ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1981, p. 314-320
0066-4804/81/090314-07$02.00/0

Vol. 20, No. 3

Resistance to Tetracycline, Erythromycin, and Clindamycin in the *Bacteroides fragilis* Group: Inducible Versus Constitutive Tetracycline Resistance

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Received 14 July 1980/Accepted 17 June 1981

The transferability of plasmid-mediated tetracycline, erythromycin, and clindamycin resistance was studied in 63 clinical isolates of the *Bacteroides fragilis* group. Of 48 strains which were tetracycline resistant (Tc^r), the regulation of both the expression of Tc^r and its transferability was shown to be under inducible control by tetracycline. In 29 of the strains, Tc^r was transferable; in the majority of these (26 strains), transferability was inducible (Tra^i) and it was constitutive (Tra^c) in only 3 strains. All four possible phenotypes were found (Tc^i Tra^i, Tc^i Tra^c, Tc^c Tra^i, and Tc^c Tra^c), which indicates independent control of both Tc^r expression and its transferability. Resistance to erythromycin and clindamycin was cotransferred with Tc^r in 14 of the 48 Tc^r strains and transferred independently of Tc^r in only 1 strain.

Bacteroides fragilis is the most frequently isolated anaerobic organism in human infections. This organism is intrinsically susceptible to a restricted number of antibiotics, and the frequency of isolation of strains resistant to clinically useful antibiotics appears to be increasing. Recently, tetracycline and macrolide-lincosamide-streptogramin (MLS) resistances have been demonstrated by us and by other groups to be plasmid mediated (17, 19, 22). These resistances appear to be transferable to susceptible *Bacteroides* strains belonging to the same species or to different species through a mechanism which may be conjugation.

Plasmid-mediated tetracycline resistance (Tc^r) has been studied (4, 7) in many aerobic and anaerobic bacterial species carrying different plasmids, and the full expression of the resistance is generally inducible by subinhibitory concentrations of the antibiotics (8). Constitutive mutants can be selected. An inducible resistance was also observed with tetracycline in strains of *B. fragilis* initially studied by us. It was also shown that the transfer of tetracycline resistance could easily be achieved from these strains to a susceptible strain when the donor bacteria were grown in the presence of tetracycline before mating. The phenotype Tc^i Tra^i was assigned to these strains (inducible tetracycline resistance, Tc^i, and inducible tetracycline transfer ability, Tra^i). Constitutive mutants for both properties were obtained (Tc^c Tra^c strains), suggesting a common regulatory mechanism for the expression of tetracycline resistance and conjugative ability of a tetracycline resistance plasmid in *B. fragilis* (18).

These initial results led us to further investigation to determine: (i) the frequency of the different antibiotic resistance phenotypes in clinical isolates of *B. fragilis* and related species, (ii) the frequency of resistance transfer among antibiotic-resistance strains of this group, and (iii) the role of antibiotic administration in the emergence and spread of these antibiotic resistance phenotypes.

In this paper, we report an epidemiological study on inducibility versus constitutivity of tetracycline resistance and tetracycline transferability with recent isolates of the *B. fragilis* group together with data on their ability to transfer or cotransfer resistance to antibiotics of the MLS group. We report also experiments made in gnotoxenic mice showing that tetracycline resistance and transferability are induced in vivo by subinhibitory concentrations of the antibiotic and that higher concentrations of tetracycline lead to the isolation of constitutive mutants.

MATERIALS AND METHODS

Bacterial strains. Sixty-three strains were studied. They were clinical strains isolated in 1978 and 1979, except for strains 92 and BEN, which were isolated in 1977. The strains were from various parts of France, except three strains from Austria. Places from which more than a single strain were isolated included: Vil-

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leneuve Saint Georges, (Dublanchet, 12 strains); Paris, various hospitals (9 strains); Roanne (Dugelay, 7 strains); Saint Germain en Laye (General Hospital, 6 strains); Strasbourg (Tytgat, 6 strains); and Charleville-Mézières (General Hospital, 3 strains).

General techniques. Techniques of growth, bacterial mating, minimal inhibitory concentration (MIC) determinations, and study of expression of tetracycline resistance were those used by Privitera et al. (17, 18). The recipient strain used in all matings was a plasmid-free *B. fragilis* strain resistant to rifampin (638 rfm). Identification was made by the method of Holdeman and Moore (11). Transcipients were also identified according to their phage sensitivity pattern.

In vivo studies. C3H/HE axenic mice weighing 25 g each were used. The animals were maintained in sterile plastic chambers (Celster-Isothermie) and were fed sterile food free of antibiotics and sterile water. The absence of bacterial and yeast contamination was checked at the beginning of each experiment and at each sampling. Bacterial strains and antibiotics were introduced into the chambers in sealed glass vials sterilized by vapors of 5% peracetic acid and were fed to the animals by addition to the drinking water. The implantation of strains 92 and 638 rfm administered to the axenic mice was checked 48 h after the introduction of each strain. Fresh fecal samples (40 to 60 mg) were collected in sterile glass tubes, immediately dispersed in reduced tryptone-glucose-yeast extract (TGY) broth, and diluted 10-fold in the same medium. For total and selective bacterial counts of parental strains and transcipients, fixed amounts of each dilution were plated on TGY agar supplemented when necessary with antibiotics at the following concentrations: rifampin, 25 μg/ml; clindamycin, 15 μg/ml; and tetracycline, 2 μ/ml. Three plates were inoculated with each dilution.

To study the Tc^r inducibility and transferability, tetracycline was added (10 μg/ml) to the drinking water of mice monocontaminated with strain 92, and the exposure to the antibiotic was maintained for 4 days. A sample of fresh feces was then collected, dispersed in 0.5 ml of TGY broth, and used for the following experiments: (i) two 10-ml tubes of TGY broth, one containing 5 μg of tetracycline per ml, were inoculated with 0.1 ml of the stool suspension; (ii) a sample of the stool suspension was grown for 10 generations in the absence of tetracycline for deinduction of tetracycline resistance and then subcultured with and without the antibiotic as described above, cultures were incubated at 37°C in an anaerobic atmosphere, and the absorbance was measured at hourly intervals with a Klett photocolorimeter (filter no. 54; Klett Manufacturing Co.); (iii) samples of the direct stool suspension and of the subculture grown in the absence of tetracycline were also used for in vitro matings with strain 638 rfm (see Table 4).

RESULTS

Epidemiological studies. Some strains were sent for identification to the Reference Center on Anaerobes, and their antibiotic resistance was noticed because antibiotic susceptibility determination was made during this period of time for all strains received. Others were received as being antibiotic resistant from colleagues who knew of our interest in this problem. Consequently, the percentage of antibiotic resistance in the *B. fragilis* group may be calculated from the strains of the first origin only. Over a period of 2 months, we received 40 strains of the *B. fragilis* group; 33 strains (82.5%) were tetracycline resistant, and 1 strain (2%) was MLS resistant.

The MICs of clindamycin and erythromycin were ≥128 μg/ml in resistant strains and ≤2 μg/ml in susceptible strains. The MICs of tetracycline were ≥16 μg/ml in resistant strains and ≤0.1 μg/ml in susceptible strains. Intermediate values of 4 to 8 μg/ml were infrequent and were included with resistant strains because of their inducibility and transferability of tetracycline resistance and because of the tetracycline level generally attained in human sera after administration of therapeutic doses.

The distribution of resistance phenotypes in all 63 strains of the *B. fragilis* group is indicated in Table 1. The phenotype Tc Em Cl is significantly more frequent in species other than *B. fragilis*. Only one isolate, a strain of *B. ovatus*, was erythromycin and clindamycin resistant without associated tetracycline resistance.

All tetracycline-resistant strains were studied for inducibility versus constitutivity for expression of tetracycline resistance by the method of Privitera et al. (18); the phenotype Tc^i or Tc^c was easily assigned to all strains.

Figure 1 shows the distribution of inducible and constitutive Tc^r strains in various classes of MICs. Three strains had tetracycline MICs of 128 μg/ml and were tetracycline constitutive. A higher proportion of tetracycline-constitutive strains was found in the MIC class of 32 μg/ml, but there was no strict correlation between the Tc^c trait and a high MIC, as the Tc^c phenotype

TABLE 1. *Distribution of antibiotic resistance phenotypes in 63 strains of the B. fragilis group*

Species	No. of strains resistant to:[a]		
	Tc	Em and Cl	Tc, Em, and Cl
B. fragilis	41	0	8
B. distasonis	4	0	2
B. ovatus	2	1	0
B. thetaiotaomicron	1	0	0
B. vulgatus	0	0	1
B. uniformis	0	0	2
B. eggerthii	0	0	1

[a] Tc, Tetracycline; Em, erythromycin; Cl, clindamycin.

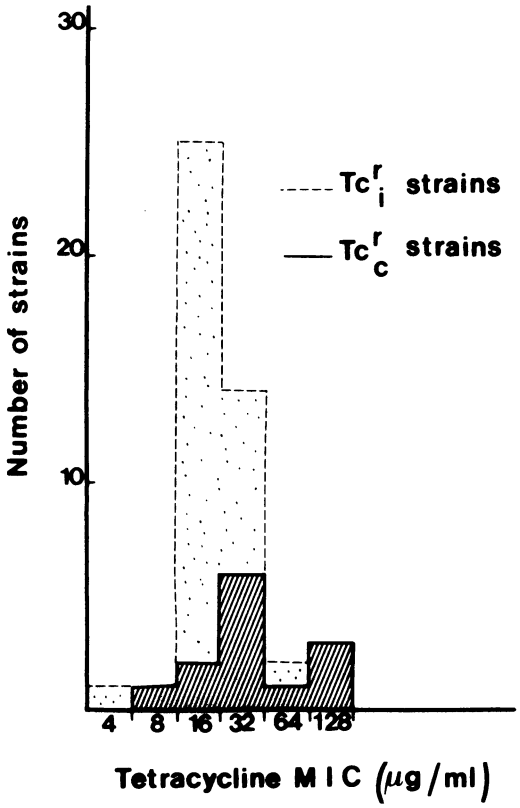

FIG. 1. *Tetracycline MICs in the 62 tetracycline-resistant strains studied.*

was also found in strains with the lowest MIC. Table 2 shows that 58.7% of all Tc^r strains have tetracycline transferability (Tra^+ strains). Various phenotypes of inducibility versus constitutivity for tetracycline resistance and for transferability were found. About two-thirds of the Tra^+ strains were Tc^i Tra^i; but Tc^c Tra^c, and dissociated phenotypes were also observed, either in tetracycline-resistant or tetracycline-, erythromycin-, and clindamycin-resistant strains. Among the strains which did not transfer tetracycline resistance (Tra^- strains), both Tc^i and Tc^c phenotypes were found.

During the 2-month period when we tested all 40 strains received, 33 of the strains were found to be tetracycline resistant and 20 of them were found to have tetracycline transferability, i.e., 60% of the Tc^r strains and 50% of the isolates.

In contrast to strain 92, the tetracycline-, erythromycin-, and clindamycin-resistant strain BEN did not transfer erythromycin or clindamycin resistance independently; nevertheless, when selection after mating was made on tetracycline, a certain number of transcipients were tetracycline, erythromycin, and clindamycin resistant; when selection was made on erythromycin or clindamycin, all transcipients were tetracycline, erythromycin, and clindamycin resistant. These results suggested a cotransfer of erythromycin and clindamycin resistance. To confirm this hypothesis, all donor strains were used in mating after growth in broth without tetracycline (tetracycline-uninduced cells) or after three subcultures on tetracycline at a noninhibitory concentration (tetracycline-induced cells). In both cases, transcipients were selected on media containing rifampin (25 μg/ml) and either tetracycline (2 μg/ml) or clindamycin (or erythromycin) (10 μg/ml). After reisolation of transcipients, antibiotic resistance phenotypes were studied for the three antibiotics. The percentage of tetracycline, erythromycin, and clindamycin transcipients was calculated from 15 to 30 transcipients chosen randomly among transcipients selected for tetracycline resistance. Of 13 strains studied, 6 did not transfer the erythromycin or clindamycin resistance either independently or by tetracycline cotransfer (Table 3). No strain except strain 92 was capable of independent transfer of the Em Cl plasmid. Six strains were able to cotransfer erythromycin and clindamycin resistance with the tetracycline resistance, whether inducible or constitutive for tetracycline transferability. In the Tra^i strains, the number of all transcipients was increased by tetracycline induction, but for a given strain the percentage of erythromycin and clindamycin resistance cotransfer was remarkably stable in both conditions and in various experiments.

In vivo studies. A low dose of tetracycline administered to the monoxenic mice colonized with strain 92 was able to induce tetracycline resistance in this strain. If employed as a tetracycline donor in in vitro matings, the in vivo-induced strain was able to transfer the tetracycline resistance to a susceptible strain (Table 4). The induction of tetracycline resistance and

TABLE 2. *Tetracycline transferability: inducibility versus constitutivity of transferability and expression of tetracycline resistance*

Tetracycline transferability	Inducible vs constitutive tetracycline resistance expression and transferability	No. of Tc^r strains	No. of Tc^r, Em^r, Cr^r strains
Tra^-	Tc^c	6	1
	Tc^i	13	4
Tra^+	Tc^c Tra^c	0	1
	Tc^c Tra^i	2	3
	Tc^i Tra^c	3	1
	Tc^i Tra^i	24	4

TABLE 3. *Resistance transfer properties of Tc^r Em^r Cl^r stains of the B. fragilis group*

Strain	Species	MIC of tetracycline (μg/ml)	Tc^r inducibility[a]	Tc^r transfer inducibility[b]		Em^r Cl^r cotransfer[c]	% of Em^r Cl^r cotransfer[d]
				−Tc/+Tc	Phenotype		
92	*B. fragilis*	16	i	−/++ (10^6)	Tra^i	−	
BEN	*B. distasonis*	16	i	−,±/++ (0–10/10^6)	Tra^i	+	25
691-78	*B. distasonis*	32	c	−/+	Tra^i	−	
140-79	*B. fragilis*	32	i	−/−	Tra^-		
182-79	*B. fragilis*	32	c	+/+ (300/200)	Tra^c	+	NT
SP 95	*B. fragilis*	32	i	−/+	Tra^i	−	
Du 10	*B. eggerthii*	128	c	+/++ (400/2 × 10^6)	Tra^i	+	96
Du 11	*B. uniformis*	32	c	−/++ (10^6)	Tra^i	+	15
Du 12	*B. uniformis*	64	i	+/++ (100/2 × 10^5)	Tra^i	+	70
Du 13	*B. fragilis*	32	i	−/+	Tra^i	+	66
Ty 50	*B. vulgatus*	16	i	−/−	Tra^-		
Ty 96	*B. fragilis*	16	i	−/+	Tra^i	−	
Ty 130	*B. fragilis*	64	c	−/−	Tra^-		

[a] Inducibility (i) and constitutivity (c) for tetracycline resistance tested according to conditions from Fig. 1 (18).

[b] Ability to transfer tetracycline resistance without (−Tc) and with (+Tc) tetracycline induction before mating. For tetracycline induction, cells were grown serially in TGY broth at a noninhibitory concentration (three passages at 0.5, 2, and 5 μg/ml), and mating with the recipient strain (538 rfm) was made by the method of Finegold (6) and Foster and Walsh (7). After an overnight incubation, the mixed culture was scraped and suspended in 2 ml of TGY, and various dilutions of the suspension were spread onto agar plates supplemented with rifampin at 25 μg/ml and either tetracycline at 2 μg/ml or erythromycin or clindamycin at 10 μg/ml. The number of transcipients per milliliter of this suspension is indicated in parentheses.

[c] Presence (+) or absence (−) or erythromycin and clindamycin resistance cotransferred with Tc^r. Strain 92 was the only strain capable of independent transfer of erythromycin and clindamycin resistance.

[d] Percentage of Tc Em Cl transcipients among transcipients selected for tetracycline resistance. NT, Not tested.

TABLE 4. *In vitro matings between B. fragilis strains 92 and 638 rfm after in vivo induction of donor strain 92*

Expt[a]	No. of transcipients (CFU/ml of mating mixture)	
	MLS^r	Tc^r
1	5×10^5	1.7×10^5
2	4.5×10^4	0

[a] In mating experiment 1, the direct suspension of the stools of mice administered strain 92 orally and fed with tetracycline was used as donor. In mating experiment 2, a sample of this stool suspension was subcultured in TGY broth for 10 generations before being used in mating as the donor.

transferability in strain 92 disappeared by cultivating the strain in the absence of tetracycline (deinduction); these properties appeared to be quickly lost in vivo also when the antibiotic was withdrawn either by interrupting the administration of tetracycline to the monoxenic animals some hours before the introduction of the recipient strain (Table 5, experiment 1) or by administering the in vivo-induced strain 92 to mice previously colonized with the recipient strain which were not receiving tetracycline (Table 5, experiment 2). Under these conditions, there was no Tc^r transfer at a detectable level ($\leq 10^2$ transcipients per g of feces).

When both donor and transcipient strains were colonizing the same animals, no transfer could be observed over an observation period of 2 weeks in the absence of antibiotic administration (Table 5). Tc^r transcipients (1.5×10^3 colony-forming units [CFU] per g of feces) could, however, be detected in the stools of mice 72 h after the addition of tetracycline (10 μg/ml) to the drinking water (Table 5, experiment 3). Transfer frequency was 1.5×10^{-8} per donor. During the observation period of 1 week and under the same experimental conditions, the transcipient counts appeared to be stable; an explanation could be that the subinhibitory concentration of the antibiotic reached in the gut of the mice by adding 10 μg of tetracycline ml to the drinking water was adequate to induce the expression of tetracycline resistance and the transferability in strain 92, but did not exert a selective pressure sufficient to allow the emergence of a numerically relevant population of Tc^r transcipients.

When tetracycline was added to the drinking water at a concentration of 200 μg/ml (Table 5, experiment 4), which should give an intestinal concentration of the antibiotic equivalent to that

TABLE 5. *In vivo transfer of resistance markers to a recipient B. fragilis strain 638 rfm*

Expt (strains 92 × 638 rfm)[a]	Tetracycline administered (μg/ml)[b]	No. of markers transferred (CFU/g of feces) in:[c]			
		Donor	Recipient	Transcipients[d]	
				MLS	Tc
1	10 (8 days)	3×10^{10}	2×10^{9}	5×10^{2}	
2	0	5×10^{8}	5×10^{10}		
3	10 (72 h)	1×10^{11}	1×10^{10}		1.5×10^{3}
4	200 (48 h)	1×10^{11}	1×10^{4}		

[a] In mating experiment 1, the donor strain was given orally together with tetracycline, and the tetracycline administration was stopped 8 h before introducing the recipient strain; in mating experiment 2, the donor strain was a stool suspension from a mouse which received strain 92 orally and was supplemented with tetracycline (in vivo-induced donor strain); in mating experiments 3 and 4, both donor and recipient strains were administered to the mice at the same time.

[b] Expressed as micrograms of tetracycline per milliliter of drinking water; the length of time of tetracycline administration before the donor strain was given is indicated in parentheses.

[c] Mean of three determinations.

[d] MLS, Macrolide lincosamide streptogramin; Tc, tetracycline.

attained in humans with therapeutic doses, the recipient counts dropped to $<10^4$ CFU/g of feces, whereas the donor counts were unchanged and transcipients were not observed ($\leq 10^3$ CFU/g of feces). On day 7, however, some clones of strain 92 were obtained by direct selection on plates supplemented with 20 μg of tetracycline per ml, and these clones turned out to be constitutive for both resistance and transferability when checked after further culture for ~40 generations in the absence of tetracycline. We also studied the in vivo transfer of the MLS^r plasmid carried by strain 92. In the absence of antibiotic-selective pressure, MLS^r transcipients could be detected in the feces of mice 72 h after the administration of the susceptible recipient strain to mice previously colonized with the donor strain. Transcipient counts were 500 CFU/g of feces, and the transfer frequency was 1.7×10^{-8} per donor. On day 12, the transcipient counts were 4×10^3 CFU/g of feces, and the transfer frequency was 4.4×10^{-8} per donor. As was observed in vitro, the in vivo segregation rate of MLS resistance in strain 92 was 1%, and the transfer frequency appeared to be independent of the transfer of tetracycline resistance.

DISCUSSION

Of the 63 strains of the *B. fragilis* group studied, 40 were received for identification and, as far as tetracycline resistance in this group is concerned, these 40 strains may be considered representative of the situation in France. Thus, 82.5% of the strains were tetracycline resistant; this value is comparable to results from other countries (6, 12). Of the 63 strains included in our study 15 were MLS^r, but most of the MLS^r strains were especially collected in view of their unusual antibiotic resistance, and they are certainly not representative of the situation in France. The survey of the resistance in our 40 unselected strains shows a 2% prevalence for the MLS^r phenotype. With the exception of one strain (*B. ovatus*), MLS resistance is associated in French strains with tetracycline resistance. These results are somewhat different from those published elsewhere and suggest limited extension of MLS resistance (2, 19).

The tetracycline resistance of the strains studied is frequently transferable (38 of 62 Tc^r strains). Tetracycline resistance expression and transferability of the strains are generally inducible by tetracycline (Tc^i Tra^i strains), but dissociated phenotypes (Tc^c Tra^i and Tc^i Tra^c) and the phenotype Tc^c Tra^c were also found. Moreover, among the so-called Tra^i strains showing an increased transfer frequency after tetracycline induction, some strains (e.g., strain Du 10 [Table 3]) have a noticeable level of constitutive transfer, and such strains could transfer tetracycline resistance in vivo.

The existence of strains with a constitutive tetracycline resistance in a group whose representatives are generally tetracycline inducible is a noticeable situation because in facultative anaerobes, plasmid-mediated tetracycline resistance expression is generally inducible except in streptococci, in which a plasmid-mediated tetracycline resistance is constitutive (5). To our knowledge, no other Tc^r inducible transfer has been described at the present time, but Tomich et al. (20) have shown that exposure of a strain of *Streptococcus faecalis* to an inducing concentration of erythromycin enhances the frequency of erythromycin-resistant transconjugants appearing during mating; the phenomenon is correlated to enhanced transposition of the erythromycin determinant on a conjugative plasmid

present in the strain. This is not the case in *B. fragilis* strain 92, because tetracycline and MLS resistance are transferred independently, and transposition of tetracycline resistance on the MLS plasmid pIP410 has never been observed (unpublished data). In other strains in which MLS resistance is cotransferable with tetracycline resistance, no plasmid deoxyribonucleic acid can be detected, and the molecular mechanism of transfer inducibility is still unknown. Although the tetracycline transfer inducibility seems at the present time to be unique to *Bacteroides* spp., substrate induction of conjugative ability has been reported for metabolic plasmids (15, 16).

The high percentage of tetracycline-resistant strains in the *B. fragilis* group could be partly considered as a long-term consequence of the wide clinical use of tetracycline in therapy, particularly the prolonged administration of the antibiotic on a large scale in acne treatment (14). The prescription of tetracycline has decreased considerably in France in recent years. For 32 strains, data on antibiotics administered previously to the patients were available and the patients did not receive tetracycline, although two of these strains were Tc^c Tra^c. Nevertheless, the experimental data obtained show that an indirect selective pressure may be assumed by tetracycline on the MLS plasmids, an observation which is potentially important from a clinical point of view, as macrolides and lincosamides are useful antibiotics in human medicine. Among the erythromycin and clindamycin strains studied, only strain 92 was capable of independent transfer of MLS resistance. The MLS resistance carried by the other strains is either nontransferable or cotransferable with the tetracycline resistance. The cotransfer of MLS resistance thus appears to be a more general mechanism than independent transfer.

In vivo transfer of antibiotic resistance has been demonstrated to occur in the intestines of mice and other laboratory animals (9, 21). There is evidence in enterobacteria that it may also take place in humans (1).

Our results show that induction of tetracycline resistance and transferability may occur in vivo. This and the fact that a common regulatory mechanism controlling both characters is a widespread property in *B. fragilis* suggest that this mechanism may play a part in the spread of antibiotic resistance in nature. These Tc^r plasmids may also be involved in more extended genetic exchanges. With the strains studied, in vivo transfer appears at a very low frequency, in spite of the high counts observed for parental strains (10^{10} to 10^{11} CFU/g of feces); in our study, an optimal donor/recipient ratio (10 to 1) appears to be essential to the transfer of antibiotic resistance (unpublished data). This fact might constitute an additional obstacle to genetic exchanges in nature between *Bacteroides* spp. and anaerobic facultative bacteria; the latter are represented in the intestinal flora in 2 to 3 orders of magnitude lower levels.

Although the in vitro transfer of multiple antibiotic resistance has been reported between *Escherichia coli* and members of the *Bacteroidaceae* (3, 10) and Mancini and Behme (13) reported the stable maintenance in *E. coli* of a Tc^r determinant transferred from *B. fragilis*, thus far we have not been able to observe any in vitro genetic exchange between a variety of strains of *E. coli* and *Bacteroides* (unpublished data). In vivo experiments with appropriate *E. coli* recipients and strain 92 as a donor gave similar negative results in spite of the high counts of the *E. coli* strains and an optimal donor/recipient ratio, but this result could be inherent to the *Bacteroides* strains used.

With strain 92, which in vitro proved to be capable of independent transfer of MLS resistance, we observed spontaneous in vivo transfer of this character. We did not study the effect of antibiotic administration on the implantation of MLS resistance. A similar study has been done on axenic mice by Tally and co-workers (personal communication), using a strain of *B. fragilis* Cl^r Em^r carrying a different plasmid. In their study, the administration of clindamycin to mice led to the stable implantation of a transcipient population at 10^{10} CFU/g of feces.

In conclusion, we summarize the three main conclusions of this study: (i) we observed the constitutive type of tetracycline resistance in clinical isolates, which raises the question of a previous contact of these strains with tetracycline; (ii) low tetracycline concentrations seem to be sufficient in vivo for induction of tetracycline resistance and transferability in *B. fragilis*; (iii) we were able to isolate constitutive mutants for both characters from the feces of mice receiving tetracycline. These observations lead us to stress the risk, for both the individual patient and the community, of the use of subinhibitory concentrations of antibiotics such as those attained during inappropriate therapy or through the use of antibiotics as supplements in animal feeds, in agriculture, and as preservatives.

ACKNOWLEDGMENTS

G.P. was supported by an EMBO fellowship.

We are indebted to P. Raibaud and R. Ducluzeau for axenic animal facilities and useful discussion.

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