Plasmid-Mediated High-Level Resistance to Erythromycin in *Escherichia coli*

ANTOINE ANDREMONT,[1]* GUY GERBAUD,[2] AND PATRICE COURVALIN[2]

Service de Microbiologie Médicale, Institut Gustave-Roussy, 94805 Villejuif Cedex,[1] and Unité des Agents Antibactériens, U.A. Centre National de la Recherche Scientifique, Institut Pasteur, 75724 Paris Cedex 15,[2] France

Escherichia coli BM2195 was found to be resistant to high levels of erythromycin. This new resistance phenotype was due to the constitutive synthesis of an erythromycin esterase which inactivates the antibiotic. The gene conferring resistance to erythromycin in this strain is carried on a 61-kilobase self-transferable plasmid, pIP1100, belonging to incompatibility group X.

Enterobacteria, including *Escherichia coli*, are intrinsically resistant to low levels of macrolide, lincosamide, and streptogramin antibiotics (MICs of ≤500 μg/ml) (2). However, higher local antibiotic concentrations, in particular of erythromycin, are obtained in the lumen of the intestinal tract after oral administration of recommended therapeutic doses, and a reduction of the aerobic gram-negative flora of the intestinal tract is achieved (2). Thus, erythromycin has been successfully used for the prevention of travellers diarrhea (2) and has been proposed for the microbial modulation of the intestinal tract in immunocompromised patients (1). Under these conditions, strains of enterobacteria resistant to these high levels of erythromycin can be selected. Mutants of *E. coli* resistant to high concentrations of erythromycin and other macrolides have been isolated and shown to possess mutations affecting ribosomal protein L4 or L22 (28). Resistance of gram-positive cocci to macrolides is almost always associated with coresistance to lincosamides and streptogramin B-type antibiotics (6, 27). Plasmid-mediated resistance to macrolides, lincosamides, or streptogramins has not yet been reported in enterobacteria. In this study we describe an *E. coli* strain highly resistant (MIC >2 mg/ml) to erythromycin.

The properties of the strains used are listed in Table 1. Strain BM2195 was a blood isolate from a patient treated with erythromycin. *E. coli* IGR42, which is highly resistant to erythromycin and does not inactivate the antibiotic, was of human fecal origin (1). The method of Steers et al. (25) was used to determine the MICs of the antibiotics on Mueller-Hinton agar. The upper limiting factor of MIC determination was the solubility of the drugs. The inactivation of erythromycin by growing (16) or resting (4) cells and assay for aminoglycoside-modifying enzymes by the phosphocellulose paper-binding technique were as described (17). Isoelectric focusing in polyacrylamide gels of S100 preparations (21), purification of high-molecular-weight plasmid DNA (19), and agarose gel electrophoresis (24) were as described.

E. coli BM2195 encodes resistance to ampicillin, chloramphenicol, gentamicin, streptomycin, sulfonamide, tetracycline, and trimethoprim and to high levels of erythromycin (Table 1). In curing experiments with acridine orange and ethidium bromide (5), erythromycin and gentamicin resistances were lost en bloc (approximately 3% of 226 colonies

tested). These two characters were also lost spontaneously at a very low frequency (0.12% of 1,617 colonies tested). One cured isolate was studied further (strain BM2195-1). The genes conferring resistance to ampicillin, erythromycin, gentamicin, and streptomycin were transferred en bloc from BM2195 to *E. coli* BM694 by conjugation at a frequency of 10^{-4}. Selection for transfer of erythromycin or gentamicin resistance revealed cotransfer of all four resistances (in the 10 clones studied). One transconjugant, strain BM2507, was studied further. The MICs of macrolide, lincosamide, and streptogramin antibiotics for strains BM2195, BM2195-1, BM694, and BM2507 are shown in Table 2. Plasmid DNA from strain BM2195 was purified by ultracentrifugation in cesium chloride-ethidium bromide and used to transform *E. coli* HB101 (12). Three major classes of transformants were obtained: one had acquired resistance to ampicillin, erythromycin, gentamicin, and streptomycin (149 clones), another had acquired resistance to ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline (16 clones), and the third had acquired resistance to only ampicillin (937 clones). One transformant of each class (strains BM2526, BM2527, and BM2528 in Table 1) was studied further. Incompatibility testing (8) of the transconjugant BM2507 revealed that all of the acquired characters, i.e., resistance to ampicillin, erythromycin, gentamycin, and streptomycin, were borne by a single plasmid belonging to incompatibility (Inc) group X (18).

The plasmid DNA from strain BM2195, its cured derivative BM2195-1, transconjugant BM2507, and transformants BM2526, BM2527, and BM2528 was purified by ultracentrifugation and analyzed by agarose gel electrophoresis before and after digestion with *Hin*dIII endonuclease (data not shown). Comparative analysis of the phenotypes with the plasmid content in the individual strains led us to conclude that the wild-type strain BM2195 harbors three resistance plasmids (Table 1): plasmid pIP1100, encoding resistance to ampicillin, erythromycin, gentamicin, and streptomycin, with a molecular size of 61 kilobases and 11 *Hin*dIII-generated DNA fragments; pIP1101, encoding resistance to ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline, with a molecular size of approximately 123 kilobases; and pIP1102, encoding resistance to ampicillin, with a molecular size of 25 kilobases. Strain BM2195-1 was susceptible to erythromycin and gentamicin and had lost pIP1100. *E. coli* strain BM2507 was resistant to ampicillin, erythromycin, gentamicin, and strep-

---

* Corresponding author.

TABLE 1. Properties of the bacterial strains used

E. coli strain	Relevant characteristics and plasmid content[a]	Source (reference)
BM2195	Prototroph, mucoid, pIP1100, pIP1101, pIP1102	Wild-type strain
BM2195-1	Prototroph, mucoid, pIP1101, pIP1102	Curing of BM2195
BM694	Prototroph, gyrA	Spontaneous mutant of Cla (19)
HB101	hsdR⁻ hsdM⁻ recA13 supE44 lac⁻24 leuB6 proA2 thi-1 rpsL20	Boyer and Roulland-Dussoix, 1969 (12)
IGR42	Prototroph, Em	Wild-type strain
BM2507	Prototroph, gyrA, pIP1100	Conjugation BM2195 × BM694
BM2526	hsdR⁻ hsdM⁻ recA13 supE44 lac⁻24 leuB6 proA2 thi-1 rpsL20, pIP1100	Transformation pIP1100 × HB101
BM2527	hsdR⁻ hsdM⁻ recA13 supE44 lac⁻24 leuB6 proA2 thi-1 rpsL20, pIP1101	Transformation pIP1101 × HB101
BM2528	hsdR⁻ hsdM⁻ recA13 supE44 lac⁻24 leuB6 proA2 thi-1 rpsL20, pIP1102	Transformation pIP1102 × HB101

[a] Genetic symbols are as described by Bachmann (3) and Novick et al. (22). pIP1100 encodes Tra⁺ IncX Ap Em Gm Sm; pIP1101 encodes Tra⁺ Ap Cm Sm Su Tc; pIP1102 encodes Tra⁻ Ap.

tomycin after acquisition of pIP1100. Plasmids pIP1100, pIP1101, and pIP1102 were detected in strains BM2526, BM2527, and BM2528, respectively, after transformation (Table 1).

E. coli BM2195 and its derivative BM2195-1, strain BM694 and the transconjugant BM2507, and strain HB101 and transformants were examined for erythromycin-modifying activity by microbiological techniques (Fig. 1 and 2). Strains harboring plasmid pIP1100 were found to inactivate erythromycin and oleandomycin but none of the other commercially available macrolide, lincosamide, or streptogramin antibiotics. The inactivating enzyme conferred detectable resistance to erythromycin only (Table 2). Because of the high intrinsic resistance to oleandomycin, we did not see any difference in MICs for this compound. The modifying activity was found both in growing (Fig. 1) and in resting (Fig. 2) cells. The enzyme appeared extremely efficient with erythromycin as a substrate (half-time decay of 30 min). We did not find any inactivating activity in the culture medium. In addition, strains harboring plasmid pIP1100 were found to contain aminoglycoside acetyltransferase and adenylyltransferase activities but no phosphotransferase activity. Resistance to gentamicin and structurally related antibiotics was due to synthesis of a 3-acetyltransferase, and resistance to streptomycin and spectinomycin was due to the production of a 3″,9-adenylyltransferase (data not shown) (11, 20). Plasmid pIP1100 also encoded a TEM-1 beta-lactamase with an isoelectric point of about 5.4 (data not shown).

We have demonstrated that the gene conferring resistance to high levels of erythromycin in E. coli BM2195 is borne on a 61-kilobase plasmid, pIP1100, which belongs to incompatibility group X. The natural resistance of gram-negative bacteria to low levels of macrolides, lincosamides, and streptogramins appears to be due to cellular impermeability. L-forms are much more susceptible to macrolides than the corresponding normal bacteria (26), and E. coli mutants which are more than usually susceptible to these antibiotics are also permeable to other agents which do not normally affect E. coli (10). Until now, microbial degradation of erythromycin has been detected in steroid-transforming strains of Streptomyces spp. (14), in Lactobacillus spp. (13), and in Pseudomonas spp. (15) (for a recent review see reference 9). However, the biochemical and genetic bases of bacterial detoxification of the antibiotic have been poorly studied. E. coli BM2195 resisted high levels of erythromycin but remained susceptible to other macrolide, lincosamide, and streptogramin antibiotics (Table 2). This new resistance phenotype was due to the constitutive synthesis of an enzyme which inactivates the antibiotic (Fig. 1 and 2). We have determined the structure of the modified drug by physicochemical techniques (4), and the results obtained indicate that the new enzyme is an erythromycin esterase which hydrolyzes the lactone ring of the antibiotic. We have cloned and sequenced a pIP1100 DNA fragment conferring high-level resistance to erythromycin, and the resistance gene, designated ereA, was located in an open reading frame of 1,032 base pairs (23). The distribution of this new character in enterobacteria highly resistant to erythromycin which were isolated from human feces was studied by colony hybridization with an intragenic probe. The gene for

TABLE 2. MICs of various macrolide, lincosamide, and streptogramin antibiotics against E. coli strains

| Strain	MIC (μg/ml) of:								
	Erythromycin	Oleandomycin	Spiramycin	Josamycin	Midecamycin	Lincomycin	Clindamycin	Pristinamycin factor I	Pristinamycin factor II
BM2195	2,048	>2,048	512	512	1,024	2,048	512	512	512
BM2195-1	128	>2,048	512	512	1,024	1,024	512	512	512
BM694	64	2,048	256	512	512	1,024	256	512	512
BM2507	2,048	>2,048	256	512	512	1,024	256	1,024	512
IGR42	1,024	>2,048	>2,048	>512	512	1,024	256	1,024	>512
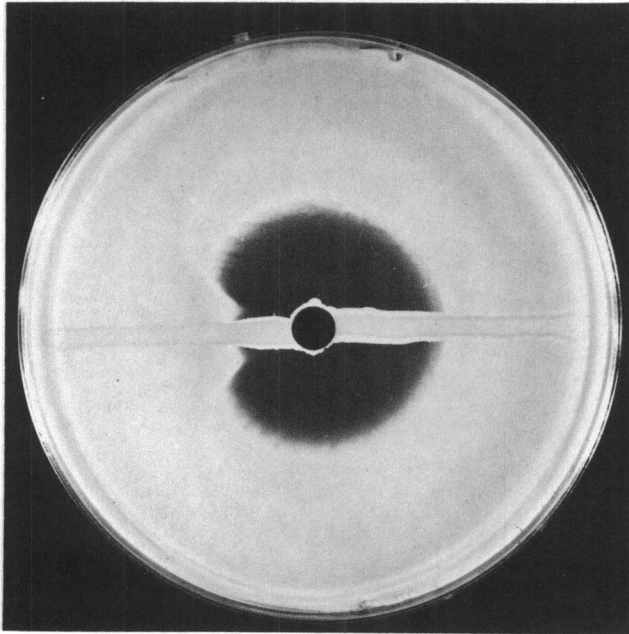
FIG. 1. Inactivation of erythromycin. To test for inactivation of erythromycin, *E. coli* BM2507 (erythromycin esterase producer) was streaked from the center leftwards and IGR42, a control nonproducer of esterase, was streaked from the center of the plate rightwards. The circular zone of inhibition of *Sarcina lutea* ATCC 9341 indicator produced by erythromycin in the center of the plate is distorted on the left side owing to destruction of erythromycin by BM2507. The paper disk contains 15 μg of erythromycin. A similar result was obtained with a 15-μg oleandomycin disk.

the erythromycin esterase was detected in strains of *E. coli* belonging to various biotypes, in *Klebsiella pneumoniae*, *Enterobacter agglomerans*, and in one strain of a coliform (M. Arthur, A. Andremont, and P. Courvalin, submitted for publication). It therefore appears that this supposedly new resistance gene is already widespread in nature.
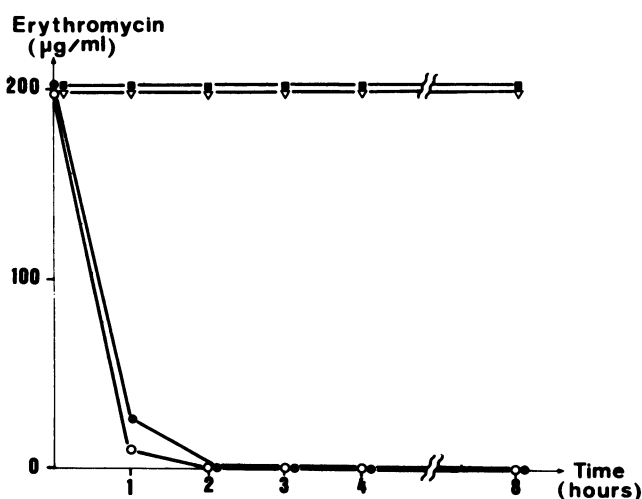

FIG. 2. Kinetics of inactivation of erythromycin by resting cells. Cells suspended in 0.1 M phosphate buffer (pH 8.0) containing 200 μg of erythromycin per ml were incubated at 37°C, and inactivation of the antibiotic was monitored by a microbiological technique (7). Symbols: ■, *E. coli* BM694; ∇, *E. coli* IGR42; ●, *E. coli* BM2507; ○, *E. coli* BM2195.

LITERATURE CITED

1. Andremont, A., P. Raibaud, and C. Tancrède. 1983. Effect of erythromycin on microbial antagonisms: A study of gnotobiotic mice associated with a human fecal flora. J. Infect. Dis. 148:579–587.
2. Andremont, A., and C. Tancrède. 1981. Reduction of the aerobic gram-negative bacterial flora of the gastro-intestinal tract and prevention of traveller's diarrhea using oral erythromycin. Ann. Inst. Pasteur 132:419–427.
3. Bachmann, B. J. 1983. Linkage map of *Escherichia coli* K-12, edition 7. Microbiol. Rev. 47:180–230.
4. Barthélémy, P., D. Autissier, G. Gerbaud, and P. Courvalin. 1984. Enzymatic hydrolysis of erythromycin by a strain of *Escherichia coli*: a new mechanism of resistance. J. Antibiot. 37:1692–1696.
5. Bouanchaud, D. H., M. R. Scavizzi, and Y. A. Chabbert. 1969. Elimination of ethidium bromide of antibiotic resistance in enterobacteria and staphylococci. J. Gen. Microbiol. 54:417–425.
6. Chabbert, Y. 1956. Antagonisme *in vitro* entre l'érythromycine et la spiramycine. Ann. Inst. Pasteur 90:787–790.
7. Chabbert, Y. A., and H. Boulingre. 1957. Modifications pratiques concernant le dosage des antibiotiques en clinique. Rev. Fr. Etud. Clin. Biol. 2:636–640.
8. Chabbert, Y. A., M. R. Scavizzi, J. L. Witchitz, G. R. Gerbaud, and D. H. Bouanchaud. 1972. Incompatibility groups and the classification of *fi⁻* resistance factors. J. Bacteriol. 112:666–675.
9. Courvalin, P., H. Ounissi, and M. Arthur. 1985. Multiplicity of Macrolide-Lincosamide-Streptogramin antibiotic resistance determinants. J. Antimicrobial Chemother. 16(Suppl.):91–100.
10. 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.
11. Davies, J., and D. I. Smith. 1978. Plasmid-mediated resistance to antimicrobial agents. Annu. Rev. Microbiol. 32:469–518.
12. Davis, R. W., D. Botstein, and J. R. Roth. 1980. Advanced bacterial genetics, p. 140. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. Dutta, G. N., and L. A. Devriese. 1981. Macrolide-lincosamide-streptogramin resistance patterns in *Clostridium perfringens* from animals. Antimicrob. Agents Chemother. 19:274–278.
14. Feldman, L. I., I. K. Dill, C. E. Holmlund, H. A. Whaley, E. L. Patterson, and N. Bohonos. 1964. Microbial transformations of macrolides antibiotics, p. 54–57. Antimicrob. Agents Chemother. 1963.
15. Flickinger, M., and D. Perlman. 1975. Microbial degradations of erythromycins A and B. J. Antibiot. 28:307–311.
16. Goldstein, F. W., A. Boisivon, P. Leclerc, and J. F. Acar. 1977. Sensibilité d'hémophilus sp. aux antibiotiques. Transfert de résistance à *Escherichia coli*. Pathol. Biol. 25:323–332.
17. Haas, M. J., and J. E. Dowding. 1975. Aminoglycoside-modifying enzymes. Methods Enzymol. 43:611–628.
18. Hedges, R. W., N. Datta, J. N. Coetzee, and S. Dennison. R factors from *Proteus morganii*. J. Gen. Microbiol. 77:249–259.
19. 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.
20. Labigne-Roussel, A., J. L. Witchitz, and P. Courvalin. 1982. Modular evolution of disseminated Inc 7-M plasmids encoding gentamicin resistance. Plasmid 8:215–231.
21. Matthew, M., A. M. Harris, M. J. Marshall, and G. W. Ross. 1975. The use of analytical isoelectric focusing for detection and identification of β-lactamases. J. Gen. Microbiol. 88:169–178.
22. 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.
23. Ounissi, H., and P. Courvalin. 1985. Nucleotide sequence of the

gene *ereA* encoding the erythromycin esterase in *Escherichia coli*. Gene **35**:271–278.

24. **Shinnick, T. M., E. Lund, O. Smithies, and F. R. Blattner.** 1975. Hybridization of labeled RNA to DNA in agarose gels. Nucleic Acids Res. **2**:1911–1929.

25. **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.

26. **Taubeneck, V.** 1962. Susceptibility of *Proteus mirabilis* and its

stable L-forms to erythromycin and other macrolides. Nature (London) **196**:195–196.

27. **Weisblum, B.** 1984. Inducible erythromycin resistance in bacteria. Br. Med. Bull. **40**:47–53.

28. **Wittmann, H. G., G. Stöffler, D. Apirion, L. Rosen, K. Tanaka, M. Tamaki, R. Takata, S. Dekio, E. Otaka, and S. Osawa.** 1973. Biochemical and genetic studies on two different types of erythromycin resistant mutants of *Escherichia coli* with altered ribosomal proteins. Mol. Gen. Genet. **127**:175.