Inhibition of *Clostridium perfringens* by an Antibiotic Substance Produced by *Bacillus licheniformis* in the Digestive Tract of Gnotobiotic Mice: Effect on Other Bacteria from the Digestive Tract

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Received for publication 28 August 1975

A strain of *Bacillus licheniformis*, established in the digestive tract of gnotobiotic mice, inhibited the subsequent establishment of a *Clostridium perfringens* strain ingested by the animals. This inhibitory effect depended on the in vivo production by *B. licheniformis* of an antibiotic substance having a number of the characteristics of bacitracin. If *C. perfringens* was the first to become established in the digestive tract of the gnotobiotic mice, *B. licheniformis* also became established but did not produce any antibiotic. Mutants of *C. perfringens* resistant to the antibiotic substance were not observed when the antibiotic was produced in situ by *B. licheniformis*, but were rapidly selected when the *Bacillus* culture filtrate or bacitracin was administered per os. *B. licheniformis* was also capable of eliminating from the digestive tract 5 of the 13 additional bacterial strains tested.

The equilibrium established among various microbes in the digestive tract of animals is undoubtedly the result of multiple control mechanisms. One such mechanism affecting the balance between organisms is thought to be mediated by the production of antibiotic substances. To date, however, there has been no demonstration in gnotobiotic animals of an antibiotic produced in situ by one bacterial strain affecting other strains in the gastrointestinal tract.

Furthermore, many studies of the effects of exogenous antibiotics (such as those incorporated in feed) have called attention to the potential difficulty inherent in the selection of antibiotic-resistant mutants. However, it has not been demonstrated whether or not such selection of mutants will occur under conditions of in situ production of antibiotic.

Therefore, in the present work, a simple ecological model was established in order to study the production of antibiotics in the gnotobiotic digestive tract and the ecological significance of such production.

MATERIALS AND METHODS

**Animals.** The animals used in these studies were adult axenic (germfree) C3H mice either reared and maintained in plastic isolators of the Trexler type or, for short-term experiments, kept in sterile glass jars as previously described (5).

The mice were fed ad libitum a commercial diet (Labena Special Duquene Purina) sterilized by autoclaving. They received either water sterilized by autoclaving or antibiotic solutions and culture filtrates passed through a membrane filter (0.22-μm pore size; Millipore Corp.).

Each animal was inoculated per os with 1 ml of a 16-h culture grown in liquid medium (see below) by esophageal intubation or by injecting 1 ml of the culture directly into the individual drinking water tubes after each animal had been deprived of water for 1 day. In the case of mice housed in the same cage in an isolator for long-term experiments, pooled, freshly passed feces were used for the enumeration of bacteria. Each pooled sample contained one fecal pellet from every animal in the group. The mice kept singly in jars were killed by cervical elongation at the end of the experiment, and one fecal pellet was collected from the colon of each animal. In some instances, as outlined below, the feces were sampled for bacteriological examination. All quantitative bacterial cultures were made immediately after sample collection from a 1:10 dilution of the sample (fecal pellets or feces) in sterile deionized water, homogenized with an Ultraturrax.

**Bacterial strains and culture media.** A strain of *Clostridium perfringens*, type A, was isolated from the digestive tract of conventional rats and was cultured in liquid medium B’ (8) under an agar plug. Selective bacterial counts were made in the same agar medium containing 0.013% neomycin (Nutritional Biochemicals Corp.) (7) and distributed in tubes (18 by 400 mm) as previously described (6).

A strain of *Bacillus licheniformis* was isolated from the digestive tract of specific-pathogen-free
mice and was cultivated on the medium described for \textit{B. subtilis} \textit{(3).} Quantitative cultures were made on the same medium in petri dishes. This strain formed an antibiotic substance in vitro when grown on medium \textit{C} \textit{(4).}

Thirteen other bacterial strains were used to define the activity spectrum of the antibiotic produced by \textit{B. licheniformis}. These included one strain each of \textit{Clostridium difficile}, \textit{Clostridium bifermantans}, \textit{Bacteroides putredinis}, \textit{Veillonella alcalens}, \textit{Staphylococcus pyogenes}, \textit{Staphylococcus epidermidis}, and \textit{Eubacterium sp.;} four strains of \textit{Clostridium} \textit{sp.}, \textit{C1}, \textit{C2}, \textit{P1}, and \textit{P2}, and two strains of \textit{Peptostreptococcus}, \textit{S1} and \textit{S2}. All of these organisms were isolated from the digestive tract of conventional rats except \textit{Clostridium} strain \textit{P1}, which came from the digestive tract of young hares, and were cultivated on medium \textit{A} \textit{(6).}

\textbf{Assay of antibiotic formation by \textit{B. licheniformis}.} The neomycin-containing \textit{B} \textit{ Igor} medium was inoculated with the test strain (\textit{C. perfringens} or other strain) and poured into petri dishes. A fresh fecal pellet was pressed into the medium while the agar was still liquid. The dishes were incubated at \textit{37 C} in anaerobic jars filled with nitrogen. After 24 h, the radius of the zone of inhibition around the fecal pellet, produced by the contained antibiotic, was measured. The radius was taken as the distance from the outside of the fecal pellet to the circular line where bacterial growth became visible. Growth of live \textit{B. licheniformis} present in the fecal pellet was prevented by the neomycin in the agar medium.

\textbf{Spectrum of activity of \textit{B. licheniformis} in vitro and in vivo.} In this experiment, \textit{B. licheniformis} and one of the other various bacterial strains were inoculated simultaneously per os into axenic animals individually housed in sterile jars. After 8 days, the inoculated test strain and the \textit{B. licheniformis} in the cecum of each mouse were enumerated and the presence or absence of an inhibitory zone around a fecal pellet was observed, using the technique described above. Each bacterial strain was tested in 2 animals.

The in vitro susceptibility of each strain to the antibiotic present in the filtrate of \textit{B. licheniformis} was determined as follows. A well was prepared in the agar medium (previously inoculated with the strain to be tested, poured into a petri dish, and allowed to solidify), and this well was filled with the filtrate of a \textit{B. licheniformis} culture. The presence or absence of a zone of inhibition was observed after 24 h of incubation at \textit{37 C}.

\textbf{RESULTS}

\textbf{In vivo antagonism between \textit{B. licheniformis} and \textit{C. perfringens}.} The strain of \textit{B. licheniformis} was introduced into one of the isolators containing \textit{12 axenic} mice, and the organisms rapidly became established in the digestive tract of the animals (Fig. 1). Two days later the animals were inoculated per os with a 12-h culture of \textit{C. perfringens}. This latter strain did not establish itself in the digestive tract of the animals. When the mice ingested a 12-h culture of \textit{C. perfringens} ad libitum, small numbers of the bacteria from the inoculum were transiently observed in the feces (Fig. 1), but as soon as the culture was no longer fed to the animals, \textit{C. perfringens} disappeared from the feces.

\textbf{Influence of the order of inoculation of the strains on the Bacillus-Clostridium antagonism.} Six gnotobiotic mice (group \textit{A}) inoculated with \textit{C. perfringens} were introduced into an isolator containing six gnotobiotic mice (group \textit{B}) previously inoculated with \textit{B. licheniformis}. \textit{B. licheniformis} rapidly became established in the animals of group \textit{A} (Fig. 2). However, during the 116 days of the experiment, this growth of \textit{B. licheniformis} did not lead to any decrease in the number of \textit{C. perfringens} in the feces, and an inhibitory zone around the fecal pellet was never observed. The feces of the animals of group \textit{B} produced an inhibitory zone during the first 100 days of the experiment; during the same period \textit{C. perfringens}, although present in group \textit{A}, did not become established in group \textit{B}. Thereafter the amount of antibiotic substance in the feces of group \textit{B} mice progressively decreased and, at the same time, \textit{C. perfringens} appeared in the feces of these animals. On day 116, the number of \textit{C. perfringens} was equivalent in the feces of the animals of the two groups.

Three other trials of the same experimental design gave similar results. In the animals in which \textit{B. licheniformis} was established first, suppression of \textit{C. perfringens} and zones of inhibition around fecal pellets were obvious. However, later these two phenomena always disappeared simultaneously, although at different times after the inoculation with \textit{B. licheniformis} in the three trials, namely 38, 65, and 69 days.
In another experiment, the feces of three animals that had first received *C. perfringens* were individually studied. In two of these animals, the number of *C. perfringens* was not modified by the establishment of *B. licheniformis*. In the third animal, the number of *C. perfringens* was 10^6 times less for 20 days and afterwards returned to the initial level.

**Influence of a B. licheniformis culture filtrate and a bacitracin solution on the C. perfringens population in the digestive tract of gnotobiotic mice.** In an isolator containing six mice monoassociated with *C. perfringens*, the drinking water was replaced for 2 days by a sterile filtrate of an 18-h culture of *B. licheniformis*. Zones of inhibition were observed in vitro around the fecal pellets (Fig. 3A), and the size of these zones was similar to that observed in animals in whom *B. licheniformis* had become established. However, the fecal population of *C. perfringens* was only slightly modified. The treatment was interrupted for 15 days and started again for another 6 days. This time no effect on the fecal population of *C. perfringens* was observed (Fig. 3A).

It is well known that some strains of *B. licheniformis* produce bacitracin. Therefore an experiment of the same type as the previous one was carried out, using this antibiotic instead of a culture filtrate. First, the drinking water was replaced by a solution containing 0.05 mg of bacitracin per ml. The zone of inhibition observed around the fecal pellets was about twice as large as the zone noticed in animals receiving the culture filtrate. The *C. perfringens* population disappeared from the feces (i.e., was less than 10^6) and returned to the initial level 4 days after the end of the antibiotic treatment. Subsequently, the animals were given a solution containing 0.01 mg of bacitracin per ml. The zone of antibiosis appearing around the fecal pellets was of the same magnitude as that observed in the animals receiving the culture filtrate. The *C. perfringens* population was transiently reduced but returned to its initial level before the end of the treatment. Finally, 4 days after the second antibiotic treatment had been stopped, a third treatment was started with a solution containing 0.05 mg of bacitracin per ml. This time no effect on the fecal population of *C. perfringens* was observed (Fig. 3B).

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**Fig. 2. Influence of the order of inoculation of the strains on the Bacillus versus Clostridium antagonism.** Symbols: open bars, number of *C. perfringens* per gram of fresh feces; hatched bars, number of *B. licheniformis* per gram of fresh feces; 1, inoculation of *C. perfringens* in mice of group A; 2, introduction of group A mice into the isolator already housing group B mice previously inoculated with *B. licheniformis*; black bars, radius (in millimeters) of the inhibitory zone around a fecal pellet.
Comparison of C. perfringens mutants resistant either to the culture filtrate or to the bacitracin. In vitro, the minimum inhibitory dose of bacitracin for the original strain of C. perfringens was 0.2 μg/ml. Furthermore this strain was inhibited by a 1:100 dilution of the culture filtrate of B. licheniformis. All the clones of C. perfringens isolated from the animals in which this bacterial strain has successfully developed despite the presence of B. licheniformis showed the same sensitivity as the original strain toward bacitracin and toward the culture filtrate. Two strains of C. perfringens were isolated from the digestive tract of mice receiving the filtrate of B. licheniformis (one isolated after the first treatment and the other after the second treatment). These two strains grew in vitro in the presence of the undiluted filtrate and in the presence of a concentration of bacitracin of 2 μg/ml. Likewise, a strain of C. perfringens was isolated from the digestive tract of the animals receiving the bacitracin solution per os between the treatment with 0.01 mg/ml and the final treatment with 0.05 mg/ml. This strain grew in the presence of the undiluted filtrate and in the presence of a concentration of bacitracin of 2 μg/ml. None of these mutants was inhibited by the antibiotic present in the feces of gnotobiotic animals carrying B. licheniformis.

Furthermore, a mutant of C. perfringens resistant to a filtrate that had been concentrated 100-fold was isolated in vitro. Simultaneously, the minimum inhibitory dose of bacitracin for this mutant increased from 0.2 μg/ml to more than 500 μg/ml.

Properties of the antibiotic substance produced by B. licheniformis. The antibiotic substance produced by B. licheniformis was found to withstand heating at 100 C for 10 min at pH 7.1 but was destroyed within 30 min at the same temperature. It was dialyzable and its activity could be preserved (judged by disk method) in solution over a pH range from 2.0 to 9.0.

In vitro and in vivo spectrum of activity of B. licheniformis. The results are reported in Table 1. The number of B. licheniformis per gram of fresh feces ranged between 1 x 10^9 and 4 x 10^9 for all animals. However, the antibiotic was not present in the feces of all animals. In three cases (Eubacterium sp., C. bifermnantans, and V. alcalescens), antibiotic production varied from one animal to another. It was observed that the six strains that were eliminated in vivo were susceptible in vitro to the filtrate of B. licheniformis. The two strains that were only slightly susceptible in vitro to the filtrate (Clostridium P, and S. pyogenes) were not eliminated in vivo.

DISCUSSION

Our results show that a strain of B. licheniformis that has established itself in the diges-
Table 1. In vitro and in vivo spectrum of activity of \( B. \) licheniformis

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Growth inhibition ( \text{in vitro} )</th>
<th>Presence or absence of antibiotic in the feces ( \text{in vivo} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C. ) difficile</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>( C. ) bifermentans</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>( C. ) perfringens</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>( C. ) sp. ( S_2 )</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>( C. ) sp. ( P_2 )</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>( V. ) licheniformis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>( S. ) epidermidis</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>( S. ) pyogenes</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>( Eubacterium ) sp.</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>( Veillonella ) alcalescens</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>( Bacteroides ) putredinis</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

*All the experiments were repeated with two mice. Only in the case of \( Eubacterium \) sp. and \( V. \) alcalescens did the results differ in the two, and in these instances both sets of data are given.

\( + \), Presence of a zone of antibiosis (radius \( > 5 \) mm) in the petri dish around a well filled with a sterile filtrate of a \( B. \) licheniformis culture; \( +\), radius is less than \( 2 \) mm; −, no visible zone of antibiosis.

\( + \), Number of bacteria from the strain under test less than \( 10^6 \) per g of cecal content in a gnotobiotic mouse inoculated 1 week earlier with \( B. \) licheniformis and with the strain under test; −, number of bacteria greater than \( 10^6 \) per g of cecal content.

\( + \), Presence in the agar medium (inoculated with and showing uniform growth of \( C. \) perfringens) of a zone of antibiosis around a fecal pellet from the colon of the gnotobiotic mouse; −, no visible zone of antibiosis.

The antibacterial effect of \( B. \) licheniformis can inhibit the subsequent implantation and multiplication of a \( C. \) perfringens strain ingested by the animal. This inhibitory effect is highly efficient, as shown by the fact that \( C. \) perfringens does not become established even after being administered per os over a prolonged interval. The antagonistic effect of \( B. \) licheniformis is not restricted to \( C. \) perfringens. It may also be seen with strains belonging to different genera, whereas strains belonging to the same genus may or may not be inhibited. The susceptibility to this effect is therefore a character related to the species, or the strain, rather than to the genus.

We have shown that the in vivo elimination of \( C. \) perfringens is correlated with the presence in the feces of the animals of an antibiotic substance produced by \( B. \) licheniformis. As soon as this substance is no longer detected in the feces, the inhibitory effect of \( B. \) licheniformis does not occur and \( C. \) perfringens can become established. We have observed that some of the physicochemical properties of this antibiotic substance are identical to those of bacitracin produced by some strains of \( B. \) licheniformis (1, 2). Furthermore, it was demonstrated that a mutant resistant to the antibiotic produced by \( B. \) licheniformis also appeared to be resistant to bacitracin and, conversely, if the mutant was selected by means of bacitracin, it was resistant to the filtrate of a \( B. \) licheniformis culture. Therefore, the antibiotic produced by our strain is most likely bacitracin. Furthermore, the fact that the mutants of \( C. \) perfringens resistant to bacitracin are also unsusceptible to the antibiotic contained in the feces of the animals carrying \( B. \) licheniformis suggests that bacitracin is produced in vivo.

Another important point relates to the apparent advantage of in situ production of bacitracin by \( B. \) licheniformis in the digestive tract as compared with continuous administration of bacitracin added to the feed. Our results show that the ingestion by gnotobiotic animals of bacitracin or of the \( B. \) licheniformis filtrate leads to a rapid selection of resistant mutants of \( C. \) perfringens. On the other hand, with antibiotic produced in situ (i.e., in the digestive tract itself) by the living \( B. \) licheniformis, we have never observed the appearance of resistant mutants in any of the animals. It may be that the antibiotic concentration present in the feces does not reflect the actual concentration in various segments of the digestive tract. Thus, \( B. \) licheniformis might grow, sporulate, and consequently produce its antibiotic only at certain levels of the digestive tract, with the antibiotic thereafter being diluted in the rest of the digestive tract. Thus, at a particular level of the digestive tract, the antibiotic could reach sufficiently high concentrations that the probability of finding a resistant mutant would be extremely small. On the other hand, if the antibiotic itself is ingested per os, it is rapidly diluted in the digestive tract. In that case, the concentrations are sufficiently low that the probability of finding a resistant mutant is much greater. Furthermore, as we observed in the case of bacitracin, the disappearance of the entire susceptible population without the appearance of resistant mutants can be obtained simply by using a solution with a concentration of 0.05 mg/ml instead of 0.01 mg/ml. That is to say, a concentration that is only five times higher than the concentration leading to selection of mutants may eliminate a given bacterial population completely.

Thus, the advantage of in situ production of antibiotic is that it does not lead to selection of
resistant mutants, whereas the disadvantage is that the production of antibiotic by \textit{B. licheniformis} requires that the microflora be previously established in the digestive tract (as observed when comparing group A and group B animals). Studies currently in progress in our laboratories are aimed at the explanation of these results in terms of the mechanisms governing antibiotic production.

**ACKNOWLEDGMENTS**

We thank G. Mocquot for his constructive criticism of the manuscript.

**LITERATURE CITED**