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# Trypsin-Dependent Production of an Antibacterial Substance by a Human *Peptostreptococcus* Strain in Gnotobiotic Rats and In Vitro

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**An antibacterial substance appeared within 1 day in feces of gnotobiotic rats harboring a human intestinal *Peptostreptococcus* strain. It disappeared when the rat bile-pancreatic duct was ligatured or when the rats ingested a trypsin inhibitor. Anaerobic cultures of the *Peptostreptococcus* strain in a medium supplemented with trypsin also exhibited an antibacterial activity, which was also inhibited by the trypsin inhibitor. In vitro the antibacterial substance from both feces and culture medium was active against several gram-positive bacteria, including other *Peptostreptococcus* spp., potentially pathogenic *Clostridium* spp. such as *C. perfringens*, *C. difficile*, *C. butyricum*, *C. septicum*, and *C. sordellii*, *Eubacterium* spp., *Bifidobacterium* spp., and *Bacillus* spp. Whatever the order of inoculation of the strains, a sensitive strain of *C. perfringens* was eliminated within 1 day from the intestine of rats monoassociated with the *Peptostreptococcus* strain. These findings demonstrate for the first time that very potent antibacterial substances can be produced through a mechanism involving intestinal bacteria and exocrine pancreatic secretions.**

Defense mechanisms against potentially pathogenic microorganisms represent a network of antagonisms involving several host components. However, little is known about the factors involved in these antagonisms. A nonindigenous strain of *Bacillus licheniformis* was found to produce a bacitracin-like substance in the gastrointestinal tracts of gnotobiotic mice, but the antibiotic was no longer detected when indigenous bacteria were associated with the *Bacillus* strain (5, 6). Bacteriocin-producing bacterial strains are commonly found in the mammalian intestinal tract. However, it has not been demonstrated that bacteriocins could be active in the intestinal tract, as bacteriocin-sensitive *Escherichia coli* strains were found to inhibit bacteriocin-producing *E. coli* strains in the gnotobiotic mouse intestine (7). A complex bacterial antagonism in the gnotobiotic mouse intestine has been described (12), but it has not been fully elucidated. In this paper we demonstrate for the first time that an antibacterial substance can be produced in the intestinal tracts of gnotobiotic rats by a mechanism involving both a human intestinal strain of *Peptostreptococcus* and the exocrine pancreatic secretions and that this substance protects the host against potentially pathogenic *Clostridium* spp.

## MATERIALS AND METHODS

**Animals and diet.** Adult axenic and gnotobiotic 2-month-old Fisher 344 rats were reared in Trexler-type isolators fitted with a rapid transfer system (La Calhène, Vélizy-Villacoublay, France). They were fed ad libitum with a commercial diet for rodents (U.A.R., Epinay-sur-Orge, France) sterilized by gamma irradiation (40 kGy).

**Ligature of the common pancreatic-bile duct.** Axenic rats were placed in a surgical sterile isolator and were fasted for 24 h before anesthesia. They were divided into two groups. One group underwent a double ligature of the common pancreatic-bile duct, and the other underwent a sham operation. After a median laparotomy, two ligatures were placed around the common duct at about 0.5 cm from the duodenum. The sham-operated rats underwent the same operation, except that the ducts were not ligated. Diet and water were given ad libitum just after the operation.

**Bacterial strains.** *Peptostreptococcus* strain E1, which produced an antibiotic-like substance, was isolated by us from the predominant bacterial flora of a healthy adult man. The target strain for demonstrating antibiotic-like activity was *Clostridium perfringens* CpA, which was a variant from our collection belonging to serotype A. It was unable to form thermoresistant spores, as checked by heating cultures and fecal suspensions of rats monoassociated with *C. perfringens* CpA at 70°C for 10 min in sealed ampoules. Strains used for studying the antibacterial spectrum were 20 *Clostridium* and 4 *Bacteroides* strains kindly provided by M. Delmée (Leuwen Universitate, Brussels, Belgium) and M. Popoff (Pasteur Institute, Paris); 94 strictly anaerobic strains isolated by us from the predominant flora of human stools and classified as members of the genera *Clostridium*, *Bacteroides*, *Veillonella*, *Fusobacterium*, *Bifidobacterium*, *Peptostreptococcus*, and *Eubacterium* by their shape, motility, Gram stain properties, shape and position of spores (if any), and catalase and volatile fatty acid production; 6 facultatively anaerobic strains isolated by us from the subdominant flora of human stools and classified as members of the genera *Lactobacillus*, *Enterococcus*, and *Escherichia* by using the API System (La Balme les Grottes, Montalieu, France); and a collection strain, *Bacillus subtilis* ATCC 6633. Spores of a strictly thermophilic *Bacillus* strain, unable to growth below 50°C, were used as a transit marker (4). When inoculated per os, these spores fail to germinate and to multiply and pass through the gut without losing viability.

**Association of rats with bacterial strains.** *C. perfringens* CpA and *Peptostreptococcus* strain E1 were grown in liquid

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brain heart infusion medium (BHI) (Difco Laboratories, Detroit, Mich.) in an anaerobic chamber for 18 and 24 h, respectively, at 37°C. A suspension containing $10^8$ spores of the transit marker per ml was admixed just before inoculation with the *C. perfringens* CpA inoculum containing $10^8$ vegetative cells. Counts of the transit marker and of *C. perfringens* allow determination of whether *C. perfringens* is subjected to a bacteriostatic effect (i.e., fails to multiply but keeps its viability as does the transit marker) or to a bactericidal effect (i.e., disappears from the feces faster than the transit marker). Rats were inoculated through the orogastric route with 1 ml of the bacterial inoculum.

**Rat samplings.** Fecal pellets were individually collected from each rat for assessment of the kinetics of bacterial establishment and elimination and of fecal proteolytic and antibiotic-like activities. At the end of experiments, rats were euthanasized with chloroform, and stomach, ileum, and cecum contents were removed for determination of proteolytic activities, antibiotic-like activities, and bacterial counts.

**Bacterial cultures and counts.** For studying the in vitro production of the antibiotic-like substance, *Peptostreptococcus* strain E1 was cultured in an anaerobic chamber in liquid BHI medium supplemented or not with the following additives: trypsin from bovine pancreas (type XIII, L-1-tosylamide phenylalanyl chloromethyl ketone treated; Sigma Chemical Co.), chymotrypsin A4 from bovine pancreas (specific activity, 90 U/mg; Boehringer, Mannheim, Germany), elastase from porcine pancreas (specific activity, 100 U/mg; Merck A.G., Darmstadt, Germany), carboxypeptidase A from bovine pancreas (specific activity, 35 U/mg; Boehringer), carboxypeptidase B from porcine pancreas (type I, chromatographically purified; Sigma Chemical Co), and trypsin inhibitor from soybean (type I-S, chromatographically prepared; Sigma Chemical Co.). Their concentrations are given elsewhere in the text and in the tables. Each medium was prereduced for 48 h in the anaerobic chamber, supplemented with enzymes just before inoculation, inoculated with 1 ml of a 24-h preculture containing $10^4$ viable cells of *Peptostreptococcus* strain E1 in 10 ml of the medium, and incubated at 37°C. Counts of *Peptostreptococcus* strain E1 were done by plating 0.1 ml of adequate serial 10-fold dilutions of the cultures on solid BHI medium prereduced for 48 h. For counting *C. perfringens* CpA and *Peptostreptococcus* strain E1 in gnotobiotic rats, feces and gastrointestinal contents were immediately diluted 10-fold and homogenized with an Ultraturrax (Osi, Paris, France) into the anaerobic chamber, and 0.1 ml of adequate dilutions was plated on solid BHI medium. Incubation at 37°C was for 24 h for *C. perfringens* CpA and 3 days for *Peptostreptococcus* strain E1. Spores of the transit marker were counted by plating on a solid medium (medium MS) containing 0.8% meat extract (Merck), 0.2% yeast extract (Difco), 0.1% glucose, 40 mg of manganese sulfate per liter, and 1% Bacto-agar (Difco) (pH 6.3). Incubation was done aerobically at 55°C for 24 h. All bacterial counts are expressed per gram (wet weight) of fresh feces or gastrointestinal contents.

**Assay for the antibiotic-like substance produced by *Peptostreptococcus* strain E1.** Plates of solid BHI medium were inoculated with $10^8$ viable cells of the target strain *C. perfringens* CpA by using the poured-plate technique. Samples from axenic and monoassociated rats (feces or gastrointestinal contents) weighing 500 ± 50 mg were gently placed into the inoculated medium just before its solidification. For assaying the in vitro production of antibiotic-like substance, 35 μl of cultures concentrated 10-fold in a Speedvac concentrator (Bioblock Scientific, Illkirch, France) was put into holes opened in the inoculated BHI medium after solidification. Plates were kept in an anaerobic jar (GasPak system; Biomerieux, Lyon, France) for 18 h at 4°C to ensure the diffusion of the antibiotic-like substance and then incubated for 8 h at 37°C. The amount of antibiotic-like substance was assayed by measuring the radius of the inhibitory zone against *C. perfringens* CpA around the holes or rat samples.

**Antibacterial spectrum of the antibiotic-like substance.** Strains to be tested were inoculated by the poured-plate technique in solid BHI medium in place of the target strain *C. perfringens* CpA. Both feces of rats monoassociated with *Peptostreptococcus* strain E1 and concentrated cultures containing the antibiotic-like substance were used. The amount of antibiotic-like substance was assayed as described above. Strains were referred to as sensitive when an inhibitory zone appeared around the feces or the holes and as resistant when the zone was absent.

**Protease assay.** Feces or cecal contents of gnotobiotic rats were diluted 10-fold in saline, homogenized, and assayed with and without trypsin inhibitor (final concentration, 1 mg/ml) for their proteolytic activity on purified pig immunoglobulin G (IgG) by the enzyme-linked immunosorbent assay (ELISA) technique described by Corthier et al. (3). IgG (5 μg/ml) was covently bound (0.1 M bicarbonate buffer, pH 9.8) to plastic microtiter plates by glutaraldehyde. After being washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (Sigma), each plate was incubated with various dilutions of fecal or cecal contents for 2 h at 37°C. After another wash with PBS containing 0.05% Tween 20, titration of the remaining bound material was performed as follows. For quantitation of pig IgG, an anti-pig IgG coupled to alkaline phosphatase (1/1,000) (Sigma) was used. Bound enzyme was revealed with *p*-nitrophenylphosphate (Sigma). The digestive protease activity titer was defined as the highest $\log_{10}$ dilution of the sample which reduced 50% of the quantity of bound material.

**Pancreatic enzyme assay.** The complete small intestine was removed, diluted threefold in saline, and homogenized for 1 min with an Ultraturrax in iced distillated water. Enzyme activities in homogenates were determined. Chymotrypsin and trypsin activities were determined as described by Reboud et al. (10). Amylase activity was measured as described by Corring and Saucier (2), and lipase activity was measured as described by Rathelot et al. (9).

**Statistical analysis.** Student's *t* test was used for a comparison of mean values of $\log_{10}$ bacterial counts, $\log_{10}$ proteolytic activity titers, and mean values of radii of inhibitory zones and of enzymatic activity titers.

## RESULTS

**Detection of inhibitory substance in gastrointestinal tracts of rats monoassociated with *Peptostreptococcus* strain E1.** A diffusible antibacterial substance against *C. perfringens* CpA appeared within 24 h after inoculation in feces of rats monoassociated with *Peptostreptococcus* strain E1 (Fig. 1). When *C. perfringens* CpA was inoculated with spores of the transit marker after *Peptostreptococcus* strain E1, *C. perfringens* was eliminated faster than the transit marker, showing that *C. perfringens* CpA lost almost all of its viability during its intestinal transit (Fig. 1A). When *C. perfringens* CpA was inoculated before *Peptostreptococcus* strain E1, it was no longer detected as soon as the diffusible antibiotic-like substance appeared in the feces (Fig. 1B). The antibiotic-like activity was also high in the ceca and colons of

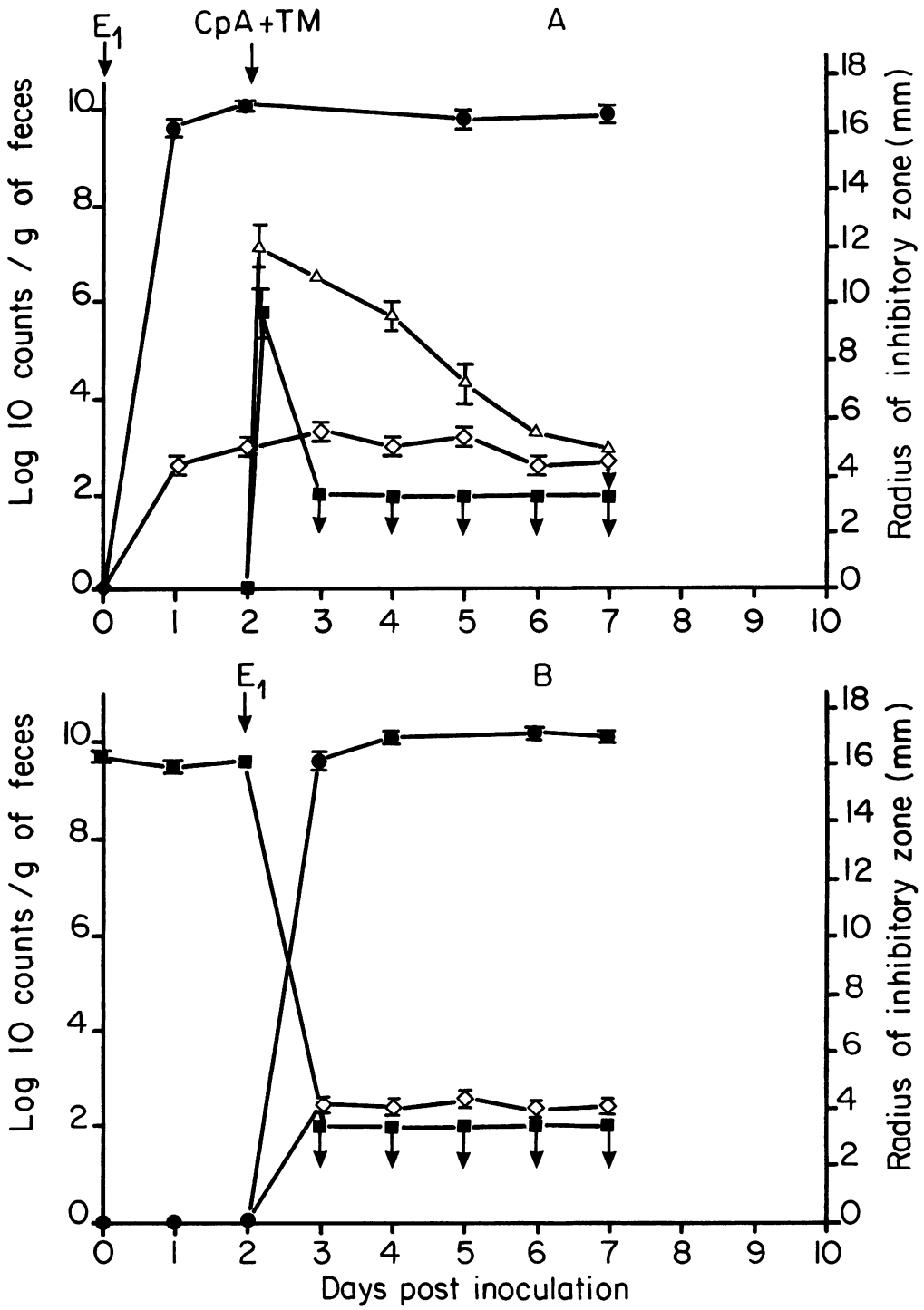


FIG. 1. Kinetics of elimination of *C. perfringens* CpA (■) and the transit marker (TM) (△) in rats monoassociated with *Peptostreptococcus* strain E1 (●). ◇, radius of inhibitory zone against *C. perfringens* CpA around the feces of monoassociated rats. Each point represents the mean ± standard error of the mean for six individual samples. Arrows indicate bacterial inoculations: (A) *Peptostreptococcus* strain E1 at day 0 and *C. perfringens* CpA plus spores of the transit marker at day 2; (B) *Peptostreptococcus* strain E1 at day 2, 6 days after *C. perfringens* CpA. ▼, limit of detection for strains (less than $10^2$ per g of fresh content or feces).

monoassociated rats, where *Peptostreptococcus* strain E1 was at the highest population level, and low in the stomach, where *Peptostreptococcus* strain E1 was at the lowest level (Table 1). By contrast, the ileum contents of both axenic and monoassociated rats exhibited an antibacterial activity against *C. perfringens* CpA.

**Role of trypsin in in vitro production of antibiotic-like substance by *Peptostreptococcus* strain E1.** As attempts to

TABLE 1. Production of an inhibitory substance active against *C. perfringens* CpA by *Peptostreptococcus* strain E1 in the gastrointestinal tracts of rats monoassociated with strain E1

| Gastrointestinal tract contents | Radius (mm) of inhibitory zone against *C. perfringens* CpA (mean ± SEM; $n$ = 6) | | $Log_{10}$ counts of strain E1/g of fresh samples (mean ± SEM; $n$ = 6) |
|---|---|---|---|
| | Axenic rats | Monoassociated rats[a] | |
| Stomach | 0.0 | 0.6 ± 0.4 | 3.4 ± 0.9 |
| Ileum | 4.0 ± 0.3 | 4.8 ± 0.7 | 4.6 ± 1.1 |
| Cecum | 0.0 | 4.4 ± 0.4 | 9.9 ± 0.1 |
| Colon | 0.0 | 4.9 ± 0.8 | 9.9 ± 0.1 |

[a] Rats were associated with strain E1 for 21 days.

obtain a diffusible antibiotic-like substance in various usual culture media, including BHI medium, failed, we decided to prepare media enriched with host components found in the axenic rat cecum, such as proteolytic pancreatic enzymes. Chymotrypsin, elastase, and carboxypeptidases A and B were assayed at concentration ranges determined by measuring enzyme activities in rat pancreas, i.e., 0.5, 0.08, 0.16, and 0.009 mg/ml, respectively. None promoted the antibiotic-like activity of *Peptostreptococcus* strain E1 at these concentrations. By contrast, Table 2 shows that trypsin was able to promote the production of a diffusible antibiotic-like substance, provided that its concentration ranged between 0.05 and 0.5 mg/ml, i.e., the range observed in rat pancreas. The diffusible antibiotic-like substance appeared in the BHI medium containing trypsin when the *Peptostreptococcus* strain E1 culture reached its maximum population level, and the growth curve of *Peptostreptococcus* strain E1 was not modified by trypsin addition (Fig. 2). An inhibitory zone never appeared in BHI medium without trypsin. No inhibitory zone appeared in noninoculated BHI medium incubated with trypsin alone. When trypsin inhibitor was included with trypsin before inoculation in BHI medium, no inhibitory zone appeared, although the *Peptostreptococcus* strain E1 counts were not modified (data not shown). In a second experiment, we introduced trypsin 12 h after the beginning of *Peptostreptococcus* strain E1 culture, i.e., when its population level was the highest. No inhibitory zone appeared during the following 36 h of incubation after inoculation (data not shown). Thus, these in vitro experiments demonstrated that the antibiotic-like activity of *Peptostreptococcus* strain E1 was trypsin dependent and that it was necessary that trypsin be present from the beginning of the *Peptostreptococcus* strain E1 growth.

TABLE 2. Effect of trypsin concentration in the culture medium on the production of antibiotic-like substance by *Peptostreptococcus* strain E1

| Trypsin concn (mg/ml)[a] | $Log_{10}$ counts of strain E1/ml of culture (mean ± SEM)[b] | Radius (mm) of inhibitory zone (mean ± SEM)[c] |
|---|---|---|
| None | 7.5 ± 0.1 | 0.0 |
| 0.005 | 7.7 ± 0.4 | 0.0 |
| 0.05 | 7.2 ± 0.1 | 3.0 ± 1.0 |
| 0.5 | 7.7 ± 0.2 | 3.0 ± 0.2 |
| 5.0 | 7.6 ± 0.3 | 0.0 |
| 0.5 | None | 0.0 |

[a] Four samples for each concentration.
[b] Incubation was for 24 h at 37°C.
[c] Amount of antibiotic-like substance was measured by the radius of the inhibitory zone against *C. perfringens* CpA (24-h cultures 10-fold concentrated were used for this measure).

**Role of trypsin in in vivo production of antibiotic substance by *Peptostreptococcus* strain E1.** Trypsin inhibitor (5 mg/ml in 0.05 M bicarbonate buffer prepared immediately before the experiment and changed daily) was given as a drink to five rats monoassociated with *Peptostreptococcus* strain E1 (Fig. 3). It has the same effect in vivo as in vitro, since the fecal inhibitory zone disappeared the day after the beginning of the treatment, without any change in the *Peptostreptococcus* strain E1 counts. *C. perfringens* CpA, inoculated 1 day after the disappearance of the inhibitory zone, became established at a high population level, although significantly lower than that obtained in rats monoassociated with *C. perfringens* CpA receiving trypsin inhibitor (7.1 ± 0.1 $log_{10}$ counts/g versus 7.9 ± 0.0 $log_{10}$ counts/g) ($P < 0.001$) (data not shown). *C. perfringens* CpA disappeared within 3 days when the treatment was stopped, whereas the fecal antibiotic-like activity concomitantly reappeared.

To assess whether trypsin was the main host component involved in in vivo production of the antibiotic-like substance, 11 rats monoassociated with *Peptostreptococcus* strain E1 underwent bile-pancreatic duct ligature, and 5 rats were sham operated. Four operated rats received trypsin (5 mg/ml in 0.05 M bicarbonate buffer prepared immediately before the experiment and changed each day) given as a drink from day 6 to 10 postoperation. All of the rats were sacrificed 10 days postoperation. In operated rats the fecal proteolytic activity was less than the detectable amount within 4 days, and the fecal antibiotic-like activity completely disappeared within 5 days (Fig. 4B). Only a slight, but significant ($P < 0.01$), decrease in the fecal proteolytic activity was observed for the sham-operated rats 3 days postoperation, without any significant change in the antibiotic-like activity (Fig. 4A). Table 3 proves the efficacy of ligature, since the activities of the four pancreatic enzymes measured dramatically decreased in the small intestines of the operated rats compared with those measured in the sham-operated rats ($P < 0.001$). Table 4 shows that (i) neither antibiotic-like nor detectable proteolytic activity was measured in the cecal contents of operated rats, whereas both activities were still present in the sham-operated rats, and (ii) *Peptostreptococcus* strain E1 counts were not modified by the operation. In the four operated rats drinking trypsin, the fecal antibiotic-like activity reappeared on the day after treatment with large individual variations, while the fecal proteolytic activity increased slowly and did not reach the level measured in feces of sham-operated rats (Fig. 4B). In the small intestines of operated rats drinking trypsin, an unexpected significant increase in amylase activity ($P < 0.01$) was observed, whereas the activities of the other enzymes, including trypsin, were not significantly different in the two groups of operated rats (Table 3). In the cecal contents of these rats, both proteolytic and antibiotic-like activities varied between individuals but remained significantly lower ($P < 0.01$) than those measured in sham-operated rats, while *Peptostreptococcus* strain E1 counts were not modified (Table 4).

**In vitro and in vivo spectra of antibiotic-like activity of *Peptostreptococcus* strain E1.** Among the 124 strains tested, most of the gram-positive strictly anaerobic strains were sensitive to the antibiotic-like substance produced by *Peptostreptococcus* strain E1 in trypsin-BHI medium and in the digestive tracts of monoassociated rats, whereas all the gram-negative strains were resistant (Table 5). Among mem-

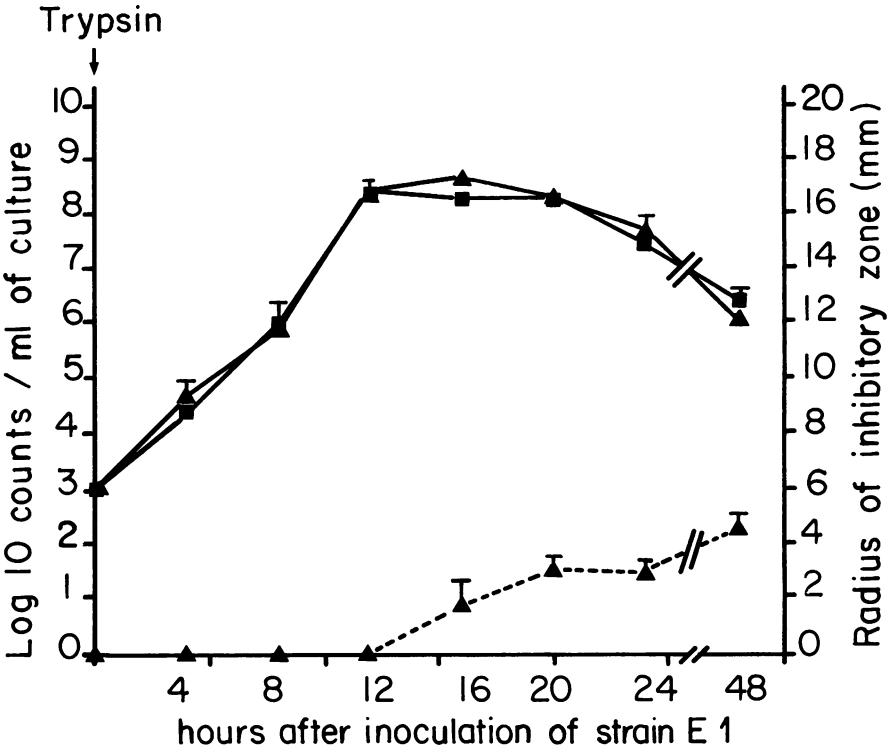


FIG. 2. Growth curve of *Peptostreptococcus* strain E1 in BHI medium with trypsin (0.5 mg/ml) (▲——▲) or without trypsin (■) and content of antibiotic-like substance in BHI medium with trypsin (0.5 mg/ml) measured by radius of inhibitory zone against *C. perfringens* CpA (▲----▲). Each point represents the mean ± standard error of the mean for three samples). The arrow indicates the trypsin addition.

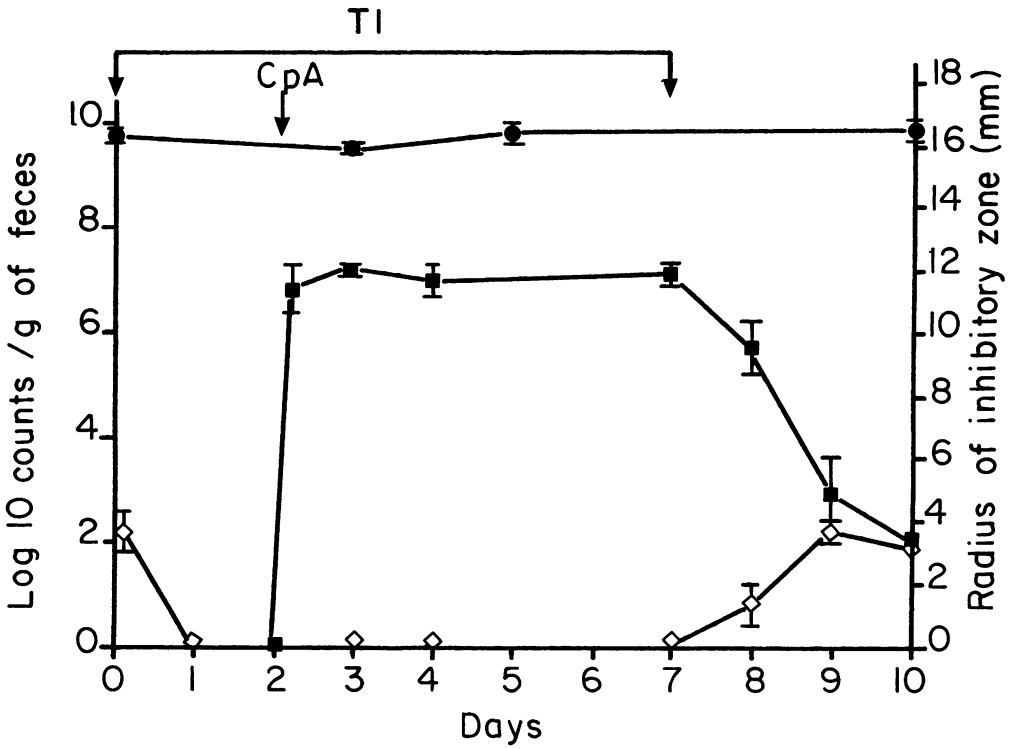


FIG. 3. Effect of trypsin inhibitor (TI) on the inhibitory zone against *C. perfringens* CpA (◇) and on the amount of *C. perfringens* CpA (■) in rats monoassociated with *Peptostreptococcus* strain E1 (●) for 10 days. Each point represents the mean ± standard error of the mean for five individual samples. The arrow indicates CpA inoculation at day 2. The horizontal bar with arrows shows the administration of oral trypsin inhibitor from day 0 to 7.

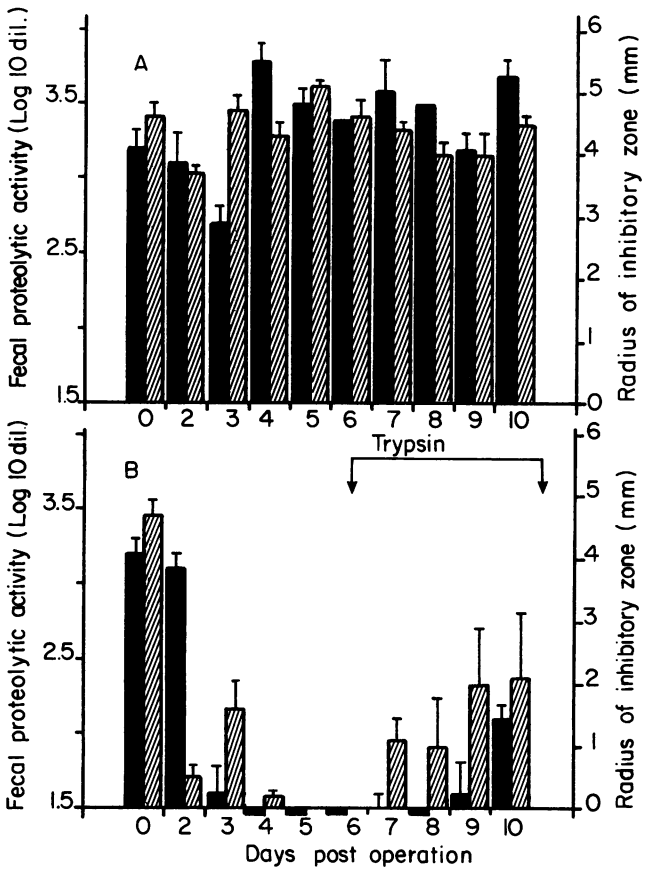


FIG. 4. Fecal proteolytic activity (solid bars) and radius of inhibitory zone (hatched bars) against *C. perfringens* CpA in feces of rats monoassociated with *Peptostreptococcus* strain E1. (A) sham-operated rats; (B) rats which have undergone a ligature of the common bile-pancreatic canal. The horizontal bar with arrows shows the administration of oral trypsin from day 6 to 10. Each bar represents the mean ± standard error of the mean of individual values from 5 sham-operated rats and from 11 operated rats from day 0 to 6 and thereafter of values from 4 operated rats drinking trypsin solution. Both proteolytic and antibiotic-like activities were less than the detectable amount for the seven operated rats drinking water after day 6 postoperation.

bers of the *Clostridium* genus, potentially pathogenic strains, including other *C. perfringens* strains (four strains), *C. difficile* (nine strains), *C. sordellii* (five strains), *C. butyricum* (eight strains), and *C. septicum* (one strain), were shown to be sensitive, as were *C. bifermentans* (one strain), *C. ramosum* (one strain), and *C. tertium* (one strain). Strains of *Lactobacillus* (two strains), *Enterococcus* (one strain), and *E. coli* (two strains) were found to be resistant, whereas *B. subtilis* was sensitive.

Two groups of three rats monoassociated with *Peptostreptococcus* strain E1 were challenged with either a sensitive toxinogenic strain of *C. difficile* or a resistant *Bacteroides* strain. These strains were inoculated and counted as for *Peptostreptococcus* strain E1. Fecal elimination of the *C. difficile* strain was as rapid as that of *C. perfringens* CpA (data not shown). By contrast, the *Bacteroides* strain became established within 1 day postinoculation and outnumbered *Peptostreptococcus* strain E1 at the end of experiment (10.5 ± 0.0 $\log_{10}$ counts/g versus 9.5 ± 0.1 $\log_{10}$ counts/g),

TABLE 3. Effect of bile-pancreatic canal ligature on enzyme activities in the small intestines of rats monoassociated with *Peptostreptococcus* strain E1

| Rat group (*n*) | Total U of enzyme in small intestine (mean ± SEM) | | | |
|---|---|---|---|---|
| | Amylase[a] | Lipase[b] | Trypsin[c] | Chymotrypsin[d] |
| Sham operated (5) | 13,786 ± 1,401 | 660 ± 155 | 234 ± 19.9 | 320 ± 51 |
| Operated (7) | 181.2 ± 21.6 | 25.0 ± 4.8 | 26.7 ± 2.8 | 21.3 ± 1.3 |
| Operated + trypsin (4) | 680.5 ± 124.7 | 30.2 ± 9.4 | 34.5 ± 12.6 | 18.7 ± 7.8 |

[a] One unit is the amount which catalyzes digestion of 1 mg of soluble starch in 30 min at 37°C.
[b] Units are micromoles of fatty acids released per minute.
[c] Units are micromoles of benzoylarginine ethylester hydrolyzed per minute.
[d] Units are the micromoles of acetyltyrosyl ethylester hydrolyzed per minute.

although the antibiotic-like activity was still present in feces of rats (radius of inhibitory zone, 3.6 ± 0.3 mm) (data not shown).

## DISCUSSION

Our results clearly demonstrate that *Peptostreptococcus* strain E1 elicits an antibiotic-like activity against *C. perfringens* strain CpA which is trypsin dependent. In vivo, the antibiotic-like activity was evident only in the ceca, colons, and feces of rats monoassociated with *Peptostreptococcus* strain E1. The presence of an antibacterial activity against *C. perfringens* CpA in the ileum contents of both axenic and monoassociated rats could be due to some endogenous compounds, such as those reported by Fuller and Moore (8), or to the antibacterial activity of the pancreatic fluid described by Rubinstein et al. (11). However, such inhibitory compounds did not inhibit the *C. perfringens* CpA establishment in our axenic rats. As the *Peptostreptococcus* strain E1 count in the rat ileum was low, it is not surprising that the ileal inhibitory substances were still present in monoassociated rats.

The commercially available purified trypsin inhibitor used was able to suppress the antibiotic-like activity in monoassociated rats. Given the high specificity of action of this inhibitor, it can be concluded that trypsin is absolutely required by *Peptostreptococcus* strain E1 for the in vivo production of the antibiotic-like substance. Furthermore, the antibiotic-like and proteolytic activities were no longer detected when trypsin was no longer available in the large intestines of monoassociated rats following the bile-pancre-

TABLE 4. Effect of bile-pancreatic canal ligature on production of antibiotic-like substance by *Peptostreptococcus* strain E1, its population level, and the proteolytic activity of cecal contents of rats monoassociated with strain E1

| Rat group (*n*) | Radius (mm) of inhibitory zone (mean ± SEM)[a] | Cecal proteolytic activity/liter (mean ± SEM) | $\log_{10}$ counts of strain E1/g of cecal content (mean ± SEM) |
|---|---|---|---|
| Sham operated (5) | 4.7 ± 0.2 | 3.6 ± 0.1 | 9.5 ± 0.1 |
| Operated (7) | 0.0 | <0.9 | 9.6 ± 0.1 |
| Operated + trypsin (4) | 1.6 ± 1.0 | 1.8 ± 0.2 | 9.4 ± 0.1 |

[a] The amount of antibiotic-like substance was measured by the radius of the inhibitory zone against *C. perfringens* CpA.

TABLE 5. Antibacterial spectrum of the antibiotic-like substance produced in vivo and in vitro by *Peptostreptococcus* strain E1 against various human intestinal strictly anaerobic strains

| Bacterial genus | No. of sensitive strains/no. tested |
|---|---|
| *Bacteroides* | 0/17 |
| *Fusobacterium* | 0/3 |
| *Veillonella* | 0/1 |
| *Clostridium* | 39/49 |
| *Bifidobacterium* | 26/26 |
| *Peptostreptococcus* | 9/16 |
| *Eubacterium* | 1/6 |

atic common ligature. As there was no proteolytic activity, as detected by the ELISA method, in feces of operated rats monoassociated with *Peptostreptococcus* strain E1 (Fig. 4), it can be ascertained that *Peptostreptococcus* strain E1 does not exhibit a proteolytic activity. Using common duct-ligated axenic rats, Corring et al. (1) demonstrated that in the absence of pancreatic hydrolysis, a large part of dietary casein was still degraded. These authors suggest that after ligature either the pepsin hydrolysis goes on in the intestine or there is an enhancement of this hydrolysis in the stomach. Nevertheless, our results show that pepsin would not be able to replace trypsin in the promoting effect on the production of the antibiotic-like substance by *Peptostreptococcus* strain E1, and neither would enterokinase. The significant decrease in the fecal proteolytic activity observed in the sham-operated rats on day 3 postinoculation is difficult to explain. It may be due to a stress effect leading to a decrease in the cecal trypsin concentration. However, this stress effect was no longer observed after day 3 postoperation.

When trypsin was given per os to the four operated rats, the antibiotic-like activity was not restored to the same extent in each rat. This could be a consequence of the low and variable consumption of trypsin solution by the rats or of the variable degradation of trypsin solution in the stomach. For this reason, we did not succeed in giving the same amount of trypsin per os as that supplied by the pancreas, and this explains why fecal and cecal levels of proteolytic enzymes were lower than those obtained in the intact rats. However, it should be pointed out that restoration of the antibiotic-like activity took place prior to that of the proteolytic activity. This suggests that the amount of trypsin required for the in vivo production of the antibiotic-like substance could be much lower than that supplied by the pancreas. The very fact that mainly trypsin may restore the antibiotic-like activity of *Peptostreptococcus* strain E1 seems to indicate that no other compounds from bile or pancreatic juice are required in this host-bacterium interaction. The unexpected increase in amylase activity observed in the small intestines of rats drinking trypsin remains to be explained.

Our in vitro results fully corroborate those obtained in vivo. The trypsin inhibitor has the same suppressive effect. Other pancreatic proteolytic enzymes do not replace trypsin; however, we did not use various doses of these enzymes. Furthermore, trypsin is active only within the concentration range found in rat pancreatic secretions. Nevertheless, the mode of action of trypsin remains to be elucidated. The assumption that trypsin could activate a proantibiotic produced by *Peptostreptococcus* strain E1, as it activates trypsinogen, seems to be unlikely since trypsin has to be introduced just before the incubation in order to promote the in vitro antibiotic-like activity of *Peptostreptococcus* strain E1, whereas trypsin loses its activity when introduced into the culture medium at the end of the exponential growth phase. The hypothesis that trypsin may hydrolyze proteins from culture media or cecal contents, thus providing substrates used by *Peptostreptococcus* strain E1 for producing its antibiotic-like substance, is also unlikely. The culture media we assayed which were unsuitable for this production of antibiotic-like substance contained nitrogen substances issued from trypsin digestion of meat or casein. It may be assumed that trypsin present at a suitable concentration may either activate bacterial enzymes responsible for the production of the antibiotic-like substance or hydrolyze protein inhibitors of this production. Whatever the actual mechanism involved, our results highlight the intimate relationships between an intestinal bacterial strain and its host, by way of the host's pancreatic secretions.

Although we have not yet demonstrated that the antibiotic-like activity was due to the same substance in vivo and in vitro, it is noteworthy that the antibacterial spectrum is identical whatever the origin of these substances. The fact that potentially pathogenic clostridia, such as enterotoxinogenic *C. difficile* strains, are sensitive is an interesting feature of our results. The in vivo experiments clearly demonstrate that *Peptostreptococcus* strain E1 elicits a bactericidal effect against *C. perfringens* CpA since *C. perfringens* CpA was washed out within 1 day, whatever its order of inoculation, whereas the transit marker was at its highest fecal level at the same time. Furthermore, the appearance of diffusible antibiotic-like substance in feces properly correlates with the disappearance of *C. perfringens* CpA. However, although *Peptostreptococcus* strain E1 has been isolated as a member of the predominant flora of a healthy man, the possible effect of the other predominant bacteria should be investigated to ascertain that the antibacterial activity we observed plays a role in the protection of a conventional host against potentially pathogenic *Clostridium* spp. Further experiments are now under way to investigate this point and to properly identify the antibiotic-like substance produced by *Peptostreptococcus* strain E1.

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