably responsible for this intrinsic resistance (29). The MICs of erythromycin against most enterobacteria range between 2 and 256 μg/ml (4, 24). However, higher local antibiotic concentrations (0.5 to 6.0 mg/g of feces) are obtained in the lumen of the intestinal tract after oral administration of usually recommended doses (4). The subsequent reduction of the aerobic gram-negative flora of the intestinal tract (2, 4) has recently found therapeutic applications (3, 4). The intestinal carriage of enterobacteria highly resistant to erythromycin (MICs ≥500 μg/ml) occurs frequently and is usually associated with the previous intake of erythromycin (3).

We recently described *Escherichia coli* BM2195, which is resistant to high levels of erythromycin (MIC >2,000 μg/ml) (1). Resistance in this strain is due to the synthesis of an erythromycin esterase which hydrolyzes the lactone ring of the antibiotic (7). The nucleotide sequence of the plasmid gene *ereA* which encodes the esterase was determined (22). The study of the distribution of the gene *ereA* by colony hybridization revealed three classes of clinically isolated enterobacteria that were highly resistant to erythromycin (5). In the first class, the gene *ereA* was detected in strains of *E. coli* of several biotypes and in *Klebsiella pneumoniae* and *Enterobacter agglomerans*, which were resistant to erythromycin by inactivation. Bacteria of the second class were also resistant to erythromycin by inactivation but did not hybridize with the specific probe for the gene *ereA*. Resistant bacteria of the third class did not inactivate erythromycin.

The aim of this work was to study the newly detected class of genes which specify resistance to erythromycin by inactivation but lack homology with *ereA*. We studied *E. coli* BM2570, which was highly resistant to erythromycin and inactivated erythromycin and oleandomycin but did not share detectable homology with the gene *ereA*. We have cloned two resistance determinants which were found to contribute in more than an additive fashion to the erythromycin resistance of BM2570 by two different mechanisms.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The sources and properties of the strains are listed in Table 1. The plasmids used are listed in Table 2 and diagrammed in Fig. 1. Strain BM2570 was previously designated no. 1 (5).

Media. Brain heart infusion broth (Difco Laboratories) and Mueller-Hinton agar (Diagnostics Pasteur) were used. All incubations were at 37°C.

Determination of MICs. The method of Steers et al. (28) was used to determine the MICs of the antibiotics.

Inactivation of macrolides. Inactivation of macrolides was screened by the Gots test (16), modified as described previously (1).

Induction of resistance to erythromycin and lincomycin. Cells of *E. coli* DB10 harboring various plasmids were grown in the presence of subinhibitory concentrations of erythromycin or lincomycin (one-fourth the MIC in liquid medium of the plasmid-free strain). After 1 h, erythromycin or lincomycin was added to a high final concentrations, and growth was monitored with a Klett spectrophotometer.

Thin-layer chromatography. Resting cells prepared as described previously (7) were incubated for 18 h at 37°C in 0.1 M phosphate buffer (pH 7.0) containing erythromycin (4 mg/ml) or oleandomycin (0.8 mg/ml). Samples corresponding to 4 μg of modified antibiotic were deposited on precoated Merck silica gel plates 60 F 254. The solvent systems used are indicated in Table 4. To detect antibiotics or modified antibiotics, plates were sprayed with H_2SO_4 in ethanol and heated at 100°C for 15 min (7).

Preparation of plasmid DNA and agarose gel electrophoresis. High-molecular-weight (17) and low-molecular-weight (14) plasmid DNAs were prepared as described previously. The restriction DNA fragments were separated by electrophoresis in horizontal slab gels (20 by 20 by 0.7 cm) containing 0.7 or 2.0% type II agarose (Sigma Chemical Co.). The plasmid copy numbers were estimated by deter-

TABLE 1. Properties of the *E. coli* strains used

Strain	Relevant characteristics and plasmid content[a]	Source or reference
AR1062	*thi leu lac gal xyl mal mtl hsdS*	Rambach and Hogness (23)
BM694	Prototroph, *gyrA*	Labigne-Roussel et al. (17)
BM2507	Prototroph, *gyrA* pIP1100 Tra^+ IncX *ereA* Ap^r Gm^r Sm^r	Andremont et al. (1)
BM2570	pIP1527 Tra^+ *erxA ereB* Cm^r Tc^r pIP1530 Tra^- Ap^r	Arthur et al. (5)
BM2571	Prototroph, *gyrA* pIP1527 Tra^+ *erxA ereB* Cm^r Tc^r	Conjugation, BM2570 × BM694
BM2572	*thiA leu rna pnp gyrA rpsL* Fa^s MLS^s pIP1527 Tra^+ *erxA ereB* Cm^r Tc^r	Conjugation, BM2571 × DB10
BM2573	Prototroph, *gyrA* pIP1527-1, Tra^- *erxA ereB*	Transformation, pIP1527 × BM694
BM2575	Prototroph, *gyrA* pIP1527-2 Tra^- *erxA*	Transformation, pIP1527 × BM694
DB10	*thiA leu rna pnp gyrA rpsL* Fa^s MLS^s	Datta et al. (13)

[a] Genetic symbols are those of Bachmann (6), and phenotypic characters of plasmids are those of Novick et al. (21).

mining the ratios of plasmid to chromosome DNA as described previously (11). DNA concentrations were estimated by microphotodensitometric analysis (17).

Analysis of minicells. Plasmids were introduced by transformation into the minicell-producing strain AR1062, and minicells were purified as described previously (23). Labeling of polypeptides with L-[^{35}S]methionine and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were by the method of Laemmli (18). ^{135}I-labeled proteins were used as M_r standards.

Enzymes and reagents. Restriction endonucleases *Bam*HI, *Cla*I, *Eco*RI, *Hin*dIII, *Pst*I, and *Sau*3A and T4 DNA ligase (Boehringer Mannheim Biochemicals) were used according to the manufacturer's recommendations. Lysozyme was obtained from Sigma. The antibiotics were provided by the following laboratories: clindamycin and lincomycin, The Upjohn Co.; spiramycin and pristinamycins I and II, Rhône-Poulenc; oleandomycin, Pfizer Inc.; erythromycin, Roussel-Uclaf; josamycin, Spret-Mauchant; midecamycin, Clin-Midy.

RESULTS

Plasmid-mediated characters expressed by *E. coli* BM2570. *E. coli* BM2570 is resistant to ampicillin, chloramphenicol, streptomycin, sulfonamide, tetracycline, trimethoprim, and high levels of erythromycin. The genes conferring resistance to chloramphenicol, erythromycin, and tetracycline were transferred from BM2570 to *E. coli* BM694 by conjugation. Selection for transfer of either chloramphenicol (20 μg/ml), erythromycin (400 μg/ml), or tetracycline (10 μg/ml) resistance revealed cotransfer of these three resistance characters (24 colonies tested on each selector). In two independent 1-h matings, transfer frequencies of 5×10^{-6} to 2×10^{-5} were obtained for these three selectable markers. One transconjugant, strain BM2571, was further studied. Transfer of erythromycin resistance from BM2571 to *E. coli* DB10, a mutant highly susceptible to MLS antibiotics, was also obtained by conjugation. This confirmed the cotransfer of resistance to not only chloramphenicol tetracycline, and erythromycin but also to other MLS antibiotics (Table 3). We detected inactivation of erythromycin and oleandomycin in the transconjugants and in BM2570 by a microbiological test (1, 16). These strains did not inactivate other MLS antibiotics. Resistance to certain MLS antibiotics could not be detected in BM694 since the MICs for this strain are similar to the solubility limits of the drugs.

Plasmid DNA was isolated from strains BM2570 and BM2571 and analyzed by agarose gel electrophoresis. Strain BM2571 carried a single plasmid, pIP1527, of approximately 150 kilobases (kb). Strain BM2570 contained two plasmids, pIP1527 and pIP1530 (data not shown). Plasmid pIP1530, approximately 6 kb, was not transferable by conjugation but could be introduced by transformation in BM694, where it conferred resistance to ampicillin only. Plasmid DNA purified from the wild-type strain BM2570 was used to transform BM694, and clones were selected on plates containing erythromycin or tetracycline. Analysis of transformants selected on tetracycline revealed 100% cotransfer of resistance to chloramphenicol, erythromycin, and tetracycline. Of 30

TABLE 2. Plasmids and their origins

Plasmid	Relevant characters[a]	Origin	Source or reference
pIP1100	Tra^+ IncX *ereA* Ap^r Gm^r Sm^r	Natural plasmid	Andremont et al. (1)
pIP1527	Tra^+ *erxA ereB* Cm^r Tc^r	Natural plasmid	This paper
pIP1527-1	*erxA ereB*	In vivo deletion in pIP1527	This paper
pIP1527-2	*erxA*	In vivo deletion in pIP1527	This paper
pBR322	Tra^- Mob^- Ap^r Tc^r	In vitro construction	Bolivar et al. (8)
pBR329	Tra^- Mob^- Ap^r Tc^r Cm^r	In vitro construction	Covarrubias et al. (12)
pUC8	Tra^- Mob^- Ap^r	In vitro construction	Vieira and Messing (33)
pAT63	Tra^- Mob^- Ap^r *ereA*	pBR322Ω(pIP1100 partial *Sau*3A [*ereA*], 1.66 kb)	Ounissi and Courvalin (22)
pAT69	Tra^- Mob^- Ap^r *erxA*	pUC8Ω(pIP1527 *Hin*dIII-*Pst*I [*erxA*], 1.8 kb)	This paper
pAT72	Tra^- Mob^- Ap^r *ereB*	pUC8Ω(pIP1527 partial *Sau*3A [*ereB*], 1.9 kb)	This paper
pAT75	Tra^- Mob^- Ap^r *erxA*	pBR322Ω(pIP1527 *Hin*dIII [*erxA*], 3.0 kb)	This paper

[a] Nomenclature of phenotypic characters of plasmids is that of Novick et al. (21).

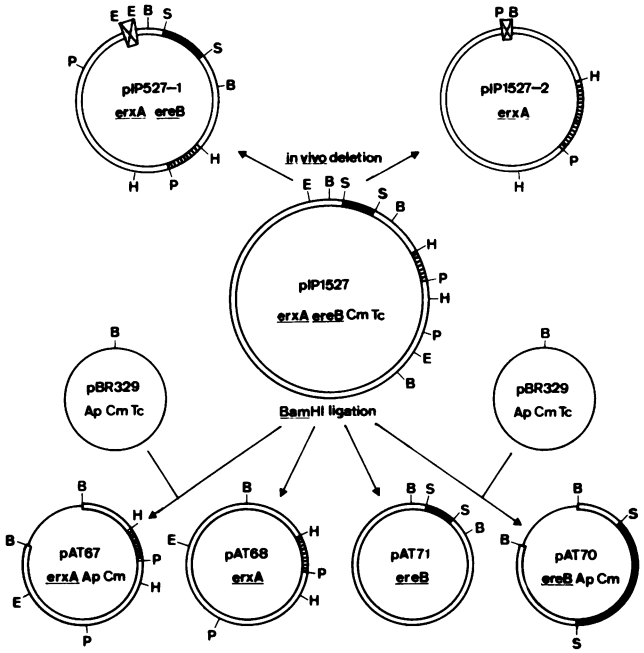

FIG. 1. Relationship of various plasmids employed in this study. Restriction sites: B, *Bam*HI; *E*, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sau*3A. Symbols: (═══) pIP1527, (——) pBR329, (▪▪▪▪▪) *erxA* region, (▬▬) *ereB* region, (=⊠=) fragment of pIP1527-1 or pIP1527-2 resulting from a deletion in pIP1527. Ap, ampicillin resistance; Cm, chloramphenicol resistance; Tc, tetracycline resistance. Not drawn to scale. Only relevant restriction sites are shown.

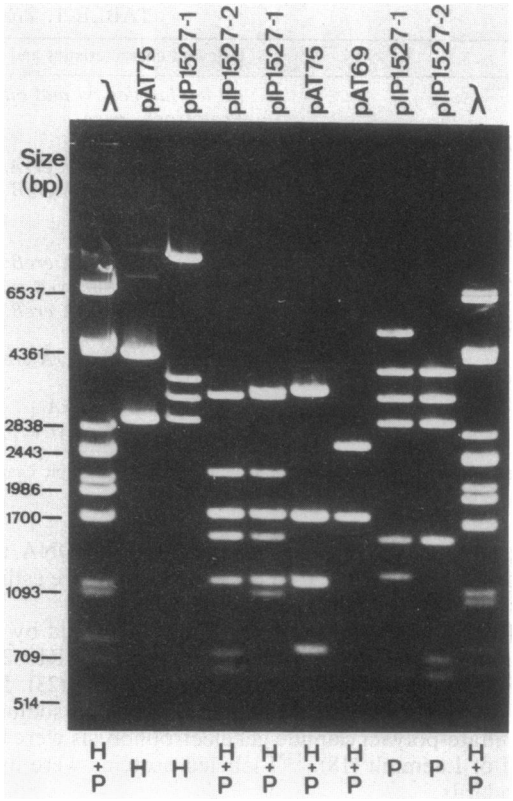

FIG. 2. Analysis of plasmid DNA by agarose gel electrophoresis. Two to three micrograms of plasmid DNA were digested with the restriction endonucleases indicated at the bottom (H, *Hin*dIII; P, *Pst*I). Fragments obtained by digestion of bacteriophage λ cI857 DNA with *Hin*dIII plus *Pst*I served as internal standards (25). The sizes of certain λ DNA fragments are indicated in base pairs at the left.

clones selected on erythromycin, 28 were resistant to the three antibiotics. Two clones, BM2573 and BM2575, that were resistant to erythromycin and susceptible to chloramphenicol and tetracycline were studied further. Both resistance to MLS antibiotics and inactivation of erythromycin in BM2573 were specified by a 18.7-kb plasmid designated pIP1527-1. Resistance to MLS antibiotics, which was encoded by a 13.3-kb plasmid, pIP1527-2, in BM2575 was not accompanied by inactivation of erythromycin.

To investigate the origin of plasmids pIP1527-I and pIP1527-2, we compared them by electrophoresis in an agarose gel which was overloaded with plasmid DNA isolated from strain BM2570. No DNA band comigrated with those corresponding to pIP1527-1 and pIP1527-2, indicating that these two structures were not resident plasmids of strain BM2570. However, comparative analysis of cleavage patterns of plasmids pIP1527, pIP1527-1, and pIP1527-2 with four restriction endonucleases, *Bam*HI, *Hin*dIII, *Eco*RI, and *Cla*I, indicated that pIP1527-1 and pIP1527-2 were derived from pIP1527. In every restriction system, all but one DNA fragment of either pIP1527-1 or pIP1527-2 comigrated with pIP1527 fragments. Therefore, these two plasmids result from a deletion in pIP1527, most probably associated with transformation. Comparison of the cleavage patterns (Fig. 2) and restriction maps (Fig. 3) of pIP1527-1 and pIP1527-2 confirmed that these two plasmids contained common sequences. The relationship between these plasmids is described in Fig. 1.

TABLE 3. MICs of various MLS antibiotics against *E. coli* DB10 derivatives harboring various plasmids[a]

Plasmid	Relevant genotype	Copy no.	MIC (μg/ml)									
			Ery	Ole	Spi	Jos	Mid	Lin	Clin	PriI	PriII	Pri
None			4	16	8	8	8	8	0.5	128	4	2
pIP1527, pIP1527-1	*erxA ereB*	Low	1,024	256	16	16	32	4,096	128	128	4	2
pIP1527-2, pAT68	*erxA*	Low	16	32	16	16	32	4,096	128	128	4	2
pAT67, pAT69	*erxA*	High	64	512	64	64	128	4,096	512	256	4	2
pAT71	*ereB*	Low	128	256	8	8	8	8	0.5	128	4	2
pAT70, pAT72	*ereB*	High	128	256	8	8	8	8	0.5	128	4	2
pIP1100	*ereA*	Low	128	2,048	8	8	8	8	0.5	128	4	2
pAT63	*ereA*	High	128	2,048	8	8	8	8	0.5	128	4	2

[a] Abbreviations: Ery, erythromycin; Ole, oleandomycin; Spi, spiramycin; Jos, josamycin; Mid, midecamycin; Lin, lincomycin; Clin, clindamycin; PriI, pristinamycin factor I; PriII, pristinamycin factor II; Pri, pristinamycin.

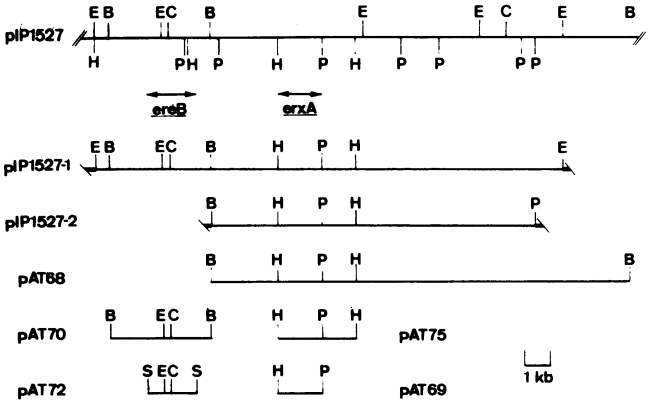

FIG. 3. Partial restriction maps of pIP1527 and of the inserts in hybrid plasmids. Restriction sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sau*3A. Double arrows indicate the shortest DNA restriction fragments necessary for expression of resistance to erythromycin. The heavy line in plasmids pIP1527-1 and pIP1527-2 indicates the DNA restriction fragments generated by the deletions in pIP1527. Only relevant restriction sites are shown.

Cloning of the erythromycin resistance determinants of pIP1527. DNAs of plasmids pIP1527 and pBR329 were mixed, cleaved with *Bam*HI, ligated, and transformed into BM694. Transformants were selected on plates containing erythromycin (400 μg/ml). The plasmids carried by clones resistant to erythromycin were purified and transformed into DB10 with selection by erythromycin (20 μg/ml) or lincomycin (100 μg/ml). This procedure allowed the detection and the separation by cloning of two genes, *erxA* and *ereB*, specifying resistance to MLS antibiotics and erythromycin, respectively, in the original strain BM2570 (Fig. 1).

(i) Cloning of the gene *erxA*. Plasmid pAT68 consisted of a single 16.0-kb *Bam*HI fragment of pIP1527 and conferred resistance to MLS antibiotics. Since no pBR329 DNA was present in this plasmid, this *Bam*HI fragment must contain an origin of replication. Plasmid pAT67 conferred resistance to ampicillin, chloramphenicol, and MLS antibiotics. Comparison of the digestion patterns of pAT67, pAT68, and pBR329 showed that pAT67 was a recombinant formed between pBR329 and pAT68.

To subclone the gene *erxA*, DNA of plasmids pAT68 and pBR322 was digested with *Hin*dIII and ligated. One transformant that was resistant to MLS and ampicillin contained plasmid pAT75, which consisted of a 3.0-kb fragment of pAT68 cloned into pBR322. The gene *erxA* was further subcloned on a 1.8-kb *Pst*I-*Hin*dIII fragment into pUC8. This plasmid, pAT69, was constructed by ligating pUC8 DNA, digested by *Hin*dIII plus *Pst*I, with the purified insert of pAT75, digested by *Pst*I (Fig. 2).

(ii) Cloning of the gene *ereB*. Plasmid pAT70 conferred resistance to ampicillin, chloramphenicol, and erythromycin by inactivation. This plasmid consisted of a 3.8-kb *Bam*HI fragment of pIP1527 inserted into pBR329. Plasmid pAT71 specified resistance only to erythromycin by inactivation and did not contain pBR329 DNA. It consisted of 3.8- and 21.0-kb *Bam*HI fragments of pIP1527. The 3.8-kb fragment was indistinguishable from the insert in pAT70. The origin of replication of pAT71 was probably located in the 21.0-kb DNA fragment, since we were consistently unable to obtain transformants resistant to erythromycin and harboring the 3.8-kb pIP1527 fragment alone. At least two DNA fragments of pAT71 obtained by digestion with either *Eco*RI, *Hin*dIII, or *Cla*I did not comigrate with the fragments of pIP1527 DNA digested with the same endonucleases. Therefore, the two *Bam*HI fragments of pAT71 were not ligated in the same relative order as that in pIP1527.

To subclone the gene *ereB*, plasmid pAT70 DNA was digested partially with *Sau*3A, and the resulting fragments were ligated to pUC8 DNA digested with *Bam*HI. Plasmid DNA of transformants resistant to erythromycin and ampicillin was purified and analyzed by agarose gel electrophoresis. The smallest plasmid, designated pAT72, was further characterized. Plasmid pAT72 consists of a 1.9-kb fragment of pAT70 inserted in pUC8.

Comparison of the different plasmids allowed the construction of a restriction map of the region of pIP1527 containing the genes *erxA* and *ereB* (Fig. 3).

Resistance phenotypes conferred by the genes *erxA* and *ereB*. The MLS resistance phenotypes conferred by genes *erxA* and *ereB* were determined for *E. coli* DB10 harboring plasmid pIP1527 or various derivatives (Table 3). The copy number of the plasmids was estimated by microphotodensitometry analysis (11, 17). Plasmids having the origin of replication of ColE1 factor were present at more than 20 copies per chromosome, whereas plasmids pAT68, pAT71, pIP1100, pIP1527, and its deletion derivatives were present at less than 5 copies per chromosome. The multicopy hybrid plasmids pAT67 and pAT69 bearing the gene *erxA* conferred high-level resistance to MLS B-type antibiotics. However, when present on plasmids pIP1527-2 and pAT68, the gene *erxA* conferred resistance to high levels of lincosamides only. Resistance to macrolides and streptogramin B-type antibiotics was low or not detectable. This difference in resistance levels correlates with the linkage of *erxA* to high- or low-copy-number replicons.

This gene dosage effect was not observed for *ereB* and erythromycin. Levels of resistance to erythromycin conferred by genes *ereB* and *ereA*, the gene encoding the erythromycin esterase (22), are similar and independent of the copy number of the plasmid. However, *ereB* conferred lower levels of resistance to oleandomycin, and complete inactivation of this antibiotic by resting cells of strain BM694(pAT72) was difficult to achieve (see below).

The separation of genes *erxA* and *ereB* on low-copy-number plasmids enabled the study of the contribution of these two genes to the high-level erythromycin resistance encoded by pIP1527. The MIC of erythromycin for strain DB10 harboring gene *erxA* and *ereB* was 16 or 128 μg/ml, respectively. However, the MIC increased to 1,024 μg/ml when the two genes were present together. Thus both genes contributed in more than an additive fashion to the high degree of resistance to erythromycin encoded by pIP1527.

Inactivation of macrolides. Inactivation of erythromycin and oleandomycin by cellular or subcellular fractions of BM694 harboring pAT63 (*ereA*), pAT72 (*ereB*), or pAT69 (*erxA*) was studied. Growing cells, resting cells, the supernatant of a bacterial sonic extract (S100), and the corresponding pellet were incubated with antibiotics in solution. Inactivation of erythromycin and oleandomycin was monitored by a microbiological technique (10), and the products of inactivation were separated by thin-layer chromatography. Cellular and subcellular fractions of strain BM694 harboring plasmids pAT63 (*ereA*) and pAT72 (*ereB*) inactivated both antibiotics. After 24 h of incubation with erythromycin (4,000 μg/ml), no residual antibiotic activity (<40 μg/ml) was detected by the microbiological technique. Incubation of oleandomycin (800 μg/ml) with preparations of BM694(pAT63) (*ereA*) also resulted in complete inactiva-

tion of the antibiotic (<80 μg/ml) after 24 h. However, in most experiments inactivation of oleandomycin by BM694(pAT72) (*ereB*), although exceeding 60%, was incomplete. The detoxification products of erythromycin or oleandomycin by both strains were indistinguishable by thin-layer chromatography (Table 4). Under the same conditions, we were consistently unable to detect modification of the antibiotics by preparations of strain BM694 harboring the gene *erxA*.

Induction of resistance. Induction of resistance to erythromycin and to lincomycin by these two antibiotics was studied for DB10 derivatives harboring plasmids pIP1527 (*erxA ereB*), pAT69 (*erxA*), and pAT72 (*ereB*). The levels of resistance of DB10 strains was unaffected by preexposure to subinhibitory concentrations of erythromycin or lincomycin.

Analysis of the polypeptides encoded by genes *erxA* and *ereB* in minicells. Plasmids pUC8, pAT69 (*erxA*), and pAT72 (*ereB*) were introduced into *E. coli* AR1062 (23), which produces minicells. After purification of the minicells and labeling of the polypeptides, proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 4). One band, which must correspond to the erythromycin-modifying enzyme, with an estimated M_r of approximately 51,000 was present with pAT72 (*ereB*) but not with pUC8. The insert in pAT69 (*erxA*) encodes a single polypeptide with an M_r of approximately 27,000. A band corresponding to a polypeptide of M_r 22,500 was also present in the pAT72 lane. Since this band was also observed with pAT69 and pUC8 after exposure for longer periods of time, it is unlikely that this polypeptide was encoded by the insert in pAT72.

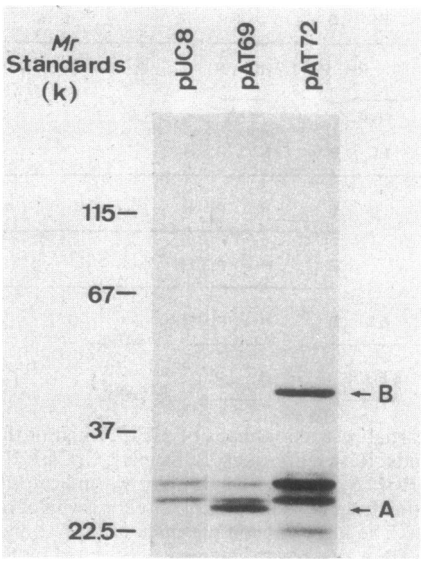

FIG. 4. Autoradiogram of L-[^{35}S]methionine-labeled polypeptides from a minicell experiment after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The two bands present with the three plasmids are the beta-lactamase and prebeta-lactamase, M_r 28,00 and 31,000, respectively. A, Polypeptide encoded by the gene *erxA*, M_r 27,000; B, polypeptide encoded by the gene *ereB*, M_r 51,000.

DISCUSSION

E. coli BM2570 was isolated from a clinical specimen. Its salient feature is high-level resistance to erythromycin (MIC >2,000 μg/ml) by two different mechanisms. The genes conferring resistance to chloramphenicol, tetracycline, MLS antibiotics, and erythromycin by inactivation were carried by pIP1527, a 150-kb plasmid. This plasmid was self-transferable to other *E. coli* strains and had at least two origins of replication. Two genes of pIP1527 encoding erythromycin resistance were separately cloned and characterized.

The gene *erxA* was cloned on a 1.8-kb DNA fragment (Fig 2) which encoded a single polypeptide with an M_r of 27,000 (Fig. 4). Based on negative results obtained with microbiological techniques, resistance to macrolides, lincosamides, and streptogramin B-type antibiotics encoded by *erxA* is probably not due to detoxification of the drugs. In addition, we did not detect any erythromycin- or oleandomycin-modifying activity in whole cells or subcellular fractions of bacteria harboring *erxA* by thin-layer chromatography (Table 4). To our knowledge, this is the first report of an MLS resistance phenotype in a naturally occurring gram-negative strain.

TABLE 4. R_fs of the modified antibiotics by thin-layer chromatography[a]

Plasmid	Relevant genotype	R_f in solvent 1		R_f in solvent 2		R_f in solvent 3	
		Ery	Ole	Ery	Ole	Ery	Ole
pAT63	*ereA*	0.18	0.14	0.05	0.10	0.37	0.36
pIP1527	*erxA ereB*	0.18	0.14	0.05	0.10	0.37	0.36
pAT72	*ereB*	0.18	0.14	0.05	0.10	0.37	0.36
pAT69	*erxA*	0.52	0.48	0.07	0.08	0.28	0.22
		0.52	0.48	0.07	0.08	0.28	0.22

[a] Ery, Erythromycin; Ole, oleandomycin. Solvent 1, Ammonium hydroxide-isopropanol (1:10, vol/vol); solvent 2, butanol-acetic acid-water (1:3:1, vol/vol/vol); solvent 3, methanol-water (4:1, vol/vol).

MLS antibiotics differ greatly with regard to their chemical structure (15) but are closely related in their mode of action. A modification of the 50S subunit of the ribosome, which is the common target of MLS, appears therefore to be the most probable resistance mechanism specified by the gene *erxA*. It is indeed unlikely that a single enzyme can detoxify or specifically modify the transport of antibiotics as chemically distinct as the MLS antibiotics. Conversely, two modifications in the 23S rRNA are known to confer an MLS resistance phenotype to *E. coli*. By in vitro mutagenesis a transversion from A-T to T-A was obtained in a gene encoding 23S rRNA (27). Resistance to MLS antibiotics can also be obtained after cloning in *E. coli* of the gene encoding the methylase from gram-positive cocci (9, 20). Both modifications involve the same position, 2058, in the 23S rRNA sequence (27, 30). The resistance phenotypes toward MLS antibiotics conferred in *E. coli* by the gene *erxA* or by the 23S rRNA methylase gene are indistinguishable. We are currently testing the possibility that gene *erxA* encodes an RNA methylase and originates in gram-positive cocci. Transfer of a resistance gene between gram-positive and gram-negative bacteria under natural conditions has recently been reported (19, 31).

The gene *ereB* encoded high-level resistance to erythromycin and oleandomycin. This resistance was due to the synthesis of an enzyme which inactivated both antibiotics but none of the other commercially available macrolides. The previously characterized erythromycin esterase (7) encoded by the gene *ereA* (22) has the same specificity toward 14-membered macrolides. Moreover the products obtained after the detoxification of erythromycin and oleandomycin by the two enzymes are indistinguishable by thin-layer chromatography (Table 4). It is therefore likely that genes

ereA and *ereB* encode two erythromycin esterases. Despite these similarities, the genes *ereA* and *ereB* appear to be very different. They share no detectable homology by colony hybridization (5), and the estimated molecular weight (51,000) of the enzyme encoded by *ereB* (Fig. 4) is significantly higher than that (37,765) deduced from the nucleotide sequence of the gene *ereA* (22).

The comparison of the resistance levels conferred by either *erxA* or *ereB* when borne by different replicons was a prerequisite to study the contribution of both genes to the high-level resistance to erythromycin encoded by pIP1527 (Table 3). In the case of the gene *erxA*, resistance levels to erythromycin and MLS antibiotics as well dramatically increased when the gene was linked to high-copy-number vectors. We believe that this difference is due to a gene dosage effect, as already reported for other resistance genes (26, 32). This effect was observed neither for the gene *ereB* nor for the gene *ereA*, which encodes the erythromycin esterase. Taken together, our results indicate that the genes *erxA* and *ereB* contribute in more than an additive fashion to the high-level resistance to erythromycin mediated by plasmid pIP1527 in DB10 (Table 3) and also in BM694 (data not shown). This is possibly due to the fact that, in the presence of high concentrations of erythromycin, synthesis of the inactivating enzyme by the putatively modified ribosomes occurs even at antibiotic concentrations which are bacteriostatic for strains resistant only by the presence of the gene *erxA*. Subsequent inactivation allows bacterial growth.

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LITERATURE CITED

1. **Andremont, A., G. Gerbaud, and P. Courvalin.** 1986. Plasmid-mediated high-level resistance to erythromycin in *Escherichia coli*. Antimicrob. Agents Chemother. **29**:515–518
2. **Andremont, A., P. Raibaud, and C. Tancrede.** 1983. Effect of erythromycin on microbial antagonisms: a study in gnotobiotic mice associated with a human fecal flora. J. Infect. Dis. **148**: 579–587.
3. **Andremont, A., H. Sancho-Garnier, and C. Tancrede.** 1986. Epidemiology of intestinal colonization by members of the family *Enterobacteriaceae* highly resistant to erythromycin in a hematology-oncology unit. Antimicrob. Agents Chemother. **29**:1104–1107.
4. **Andremont, A., and C. Tancrede.** 1981. Reduction of the aerobic Gram-negative bacterial flora of the gastro-intestinal tract and prevention of traveller's diarrhea using oral erythromycin. Ann. Microbiol. (Paris) **132B**:419–427.
5. **Arthur, M., A. Andremont, and P. Courvalin.** 1986. Heterogeneity of genes conferring high-level resistance to erythromycin by inactivation in enterobacteria. Ann. Microbiol. (Paris) **137A**:125–134.
6. **Bachmann, B. J.** 1983. Linkage map of *Escherichia coli* K-12, edition 7. Microbiol. Rev. **47**:180–230.
7. **Barthélémy, P., D. Autissier, G. Gerbaud, and P. Courvalin.** 1984. Enzymic hydrolysis of erythromycin by a strain of *Escherichia coli*: a new mechanism of resistance. J. Antibiot. **37**: 1692–1696.
8. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
9. **Carlier, C., and P. Courvalin.** 1982. Resistance of streptococci to aminoglycoside-aminocyclitol antibiotics. *In* D. Schlessinger (ed.), Microbiology—1982. American Society for Microbiology, Washington, D.C.
10. **Chabbert, Y., and H. Boulingre.** 1957. Modifications pratiques concernant le dosage des antibiotiques en cliniques. Rev. Fr. Etud. Clin. Biol. **2**:636–640.
11. **Courvalin, P. M., C. Carlier, O. Croissant, and D. Blangy.** 1974. Identification of two plasmids determining resistance to tetracycline and erythromycin in group D *Streptococcus*. Mol. Gen. Genet. **132**:181–192.
12. **Covarrubias, L., and F. Bolivar.** 1982. Construction and characterization of new cloning vehicles. VI. Plasmid pBR329, a new derivate of pBR328 lacking the 482-base pair inverted duplication. Gene **17**:79–89.
13. **Datta, N., R. W. Hedges, D. Becker, and J. Davies.** 1974. Plasmid-determined fusidic acid resistance in the *Enterobacteriaceae*. J. Gen. Microbiol. **83**:191–196.
14. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Large scale isolation of *E. coli* plasmid DNA, p. 116–119. *In* Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Gale, E. F., E. Cundliffe, P. E. Reynolds, M. H. Richmond, and M. J. Waring.** 1981. Antibiotic inhibitors of ribosome function, p. 468–485. *In* The Molecular basis of antibiotic action, 2nd ed. John Wiley & Sons, Inc., New York.
16. **Gots, J. S.** 1945. The detection of penicillinase-producing properties of microorganisms. Science **102**:309.
17. **Labigne-Roussel, A., G. Gerbaud, and P. Courvalin.** 1981. Translocation of sequences encoding antibiotic resistance from the chromosome to a receptor plasmid in *Salmonella ordonez*. Mol. Gen. Genet. **182**:390–408.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
19. **Lambert, T., G. Gerbaud, P. Trieu-Cuot, and P. Courvalin.** 1985. Structural relationship between the genes encoding 3'-aminoglycoside phosphotransferases in *Campylobacter* and gram-positive cocci. Ann. Microbiol. (Paris) **136B**:135–150.
20. **Malke, H., and S. E. Holm.** 1981. Expression of streptococcal plasmid-determined resistance to erythromycin and lincomycin in *Escherichia coli*. Mol. Gen. Genet. **184**:283–285.
21. **Novick, R. P., R. C. Clowes, S. N. Cohen, R. Curtiss III, N. Datta, and S. Falkow.** 1976. Uniform nomenclature for bacterial plasmids: a proposal. Bacteriol. Rev. **40**:168–189.
22. **Ounissi, H., and P. Courvalin.** 1985. Nucleotide sequence of the gene *ereA* encoding the erythromycin esterase in *Escherichia coli*. Gene **35**:271–278.
23. **Rambach, A., and D. S. Hogness.** 1977. Translation of *Drosophila melanogaster* sequences in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **74**:5041–5045.
24. **Sabath, L. D., V. Lorian, D. Gerstein, P. B. Loder, and M. Finland.** 1968. Enhancing effect on alkalinisation of the medium on the activity of erythromycin against Gram-negative bacteria. Appl. Microbiol. **16**:1288–1292.
25. **Sanger, F., A. R. Coulson, G. F. Hong, D. F. Hill, and G. B. Petersen.** 1982. Nucleotide sequence of bacteriophage lambda DNA. J. Mol. Biol. **162**:729–733.
26. **Schmitt, R., E. Bernhard, and R. Mattes.** 1979. Characterization of Tn*1721*, a new transposon containing tetracycline resistance genes capable of amplification. Mol. Gen. Genet. **172**:53–65.
27. **Sigmund, C. D., M. Ettayebi, and E. A. Morgan.** 1984. Antibiotic resistance mutations in 16S and 23S ribosomal RNA genes of *Escherichia coli*. Nucleic Acids Res. **12**:4653–4663.
28. **Steers, E., E. L. Foltz, B. S. Gravies, and J. Riden.** 1959. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. Chemother. **9**:307–311.
29. **Taubeneck, U.** 1962. Susceptibility of *Proteus mirabilis* and its stable L-forms to erythromycin and other macrolides. Nature (London) **196**:195–196.
30. **Thakker-Varia, S., A. C. Ranizini, and D. T. Dubin.** 1985. Ribosomal RNA methylation in *Staphylococcus aureus* and *Escherichia coli*: effect of the "MLS" (erythromycin resistance) methylase. Plasmid **14**:152–161.
31. **Trieu-Cuot, P., G. Gerbaud, T. Lambert, and P. Courvalin.** 1985. *In vivo* transfer of genetic information between gram-

positive and gram-negative bacteria. EMBO J. **4**:3583–3587.
32. **Uhlin, B. E., and K. Nordström.** 1977. R plasmid gene dosage effects in *Escherichia coli* K-12: copy mutants of the R plasmid R1*drd-19*. Plasmid **1**:1–7.
33. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
34. **Weisblum, B.** 1985. Inducible resistance to macrolides, lincosamides, and streptogramin type B antibiotics: the resistance phenotype, its biological diversity, and structural elements that regulate expression—a review. J. Antimicrob. Chemother. **16**(Suppl. A):63–90.