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D. E. Corpet, S. Lumeau, F. Corpet. Minimum antibiotic levels for selecting a resistance plasmid in a gnotobiotic animal model.. Antimicrobial Agents and Chemotherapy, 1989, 33 (4), pp.535-40. hal-00433273

**HAL Id: hal-00433273**

**<https://hal.science/hal-00433273>**

Submitted on 18 Nov 2009

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## Minimum Antibiotic Levels for Selecting a Resistance Plasmid in a Gnotobiotic Animal Model

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**The minimum antibiotic concentrations for selecting an R plasmid in vivo were determined in germfree rates colonized by two isogenic strains of *Escherichia coli*, one of which carried an R plasmid. Seventy groups of three gnotobiotic mice were given low doses of ampicillin, colistin, flumequin, gentamicin, tetracycline, or streptomycin via drinking water for 2 weeks. The equilibrium between susceptible and resistant populations of bacteria was monitored daily in feces and compared with that of control mice given pure water. This model yielded reproducible data, and dose and response were strongly correlated. The minimum selecting doses ranged from 0.9 to 12.8 µg/ml of water, depending on the antibiotic and the R plasmid. The use of mathematical models and complementary in vitro experiments accounted for the effect of the low antibiotic levels.**

Antibiotic residues may be found in meat or milk after antimicrobial chemotherapy (19). Surveys indicate that these residues are of rare occurrence and at concentrations generally between 0.1 and 1 µg/g (27): for example, an antibiotic could be detected in 5.3% of the pig and calf carcasses in the United States in 1973, and 81% of these contaminated carcasses contained less than 1 µg of antibiotic (usually streptomycin) per g of meat (26). Can these residues be a hazard for the human consumer? What is the no-effect level of an antibiotic on the proportion of fecal R-plasmid-bearing bacteria? To answer these questions, experiments have been conducted in human volunteers and in vitro, but their results appear to be contradictory.

In human volunteers, per os administration of 1.5 mg of ampicillin or 1 mg of oxytetracycline per day failed to produce any significant effect on resistant bacteria (1, 4). When volunteers were given low levels of oxytetracycline for long periods, resistant enterobacteria occurred sporadically in some of the individuals who ingested 10 mg of the drug per day, but smaller intakes had no effect (13). However, because of contaminations from food, the concentration of resistant *Escherichia coli* in feces varies enormously in humans. Thus, in the same volunteer it ranged from an undetectable level to a level at which resistant bacteria overcome susceptible *E. coli* (4, 5).

Hence, a very large sample of volunteers would be required to evidence any significant change in the level of resistance in human gut flora.

In a continuous-flow culture of three clones of *E. coli* K-12 strains, one of which carried an R plasmid, the minimum antibiotic concentration that selected an R plasmid was found to be 0.25 µg/ml (15). However, this in vitro model did not take into account three interfering ecological factors: in vivo, antibiotic residues are absorbed or inactivated; the drug may alter the dominant anaerobic microflora; and the ecological conditions are strict anaerobiosis, low flow rate, and limiting substrate (3). There is good evidence that bacteria multiply much more slowly in an experimental animal than in vitro. The generation time may be lengthened from 30 min up to 20 h (8), which might explain the discrepancies between the results of in vitro and in vivo experiments.

To overcome the experimental difficulties encountered in studies of humans, animal models have been used, with the following results. (i) A selective pressure was demonstrated in beagles given food containing 10 µg of oxytetracycline per g but not with food containing 2 µg of oxytetracycline per g (24). (ii) When germfree mice were dosed with the complex fecal flora of a human donor, their fecal flora was similar to that of the human donor (22). When such gnotobiotic mice were

continuously given 0.5 µg of chlortetracycline or ampicillin per ml of water, a mean 30-fold increase in the proportion of resistant *E. coli* was observed in their feces, compared with the levels for control mice harboring the same flora (4). This antibiotic level may correspond to that ingested by a human consuming 150 g of meat containing 3 µg of residue per g (4). The main drawback of this last experimental system is that it is impossible to obtain exactly the same flora twice, and hence to duplicate an experiment, because of the variability of the human donor flora.

In this study, we determined the minimum antibiotic concentrations selecting a resistance plasmid *in vivo* by using a new animal model. It combined germfree mice with two isogenic strains of *E. coli*, one of which carried an R plasmid. The gnotobiotic mice were given, via drinking water, small doses of ampicillin, colistin, flumequin, gentamicin, tetracycline or streptomycin, antibiotics which are widely used in veterinary practice. With this simple model, reproducible data were obtained, and the minimum selecting doses ranged from 0.9 to 12.8 µg/ml of water. The use of mathematical models and complementary *in vitro* experiments enabled us to account for the effects of these doses.

## MATERIALS AND METHODS

**Bacterial strains.** *E. coli* plasmid-free EMO was used in all experiments. It was isolated from the stool of a healthy human and cured of a cryptic plasmid. It was susceptible to all the antibiotics tested, it did not carry any chromosomal marker of resistance but could be detected by its ability to ferment raffinose, and it did not contain any covalently closed circular form of DNA (9). In most experiments, we used an R-plasmid-bearing strain obtained by conjugating the EMO strain with a multi-resistant strain isolated from human urine. It carried the pYD1 conjugative plasmid, which confers resistance to ampicillin, gentamicin, streptomycin, tetracycline, and 10 other antibiotics. This plasmid was 72 to 79 megadaltons in size when determined by agarose gel electrophoresis or contour length measurement (10). The *in vivo* elimination of a pYD1-

bearing strain by its plasmid-free counterpart has been demonstrated previously (9).

Other plasmid-bearing strains were obtained by mating strain EMO with tetracycline-resistant *E. coli* strains obtained in our laboratory either from collections (plasmids RP4 [Tra<sup>+</sup> Amp<sup>r</sup> Kan<sup>r</sup> Tet<sup>r</sup>, 36 megadaltons] [25] and R270 [Tra<sup>+</sup> Suf<sup>r</sup> Tet<sup>r</sup>, 70 megadaltons] [15]) or from fresh human stools (plasmid pC1 [Tra<sup>+</sup> Tet<sup>r</sup>]). No chromosomal mutant was used, so that all strains were isogenic. They were all susceptible to colistin and flumequin.

All strains were kept at -25°C in a 45% glycerol broth supplemented with 10 µg of tetracycline per ml for the plasmid-bearing strains. Before inoculation into the mice, all strains were cultured overnight at 37°C in an antibiotic-free nutrient broth and diluted in phosphate buffer (0.75 g of KH<sub>2</sub>PO<sub>4</sub> per liter -- 3 g of K<sub>2</sub>HPO<sub>4</sub> per liter) on the basis of their optical density at 400 nm.

**In vitro plasmid transfer.** The transfer rate constant of plasmids was measured by mating the exponentially growing cells at a donor/recipient ratio of 1:1 in liquid brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at 37°C for 30 min. The donor strains were EMO carrying pYD1, RP4, R270, and pC1, and the recipient strain was a rifampicin-resistant mutant of the plasmid-free EMO strain. Rifampicin (100 µg/ml) and tetracycline (20 µg/ml) were used to select transconjugants. Appropriate dilutions were plated onto selective media. The transfer rate constant was estimated by the ratio of transconjugants to donor cells measured at 1 h. It is an approximation of the rate proposed by Levin et al. (16), but it allows for a rough comparison of the relative fertilities of the plasmids considered.

**Experimental design.** Each experiment was conducted on three germfree mice maintained in one isolator. The mice were given a first inoculum containing 10<sup>6</sup> viable cells of plasmid-free *E. coli* and, 3 days later, a second inoculum containing 10<sup>4</sup> cells of R-plasmid-bearing *E. coli*. Antibiotic treatment started 6 h after the second bacterial inoculation. For the control groups, the drinking water in the isolators was drug free, and for the experimental groups, it was supplemented with ampicillin, colistin, flumequin, gentamicin, tetracycline, or streptomycin, at concentrations

ranging from 0.5 to 256 µg/ml. In all, 70 experiments were conducted, one on each of 70 groups of three mice. Antibiotic concentrations in drinking water and feces were assayed by a bacteriological method. The number of total and resistant *E. coli* organisms in the feces of the mice was determined daily for 10 to 15 days by comparative plate counting. The kinetics of the R-plasmid-bearing *E. coli* populations in the experimental and control groups were compared.

**Gnotobiotic mice.** Two hundred and ten adult OF1 germ-free mice (IFFA Credo, Lyon, France) were maintained in plastic isolators, fed ad libitum a commercial diet sterilized by irradiation (4 Mrad), and supplied with autoclaved acidified drinking water (pH 2.5). Before the start of an experiment, three mice were transferred into a separate isolator. After confirmation that they were germfree or monocolonized (i.e., germfree but harboring a single pure strain), the mice were given 0.5 ml of a calibrated inoculum at 11 a.m. It was administered directly into the stomach by gavage. Freshly voided feces were collected at the anus of the mice 5 days a week at 10 a.m., and the pooled fecal pellets were processed within 30 min of collection.

**Bacterial counts.** Fresh pooled feces were diluted 10-fold in sterile saline, and 0.2-ml fractions were plated onto deoxycholate agar (DCA; Difco) in petri dishes containing either no antibiotic or 20 µg of tetracycline per ml of agar. The total viable number of *E. coli* isolates per gram of fresh feces was determined from drug-free DCA plates, and the number of resistant *E. coli* isolates was determined from the drug-supplemented plates. During each experiment, we checked twice that there were the same numbers of fecal tetracycline-resistant bacteria and of streptomycin- and ampicillin-resistant bacteria. Hence, the number of tetracycline-resistant bacteria was considered a good indicator of the number of plasmid-bearing bacteria. We also checked that the DCA medium and the antibiotics tested against *E. coli* strains had no synergistic inhibitory effects. Results were expressed as the decimal logarithms of the number of bacteria.

**Antibiotic administration and assays.** Except for the ampicillin, which was given every 2 days, the antibiotics were added to the

drinking water twice a week, always after sterilization by filtration (pore size, 0.45 µm). The supplemented drinking water samples were assayed by the cylinder plate method with *Bacillus subtilis* (or with *Bacillus cereus* for tetracycline). Fecal samples were assayed for their antibiotic concentrations after 1/3 dilution and centrifugation at 800 x g for 5 min. Feces were diluted in distilled water (pH 6.8 for ampicillin and free tetracycline), in pH 4.5 buffer for total tetracycline, or in pH 7.9 buffer for streptomycin and gentamicin (19). Fecal supernatants were assayed in a 100-µl stainless steel cylinder with the above-mentioned germs.

**In vitro experiments.** Strains were grown in a liquid medium containing, per liter, 2 g of yeast extract (Difco), 2 g of casein hydrolysate (Difco), 4 g of NaCl, 0.5 g of KH<sub>2</sub>PO<sub>4</sub> and 1.5 g of K<sub>2</sub>HPO<sub>4</sub> (pH 7.0). A log-phase culture of the strain tested was inoculated in 30 ml of the medium and incubated for 24 h at 37°C in a shaking water bath. Samples were taken every 30 min and measured for optical density at 400 nm. The number of CFU was determined on DCA plates at 0, 2, 8, and 24 h. Data were plotted and computerized to determine curve coefficients.

**Mathematical models.** The competition between the plasmid-free and plasmid-bearing strains in the gut of mice was simulated by the mathematical model proposed by Freter (11) with some modifications (3). In this model, the number of newly formed plasmid-free bacteria per unit of time is augmented by the growth and reduced by the flow rate washout, and these changes can be formalized by the following equation:

(1)

$dN_s/dt = N_s(\psi_s - \rho)$  where  $N_s$  is the number of susceptible plasmid-free bacteria per gram of gut content,  $dN_s/dt$  indicates the differentiation of  $N_s$  with respect to time,  $\psi_s$  is the growth rate of the susceptible bacteria, and  $\rho$  is the flow rate due to intestinal transit.

(2)

$dN_r/dt = N_r(\psi_r - \rho)$  is the same equation applied to the number of resistant plasmid-bearing bacteria per gram,  $N_r$ , which is augmented by the growth rate,  $\psi_r$ , and reduced by the flow rate,  $\rho$ .

The concentration of the limiting substrate is increased by the intestinal transit which supplies new nutrients and reduced by the growth of both the susceptible and resistant bacterial populations:

$$dL/dt = \rho(L_i - L) - (\psi_s \cdot N_s + \psi_r \cdot N_r)/Y \quad (3)$$

where  $L$  is the concentration of the limiting substrate in the compartment considered,  $L_i$  is the initial concentration of substrate entering the compartment, and  $Y$  is the yield constant of the limiting substrate (i.e., the number of bacteria formed divided by the weight of the limiting substrate).

Both growth rates follow the classical Monod equation (20):

$$\psi_s = \mu_s \cdot L/(Ks_s + L) \quad (4)$$

$$\psi_r = \mu_r \cdot L/(Ks_r + L) \quad (5)$$

where  $\psi_s$  and  $\psi_r$  are the growth rates of susceptible and resistant bacteria, respectively,  $\mu_s$  and  $\mu_r$  are the maximum growth rates (at the optimal level of the limiting substrate), and  $Ks_s$  and  $Ks_r$  are the saturation constants (which are numerically equal to the substrate concentration at which half the maximum growth rates are obtained). We previously built more sophisticated models which took into account the plasmid transfer and segregation (16) and the attachment of bacteria to the intestinal wall (11), but these factors appeared to be of minimal influence in the present study. To simulate in vitro batch experiments, the present model was also used in simplified form by setting the flow rate at zero.

A computer program was devised and used to evaluate the data from in vivo and in vitro experiments by numerical simulation methods. It was based on a fifth-order Runge-Kutta kernel, written in Turbo Pascal, and used on an 8086 XT microcomputer. The numerical values of the parameters were taken from data previously obtained in vitro (14) or in vivo (11) and modified by trial and error to fit the present experimental data.

## RESULTS

### Equilibrium in the absence of antibiotic.

When plasmid-free *E. coli* EMO alone was inoculated into germfree mice, it became established 1 day later at the high population level of  $10^{9.5}$  cells per g of feces. After subsequent inoculation of these mono-colonized mice with a tetracycline-resistant strain of *E. coli* EMO containing either R plasmid RP4 or R270, the resistant strains became dominant and rapidly outnumbered the plasmid-free strain. In contrast, when the mice mono-colonized by the plasmid-free strain were given *E. coli* EMO harboring plasmid pC1 or pYD1, these resistant populations were antagonized by EMO and their number slowly decreased (Fig. 1, CON). This control experiment was repeated 10 times with pYD1 and 4 times with pC1 (Table 1).

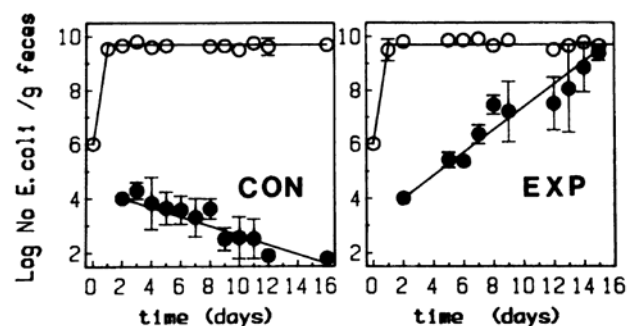


FIG. 1. Competition between two isogenic strains of *E. coli* in gnotobiotic mice drinking pure water (CON) or 2  $\mu$ g of streptomycin per ml of water (EXP).

Germfree mice were inoculated on day zero with  $10^6$  plasmid-free *E. coli* EMO bacteria (O) and on day 2 with  $10^4$  plasmid pYD1-bearing bacteria (●). Each point represents six groups (CON) or two groups (EXP) of three mice each. Curves in panel CON were calculated with the mathematical model given in the text, with the following numerical values:  $p = 0.08$  per h;  $\mu_s = \mu_r = 1.66$  per h;  $Ks_s = 0.0009$  g/liter;  $Ks_r = 0.001$  g/liter;  $L_i = 0.005$  g/liter;  $Y = 10^{12}$  bacteria per g. Equation 6 replaces equation 1 for calculation of the curves in panel EXP, and the numerical value of AB was set at 0.06 per h.

TABLE 1. Effects of antibiotics on the number of resistant *E. coli* cells in germfree mice colonized by two isogenic *E. coli* strains

Concn ( $\mu\text{g/ml}$ )	Log no. of resistant <i>E. coli</i> with drug <sup>a</sup> :														
	Tetracycline			Gentamicin			Streptomycin			Ampicillin			Tetracycline <sup>b</sup>		
	Mean	SD	No. of expts	Mean	SD	No. of expts	Mean	SD	No. of expts	Mean	SD	No. of expts	Mean	SD	No. of expts
0	3.0 <sup>c</sup>	1.6	10	3.0 <sup>c</sup>			3.0 <sup>c</sup>			3.0 <sup>c</sup>			1.9	1.4	4
0.5			0			0			0	3.4		1			0
1			0			0	5.1	0.8	2	4.9		1			0
2	3.4	1.2	7	4.8	0.2	2	6.4	0.4	2	6.7		1			0
4	3.3		1	5.9		1	7.5	1.8	3	6.4	1.3	2			0
8	5.9	1.8	3	7.9		1	8.9		1	9.4		1	3.3	0.9	2
16	6.5	0.7	2	9.2		1	9.4	0.0	2			0	5.4	0.9	2
32	7.4	1.3	2			0			0			0	9.0	0.7	2
64	9.4		1			0			0			0			0

a. Data are log number of resistant *E. coli* in pooled feces of gnotobiotic mice harboring a plasmid-free strain, 5 days after inoculation with  $10^4$  pYD1 plasmid-bearing cells. Drugs were given via drinking water.

b. Data shown in this column were obtained with plasmid pC1 instead of pYD1.

c. This datum for control groups was copied from the first tetracycline column, since the 10 experiments were not repeated.

**Effect of antibiotics on kinetics.** When 2  $\mu\text{g}$  of streptomycin per ml was added to the drinking water of mice di-colonized by the plasmid-free strain and the strain bearing plasmid pYD1, the number of resistant bacteria in feces increased slowly (Fig. 1, EXP). With 4  $\mu\text{g}$  of streptomycin per ml, the population of resistant bacteria grew rapidly, and after 6 days of drug administration, the plasmid-bearing cells out-numbered the plasmid-free cells. Similar kinetics were observed with ampicillin, gentamicin, and tetracycline (data not shown).

**Minimum selecting level.** The number of drug-resistant bacteria in the feces of mice at 5 days after inoculation with  $10^4$  plasmid-bearing bacteria was chosen so as to reflect the information obtained from the kinetic experiments. Ampicillin, gentamicin, tetracycline, and streptomycin increased the number of resistant bacteria (Table 1), with a strong correlation between the number at day 5 and the  $\log_2$  of the drug concentration in water (r ranged from 0.86 to 0.99). The minimum selecting level was defined as the drug

concentration in water which led to a resistant bacterial population of  $10^5$  bacteria at day 5, which corresponds to a 10-fold increase in the resistant population compared with that of the inoculum and to a 100-fold increase compared with the control group. Minimum selecting levels were, per ml of drinking water, 0.9  $\mu\text{g}$  of streptomycin, 1  $\mu\text{g}$  of ampicillin, 2.3  $\mu\text{g}$  of gentamicin, 6.5  $\mu\text{g}$  of tetracycline with the pYD1 plasmid, and 12.8  $\mu\text{g}$  of tetracycline with the pC1 plasmid.

To assess the effects of non-selecting antibiotics by using the same animal model, colistin and flumequin were administered to groups of mice via the drinking water at concentrations ranging from 1 to 64  $\mu\text{g/ml}$  (flumequin) or 1 to 256  $\mu\text{g/ml}$  (colistin). In 13 experiments lasting 2 weeks or more, these drugs did not reduce the level of the EMO plasmid-free strain, nor did they increase the fecal excretion of pYD1-bearing bacteria (data not shown). Also, in the three groups of mice which were given more than 64  $\mu\text{g}$  of colistin per ml of water, the plasmid-bearing bacteria

were eliminated faster than in the control groups and no plasmid-bearing *E. coli* cells were detected in feces at day 5.

#### Antimicrobial concentrations in feces.

Antibiotic concentrations were measured in drinking water and in fecal samples from some experimental groups of mice. In the water, a mean proportion of 95% of the theoretical dose was found (Table 2). In fecal samples, the tetracycline and streptomycin concentrations averaged 34% of the concentration in the water, and gentamicin and ampicillin concentrations averaged 13% (Table 2). The groups of mice given water supplemented with 8 µg of ampicillin per ml constituted an exception, because only 49% of the dose was found in the water and only 2% of the concentration in the water was found in the feces. This might be due to antibiotic absorption or to destruction by the resistant strain, both of which have already been studied in gnotobiotic animals (7).

TABLE 2. Antibiotic concentrations in feces of gnotobiotic mice during drug administration via the drinking water

Drug	Water dose (µg/ml)	Concentration (µg/ml) in water			Concentration (µg/g) in feces		
		Mean	SD	No. of assays	Mean	SD	No. of assays
Tetra-cycline	8	6.0	4.6	5	1.9	0.8	5
	16			0	2.8	0.4	3
	32	30.7		1	12.3	1.6	6
Genta-micin	4	4.6	3.0	10	0.6	0.5	12
	8	5.0	4.8	4	0.7	0.3	5
Strepto-mycin	4	4.3	1.4	8	1.4	1.0	15
	8	9.6	7.3	3	2.8	1.7	6
Ampi-cillin	4	3.5	1.7	14	0.4	0.2	13
	8	3.9	1.1	14	0.1	0.1	6

A complementary experiment showed that an ampicillin dose of 1.2 mg per day was detoxified in seven gnotobiotic mice harboring a resistant *E. coli* strain, since the concentration of ampicillin in ceca was 1.3% of the concentration in the drinking water, versus 50% in eight mice monocolonized by a susceptible strain (full data not shown). The very low ampicillin concentration in mouse feces may thus reflect the concentration in the cecum (Table 2). Since streptomycin, gentamicin, and tetracycline are minimally absorbed from the large intestine and are not inactivated outside the bacterial cells, the antibiotic concentration in the cecum can be about half the concentration in feces because of water absorption in the colon. The concentration of free tetracycline in feces which was assayed in distilled water, which was likely to be the only active fraction of the antibiotic, was about 20% of the total concentration. This difference could be due to the chelation of tetracycline by divalent cations, which inhibit the antibiotic (23).

**In vitro experiments.** In liquid medium, the MICs for the plasmid-free *E. coli* strain ranged between 1 and 4 µg/ml (Table 3). The effect of low antibiotic concentration on bacterial growth rate and the number of bacteria at equilibrium was measured in batch cultures. In media containing more than 1 µg of ampicillin, gentamicin, or colistin per ml, there was a large discrepancy between the number of bacteria estimated on the one hand from the optical density after 8 h and 24 h of incubation and on the other hand by plate counting after the same incubation periods, as already reported by Lorian (17). Sub-inhibitory concentrations of antibiotics reduced the total number of bacteria at equilibrium compared with that in the control (Table 3). At concentrations of 2 µg/ml and above, the population increased for 1 or 2 h and then decreased (data not shown).

The in vitro plasmid transfer rate constant between plasmid-bearing EMO and rifampicin-resistant EMO, in exponentially growing cultures, was  $3.0 \times 10^{-11}$  ml per cell per h for plasmid R270,  $2.6 \times 10^{-12}$  ml per cell per h for RP4,  $1.1 \times 10^{-12}$  ml per cell per h for pYD1, and  $7.7 \times 10^{-13}$  ml per cell per h for pC1.

TABLE 3. Inhibition by antibiotics of the growth of a susceptible *E. coli* strain in vitro

Concn μg/mU	Mean no. of bacteria at 8 h of exposure to drug <sup>a</sup> :					
	Tetracycline (MIC=1)	Gentamicin (MIC=1)	Streptomycin (MIC=1)	Ampicillin (MIC=2)	Flumequin (MIC=4)	Colistin (MIC=1)
0.1	55	107	116	99	ND <sup>b</sup>	ND
0.2	30 <sup>c</sup>	91	124	72	ND	ND
0.4	34 <sup>c</sup>	0.6 <sup>c</sup>	62	64	61	69
0.8	7 <sup>c</sup>	ND	25 <sup>c</sup>	9.1 <sup>c</sup>	ND	ND
2	ND <sup>b</sup>	0 <sup>c</sup>	10 <sup>c</sup>	0.2 <sup>c</sup>	1 <sup>c</sup>	0.32 <sup>c</sup>
4	ND	0 <sup>c</sup>	4 <sup>c</sup>	0.05 <sup>c</sup>	0.6 <sup>c</sup>	0.08 <sup>c</sup>

a. Counted on DCA plates as a percentage of that of the control ( $86 \pm 28 \times 10^7$  CFU/ml, n = 27). MICs are in micrograms per milliliter.

b. ND, Not determined.

c. Different from control (P < 0.01, Student's t test).

**Mathematical modeling.** The mathematical model matched the population levels observed in experiments conducted on control groups of mice (Fig. 1, CON). We tested four possible effects of low antibiotic concentrations on the in vivo equilibrium between plasmid-free and plasmid-bearing strains (the antibiotic is represented in the following equations by AB, as a function of concentration). For this purpose, it was alternatively assumed that the drug would kill some plasmid-free cells, thus changing equation 1 to:

$$dN_s/dt = N_s(\psi_s - \rho - AB) \quad (6)$$

reduce the ability of susceptible cells to use a limiting substrate, hence increasing the saturation constant and changing equation 4 to:

$$\psi_s = \mu_s \cdot L/(Ks_s + AB) + L \quad (7)$$

reduce the maximum growth rate of plasmid-free cells ( $\mu_s$ ), thus changing equation 4 to:

$$\psi_s = (\mu_s - AB) \cdot L/(Ks_s + L) \quad (8)$$

or enhance the rate of R plasmid transfer from resistant to susceptible strains, leading to a more complex model that we also tested (equations not shown).

Although it was not necessary to imagine more sophisticated explanations, we were unable to eliminate any one of these hypotheses on the basis of numerical simulations of in vivo competition, since the results obtained with all these drug effect

models matched the experimental data obtained in vivo. For example, the effect of 2 μg of streptomycin was simulated by setting the numerical value of the parameter AB at 0.06 per h in equations 6 (Fig. 1, EXP) and 8 or at 0.0006 g/liter in equation 7.

The same mathematical model was used to simulate in vitro experiments with the same intrinsic bacterial constants, by setting the flow rate at zero and adjusting the numerical value of substrate concentration to fit the experimental density. Comparison of the effect of the antibiotics on the plasmid-free strain in vitro with the simulated growth curve obtained by computer indicated that the main effect of the antibiotics was the death of plasmid-free cells (equation 6), since simulations based on equation 7 or 8 or on the increase in the R plasmid transfer did not fit the late decrease in the susceptible population observed in vitro. This explanation is simple, but it may not apply to the in vivo situation, since the physiological states of the cells are different. Furthermore, when the numerical value of the parameter AB was set at 0.06, the effect on the simulated growth rate and population number was minor and corresponded to undetectable experimental changes in vitro (e.g., the doubling time increased from 27 to 28 min with equation 6).

In other words, the mathematical model shows that a small antibiotic concentration which is not sufficient either to reduce the in



vitro growth values significantly or to eliminate a susceptible bacterium from the gut of mono-colonized mice can suffice to permit the outgrowth of an R-plasmid-bearing strain in di-colonized mice.

## DISCUSSION

The experimental model proposed here is simple and gives reproducible results. The three main advantages of gnotobiotic models over previous experimental situations are the isolation from the bacterial environment, ensuring the absence of contamination; the use of a well-defined flora, permitting truly identical control and experimental groups of mice as well as identical sequential experiments; and the physiological ecological conditions arising from the *in vivo* situation. However, although the principles that govern bacterial interactions in the mouse intestine may also be expected to have their counterpart in humans, the ecological situation of *E. coli* strains in the gut of a germfree mouse is far from the situation of bacteria in the guts of humans because of absence of dominant anaerobes. Consequently, the model would be improved if a known flora of human origin were inoculated into the digestive ecosystem of the mouse, in order to mimic the role of dominant anaerobic flora in humans.

The RP4 and R270 plasmids conferred an apparent ecological advantage on the carrying strain in the di-colonized mice drinking pure water, while the pYD1 and pC1 plasmids were detrimental to the bacteria. The advantage thus afforded by some plasmids to the host bacteria might be due to encoded plasmid proteins, an adaptation period of the bacterium-plasmid association conferring increased fitness of the host (2). However, most R-plasmid-bearing strains are disadvantaged compared with their plasmid-free counter-parts, probably because their *in vivo* growth rate is reduced (28) by the rise in the saturation constant for the limiting substrate (11). Then, the dominance of RP4 and R270 is likely due to a high rate of plasmid transfer leading to the spread of the plasmid to susceptible cells, as previously shown in gnotobiotic mice colonized by *E. coli* K-12 strains with chromosomal markers, one of which bore plasmid R270 (6).

We chose here to study isogenic strains, because of the detrimental effect of chromosomal mutations on strains *in vivo* (6, 21). It was therefore not possible to detect *in vivo* plasmid transfer, since the parent strains had no markers. The *in vitro* values of transfer rate constant show that the four plasmids are conjugative and repressed for transfer (16). Plasmids pYD1 and pC1, which fail to predominate in the gut, show lower frequencies of transfer *in vitro* than R270 and RP4, though the difference between RP4 and pYD1 was small. However, these differences are only indicative of what might take place in the gut, since the value of the transfer rate constant in exponentially growing cultures is considerably greater than its corresponding value in slowly dividing cultures or in mouse cecal flora (11, 16).

An attempt to explain the *in vivo* effect of low antibiotic levels on the equilibrium among *E. coli* strains was made by comparing the antibiotic concentrations in feces and the *in vitro* susceptibility of the strain. For example, the minimum selecting level for tetracycline *in vivo* was 6.5 µg/ml of water, which produced a free concentration of 0.5 µg/g in feces (calculated from data in Table 2). This concentration is one-half the MIC for the plasmid-free strain and was found to reduce the number of bacteria *in vitro* (Table 3). The same speculation might well apply to gentamicin, but for streptomycin and ampicillin the calculated concentrations in feces which corresponded to the minimum selecting level *in vivo* were about 1/20 of the MICs and produced no detectable effect *in vitro*. In studies by Lorian and De Frietas (18), the minimal concentration of ampicillin and gentamicin which reduced the number of viable cells 10-fold was about 1/20 of the MIC for *E. coli*. This might explain the *in vivo* effect of low drug concentrations. For all tested drugs, however, the concentration which selected resistance *in vivo* was lower than MIC and did not directly eliminate the susceptible bacteria from the gut. A possible explanation for this phenomenon was provided by modeling the population dynamics.

Garber (12) has proposed a mathematical model, based on the logistic equation, to simulate the effects of antibiotics on growth of mixed bacterial populations. This

model, however, did not explicitly take into account the flow due to intestinal transit, the effect of limited substrate concentrations, and the difference between the growth rates of competing strains. We therefore adapted Freter's equations so as to mimic the effects of low antibiotic concentrations in vivo, on the basis of a minimum of simple postulates (11). Evaluation of our experimental data by mathematical models led to a conclusion which could not have been reached otherwise; antibiotic concentrations which have little or no measurable effect in vivo and in vitro on a susceptible strain can "select" an R-plasmid-bearing strain in vivo, and this interaction might be the consequence of small differences between the net growth rates of the interacting bacteria and their plasmid.

We defined the minimum selecting level in vivo arbitrarily as the dose which led to a 10-fold increase in the number of plasmid-bearing cells 5 days after inoculation. The minimum antibiotic concentration defined by Lorian and De Frietas in vitro rests on a similar basis (18). In the long term, this concentration led to the predominance of the plasmid-bearing strain in the gut. In the animal model used here, the minimum selecting levels ranged from 0.9 to 12.8 µg/ml of drinking water and were in the same ranges as the MICs (Table 3). The selecting effect seems to be specific, since administration of flumequin and colistin produced no selection. An attempt was made to extrapolate the minimum selecting levels from mice to humans by comparative assay of the drug concentrations in the feces of mice and in the stools of a single volunteer who for 5 days ingested 10 mg of the same kinds of antibiotics per day that were given to the mice. Then, the calculated residues per gram of meat which might exert a selecting effect in humans would range from 1 to 25 µg, which is higher than the levels of the residues found in meat (19, 26, 27). The main limitations of the study are the lack of data about the effective antibiotic concentrations in the guts of mice and the inability to assess the role of plasmid transfer in vivo. Because of these problems, interpretations of results are speculative. Besides, the question remains of the possible public health hazard due to the increase in the number of resistant

nonpathogenic bacteria in the guts of humans (5).

## ACKNOWLEDGMENTS

We thank Yvonne Duval-Iflah for strain EMO and plasmid pYD1, J. Denarie for plasmid RP4, and G. Lebek for plasmid R270.

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