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Antagonism Exerted by an Association of a *Bacteroides thetaiotaomicron* Strain and a *Fusobacterium necrogenes* Strain against *Clostridium perfringens* in Gnotobiotic Mice and in Fecal Suspensions Incubated In Vitro

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Antagonism between an association of *Bacteroides thetaiotaomicron* and *Fusobacterium necrogenes* strains and two strains of *Clostridium perfringens* was evidenced both in vivo in gnotobiotic mice and ex vivo in fecal suspensions incubated for 22 h at 37°C. Several features of this antagonism were similar in and ex vivo. (i) An obligate and continuous synergy between *B. thetaiotaomicron* and *F. necrogenes* was required; (ii) the two *C. perfringens* strains did not respond to the same extent to this antagonism; and (iii) expression of the antagonism was host and diet dependent. Neither diffusible nor soluble inhibitory substances were detectable in feces of gnotobiotic mice, nor could depletion of nutrients be identified as causing antagonism in both in and ex vivo experiments. Our findings support the hypothesis that a reversible bacteriostasis induced by the inhibitory strains acting together continuously, and hindering the target strain from utilizing available nutrients, was responsible for this antagonism.

The mechanisms by which an undisturbed intestinal flora protects against potentially pathogenic bacterial strains or hinders exogenous bacterial strains from being established are far from being known. In vitro studies often lead to conflicting results (11), and it is unlikely that they could reflect the in vivo ecological situation. However, the continuous-flow culture system (10) gives in vitro results which are consistent with those obtained in gnotobiotic mice when *Escherichia coli* is the target organism. Wilson and Freter (18) demonstrated that such an in vitro experimental model was also efficient for studying interactions of *Clostridium difficile* with intestinal floras when a soluble extract of fecal pellets from axenic mice was used as a culture medium. This result stresses the fact that fecal endogenous components, including nondigestible dietary substances and secretions and excretions of the host, could play a role in the mechanisms of bacterial antagonism. The limitation of the in vitro models developed by Freter and co-workers was that a collection of >100 bacterial strains was needed for simulating the in vivo models. Such complex floras, although synthetic, do not allow an analytical approach to the actual mechanisms of bacterial antagonism. Recently, we described (19) a bacterial antagonism in which a target strain of *C. perfringens* was washed out from the intestine of gnotobiotic mice by only three anaerobic strains. In addition, we demonstrated that fecal suspensions prepared from mice associated with these three inhibitory strains and incubated in vitro exerted a strong bacteriostatic effect against the target strain. The aims of the present work were to investigate further the mechanism by which a target strain can be eliminated from the intestine with synthetic floras as simply as possible and to demonstrate that an ex vivo experimental model, i.e., a static incubation of fecal suspensions, may reproduce the characteristics of the in vivo antagonism.

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MATERIALS AND METHODS

Animals and diets. Adult axenic C3H/HeJ mice and Fischer 344 rats were reared in Trexler-type isolators fitted with a rapid transfer system (La Calhène, Vélizy-Villacoublay, France). They were fed ad libitum a commercial diet for rodents (Usine d'alimentation rationnelle, Epinay-sur-Orge, France) sterilized by gamma irradiation (40 kGy). In one experiment, mice were fed a milk replacer containing the following (in grams per kilogram): refatted milk powder, 500; skim milk powder, 200; barley, 190; fish proteins, 70; calcium carbonate, 15; calcium phosphate, 5; and vitamin mixture, 20; sterilized by gamma irradiation.

Bacterial strains. *Bacteroides thetaiotaomicron* BT, *B. vulgatus* BV, and *Fusobacterium necrogenes* FN were isolated by Corpet and Nicolas (1) from the feces of a 48-h-old healthy piglet. We isolated *F. varium* Fu from a conventional rat. Strain CpA was a variant of a *C. perfringens* strain belonging to serotype A which was unable to form thermoresistant spores in the intestine of monoassociated mice and was devoid of lecithinase. *C. perfringens* strain CpC, belonging to serotype C and kindly supplied by M. Sebald (Pasteur Institute, Paris, France) was highly pathogenic for axenic mice (13). Spores of a thermophilic *Bacillus subtilis* strain were used as transit marker, as described previously (3).

Association of axenic rodents with bacterial strains. All strains were grown in soft medium B′ (17) containing 1.5% Bacto-Peptone (Difco Laboratories, Detroit, Mich.), 1% autolyzed yeast (Difco), 1% tryptone (Difco), 0.1% Tween 80, and 0.18% agar, pH 6.5, and placed in a boiling-water bath for 30 min before use. A 6-ml portion of an 18-h (strains CpA, FN, and FV) or 48-h (strains BT and BV) culture was given as drinking water to axenic rats or mice after an 18-h water deprivation. For CpC, because of its toxigenicity, an 18-h culture in liquid medium B′ incubated in an anaerobic chamber was centrifuged and the pellet was diluted in B′ medium to obtain 10^6 viable cells per ml before challenge. Diassociated rodents were always inoculated with a mixed

TABLE 1. Comparative efficiency of a one-strain and a two-strain association in reducing the *C. perfringens* CpA population level in gnotobiotic mice

Group[a]	Strain	Counts (mean $\log_{10}$ ± SD)/g in:		
		Small intestine[b]	Cecum[b]	Feces[c]
I	BT	7.3 ± 0.5	10.9 ± 0.2	10.8 ± 0.2
	FN	5.2 ± 1.3	9.9 ± 0.2	9.9 ± 0.2
	CpA	<2.0	<2.0	<2.0
II	BT	6.9 ± 0.7	10.8 ± 0.2	10.9 ± 0.2
	CpA	5.8 ± 0.7	8.8 ± 0.1	9.0 ± 0.1
III	FN	5.2 ± 1.6	9.8 ± 0.1	9.8 ± 0.2
	CpA	5.1 ± 1.4	9.0 ± 0.2	9.1 ± 0.2
IV	CpA	6.5 ± 0.6	9.5 ± 0.2	9.7 ± 0.2

[a] Axenic mice (six in each group) were first associated with *B. thetaiotaomicron* BT and *F. necrogenes* FN (group 1), BT (group II), or FN (group III) and then challenged with strain CpA 7 days later. CpA was inoculated alone in group IV.

[b] Mice were sacrificed 25 days after CpA challenge. Small intestine and cecum were removed individually.

[c] Feces were collected individually on days 7, 14, and 21 after CpA challenge. Values are the mean $\log_{10}$ ± SD of the six samples taken on all 3 days.

culture of the two strains prepared extemporaneously. Rodents were challenged with CpA and CpC as indicated in the text. *Bacillus subtilis* spores were prepared by plating a spore suspension on agar medium MS containing 0.8% meat extract (Merck AG, Darmstadt, Federal Republic of Germany), 0.2% yeast extract (Difco), 0.1% glucose, 40 mg of manganese sulfate per liter and 1% Bacto-Agar (Difco), pH 6.3. A suspension containing 10^8 spores per ml was extemporaneously admixed with the CpA inoculum before challenge. Feces were individually collected from the anuses of animals and submitted for bacterial counts within 1 h. Sampling frequency is reported in the text. At the end of experiments, animals were killed by ether inhalation, and small intestines and ceca were removed and submitted for bacterial counts within 1 h.

Bacterial counts. Feces were diluted 100-fold in diluting medium LCY (16) and homogenized by hand. Intestinal samples were diluted 10-fold in LCY and homogenized with an Ultraturrax (OSI, Paris, France). A 1-ml amount of adequate serial 10-fold dilutions mixed with 14 ml of solid B′ medium was poured into a tube (8 by 400 mm) and immediately cooled in running tap water to ensure prompt solidification (16).

Incubation was done at 37°C. *Clostridium* colonies and *Bacteroides* and *Fusobacterium* colonies were counted 1 and 7 days later, respectively. *Bacteroides* strains formed small, lenticular, opaque colonies (<1 mm in diameter); *Fusobacterium* strains formed large, transparent, lenticular colonies (>3 mm in diameter). They were easily recognizable. Spores of the transit marker were counted by plating on MS medium.

Fecal filtrates and suspensions. Various types of fecal suspensions were used. They were prepared from pooled, freshly passed feces diluted in sterile deionized water or APT medium, which is B′ medium without Tween 80. Centrifugation was done at 12,000 × *g* for 15 min. In some experiments supernatants were filter sterilized. A 1-ml portion of fecal suspension was poured into a screw-cap tube (18 by 120 mm); tubes were inoculated with the target strain and placed in an anaerobic glove box (La Calhène). Incubation was for 22 h at 37°C, and CpA and CpC inocula were 0.1 ml of a 10^{-4} dilution of an 18-h culture, unless otherwise stated. Division numbers (*N*) of CpA and CpC were calculated from the formula $n = n_0 \times 2^N$, where n is the number of target strain colonies at the end of incubation and n_0 is the initial number. n_0 was always between 10^3 and 8×10^3 per ml. Six replicate determinations were made for all assays, and data are expressed as the mean ± standard deviation (SD) of the division numbers.

Continuous-flow cultures were made in enriched veal infusion broth as described by Freter et al. (10). The flow rate was 1/6 h^{-1}.

Detection of inhibitory diffusible substances by an in vitro test. About 3 ml of melted medium B′ inoculated with 10^4 cells of CpA and CpC per ml was poured into a Veillon tube (8 by 180 mm) and promptly solidified. A twofold dilution of freshly passed feces from gnotobiotic mice diassociated with strains BT and FN was prepared in melted solid medium B′, and 2 ml of this fecal suspension was poured into the Veillon tube. After solidification, the tube was filled with melted solid medium B′, kept at 4°C for 24 h, and then incubated for 18 h at 37°C.

Statistical analysis. Analysis of variance (Fisher *F* test) was used to compare mean values of a given bacterial population level or division number converted into $\log_{10}$ before statistical analysis for each in or ex vivo experiment. Statistical evaluation of the significance of differences was performed with Student's *t* test.

TABLE 2. Comparative efficiency of the association of *B. thetaiotaomicron* BT and *F. necrogenes* FN in reducing the *C. perfringens* CpA and CpC population sizes in feces of gnotobiotic mice

Group[a]	Strain	Fecal counts (mean $\log_{10}$ ± SD)/g on given days[b]					
		1	2	3	7	14	21
V	BT	10.9 ± 0.1	10.8 ± 0.1	10.8 ± 0.1	11.0 ± 0.2	11.1 ± 0.3	11.0 ± 0.2
	FN	9.9 ± 0.1	9.8 ± 0.1	9.9 ± 0.2	10.0 ± 0.2	10.2 ± 0.3	10.1 ± 0.2
	CpA	6.5 ± 0.5	4.3 ± 0.2	3.4 ± 1.2	<2.0	<2.0	<2.0
	TM[c]	7.9 ± 0.4	6.5 ± 0.4	5.1 ± 0.3	4.8 ± 0.4	3.4 ± 0.6	3.0 ± 0.5
VI	BT	11.1 ± 0.2	10.9 ± 0.1	11.1 ± 0.2	10.2 ± 0.2	10.8 ± 0.3	10.9 ± 0.4
	FN	9.9 ± 0.3	9.8 ± 0.1	9.9 ± 0.2	9.4 ± 0.2	10.1 ± 0.2	9.8 ± 0.2
	CpC	4.2 ± 0.6	3.5 ± 0.8	3.7 ± 1.0	4.2 ± 1.0	6.1 ± 1.0	6.6 ± 1.2

[a] Axenic mice (six in each group) were first associated with strains BT and FN and then challenged 7 days later with 10^8 CpA and 10^8 *Bacillus subtilis* spores (group V) or CpC (group VI).

[b] Days after CpA or CpC challenge.

[c] TM, Transit marker.

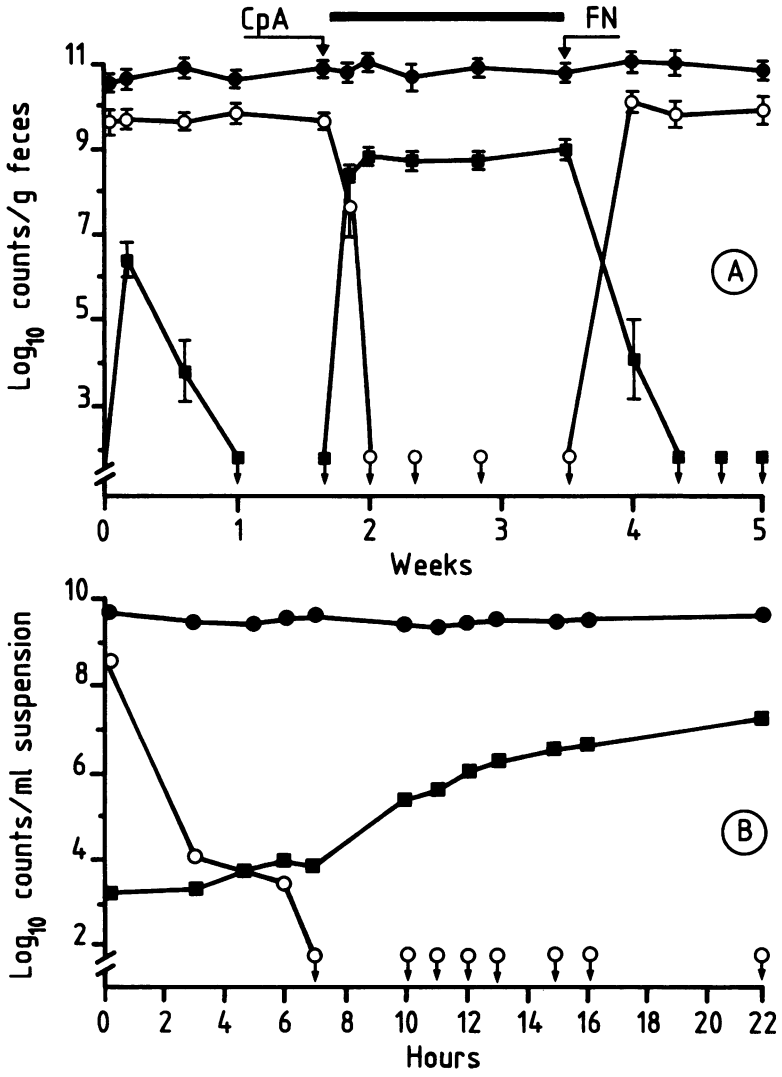

FIG. 1. Effect of colistine sulfate (R. Bellon Laboratories, Paris, France) on antagonism exerted by strain BT (●) associated with strain FN (○) against target strain CpA (■) in feces of six gnotobiotic mice. (Each point represents the mean $\log_{10} \pm$ SD of bacterial counts.) Arrows indicate CpA and FN reinoculation. Horizontal bar shows the length of oral antibiotic administration (A) or, in fecal suspension FS1 (Table 6), the length of admixture of colistine sulfate (1 mg/ml) (B).

RESULTS

Synergistic antagonism of the two-strain (*B. thetaiotaomicron* and *F. necrogenes*) association against *C. perfringens* CpA. Table 1 clearly shows that a synergy between *B. thetaiotaomicron* BT and *F. necrogenes* FN was required for eliminating strain CpA from the intestines of gnotobiotic mice. In mice associated with each strain alone, the fecal and cecal population levels of CpA were only slightly, but significantly (9.1 ± 0.2 versus 9.7 ± 0.2; $P < 0.001$), reduced compared with those observed in mice monoassociated with CpA, whereas CpA was no longer detected when mice harbored the two-strain association. In the small intestine, population levels of the inhibitory and target strains were >100-fold lower than in the ceca and feces.

Ten other groups of six mice each diassociated with strains BT and FN were challenged with CpA, which was always eliminated within 7 days. A group of 18 mice harboring BT and FN was kept in the same isolator for 12 months and then challenged with CpA, the elimination of which also occurred within 7 days (data not shown). There was no difference between male and female mice with respect to CpA elimination.

Comparison of the rate of fecal elimination of CpA and the transit marker shows (Table 2) that CpA inoculum (10^8 cells) was eliminated slightly faster than transit marker inoculum (10^8 cells) since the changes in mean $\log_{10}$ counts between days 2 and 1 after inoculation were 2.2 and 1.4 for CpA and transit marker, respectively.

In the former experiments, CpA was inoculated after strains BT and FN. When CpA was inoculated first, it was also eliminated. The only difference was that its elimination was achieved within 14 instead of 7 days.

Figure 1A shows that synergy between strains BT and FN was an absolute requisite throughout the experiment, since strain CpA became established in mice given colistine sulfate as soon as the number of FN was $<10^8$ per g. CpA was in turn eliminated when antibiotic administration was stopped and FN became reestablished.

Effect of two-strain (*B. thetaiotaomicron* BT and *F. necrogenes* FN) association on *C. perfringens* CpC. The target strain

TABLE 3. Effect of two other two-strain associations, host, and diet on *C. perfringens* CpA population levels in gnotobiotic mice and rats

Group (host, diet)[a]	Strain	Fecal counts (mean $\log_{10}$ ± SD)/g on given days[b]					
		1	2	3	7	14	21
VII (mice, commercial diet)	BT	11.2 ± 0.1	10.9 ± 0.2	10.9 ± 0.2	10.8 ± 0.2	10.6 ± 0.2	11.2 ± 0.2
	FV	10.4 ± 0.1	10.1 ± 0.1	10.2 ± 0.1	10.0 ± 0.2	9.9 ± 0.2	10.5 ± 0.2
	CpA	5.4 ± 2.0	5.2 ± 1.3	5.3 ± 1.5	7.4 ± 0.5	7.4 ± 0.8	8.0 ± 0.8
VIII (mice, commercial diet)	BV	10.5 ± 0.2	10.4 ± 0.3	10.2 ± 0.3	10.5 ± 0.3	10.2 ± 0.1	10.2 ± 0.2
	FN	9.9 ± 0.1	9.9 ± 0.2	9.7 ± 0.3	9.8 ± 0.2	9.7 ± 0.2	9.7 ± 0.2
	CpA	6.1 ± 1.3	7.2 ± 0.2	7.1 ± 0.2	7.3 ± 0.2	7.5 ± 0.2	7.5 ± 0.1
IX (mice, milk diet)	BT	10.6 ± 0.3	10.7 ± 0.5	10.3 ± 0.5	10.6 ± 0.3	10.6 ± 0.3	10.4 ± 0.6
	FN	9.6 ± 0.2	9.5 ± 0.5	9.5 ± 0.3	9.9 ± 0.3	9.8 ± 0.2	9.2 ± 0.8
	CpA	8.1 ± 1.1	7.1 ± 0.4	5.8 ± 0.3	7.6 ± 0.3	8.2 ± 0.2	7.7 ± 0.3
X (rats, commercial diet)	BT	10.5 ± 0.1	10.8 ± 0.3	10.7 ± 0.1	10.6 ± 0.1	10.5 ± 0.1	10.5 ± 0.3
	FN	9.3 ± 0.2	9.7 ± 0.2	9.6 ± 0.1	9.7 ± 0.1	9.4 ± 0.3	9.6 ± 0.3
	CpA	6.6 ± 0.1	5.0 ± 0.7	5.0 ± 0.4	6.5 ± 0.4	6.6 ± 0.2	6.9 ± 0.3

[a] Axenic mice (six in each group) were inoculated with *B. thetaiotaomicron* BT and *F. varium* FV (group VII), *B. vulgatus* BV and *F. necrogenes* FN (group VIII), or BT and FN (group X) and then challenged 7 days later with CpA. Axenic rats (group X, six rats) were inoculated like mouse group IX. Counts of CpA in monoassociated rats were 8.6 ± 0.1 (mean ± SD) per g of feces 14 days after challenge.
[b] Days after CpA challenge.

CpC did not behave like CpA, since the fecal CpC population level was reduced, not eliminated, by the two-strain association even though the population levels of both BT and FN were not significantly different in groups V and VI (Table 2). On day 21, one mouse harbored 5×10^8 CpC per g of feces and died 2 days later, whereas the remaining five mice harbored 10^6 to 10^7 CpC per g. They were sacrificed healthy on day 25, and no sign of enteritis was observed. Cecal CpC counts also ranged between 10^6 and 10^7 per g.

Specificity of *B. thetaiotaomicron* BT and *F. necrogenes* FN in ensuring prompt *C. perfringens* CpA elimination. When *B. thetaiotaomicron* was associated with *F. varium* or *F. necrogenes* was associated with *B. vulgatus*, both two-strain associations were significantly less efficient than the association of *B. thetaiotaomicron* and *F. necrogenes*, since CpA remained at a rather high level after its challenge in groups VII and VIII: 8.0 ± 0.8 and 7.5 ± 0.1 (Table 3) versus <2.0 (Table 1). The fecal population levels of strains BT and FN in both two-strain associations were not significantly different compared with those of strains BT and FN in group V (Tables 2 and 3). Mice were sacrificed on day 25, and counts in small intestines and ceca similar to those shown in Table 1 were obtained.

Effects of host and diet on antagonism. The synergistic antagonism exerted by the two-strain association (*B. thetaiotaomicron* BT and *F. necrogenes* FN) against *C. perfringens* CpA was significantly different in gnotobiotic rats and mice receiving the same diet. CpA was not washed out in diassociated rats as it was within 7 days in diassociated mice, although CpA population levels were significantly lower in rats than in mice when they were monoassociated with CpA (8.6 ± 0.1 versus 9.7 ± 0.2; $P < 0.001$). However, CpA population levels remained lower in the feces of rats diassociated with BT and FN than in those of rats monoassociated with CpA (6.6 ± 0.2 versus 8.6 ± 0.1, on day 14; $P < 0.001$). Populations of both strains BT and FN were only slightly higher in mice than in rats (Tables 2 and 3).

In addition, a milk diet was found to block the antagonistic effect of the two-strain association against CpA in mice (Table 3). Again, BT and FN population levels were only slightly lower in mice fed a milk diet than in mice fed a commercial diet.

Continuous-flow culture experiments. The two-strain association failed to hinder the growth of both target strains CpA and CpC in a continuous-flow culture system (Table 4).

Attempts to detect nutrient depletion or diffusible inhibitory substances in feces of gnotobiotic mice harboring *B. thetaiotaomicron* and *F. necrogenes*. We failed to detect any diffusible substances able to inhibit CpA and CpC growth in an agar medium from feces of diassociated mice. Furthermore,

TABLE 4. Comparative population levels of *B. thetaiotaomicron* BT, *F. necrogenes* FN, and *C. perfringens* CpA and CpC in a continuous-flow system

Strain association	Counts[a] of:			
	BT	FN	CpA	CpC
BT + FN + CpA	9.3 ± 0.1	8.6 ± 0.3	7.9 ± 0.2	
BT + FN + CpC	9.3 ± 0.2	8.4 ± 0.1		8.7 ± 0.2
CpA			8.2 ± 0.2	
CpC				8.7 ± 0.2

[a] Mean $\log_{10}$ ± SD of five samples taken on days 2, 3, 4, 7, and 10 after CpA or CpC challenge. Strains BT and FN were inoculated 10 days before challenge.

TABLE 5. Attempts to detect inhibitory substances or depletion of growth factors for *C. perfringens* CpA and CpC in filtrates prepared from feces of mice diassociated with *B. thetaiotaomicron* and *F. necrogenes*

Target strain	Division no. in:				
	APT medium	Filtrates[a] from:		Enriched filtrates[b] from:	
		Axenic mice	Diassociated mice	Axenic mice	Diassociated mice
CpA	17.7 ± 0.5	11.1 ± 0.7	17.4 ± 0.9	17.1 ± 0.8	17.7 ± 0.8
CpC	17.6 ± 0.4	11.0 ± 0.9	18.2 ± 1.7	17.3 ± 0.4	17.3 ± 0.4

[a] Prepared from feces diluted fourfold in sterile deionized water inside an anaerobic chamber.
[b] Prepared from feces diluted fourfold in APT medium. $P < 0.001$ between data for filtrates prepared from axenic mice and all other data.

TABLE 6. Influence of amount of soluble fecal substances in various suspensions prepared from mice diassociated with *B. thetaiotaomicron* BT and *F. necrogenes* FN on division numbers of *C. perfringens* CpA and CpC

Fecal suspension[a]	Division no. of:		Initial counts (log_{10} means ± SD) of:	
	CpA	CpC	BT	FN
FS1	0.9 ± 0.7	4.9 ± 0.9	10.2 ± 0.1	9.1 ± 0.1
FS2	1.7 ± 1.6	0.3 ± 1.3	10.2 ± 0.1	9.3 ± 0.1
FS3	3.0 ± 0.7	11.0 ± 2.0	10.4 ± 0.1	9.3 ± 0.2

[a] FS1 was prepared by diluting feces of diassociated mice 10-fold in APT medium, centrifuging, and diluting pellets fourfold in the supernatant. FS2 was prepared by diluting feces of diassociated mice fourfold in APT medium, and FS3 was prepared by diluting the feces 10-fold in sterile deionized water, discarding the supernatant, centrifuging, and diluting pellets fourfold in APT medium. $P < 0.001$ between data from both CpA and CpC in FS1 and FS3, between data from both FS1 and FS3 for CpA and CpC, and between data from FS1 and FS2 for CpC only.

CpA and CpC division numbers were not significantly different in APT medium and in filtrates prepared from feces of diassociated mice (Table 5). By contrast, filtrates prepared from feces of axenic mice allowed significantly lower division numbers of CpA and CpC than did the former filtrates. These differences disappeared when filtrates from feces of axenic mice were prepared from suspensions in APT medium (Table 5).

Bacteriostatic effect of suspensions prepared from feces of mice diassociated with *B. thetaiotaomicron* BT and *F. necrogenes* FN against *C. perfringens* CpA and CpC. FS1 suspensions were prepared as described in Table 6 (footnote *a*) and inoculated with CpA and CpC. The CpA division numbers were 0.9 ± 0.7, whereas they were 16.7 ± 0.4 in FS1 heated at 70°C for 10 min in sealed ampoules. The CpC division numbers were significantly ($P < 0.001$) higher than those obtained for CpA in fresh FS1 (4.9 ± 0.9 versus 0.9 ± 0.7) but not significantly different in heated FS1 (16.1 ± 0.1 versus 16.7 ± 0.4) (data not shown). Such an ex vivo model was thus in agreement with the results observed in vivo.

Attempts to maximize this in vitro antagonism by testing various combinations of soluble fecal substances (supernatant) and fecal solids revealed that some soluble fecal substances were needed (Table 6, lines 1 and 3). However, an excess led to artifacts, since CpC division numbers were significantly reduced in FS2 (Table 6, lines 1 and 2). Suspension FS1 gave the best response and was the preparation used subsequently.

Incidence of various parameters on bacteriostatic effect of FS1 fecal suspensions against *C. perfringens* CpA and CpC. A significant inverse correlation was observed between *B. thetaiotaomicron* BT and *F. necrogenes* FN initial counts and CpA and CpC division numbers in serially diluted FS1 suspensions, whereas such a correlation was not observed with BT and FN final counts, i.e., after a 22-h incubation at 37°C (Table 7). CpA and CpC division numbers were also inversely correlated with BT and FN initial counts in sonicated suspension FS1, and the CpA division numbers were significantly lower ($P < 0.001$) than those of CpC in all suspensions tested, except in the sonicated one (Table 7).

The bacteriostatic effect of the FS1 suspensions did not significantly differ either when CpA and CpC inocula differed by a factor of 100 or when glucose was added to FS1. Doubling the incubation time from 22 to 44 h did not significantly change the CpA division numbers (Table 8), which were 0.5 ± 0.2 after a 72-h incubation (data not shown).

Division numbers of both CpA and CpC were also significantly lower in FS1 suspensions prepared from mice diassociated with *B. thetaiotaomicron* BT and *F. necrogenes* FN than in those prepared from either mice monoassociated with BT or FN or mice diassociated with BT and *F. varium* FV or *B. vulgatus* BV and FN, or rats diassociated with BT and FN, or mice diassociated with BT and FN and receiving the milk diet (Table 9).

When colistine sulfate was added to the FS1 suspensions, the FN counts dropped to an undetectable level, whereas the BT counts were not affected and the CpA division numbers increased (Fig. 1B).

Division numbers of CpA and CpC in filtrates prepared from FS1 suspensions incubated for 24 h at 37°C (8.7 ± 0.5 and 14.0 ± 0.5, respectively) were significantly ($P < 0.001$) lower than those obtained in filtrates obtained from nonincubated suspensions (17.7 ± 0.8 and 17.3 ± 0.4, respectively; Table 5).

DISCUSSION

Our experimental model, involving an antagonism between strains belonging to the predominant intestinal genera such as *Bacteroides* and *Fusobacterium* and potentially enteropathogenic strains such as *C. perfringens*, seems to be the simplest described to date. Several interesting features have been demonstrated. The antagonism was easily reproducible in vivo and very stable, provided that mice were fed

TABLE 7. Influence of *B. thetaiotaomicron* BT and *F. necrogenes* FN initial counts in fecal suspensions prepared from diassociated mice on *C. perfringens* CpA and CpC division numbers

Counts (mean log ± SD)/ml[a]				Division no. of:	
Initial		Final			
BT	FN	BT	FN	CpA	CpC
10.4 ± 0.1	9.3 ± 0.2	10.0 ± 0.1	8.5 ± 0.2	0.9 ± 0.7	4.9 ± 0.9
9.9 ± 0.1	8.5 ± 0.1	9.6 ± 0.1	8.4 ± 0.1	2.0 ± 0.5	13.7 ± 0.4
9.7 ± 0.1	8.2 ± 0.1	9.7 ± 0.1	8.3 ± 0.2	5.0 ± 0.6	13.2 ± 0.3
9.4 ± 0.1	8.0 ± 0.2	9.5 ± 0.1	8.2 ± 0.2	8.5 ± 0.3	ND[b]
8.9 ± 0.1	7.3 ± 0.1	9.5 ± 0.1	8.1 ± 0.1	9.2 ± 0.3	16.0 ± 0.6
6.7 ± 0.1	5.4 ± 0.2	9.3 ± 0.2	8.1 ± 0.2	14.0 ± 0.2	ND
7.6 ± 0.1	5.5 ± 0.1	9.4 ± 0.1	8.4 ± 0.2	13.2 ± 0.4	14.5 ± 1.1

[a] Counts were made from FS1 (line 1) and fecal suspensions prepared as follows: supernatants from feces of diassociated mice diluted 10-fold in APT medium were used for diluting pellets 10-fold (line 2), 20-fold (line 3), 40-fold (line 4), 100-fold (line 5), and 10,000-fold (line 6). In line 7, data were from sonicated FS1 suspensions. Correlation coefficients (r) between CpA division numbers and BT and FN initial counts were −0.93 and −0.95 ($P < 0.001$), respectively. For CpC, these values were −0.84 and −0.88 ($P < 0.01$), respectively.

[b] ND, *Not done*.

TABLE 8. Influence of various parameters on *C. perfringens* CpA and CpC division numbers in FS1 fecal suspensions prepared from mice diassociated with *B. thetaiotaomicron* BT and *F. necrogenes* FN

Experimental conditions	Division no. of:		Initial counts (log_{10} means ± SD) of:	
	CpA	CpC	BT	FN
High inocula[a]	0.5 ± 0.4	3.8 ± 0.7	10.4 ± 0.1	9.3 ± 0.8
Long incubation[b]	0.6 ± 0.3	ND[c]	10.0 ± 0.1	9.1 ± 0.2
Presence of glucose[d]	0.6 ± 0.7	ND	10.4 ± 0.1	9.3 ± 0.2

[a] CpA and CpC inocula were 5.5 ± 0.1 and 5.6 ± 0.1, respectively, instead of 3.4 ± 0.1 and 3.3 ± 0.1. $P < 0.001$ between data from CpA and CpC.
[b] Incubation was for 44 instead of 22 h.
[c] ND, Not done.
[d] 0.1% glucose was added in FS1.

an adequate diet. As in the model described by Wilson and Freter (18), the target strain was suppressed even though it was inoculated first, whereas the order of inoculation has played an important role in several other reported interactions (5–7, 9, 14). The antagonistic effect of *B. thetaiotaomicron* and *F. necrogenes* acting synergistically against strain CpA was as efficient as that of a previously described (19) three-strain association and simulated that of whole piglet flora established in gnotobiotic mice (1, 15). Presumably, other bacterial strains could replace these two strains in eliminating strain CpA. However, our results show that not all strains belonging to the same genera were able to replace our inhibitory strains; we did not try to replace our two strains by other strains belonging to the same species. In contrast to the CpA strain, the CpC strain was more resistant to the antagonistic effect of the two-strain association since its growth was only repressed in gnotobiotic mice. It is noteworthy that these two strains afforded a protection to the host against the highly enteropathogenic CpC strain even though the population size of CpC was only partially reduced. That a synergy between inhibitory strains is required to antagonize a target strain in experimental models has already been described (5, 9, 12, 14), but the involvement of only two strains in such a synergy is an obvious improvement in the effort to achieve a simple model system. Assuming that it is also the case with complex floras, such as those described by Freter et al. (10) or Wilson and Freter (18), one can imagine how difficult it would be to simplify them. Indeed, if 2 of 50 strains are involved in a synergistic antagonism, the number of combinations which have to be tested in gnotobiotic mice is as much as 1,225, or 19,600 if 3 of 50 strains are involved. This emphasizes the utility of the bacterial antagonism described here. Another interesting feature of our model is that the target strain could be either eliminated or maintained at a low but rather constant population level in the same gnotobiotic animals, depending on the host (rat or mouse) and diet.

Our data clearly show that ex vivo experiments that use static incubations of fecal suspensions simulate our in vivo experiments. Thus, in and ex vivo antagonisms may be mediated by similar mechanisms. That a continuous-flow culture system failed to reproduce the bacterial antagonism we found may be due to both low population levels of strains BT and FN and the absence in our continuous-flow culture of fecal extracts. Taking into consideration that our ex vivo model appeared to be suitable for approaching the mechanism of the interaction we observed, we did not study the continuous-flow system further. Like Wilson and Freter, we demonstrated that soluble fecal extracts were needed to obtain the greatest antagonistic effect against both CpA and CpC. However, we also demonstrated that an excess of soluble fecal components or a 22-h incubation of FS1 suspensions led to production of some soluble growth inhibitors which can be considered as artifacts in our ex vivo model. Such discrepancies between ex and in vivo experiments are not surprising since bacterial metabolites such as volatile fatty acids produced in the large intestine can be readily absorbed by the intestinal wall or excreted or both, whereas the same metabolites accumulate in a closed in vitro or ex vivo static system and can thus suppress or limit the growth of the target strain. These results point out the difficulty in using in vivo models for studying bacterial antagonisms.

Concerning the mechanism of this bacterial antagonism, some assumptions may be drawn from our results, while some others should be rejected. Since the results of our ex vivo experiments simulate those of in vivo experiments, the hypothesis that competition for adhesion sites on the intestinal wall is a prerequisite for the antagonistic effect of the two inhibitory strains against CpA is unlikely. A hypothesis of depletion of essential intestinal nutrients or of production of soluble inhibitors accumulating in the feces is also unlikely. Soluble fecal inhibitors such as a bacitracin-like antibiotic or a copper-dipeptide complex have been successfully detected in other in vivo experimental models (2, 4) but were not found in our model. Bile salts are known to be deconjugated by *Bacteroides* sp. and to firmly adhere to insoluble fecal material. However, their inhibitory role could be ruled out in our experiments since heated FS1 suspensions, which should contain such substances, were not bacteriostatic for CpA.

Freter et al. (8) emphasized that some inhibitory metabolites such as H_2S can hinder target strains from utilizing

TABLE 9. Comparative efficiency of various fecal suspensions in reducing *C. perfringens* CpA and CpC division numbers

Source of feces	Diet	Strain(s) harbored[b]	Division no.[a] of:	
			CpA	CpC
Mice	Commercial	BT (10.4 ± 0.1) + FN (9.3 ± 0.2)	0.9 ± 0.7	4.9 ± 0.9
Mice	Commercial	BT (10.4 ± 0.1)	10.2 ± 0.3	12.7 ± 0.8
Mice	Commercial	FN (9.4 ± 0.1)	7.9 ± 0.3	14.5 ± 0.4
Mice	Commercial	BT (10.4 ± 0.2) + FV (9.6 ± 0.1)	6.7 ± 0.9	ND[c]
Mice	Commercial	BV (9.9 ± 0.1) + FN (9.1 ± 0.1)	4.7 ± 0.4	ND
Mice	Milk diet	BT (10.3 ± 0.1) + FN (9.1 ± 0.1)	5.0 ± 0.6	ND
Rats	Commercial	BT (10.4 ± 0.2) + FN (9.4 ± 0.1)	4.8 ± 0.8	13.4 ± 0.8

[a] $P < 0.001$ between data from line 1 and those from each of lines 2 to 7 for both CpA and CpC and between data from CpA and CpC in each line.
[b] Numbers in parentheses are the initial counts. The various fecal suspensions were prepared as described for FS1 (Table 6, footnote *a*).
[c] ND, Not done.

limiting nutrients present in their culture medium, which would otherwise support their growth. Substrates such as glucose were able to prevent such a metabolic inhibition. Our in and ex vivo data are quite different from those of Freter et al. (8, 10) because of the following. (i) We succeeded in simplifying a complex flora without any loss of antagonistic effect. (ii) The antagonism described was independent of the order of inoculation, but depended on the host and diet. (iii) H_2S or other volatile compounds cannot be involved in this antagonism, since incubations of fecal suspensions were performed in screw-cap tubes with a large headspace into which volatile compounds could easily escape. (iv) CpA growth did not resume when excess glucose was added to the fecal suspensions. (v) Continuous-flow culture did not reproduce the in vivo results. The actual mechanism by which our two-strain association antagonizes strain CpA is therefore not the same as that described by Freter et al. (8) for *E. coli* C25. However, Freter's assumption that some inhibitors, different from H_2S, could interfere with the utilization of nutrients other than macronutrients seems to be the most appropriate to explain the observation that target strains CpA and CpC enter into bacteriostasis in a medium in which available macronutrients are still present. Such inhibitors are produced synergistically by *B. thetaiotaomicron* BT and *F. necrogenes* FN in a steady state, as in the intestine and in our ex vivo model, and do not accumulate. That CpA was eliminated from the intestine of gnotobiotic mice whatever the order of inoculation suggests that CpA entered into bacteriostasis, i.e., failed to utilize available nutrients when the antagonistic strains reached their maximum population level. This assumption is supported by our ex vivo experiments in which an almost complete CpA bacteriostasis only occurred in the suspension containing the highest counts of the two inhibitory strains. A bactericidal effect against a target strain is not a prerequisite for its washout from the intestine because of the intestinal transit. In our in vivo experiments, however, some death of CpA cells occurred because the rate of CpA elimination was faster than that of the transit marker. In our ex vivo experiments, a full bacteriostasis was not achieved. The reason might be that the counts of both BT and FN strains in fecal suspension were significantly lower than in feces of diassociated mice. This bacteriostasis, however, was reversible, as in Freter's experiments, since CpA and CpC could be easily counted in feces or suspensions by diluting them. This suggests that inhibitors allowing CpA, and to a lesser extent CpC, to enter into bacteriostasis might be washed out from the target cells when they are no longer produced in the surroundings of these cells. Additional hypotheses that could be advanced to explain our results are as follows. (i) Unknown endogenous components, issued from the host or the diet or both, might modulate the synthesis or the efficiency of the bacterial metabolic inhibitors. (ii) Inhibitory bacterial cells have to produce them continuously to maintain a complete bacteriostasis of the target strains so that no accumulation will be required for antagonizing the target strains. Direct cell-to-cell contacts might also contribute to the transfer of the inhibitors to the target strains. This assumption is consistent with the fact that there was an inverse correlation between the BT and FN initial counts and the CpA and CpC division numbers in our ex vivo experiments. Further experiments are now under way to elucidate the actual mechanism of this bacterial antagonism.

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LITERATURE CITED

1. **Corpet, D., and J. L. Nicolas.** 1979. Clinical and experimental gnotobiotics. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Suppl. **7**:169–174.
2. **Dubos, F., J. P. Pélissier, C. Andrieux, R. Ducluzeau, and P. Raibaud.** 1985. Inhibitory effect of a copper-dipeptide complex on the establishment of a *Clostridium perenne* strain in the intestinal tract of gnotobiotic mice. Appl. Environ. Microbiol. **17**:415–424.
3. **Ducluzeau, R., M. Bellier, and P. Raibaud.** 1970. Transit digestif de divers inoculums bactériens introduits "per os" chez des souris axéniques et "holoxéniques" (conventionnelles): effet antagoniste de la microflore du tractus gastro-intestinal. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. I Orig. **213**:533–548.
4. **Ducluzeau, R., F. Dubos, P. Raibaud, and G. D. Abrams.** 1978. Production of an antibiotic substance by *Bacillus licheniformis* within the digestive tract of gnotobiotic mice. Antimicrob. Agents Chemother. **13**:97–103.
5. **Ducluzeau, R., M. Ladiré, C. Callut, P. Raibaud, and G. D. Abrams.** 1977. Antagonistic effect of extremely oxygen-sensitive clostridia from the microflora of conventional mice and of *Escherichia coli* against *Shigella flexneri* in the digestive tract of gnotobiotic mice. Infect. Immun. **17**:415–424.
6. **Ducluzeau, R., and P. Raibaud.** 1974. Interactions between *Escherichia coli* and *Shigella flexneri* in the digestive tract of gnotobiotic mice. Infect. Immun. **9**:730–733.
7. **Duval-Iflah, Y., P. Raibaud, and M. Rousseau.** 1981. Antagonisms among isogenic strains of *Escherichia coli* in the digestive tracts of gnotobiotic mice. Infect. Immun. **34**:957–969.
8. **Freter, R., H. Brickner, M. Botney, D. Cleven, and A. Aranki.** 1983. Mechanisms that control bacterial populations in continuous-flow culture models of mouse large intestinal flora. Infect. Immun. **39**:676–685.
9. **Freter, R., H. Brickner, J. Fekete, M. M. Vickerman, and F. E. Carey.** 1983. Survival and implantation of *Escherichia coli* in the intestinal tract. Infect. Immun. **39**:686–703.
10. **Freter, R., E. Stauffer, D. Cleven, L. V. Holdeman, and W. E. C. Moore.** 1983. Continuous-flow cultures as in vitro models of the ecology of large intestinal flora. Infect. Immun. **39**:666–675.
11. **Hentges, D. J., and R. Freter.** 1962. *In vivo* and *in vitro* antagonism of intestinal bacteria against *Shigella flexneri*. I. Correlation between various tests. J. Infect. Dis. **110**:30–37.
12. **Hudault, S., H. Bewa, C. Bridonneau, and P. Raibaud.** 1985. Efficiency of various bacterial suspensions derived from cecal floras of conventional chickens in reducing the population level of *Salmonella typhimurium* in gnotobiotic mice and chicken intestines. Can. J. Microbiol. **31**:832–838.
13. **Hudault, S., C. Bridonneau, and P. Raibaud.** 1983. Pouvoir pathogène de différents types toxinogènes de *Clostridium perfringens* inoculés *per os* à des souris axéniques et holoxéniques. Ann. Microbiol. (Paris) **134B**:277–283.
14. **Hudault, S., P. Raibaud, R. Ducluzeau, and C. Bridonneau.** 1982. Effet antagoniste à l'égard de *Clostridium perfringens* exercé par des souches de *Clostridium* isolées de la·microflore de souris holoxéniques dans le tube digestif de souris gnotoxéniques. Ann. Microbiol. (Paris) **133A**:443–459.
15. **Nicolas, J. L., P. Raibaud, R. Ducluzeau, and D. Corpet.** 1981. Antagonism against *Clostridium perfringens* in the digestive tract of gnotoxenic mice and piglets exerted by a small number of anaerobic bacteria isolated from the faecal flora of a conventional piglet, p. 201–205 *In* S. Sasaki, A. Ozawa, and K. Hashimoto (ed.), Recent advances in germfree research. Proceedings of the VIIth International Symposium on Gnotobiology. Tokai University Press, Tokyo.
16. **Raibaud, P., A. B. Dickinson, E. Sacquet, M. Charlier, and G. Mocquot.** 1966. La microflore du tube digestif du rat. I. Techniques d'étude et milieux de culture proposés. Ann. Inst.

Pasteur (Paris) **110**:568–590.

17. **Sacquet, E., P. Raibaud, and J. Garnier.** 1971. Etude comparée de la microflore de l'estomac, de l'intestin grêle et du caecum du rat "holoxénique" (conventionnel), et de ses modifications à la suite de diverses interventions chirurgicales: anse aveugle jéjunale, déviations biliaires. Ann. Inst. Pasteur (Paris) **120**: 501–524.
18. **Wilson, K. H., and R. Freter.** 1986. Interaction of *Clostridium difficile* and *Escherichia coli* with microfloras in continuous-flow cultures and gnotobiotic mice. Infect. Immun. **54**:354–358.
19. **Yurdusev, N., J. L. Nicolas, M. Ladiré, R. Ducluzeau, and P. Raibaud.** 1987. Antagonistic effect exerted by three strictly anaerobic strains against various strains of *Clostridium perfringens* in gnotobiotic rodent intestines. Can. J. Microbiol. **33**: 226–231.