THE ANAEROBIC BACTERIAL FLORA OF THE MOUSE CECUM*

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The study of the anaerobic bacteria which inhabit the intestinal tract has been greatly facilitated by two methodological advances: (*a*) the use of strict anaerobic techniques developed by Hungate (1) for the study of rumen bacteria has increased the yield of colonies recovered from cultures of the intestine and has made possible for the first time the cultivation of certain anaerobic species: (*b*) the production of so-called specific-pathogen-free (SPF) animals, especially those derived from germfree animals, has provided experimental systems in which the variability of the intestinal flora is reduced to a manageable level.

It has long been known that when the contents of the cecum of normal mice are observed under the microscope, they are found to contain immense numbers of bacilli with pointed ends—the so-called "tapered rods" (2, 3). Although bacteria of this morphologic type greatly outnumber all other species, they rarely appear in ordinary anaerobic cultures of cecal contents or fecal materials, or at best, in small numbers. We have outlined elsewhere a method which permits the cultivation of some, but not all, of these tapered rods (4).

In the present communication, we shall describe in detail improvements of this method based on Moore's modification of Hungate's anaerobic technique (5). We shall also present information concerning some of the anaerobic species which have been isolated from the cecum of SPF mice.

Materials and Methods

Experimental Animals.—The mice used were adult animals of the COBS-CD-1 strain produced by Charles River Breeding Laboratories, Inc., Wilmington, Mass. This colony was initiated from mice obtained by caesarian section and raised at first under germfree conditions. At a set time, the animals were colonized with a bacterial flora consisting of one strain each of lactobacillus, enterococcus, anaerobic streptococcus, slow lactose fermenting coliform, and two strains of bacteroides, all isolated by Dr. R. W. Schaedler from NCS mice at Rockefeller University. These animals were used as breeders to develop a colony which was maintained in an environment designed to minimize contact with bacterial or viral pathogens. Filtering the room air, sterilizing all food and water, and having all personnel put on sterile clothes and perform a surgical scrub before entering the room has resulted in the creation of an SPF mouse colony with a simplified and relatively stable intestinal flora.

After arrival at our laboratory, the mice were housed in plastic boxes fitted with Isocaps, (Isocages from Lab Cages, Inc., Kennett Square, Pa.) with wood shavings for bedding. They

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were fed the SPF mouse diet D&G (supplied by Dietrich and Gambrill Inc., Frederick, Md,) and acid-water ad libitum as described in reference 2. They were killed with chloroform just before being brought into the anaerobic chamber.

Gas Preparation.—All gases used were passed through a heated copper oven (Sargent Catalog No. S-36517, Sargent-Welch Scientific Co., Skokie, Ill.) to remove the last traces of oxygen. The copper was reduced with 3% H_2–97% CO_2 (Matheson Co. Inc., East Rutherford, N. J.) as needed. The details of this procedure are described in the manual Outline of Clinical Methods in Anaerobic Bacteriology prepared by the Anaerobe Laboratory, Virginia Polytechnic Institute, Blacksburg, Va., and available from them. The methods used by the V.P.I. Anaerobe Lab are also presented in reference 5.

Media and Dilution Tubes.—The agar culture medium used was that described for the cultivation of bacteroides and clostridia in reference 2, except that the neomycin was deleted and 4% sheep's blood was added. This modified medium will be referred to hereafter as S medium. Rumen fluid-glucose-cellobiose-agar (Robbin Laboratory, Chapel Hill, N. C.) with serum added was also used for determination of colonial morphology. All plates were placed under anaerobic conditions (2) immediately after preparation and kept under these conditions for at least 24 hr before use. The diluent used for specimen preparation and serial dilutions was a prereduced liquid medium prepared under oxygen-free conditions. The technique for preparation of the prereduced medium was similar to that described in the V.P.I. Anaerobe Lab manual. The medium was the S medium mentioned previously with agar and hemin omitted and resazurin solution (2 ml per 500 ml of medium) added as an indicator of reduced conditions. The resazurin solution was prepared according to the V.P.I. manual by dissolving one resazurin tablet (Allied Chemical Catalog No. 506, Allied Chemical Co. Valley Stream, N.Y.) in 44 ml distilled water. All ingredients were mixed in an Erlenmeyer flask fitted with a removable chimney; boiling was continued until the indicator turned from pink to yellow. The flask was then cooled in ice water under 3% H_2–97% CO_2. After cooling, the medium was dispensed into tubes under oxygen-free N_2 gas and the tubes stoppered. For details of this procedure, see the V.P.I. Anaerobe Lab Manual, pp. 27 and 52. The tubes were then autoclaved. Any tube in which the indicator turned pink was discarded.

Anaerobic Chamber.—All procedures for the isolation and enumeration of intestinal bacteria were carried out inside a Fiberglas glove box (Labconco Corp., Kansas City, Mo.) that permitted the establishment of anaerobic conditions. The animals, together with culture media and other equipment, were placed in the side transfer box of the chamber. By means of two outlets, prepurified nitrogen (Matheson Chemical Co.) was run through the side transfer box for 20 min to decrease the oxygen content. The transfer box was then opened from the inside and the contents brought into the chamber. After the transfer box was closed, anaerobic conditions (verified by the complete reduction of a methylene blue indicator) were established by passing a stream of prepurified nitrogen gas through the chamber for 90 min.

Preparation of Specimens for Bacteriological Examination.—After reducing the copper with 3% H_2–97% CO_2, a mixture of 5% CO_2–95% N_2 (Matheson Chemical Co.) was used for the balance of the experiment. The mice were pinned on a dissecting board, and their ceca exposed and removed. Each cecum was placed in 5 ml of prereduced liquid medium and homogenized by hand with a Teflon grinder. The cecal suspensions were further diluted in prereduced liquid medium in 100-fold and 10-fold steps. A calibrated loopful of each dilution was put on the surface of an S agar plate and spread out evenly. The plates obtained from each group of five mice were placed in an anaerobic glass jar (GasPak, Baltimore Biological Laboratory, Cockeysville, Md.) that was already inside the chamber. The hydrogen and carbon dioxide generator of the "GasPak" system was activated and the jar sealed. At the conclusion of the experimental procedure, all unnecessary materials were removed from the chamber via the side transfer box. The box was then flushed with 5% CO_2–95% N_2 gas and a vacuum drawn. The entire chamber, with the "GasPak" jars inside, was put into a warm (37°C) room and incubation was continued for 48 hr.

Subculturing Procedure.—After the initial 48 hr incubation of the agar plates, anaerobic conditions inside the chamber were reestablished and the necessary equipment brought in as outlined above. The plates were examined under a dissecting microscope inside the chamber. Suitable colonies were picked with a platinum needle, streaked onto a fresh plate and incubated for 48 hr in a "GasPak" jar. If the resulting subculture seemed to belong to a single colonial type, several isolated colonies were picked by needle and transferred to a tube of prereduced liquid medium. Each tube was flushed with a 5% CO_2–95% N_2 mixture and tightly sealed. After removal from the chamber, all subcultures were incubated at 37°C. When turbidity was noted in any tube, anaerobic transfer to fresh liquid medium was done outside the chamber by Moore's method (5). Some subcultures were simultaneously transferred onto fresh S agar plates inside the chamber every 2–4 days.

Morphological Studies.—Colonial morphology was observed under a dissecting microscope (Bausch & Lomb Incorporated, Rochester, N. Y.). Oblique light made the colors of the colonies more prominent than did a light source placed directly above the plate.

Specimens of cecal homogenates and individual bacterial colonies were studied for bacterial morphology under the oil immersion lens of a phase microscope at a magnification of 1000. Although this was done outside the chamber, the environment remained sufficiently anaerobic to enable most bacteria in the cecal homogenates and liquid subcultures to retain their motility.

Identification of Bacteria.—Cultures were sent to the Anaerobe Lab of V.P.I. where they were assigned to a genus and species on the basis of biochemical and morphologic properties.

RESULTS

The tapered rods characteristically appeared as "speckled" or "mosaic" colonies on agar plates. Although a few types of lactobacilli and bacteroides also formed "speckled" colonies, the coarseness of their pattern rendered these easily distinguishable from the tapered rod colonies in almost every case. Table I illustrates the increase in yield of speckled colonies achieved by the modified anaerobic method in comparison with that obtained by the method described in the preceding paper (4). Granted that there was some overlap, it is clear that the new method gave colony counts two to ten times higher than the old method. In fact, the difference was more striking than indicated in the Table because, with the old technique, it was a fairly common occurrence for no "speckled" colonies to appear on the plates at the dilutions studied, whereas this did not happen in any of the more than 200 mice cultured in the present study.

There was also a qualitative difference in the results obtained by the two methods. With the old method, only the smaller tapered organisms appeared on the agar plates, although larger ones with and without spores had been seen in the cecal suspensions under the phase microscope. With the improved technique, at least two of the larger varieties were cultured, and for the first time in this laboratory, a tapered rod with spores was grown on an agar plate.

Most of the anaerobic cultures isolated from the mouse cecum by the techniques described in the present paper appear to belong to species which have not yet been identified. Only a few of their distinctive characteristics can be described at the present time.

"Tough" Tapered Rod.—The adjective "tough" was used to denote that this morphologic type exhibited a degree of oxygen sensitivity not much higher than that of the bacteroides species. Under routine anaerobic techniques, organisms of this group survived in the chamber whereas other tapered rods did not; they exhibited tapered ends and were cigar-shaped. Fig. 1 shows some "tough" tapers from a subculture that had been grown under good anaerobic conditions. If the anaerobic atmosphere during transfer was less than perfect, the rods became about half as long as those in Fig. 1 but retained the cigar-like form. The rods characteristically appeared in pairs; they formed long chains on occasion in liquid media. Although motile bacteria with this morphologic appearance were seen under phase microscopy, no motility was observed and no spores were seen in those cultured on plates. Fig. 2 shows the size relation

TABLE I

Comparative Numbers of Speckled Colonies Recovered from the Mouse Cecum by Two Different Anaerobic Techniques

Speckled colonies per individual cecum at 10^6 dilution*	
Old technique‡	New technique§
40	76
63	148
8	122
9	103
7	122
80	310
190	43
104	118
13	480
28	500

* Figures to be multiplied by 1.6×10^7 to give number of colonies per g of cecal homogenate.

‡ Described in reference 4.

§ Described in present paper.

between three "tough" tapers and a medium length tapered rod with flagellum (as described below).

On S plates, colonies were round, about 2–5 mm in diameter, convex and smooth. Most striking was the presence of several lines that ran through the colony and gave it a "speckled" or "mosaic" appearance. This speckling proved to be characteristic for tapered rods, but the basis for it remains unknown. Most colonies were grey-white but some were bright or dull pink; with continued incubation beyond 48 hr, the pink color tended to fade. No spreading growth was observed.

The so-called "tough" tapered rods have been observed in every mouse studied, their population being characteristically 10^{10-11} organisms per gm of cecal homogenate. These are gram-positive, nonsporeforming, and quickly lose their gram positivity with age. They belong to the genus *Eubacterium*, of a species different from *E. lentum*, *E. ventriosum*, or *E. limosum*.

Medium and/or Thin Tapered Rods.—These organisms tended to be two to three times longer than the "tough" tapered rods and some strains were quite slender (Figs. 3 and 4). The slender rods were uniform in size and shape; characteristically they clumped together both in pure culture and in cecal homogenate preparations. The wider rods of medium length were

more pleomorphic (Fig. 4) and did not clump. Motility was observed in some strains, but flagella could not be seen with the phase microscope. No spores were seen.

On S plates, colonies exhibited the "speckled" appearance described above. There was more variation in colonial appearance with different strains than in the case of the "tough" tapered rods. Colonies were discrete or spreading; convex or flat; grey-white, pink, or green; smooth; and the surface of some had an opalescent quality. A strain with flat spreading growth often had discrete convex colonies on transfer to a new plate if anaerobic conditions were less than optimal. Whether or not other factors influenced this colonial variability remains to be determined. Discrete colonies were 1–4 mm in diameter.

Organisms of this type were recovered from approximately 75% of mice studied in numbers of 10^{8-9} organisms per gm of cecal homogenate. Examination of cecal homogenates under phase microscopy showed that this morphologic type was present in all normal mice; the failure to recover them from 25% of the animals was probably due to the fact that their numbers were less than 10^7 organisms per gm of cecal homogenate.

The six strains identified to date have all been assigned to the genus *Fusobacterium*. There were at least three species represented, one being *F. russii* (*Sphaerophorus influenzaeformis*) and the other two being unknown. The unknown species were not *F. fusiformis*, *F. varius*, *F. necrophorus*, or *F. ridiculosus*. *F. russii*, a medium taper, formed pink, discrete, opalescent colonies 1–3 mm in diameter. One of the unknown species was a thin, medium taper and formed green colonies, either spreading in growth or discrete with a diameter of 1–2 mm. The other unknown species was a motile medium taper that produced a pink spreading growth on agar plates.

Large Tapered Rods.—These organisms (Fig. 5) were approximately twice as long and wide as the medium tapered rods. Some were motile but no spores were seen. Colonial morphology could not be determined because these organisms could not be grown on agar plates. They were present in 20–30% of mice studied and appeared to reach populations of 10^{5-6} organisms per gm of cecal homogenate.

Tapered Rods with Visible Flagella.—There were at least four types of motile tapered rods with visible flagella. The most striking was a medium length rod (Type A, Figs. 2 and 6) with flagella at both ends. Because of the different planes involved, only one flagellum could be photographed for a given organism. Often the bacterium had a halo around it (Fig. 7) and, rarely, a terminal spore was present (Fig. 8). The rod was motile with a characteristic side-to-side movement.

The second type (Type B) was smaller by about one-third, somewhat curved, and had a flagellum at one end only. On subculture (Fig. 9) the organism was slightly larger and lost most of its curve. Its movement had a jerky quality with each end rotating side to side in turn.

Another type (Type C) was about the same size as a "tough" tapered rod but was curved and had a flagellum attached at the middle of the concavity. A fourth type (Type D) was quite short and straight with a terminal flagellum. These last two types have not yet been cultured.

Types A and B were rarely obtained on S plates, but did grow consistently on rumen-fluid-glucose-cellobiose-agar (RGCA) plates. Colonies were colorless, flat, and granular-speckled, with varying amounts of spreading growth. The spreading areas ranged from a moderately thick layer in some types to a fine, nearly invisible film in others. The one time Type A appeared on an S plate; it was a discrete colony, 6–10 mm in diameter, with a broad speckled pattern.

Bacteria in this morphologic category were present in almost all normal mice, although the relative numbers of Types A–D varied from mouse to mouse. Not all types were present in every mouse. In some preparations, there were so many Type A organisms that other kinds of bacteria could not be seen on the slide. When present, the tapered rods with visible flagella achieved populations of 10^{11-10} organisms per gm of cecal homogenate.

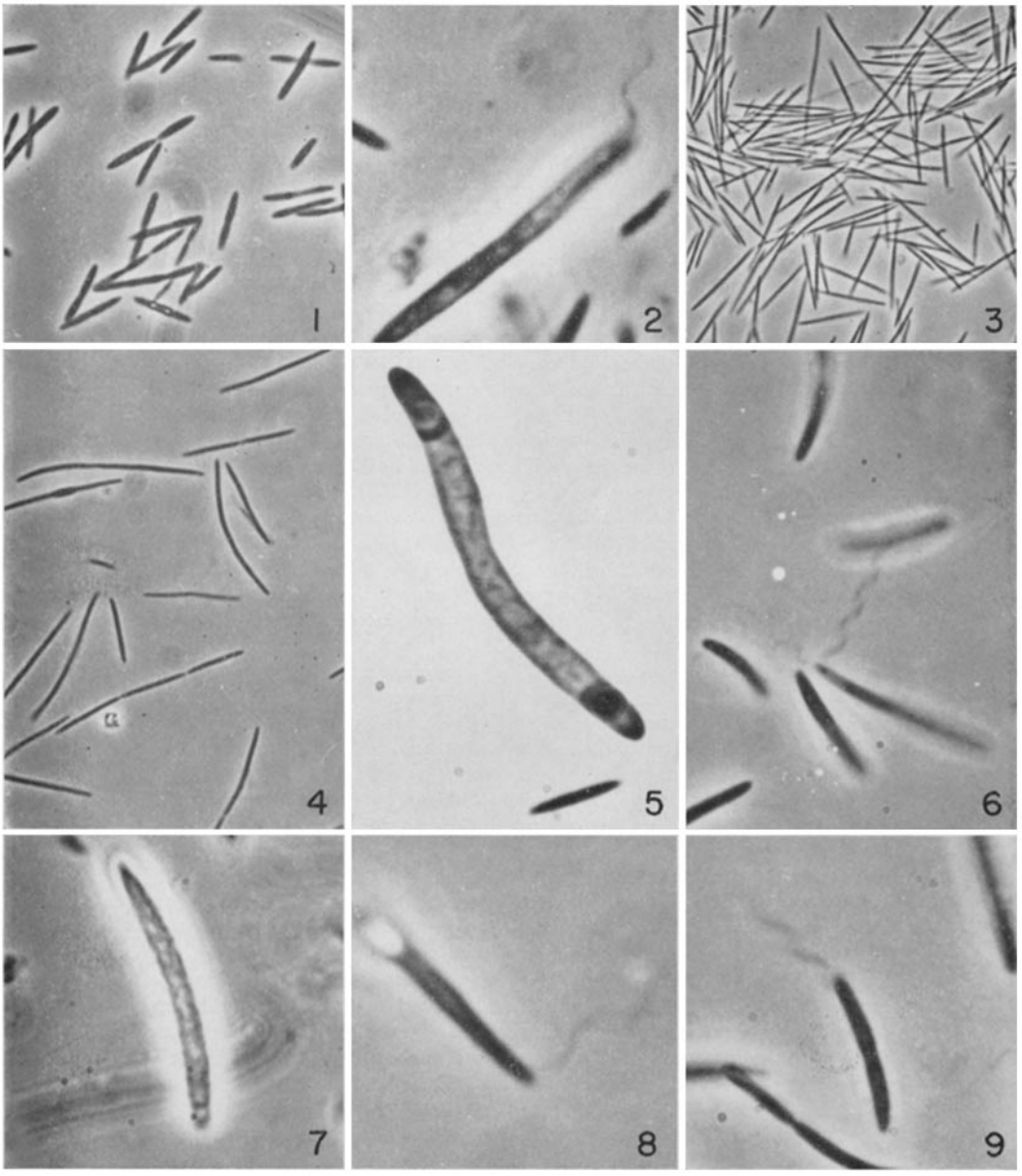

FIG. 1. "Tough" tapered rod grown under strict anaerobic conditions. × 3500.
FIG. 2. Medium-sized tapered rod with flagellum and three "tough" tapers. × 9000.
FIG. 3. Clump of uniform, medium-sized, thin tapered rods. × 2000.
FIG. 4. Pleiomorphic, medium-sized tapered rods. × 3500.
FIG. 5. Large and "tough" tapered rods. × 9000.
FIG. 6. Medium-sized tapered rod with flagellum. × 5500.
FIG. 7. Medium-sized tapered rod with flagellum (not visible) and halo. × 6500.
FIG. 8. Medium-small tapered rod with terminal spore and flagellum. × 10,000.
FIG. 9. Slightly hooked tapered rod with flagellum. × 7500.

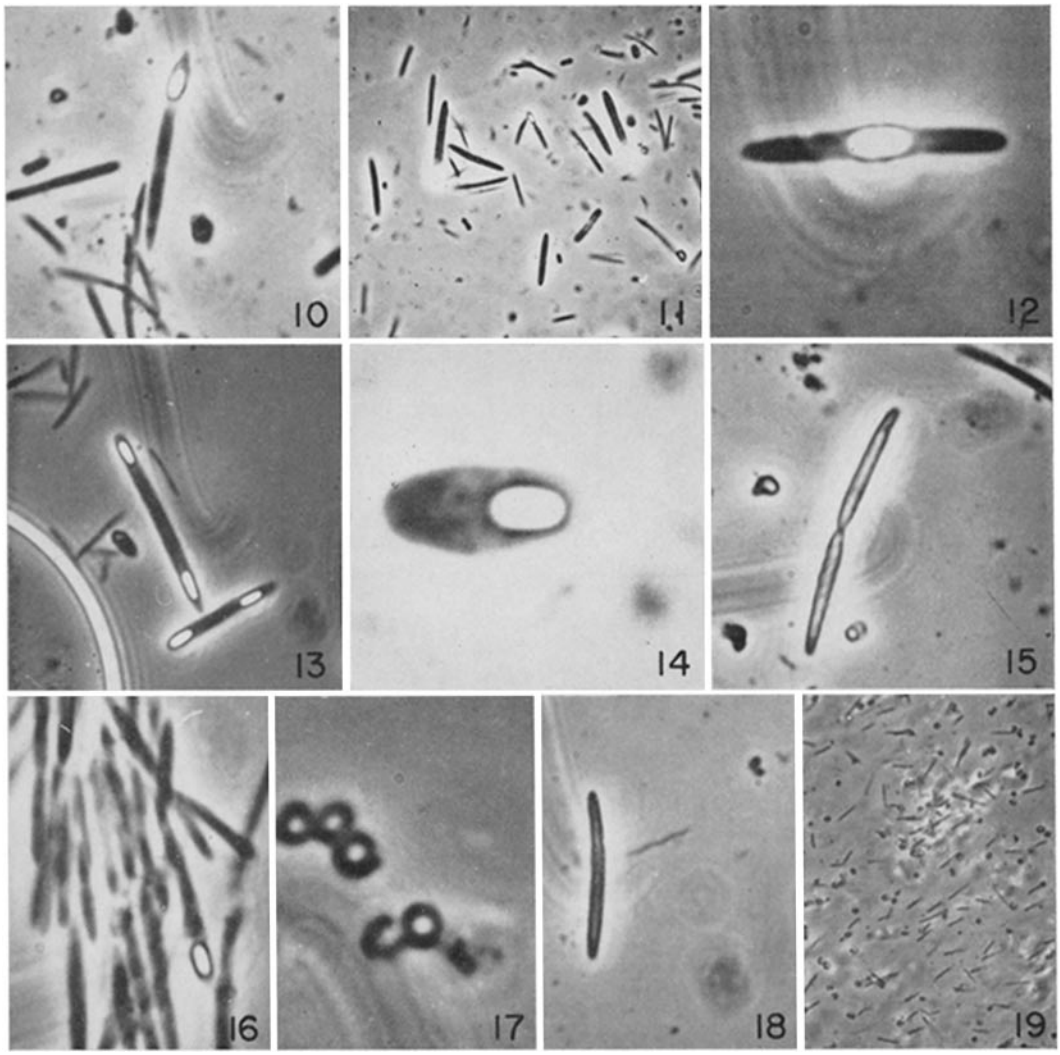

FIG. 10. Medium-sized sporeformer. × 4500.
FIG. 11. Medium and small-sized sporeformers. × 2000.
FIG. 12. Slightly refractile sporeformer with central spore grown on S agar plate. × 9000.
FIG. 13. Large sporeformer with subterminal spores at each end. × 4500.
FIG. 14. Sporeformer, rarely found in normal mice. × 16,500.
FIG. 15. Medium-sized, refractile tapered rod; formed spores on agar plates. × 5500.
FIG. 16. Clump of sporeformers. × 7500
FIG. 17. Doughnut-shaped rods: *Catenabacterium catenaforme*. × 5500.
FIG. 18. Type A spiral next to medium-sized tapered rod. × 5500.
FIG. 19. Type A spirals and circular decaying forms. × 2000.

None of these strains have been identified.

Sporeformers.—The normal mouse harbored several morphologic kinds of sporeformers among the tapered rod population. The commonest (Fig. 10) was of medium length with a subterminal spore. Another frequently present type (Fig. 11) was of the same shape but only half as long and half as wide. Some of the other distinct types seen are shown in Figs. 12–14. Some strains were motile, others not, and, as previously mentioned, some had visible flagella. Virtually all sporeformers in mice fed D&G diet had tapered ends.

Few of the tapered rods with spores have been grown on agar plates. This may reflect the lack of a suitable medium or the fact that because conditions did not favor spore formation, the organisms were grouped with the nonsporeforming tapered rods. One sporeformer isolated from a normal mouse was slightly refractile under the phase microscope (Fig. 15) and had a centrally-placed spore. On S medium, the colony was pale grey, speckled, and had thick areas of spreading growth. The only other isolated sporeformer was a slightly cigar-shaped, motile, medium length tapered rod that produced a discrete, convex, green speckled colony 2–5 mm in diameter on S medium.

Sporeformers achieved populations of 10^{7-8} organisms per gm of cecal homogenate and were present in all normal mice. Inclusion of the noncultivable sporeformers would have greatly increased this number. Many of the clumps seen in cecal homogenate preparations are made up of sporeforming organisms (Fig. 16) and this may provide a clue to the identification of the organisms that have been seen adhering in sheets to the mucous layer of the mouse large intestine (6).

The only sporeformer that has been characterized by the V.P.I. Anaerobe Lab was the second one whose colonial morphology was described above. Oval terminal spores were noted during the identification procedure, but the organism could not be placed in any of the commonly recognized species including the 20 listed in the V.P.I. Lab Manual. It was gram-negative, but, unlike other gram-negative sporeforming tapered rods, it produced butyric acid.

"Doughnut-Shaped" Rods.—These appeared as semicircular or circular forms (Fig. 17) often in groups of 5–20 organisms. On subculture in liquid medium, these organisms produced curved rods and loops with blunted ends.

The colony on S medium was yellow-white, discrete, convex, and mucoid with a diameter of 7–12 mm. It was not speckled.

This type appeared sporadically in normal mice and only rarely reached levels of 10^{7-8} organisms per gm of cecal homogenate. It has been identified as *Catenabacterium contortum*

"Spiral" Organisms.—Four kinds of "spiral" organisms were seen in normal mice. One (Type A-Fig. 18) had two or three bends as a rule and was rapidly motile. Most forms were about half the length of the one in Fig. 18 and were thinner than any bacterium. The second type (Type B) was twice as long and one-third as wide as Type A, thereby resembling true *Treponema*. This kind was also rapidly motile. A third kind (Type C) was very short with only one bend while a fourth (Type D) was twice as long and wide as Type A.

Types B, C, and D have not been grown on the surface of agar plates. Type A appeared as small (0.5 mm), grey, round, slightly elevated colony when discrete; usually it was a finely granular, spreading growth with a greening effect on blood plates. As was the case with many of the strictly anaerobic tapered rods, Type A "spirals" began to disintegrate or assume a circular shape upon exposure to room air (Fig. 19).

Type A was present in all normal mice at levels of 10^{9-10} organisms per gm of cecal homogenate. Types B, C, and D were visualized sporadically.

DISCUSSION

The procedure used in the present study has yielded a greater variety of bacterial types and more numerous colonies than the procedure described in a

preceding paper (4). Although it is not possible to state which of the refinements were responsible for the improvement in results, some information relevant to this point is available. In comparative experiments, mice were tested in identical fashion except that half the cecum was processed using prereduced liquid medium as the diluent and the other half using prereduced liquid medium that had been exposed to oxygen by having the tube stoppers removed for 3 min. The colony counts were three to five times higher in the specimens tested with the prereduced, unexposed medium. Furthermore, upon removal from the chamber, tapered rods in the still reduced liquid medium retained their motility when looked at under phase microscopy, while tapered rods in the medium exposed to oxygen did not.

The use of a copper oven to remove the last traces of oxygen from the gas mixtures was also crucial. The Matheson prepurified nitrogen preparation contains 5–15 ppm of oxygen. When this gas was used without passing it through the copper oven, the methylene blue indicator inside the chamber was not completely reduced and the recovery of tapered anaerobes on plates was impaired.

From the data presented, it can be seen that the strict anaerobes make up the majority of the intestinal flora of the mouse and that their population is a complicated one with many subgroups. Morphological criteria have been the primary means of separation in this paper because genus and species identification is, at this time, quite spotty. The organisms thus far classified belong either to unknown species of a well known genus or to a genus such as *Eubacterium* or *Catenabacterium*. It may well be that a given morphologic type will be found to contain genera and species other than those mentioned here.

More and more anaerobic intestinal species are recognized as cultural techniques improve. Tapered rods with flagella, previously ungrowable in this laboratory, have now been cultured on a medium enriched with rumen fluid. There are indications that this medium may eventually permit growth of the several kinds of flagellated tapers as well as the sporeformers.

In addition to the tapered rods, which are common and easily recognizable, the mouse intestine harbors other strict anaerobic forms such as cocci, diphtheroids, and other ill-defined organisms. The "spiral" organisms also require further study as they are numerous and exist in intimate association with the intestinal mucosa and mucous layer (7).

The overriding consideration in the study of intestinal anaerobes is to prevent exposure of the specimen to oxygen throughout the experimental procedures. This can be achieved by using an anaerobic chamber or the Hungate roll-tube method. Falsely negative results in the search for anaerobes may have their origin in the following technical errors: (*a*) Collection of specimens under nonanaerobic conditions. (*b*) Exposure of specimens to room air during plating procedure. Studies on strains isolated from the mouse intestine indicate that 5–10 min exposure to room air can be sufficient to inhibit growth.

(*c*) Exposure of the specimen to oxygen during the establishment of truly anaerobic conditions by jar catalyst. As the "GasPak" system takes 1–2 hr to establish anaerobiasis, we made it a practice in the present experiments to leave the "GasPak" jar inside the anaerobic chamber at all times. (*d*) Use of media that have not been prereduced. The oxygen content of liquid media and the presence of oxidized components in agar media can be sufficient to inhibit growth.

It will not be possible to assess the importance of intestinal bacteria in pathological processes until adequate techniques are used systematically to detect the presence of strictly anaerobic species.

SUMMARY

Refinements of the anaerobic technique such as the utilization of prereduced media and the removal of traces of oxygen from the gases used by passage through a hot copper oven resulted in quantitative and qualitative improvements in the recovery of anaerobic bacteria from the cecum of SPF mice.

The commonest morphologic types of bacteria in the mouse cecum were tapered rods. These characteristically gave "speckled" colonies on agar plates. Those identified were species of the genera *Fusobacterium*, *Eubacterium*, and *Clostridium*. Several of the morphologic types seen with phase microscopy still could not be cultivated.

The implications of these findings for the study of the intestinal flora are discussed.

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