Plasmid-Mediated Susceptibility to Intestinal Microbial Antagonisms in Escherichia coli

ANTOINE ANDREMONT,1* GUY GERBAUD,2 CYRILLE TANCÈDE,1 AND PATRICE COURVALIN2

Service de Microbiologie Médicale, Institut Gustave-Roussy, 94805 Villejuif Cedex,1 and Unité des Agents Antibactériens, U.A. Centre National de la Recherche Scientifique 271, Institut Pasteur, 75724 Paris Cedex 15,2 France

Received 18 March 1985/ Accepted 3 June 1985

Self-transferable plasmid pIP1100 confers to Escherichia coli an unusually high level of resistance (1 to 2 mg/ml) to erythromycin by production of an erythromycin esterase. The effect of pIP1100 on the destiny of E. coli strains in the intestines of gnotobiotic mice was studied. In germfree mice, pIP1100 was efficiently transferred to a plasmid-free E. coli recipient. Intestinal counts of the donor, the recipient, and the transconjugants were greater than 8.5 log CFU/g of feces. When erythromycin was added to the diet of the mice, counts of the plasmid-bearing strains were only slightly lowered and partial inactivation of erythromycin was observed in the feces. Transfer of pIP1100 also occurred in human-flora-associated mice. In this model all the E. coli strains were subject to microbial antagonisms caused by the anaerobic components of the flora. However, strains harboring pIP1100 were strongly inhibited (less than 2.5 log CFU/g of feces), whereas their plasmid-free counterparts persisted at much higher population levels (greater than 5.2 log CFU/g of feces). The ecological disadvantage conferred by pIP1100 to E. coli when a complex human flora was concomitantly present in the intestine of the mice persisted during erythromycin administration. These results provide an explanation for the low incidence of isolation of highly erythromycin-resistant E. coli strains despite the extensive use of the antibiotic.

Microbial antagonisms have been observed in the intestinal tracts of humans and of experimental animals. They can be due to the action of a single strain against another strain belonging to the same genus (11), to that of a defined mixture of strains against a strain of another genus (10), or to that of complex endogenous microflora against pathogenic (29) or nonpathogenic (1) exogenous strains. These antagonisms can be affected to various extents by antimicrobial agents (1).

The mechanisms proposed to account for the antagonisms exerted by the anaerobic intestinal flora of humans against members of the family Enterobacteriaceae include production of colicins or of volatile fatty acids, modification of bile acids, and competition for nutrients or for attachment sites on the mucosa (for a recent review see reference 15). Less attention has been paid to the factors influencing susceptibility of the target cells to bacterial antagonisms. For example, members of the family Enterobacteriaceae harboring certain antibiotic resistance plasmids survive less well in the human intestinal tract than do their plasmid-free counterparts (13). Transconjugants of an Escherichia coli strain have been shown to become established at a lower level than the susceptible parental strain in the digestive tracts of gnotobiotic mice in either the presence or the absence of human fecal bacteria (12). However, this ecological disadvantage disappeared during antibiotic administration and even after the end of the drug intake (12). Andremont et al. recently described E. coli BM2195, isolated from a human blood culture, which is resistant to high levels of erythromycin (MIC, ≥1 mg/ml) (A. Andremont, G. Gerbaud, and P. Courvalin, submitted for publication). This resistance phenotype is due to the presence of an erythromycin esterase which hydrolyzes the lactone ring of the antibiotic (4). The gene, designated ereA, encoding this new biochemical mechanism of resistance in BM2195 is borne by a 61-kilobase (kb) plasmid, pIP1100, capable of self-transfer to other E. coli cells. Plasmid pIP1100 belongs to incompatibility group X and confers resistance to ampicillin, gentamicin, and streptomycin. The nucleotide sequence of the gene ereA has been determined (H. Ounissi and P. Courvalin, Gene, in press), and this new resistance gene was detected in various members of the family Enterobacteriaceae of human fecal origin by colony hybridization with a 716-base-pair intragenic probe (M. Arthur, A. Andremont, and P. Courvalin, submitted for publication).

The destiny of strains harboring pIP1100 is of clinical importance, since intestinal strains inactivating antibiotics such as beta-lactams (24) or erythromycin (9) have been shown to interfere efficiently in vivo with antibiotic therapy. In the present study, we report the ecological disadvantage conferred by plasmid pIP1100 to strains of E. coli when they are associated with a human fecal flora in the intestines of gnotobiotic mice.

MATERIALS AND METHODS

E. coli strains. The sources and properties of the strains used in this study are listed in Table 1. Plasmid pIP135-1 (Tra+ Inc7-M Tc' Hg'; 70.4 kb) (16) was introduced into E. coli C600(pIP1100) by conjugation (6). E. coli BM212 organims harboring plasmids ColEl::Tn3 (Tra' Mob+ Ap'; 11 kb) (26) and Tp114 (Tra' IncII Km'; 62.5 kb) (14) were from our laboratory collection.

Determination of MICs. The method of Steers et al. (27) on Mueller-Hinton agar (Difco Laboratories) was used to determine the MICs of erythromycin.

Gnotobiotic mice. Adult germfree (GF) C3H mice (Centre de Sélection des Animaux de Laboratoire, Orléans, France) were maintained in plastic Trexler-type isolators. They were used either in the GF state or in the gnotobiotic state 2 weeks after weaning...
after they had been associated with a human fecal flora by intragastric and intrarectal inoculation (1). To facilitate the enumeration of *E. coli* clones, the complex flora used was depleted of all members of the family Enterobacteriaceae by selective antimicrobial modulation of the intestinal tract of the human donor with erythromycin (1). Precise analysis of the resulting flora in mice showed that total bacterial counts and composition in anaerobes were not significantly altered by this procedure and that most of the resistance to colonization by exogenous members of the family Enterobacteriaceae was retained (1). No antibiotic activity was detected in the feces of the recipient mice by a microbiological technique (7). No coliform strain could be isolated from the feces of human-flora-associated (HFA) mice by repeatedly plating the pellets either directly or after 24 h of growth in brain heart infusion broth on Drigalski agar (Institut Pasteur Production, Paris, France). Mice were given drinking water (pH 3) ad libitum and fed with a locally prepared sterile diet supplemented when necessary with erythromycin (10 g/kg of diet) (1). GF and HFA mice were inoculated intragastrically (1 ml of a 10^8-CFU/ml broth culture) with one or two *E. coli* strains. In the latter case, the plasmid-free strain was inoculated 5 days before the plasmid-carrying derivative.

**Bacterial counts in mouse pellets.** Freshly passed pellets were weighed and serially 10-fold diluted in physiological saline. *E. coli* strains were enumerated on Drigalski agar with or without erythromycin (400 mg/liter). The *E. coli* strains were discriminated on the basis of growth characteristics and lactase fermentation. Clones of *E. coli* BM21 lacking plpI100 were detected by the replica plating technique (17). In brief, colonies grown on Drigalski agar were transferred to agar medium containing 400 mg of erythromycin per liter with a velvet replicator. Clones which did not grow were considered to be lacking plasmid plpI100. Preliminary experiments indicated that this technique allows the detection of one plasmid-free clone per 10^2 bacteria in populations bearing plpI100 (data not shown). In every experiment, five clones of each phenotype were studied by the disk-agar diffusion test (20) and their plasmid content was analyzed by agarose gel electrophoresis of crude bacterial lysates (23) and by DNA-DNA hybridization (18).

**Assays of erythromycin in mouse pellets.** The concentration of erythromycin in mouse pellets was measured by the agar diffusion assay (7). Antibiotic medium 5 (Difco) at pH 8.0 and Bacillus subtilis ATCC 6633 were used. Dilutions of samples and of control erythromycin were made in phosphate-buffered saline (pH 8.0).

**Preparation of plasmid DNA.** High-molecular-weight (23) and pBR322 and derivative pAT66 plasmid DNA (8) were prepared as described previously.

**Agarose gel electrophoresis.** Agarose gel electrophoresis was done as described previously (25).

**Hybridization.** ^32^P-labeled pAT66 DNA was hybridized to bacterial colonies contained on nitrocellulose filters as described previously (19). Hybrid plasmid pAT66 consists of pBR329 (Tra^- Mob^- Ap^- Cm^- Tc^-) with a 716-base-pair EcoRI-PstI insert of plasmid plpI100 DNA internal to the structural gene *ereA* for the erythromycin esterase.

**Chemicals.** 5'-[α-^32^P]ATP, triethylammonium salt, was obtained from the Radiochemical Centre, Amersham, England. The antibiotics were provided by the following laboratories: gentamicin, Schering Corp.; ampicillin, Bristol Laboratories; streptomycin, Pfizer Inc.; chloramphenicol and erythromycin, Roussel-Uclaf; nalidixic acid, Winthrop Laboratories; Sarkosyl (sodium N-lauroyl sarcosinate), Colgate-Palmolive.

**Statistical analysis.** All counts were converted into common logarithms. The log distribution of intestinal bacterial counts has been shown to be normal (5). Student's *t* test was used for the comparison of mean values. When necessary, counts of less than 2.00 log_{10} CFU/ml were converted to 2.00

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**TABLE 1. Properties of the bacterial strains used**

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Relevant characteristics and plasmid content</th>
<th>MIC of erythromycin (mg/liter)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM2507</td>
<td>Prototroph, gyrA; plpI100 Tra^- IncX: Ap^- Em^- Gm^- Sm^-</td>
<td>2.048</td>
<td>Andremont et al. 1985</td>
</tr>
<tr>
<td>C600</td>
<td>thr leu thi lacY; plpI100 Tra^- IncX: Ap^- Em^- Gm^- Sm^-</td>
<td>32</td>
<td>E. Wollman</td>
</tr>
<tr>
<td>BM21</td>
<td>Prototroph, gyrA; plpI100 Tra^- IncX: Ap^- Em^- Gm^- Sm^-</td>
<td>32</td>
<td>E. S. Anderson</td>
</tr>
<tr>
<td>BM21(pIP1100)</td>
<td>Prototroph, gyrA; plpI100 Tra^- IncX: Ap^- Em^- Gm^- Sm^-</td>
<td>1.024</td>
<td>Conjugation in vitro BM2507</td>
</tr>
<tr>
<td>BM2507</td>
<td>BM21(pIP1100)</td>
<td>1.024</td>
<td>Conjugation in vitro BM2507</td>
</tr>
</tbody>
</table>

* Genetic symbols are by the convention of Bachmann (3) and Novick et al. (22).
* Designation of plasmid and its phenotypic characteristics are aligned.

**TABLE 2. Fecal counts of *E. coli* strains in gnotobiotic mice**

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Mono-inoculated GF mice</th>
<th>Mono-inoculated HFA mice</th>
<th>Di-inoculated GF mice</th>
<th>Di-inoculated HFA mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before treatment</td>
<td>During treatment</td>
<td>Before treatment</td>
<td>During treatment</td>
</tr>
<tr>
<td>BM21</td>
<td>9.30 ± 0.34</td>
<td>&lt;2.00</td>
<td>5.25 ± 0.30</td>
<td>&lt;2.00</td>
</tr>
<tr>
<td>C600(pIP1100)</td>
<td>9.25 ± 0.22</td>
<td>8.50 ± 0.56</td>
<td>2.70 ± 0.93</td>
<td>2.16 ± 0.36</td>
</tr>
<tr>
<td>BM21(pIP1100)</td>
<td>9.40 ± 0.12</td>
<td>8.95 ± 0.11</td>
<td>N/D</td>
<td>ND</td>
</tr>
<tr>
<td>C600</td>
<td>9.68 ± 0.70</td>
<td>&lt;2.00</td>
<td>5.27 ± 0.72</td>
<td>&lt;2.00</td>
</tr>
</tbody>
</table>

* Data are mean log_{10} CFU per gram of feces ± standard error of the mean (six mice per group).
* Inoculation with *E. coli* BM21 and C600(pIP1100).
* Ten days after the inoculation of the *E. coli* strains.
* Ten days after the beginning of erythromycin administration.
* UD, Undetected by the replica plating technique.
* ND, Not done.
* Four mice only.
RESULTS

In control experiments, *E. coli* BM21, BM21(pIP1100), C600, and C600(pIP1100) could be individually established at high population levels in the intestines of GF mono-associated mice (Table 2). By comparison, colonization was impaired by previous association of the mice with a human fecal flora. In mono-inoculated HFA mice, plasmid-free *E. coli* BM21 and C600 persisted at an intermediate level, whereas strain C600(pIP1100) was almost eliminated.

In experiments with di-inoculated animals, GF and HFA mice were inoculated at 5-day intervals with *E. coli* BM21 and *E. coli* C600(pIP1100), respectively. *E. coli* BM21 (pIP1100) transconjugants obtained in vivo could be isolated from the feces of both groups of animals (Table 2). The presence of pIP1100 in the transconjugants was confirmed by agarose gel electrophoresis of crude bacterial lysates and by DNA-DNA hybridization (Fig. 1 and 2). In GF di-inoculated mice, counts of *E. coli* BM21 and BM21(pIP1100) were high and similar to those observed in mono-inoculated GF animals (9.51 ± 0.15 and 9.00 ± 0.33 log CFU/g of feces, respectively [Table 2]). Although the counts were high, a slight inhibition of strain C600(pIP1100) was observed in di-inoculated GF mice (8.61 ± 0.53 log CFU/g of feces). By contrast, in HFA di-inoculated mice, *E. coli* BM21 persisted at a level similar to that obtained in HFA mono-inoculated mice, but both donor strain C600(pIP1100) and transconjugants BM21(pIP1100) were either hardly or not detectable (2.55 ± 0.55 and 2.00 log CFU/g of feces, respectively).

Concentrations of erythromycin obtained in the feces of gnotobiotic mice under erythromycin treatment are presented in Fig. 3. Antibiotic concentrations were significantly higher in HFA mice devoid of strains harboring plasmid pIP1100 than in mice with high fecal counts of strains carrying pIP1100 (*P* < 0.01). Concentrations of erythromycin were not significantly different in mice mono-associated with either *E. coli* BM21(pIP1100) or *E. coli* C600(pIP1100). During administration of erythromycin, *E. coli* BM21 was eliminated from both mono- and di-inoculated mice (Table 2). Conversely, strains C600(pIP1100) and BM21(pIP1100),
although at significantly lower levels, persisted in both groups of animals. Finally, in HFA mice no significant modification of the counts of the strains harboring pIP1100 was observed when erythromycin was added to the diet.

DISCUSSION

The physiological properties of strains harboring plasmid pIP1100 are of clinical importance, since dense intestinal populations of these strains partially inactive erythromycin in vivo (Fig. 3). The concentrations of erythromycin obtained in the feces of gnotobiotic mice (Fig. 3) were similar to those reported for this model (1) or observed in humans under treatment with usual therapeutic regimens of erythromycin base (21). This observation emphasizes the relevance of our results to the ecology of the intestinal tracts of humans.

Oral erythromycin has been shown to eliminate members of the family Enterobacteriaceae from the intestinal flora of humans (2). This effect has been sustained for up to 3 weeks (28). The high levels of the local concentrations of the antibiotic in the human intestinal tract, which are much higher than the MICs of erythromycin for endogenous members of the family Enterobacteriaceae (2). Elimination of members of the family Enterobacteriaceae is associated with few modifications of the intestinal microbial antagonisms exerted by the anaerobic flora against exogenous microorganisms (1). Selective antimicrobial modulation of the intestinal tract with erythromycin has been used for the prevention of infections in neutropenic patients with hematological malignancies (B. Bellaiche, H. Richet, M. C. Escande, P. H. Lagrange, and R. Zittoun, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 24th, Washington, D.C., abstr. no. 381, 1984), because intestinal colonization with aerobic gram-negative bacteria is the major harbinger of bacteremia in this clinical situation (C. Tancrède and A. Andremont, J. Infect. Dis., in press).

Erythromycin has also been used to prevent intestinal colonization by enteropathogenic members of the family Enterobacteriaceae in travelers (2). Under these conditions, the presence or of the recolonization by members of the family Enterobacteriaceae highly resistant to erythromycin is a potential hazard. High-level resistance to erythromycin in E. coli and also, most probably, in other gram-negative bacteria can be secondary to mutations affecting ribosomal proteins (30) or to the acquisition of an erythromycin-modifying enzyme (Andremont et al., submitted for publication). In fact, an E. coli mutant of clinical origin highly resistant to erythromycin was previously shown to be able to colonize the intestinal tract of HFA mice before, during, and after erythromycin treatment (1). The results of the present study clearly demonstrate that this is not the case for E. coli strains harboring plasmid pIP1100 (Table 2). Transfer of pIP1100 was observed in the intestinal tract of di-inoculated gnotobiotic mice and was not hindered by the dense anaerobic flora of HFA mice. However, both donor and transconjugants were strongly inhibited in the intestines of HFA mice, whereas the plasmid-free recipient persisted at much higher concentrations. Since the specific inhibition of E. coli strains harboring pIP1100 was not observed in mono- or di-inoculated GF mice in which no other bacteria were present, it is inferred that the presence of this plasmid confers on E. coli a specific susceptibility to the microbial antagonisms exerted by the human fecal flora harbored by the HFA mice. By contrast with results reported for other resistance plasmids (12), the ecological disadvantage mediated by pIP1100 in HFA mice did not disappear under antibiotic treatment. Under these conditions, no increase in the population levels of the strains harboring pIP1100 was observed. The presence of E. coli BM21(pIP1100) and C600(pIP1100) in counts over 6.00 log CFU/g of feces in GF mice inoculated with these two strains and treated with erythromycin indicates that administration of the antibiotic does not account for the persistence of this drastic inhibition. Erythromycin treatment had some effect on the level of colonization by strains harboring pIP1100 when the pretreatment counts were high. This effect is probably related to the fact that the MICs of erythromycin for E. coli strains bearing pIP1100 (1,024 to 2,048 mg/liter; Table 1) and the concentrations of the drug recovered from the feces of the animals (1.040 to 2.220 mg/g; Fig. 3) are within the same range. This effect is not observed with more resistant strains (MIC, >4,096 mg/liter) (1). This finding is consistent with the low prevalence of human intestinal colonization by members of the family Enterobacteriaceae highly resistant to erythromycin (A. Andremont, H. Sancho-Garnier, and C. Tancrède, submitted for publication), despite the extensive use of this antibiotic over the past 20 years. This observation favors further use of oral erythromycin for selective antimicrobial modulation of the intestinal tract.

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LITERATURE CITED