Common Mesophilic Anaerobes, Including *Clostridium botulinum* and *Clostridium tetani*, in 21 Soil Specimens

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A relatively rich medium was markedly superior to a dilute medium for the isolation of anaerobic bacteria from soil. The obligate anaerobes isolated from 21 soil samples were all clostridia and the counts ranged from $2.7 \times 10^4$ to $3.3 \times 10^8$ per g. The organisms most frequently isolated were *Clostridium subterminale*, *C. sordellii*, *C. sporogenes*, *C. indolis*, *C. bifermentans*, *C. mangenoti*, and *C. perfringens*. Seventeen other species were also recognized but almost one-third of the isolates could not be identified with any known species of *Clostridium*. *C. botulinum* type A was demonstrated in six soil samples, and type B in one. These soils were neutral to alkaline in reaction (average pH 7.9) and low in organic matter content (1.4%). The association of *C. botulinum* types A and B with neutral to alkaline soils was statistically significant ($P = 0.001$) as was their association with soils low in organic matter ($P = 0.005$). *C. botulinum* types E and F were found in one soil sample, pH 4.5, with organic matter 13.7%. *C. tetani* was isolated from two soil samples, both of intermediate pH value and higher than average organic matter content.

The obligately anaerobic bacteria have two principal habitats in nature, the alimentary tract of animals and man and the soil. Those found in the alimentary tract are primarily sporeforming rods and cocci (7, 11, 12), whereas those from soil are sporeformers. However, the obligate anaerobes of the soil have received little attention, except for special physiological groups. The nitrate and sulfate reducers, the flax retters, the nitrogen fixers, cellulose digesters, and methane formers have all been investigated (15, 16) as well as the pathogenic clostridia (14), but the occurrence of obligate anaerobes not falling into these groups has largely been overlooked, although it seems generally recognized that they are an integral part of the soil microflora. Moreover, as Garcia and McKay (4) have pointed out, we have but little information on the relationship of soil factors and the occurrence of soil bacteria.

In this investigation, we determined the relative numbers of facultative and obligately anaerobic bacteria in 21 soil samples, isolated and where possible identified to species the most commonly occurring anaerobes, and endeavored to correlate the properties of some of the soil samples with the presence of *Clostridium botulinum* and *C. tetani*.

**MATERIALS AND METHODS**

The source, pH, and organic content of the soil samples are shown in Table 1. They were obtained during the summer of 1973 by removing the top 2 to 5 cm and sampling the soil to about 15 cm and were transported in polyethylene bags to the laboratory, where they were transferred to parchment bags (2) for sufficient drying to allow sieving and sampling. Most probable number (MPN) counts were carried out by adding 1-g samples of each soil specimen to 9 ml of 1% gelatin solution, pH 6.9, dispersing with the aid of a Vortex mixer, and making decimal dilutions in the same solution. One-milliliter amounts of each dilution were inoculated, anaerobically under CO₂, into five tubes of cooked meat-glucose medium (6) which were incubated anaerobically for 3 weeks. Growth was determined by microscopic examination.

When growth first became evident in the MPN tubes, plates of Trypticase soy or of brain heart infusion agar were surface inoculated from tubes of the three higher dilution series showing growth and were incubated anaerobically in Brewer jars at 30 C to provide isolates for the determination of the proportion of facultative and obligate anaerobes in each specimen, as well as for the identification of the strains of obligate anaerobes. To obtain isolates of the slower growing bacteria, plates were also inoculated from the MPN tubes at the end of the 3-week period of incubation and incubated anaerobically. Isolated colonies were picked without selection from the isolation plates and deeply inoculated into tubes of meat infusion semi-solid medium (beef infusion with 1% Trypticase, 0.2% glucose, 0.3% agar, pH 6.8 to 7.0) which were incubated at 30 C aerobically. Those strains growing up to and on the surface of the semi-solid medium were designated as facultative anaerobes and those not growing up to the surface as obligate anaerobes. Occasionally, strains were en-
TABLE 1. Source, pH, and organic content of soil specimens

| Specimen no. | Source                      | pH  | Organic matter (%) |
|--------------|-----------------------------|-----|-------------------|
| 9            | Vegetable garden, compost pile, Virginia | 7.2 | 6.7               |
| 10           | Stream bank, Virginia       | 6.3 | 4.4               |
| 11           | Grassy field, Virginia      | 5.7 | 3.6               |
| 12           | Oak forest, Virginia        | 4.5 | 3.0               |
| 13           | Roadside clay, Virginia     | 5.1 | 0.2               |
| 14           | Wheat field, Idaho          | 7.1 | 3.1               |
| 15           | Sage brush area, Central Washington | 8.3 | 0.5               |
| 16           | Wheat field, Washington     | 7.3 | 0.8               |
| 17           | Sage brush area, Eastern Washington | 7.5 | 1.4               |
| 18           | Wheat field, Central Idaho  | 6.1 | 5.3               |
| 19           | Conifer forest, West Cascade Mountains, Washington | 5.0 | 12.9              |
| 20           | Grassland, S. Dakota       | 8.6 | 1.2               |
| 21           | Rain forest, Olympic peninsula | 4.5 | 13.7              |
| 22           | Grassland, Wyoming          | 8.0 | 1.3               |
| 23           | Grassland, East Cascade Mountains, Washington | 6.3 | 5.6               |
| 24           | Corn field, Iowa           | 5.5 | 7.5               |
| 25           | Corn field, Virginia       | 6.3 | 3.5               |
| 26           | Grassland, S. Dakota-Wyoming border | 8.2 | 1.4               |
| A            | Costa Rica, forest soil    | 6.5 | 1.6               |
| B            | Costa Rica, forest soil    | 6.5 | ND                |
| C            | Costa Rica, forest soil    | 6.1 | ND                |

*ND, Not done.

countered that grew to the surface but not above it. Such strains were surface inoculated to blood agar plates that were incubated aerobically at 30 C. Growth on such plates was taken to indicate that the strain was a facultative anaerobe. Identification to species of the obligate anaerobes was carried out by the methods of Holdeman and Moore (6).

In preliminary experiments, decimal dilutions of soil were made and aerobic and anaerobic plate counts were carried out on four soil specimens using two media and three plates per set and by incubating the cultures at 30 C until further incubation of the aerobic plates was rendered useless by the overgrowth of the agar by molds. This was usually 2 to 4 days, and incubation of the anaerobic plates was terminated at the same time, and the colonies were counted. Two media were used, brain heart infusion agar and a medium containing 0.1% peptonized milk and 1.5% agar. The latter was similar to the medium found (8) to give highest counts of aerobic bacteria from soil, except that we did not use the anti-fungal agent actidione because of lack of knowledge of its action on anaerobic bacteria.

Demonstration of the presence of C. botulinum and C. tetani in these soil specimens was determined by inoculating 10 1-g samples of each specimen into tubes of cooked meat medium and incubating at 20 to 22 C for 5 to 7 days. Five tubes were inoculated instead of 10 for specimens A, B, and C. After incubation, the tubes were frozen and held in frozen condition overnight to reduce non-botulinic deaths (1). The contents were then thawed and centrifuged. Two mice were injected intraperitoneally with 0.3 ml of the supernatant fluid from each tube and were held for 3 days. If the mice died, the experiment was repeated using culture fluid to which had been added C. botulinum antitoxin, types A or B. Occasionally, the toxic material in the cultures could not be neutralized by types A and B antitoxins; in such cases, neutralization with other antitoxins, those to C. perfringens, C. septicum, C. tetani, and to C. botulinum C, D, E, F, and G, was attempted. Neutralization of the toxic supernatant fluid was achieved in each case.

The statistical significance of the difference between two means was made by using a two-tailed Mann-Whitney U test, a nonparametric test. The hypothesis that two means were the same was rejected at the $P = 0.05$ level.

RESULTS AND DISCUSSION

The results of the experiment using nutritionally rich and nutritionally dilute media under aerobic and anaerobic conditions are given in Table 2. The short incubation time used in this experiment, 2 to 4 days, was necessitated by the overgrowth of the aerobic plates by filamentous fungi. Consequently, colonies of only the more rapidly growing organisms could be counted and the aerobic counts reported here are considerably below those of Larkin (8), who used a similar dilute medium with a fungus inhibitor and an incubation period of 10 days. Nevertheless, the results of the comparison of the two media show that the richer medium was definitely better for enumeration of anaerobes, probably a reflection of the relative inefficiency of anaerobic metabolism in obtaining energy from dilute nutrient.

TABLE 2. Plate counts on peptonized milk and brain heart infusion agar

| Soil sample | Conditions of incubation | Peptonized milk | Brain heart infusion |
|-------------|--------------------------|-----------------|---------------------|
| 9           | Aerobic                  | 1.8*            | 9.0                 |
|             | Anaerobic                | 0.0039          | 2.0                 |
| 10          | Aerobic                  | 1.4             | 0.7                 |
|             | Anaerobic                | 0.005           | 1.2                 |
| 11          | Aerobic                  | 4.2             | 3.8                 |
|             | Anaerobic                | 0.6             | 1.3                 |
| 12          | Aerobic                  | 0.81            | 0.46                |
|             | Anaerobic                | 0.0022          | 0.49                |

*All counts times $10^4$. 
The MPN count and the proportions of facultative and obligate anaerobes isolated from each soil sample are given in Table 3. The MPN count, rather than the plate count, was used for estimating the anaerobe population because of the superiority of cooked meat medium for stimulating germination of anaerobe spores. The MPN count includes both facultative and obligate anaerobes and extends over a considerable range, from fewer than 700 per g for soil 23 to more than $6 \times 10^6$ for soil 9. Repetition of the MPN procedure for soils 15 and 23 yielded much the same results. Of the 576 strains isolated under anaerobic conditions in this study, 40.3% were obligate anaerobes. The proportion of obligate anaerobes isolated from the various soil samples varied considerably, ranging from <3 to 85%. The obligate anaerobe population (MPN \times percentage of obligate anaerobes) varied from $0.00027 \times 10^6$ to $3.3 \times 10^4$ per g. This range is considerably wider than that reported by Gibbs and Freame (5), who determined the clostralid population of six soil samples, using “differential reinforced clostralid medium,” and who found counts ranging from $0.035 \times 10^4$ to $1.2 \times 10^4$. Thayer (17) found slightly more than $10^4$ clostridia per g in a Texas highplains shortgrass prairie soil, with only a slight drop in the clostralid count from the surface to 40 cm.

The identity of the 232 strains of obligate anaerobes isolated in this study, and the soil specimens from which they were isolated, are given in Table 4. No effort was made to isolate and identify all the species of anaerobes in these soil samples, only those present in greatest numbers. Isolation from soil specimen 11 was carried out twice, resulting in a larger number of isolates and more species of clostridia than the other soil specimens. All strains of obligate anaerobes were clostridia; either spores were demonstrable microscopically or the organisms were large gram-positive rods withstanding heating at 70 C for 10 min. Clostridia that did not appear to fall into any of the species listed in the eighth edition of Bergey’s Manual of Determinative Bacteriology were isolated from 17 soil specimens and made up about one-third of the isolates. Finding C. subterminale, C. sordellii, C. sporogenes, C. bifermantans, and C. perfringens among the anaerobes from the soil was not surprising, for these organisms are frequently encountered elsewhere, probably often from dust contamination. However, C. indolis and C. manganoti were isolated more frequently than was expected. The few previous isolations of C. indolis have been mostly from clinical specimens. C. manganoti is probably ubiquitous, for it has previously been reported from marshy soil from the Ivory Coast, from soil of Saigon, Siciy, and Indochina, as well as from a case of Madura foot in Tchad and the liver of a sheep in France (13). In general, the findings in this study are in some contrast to those of Matches and Liston (9) who investigated the mesophilic anaerobes

### Table 3. Numbers of facultative and obligately anaerobic bacteria isolated from 21 soil samples

| Soil sample no. | No. of colonies examined | % Obligate anaerobes | Anaerobic MPN count (per g \times 10^6) | Obligate anaerobes (per g \times 10^6) |
|-----------------|--------------------------|----------------------|----------------------------------------|-------------------------------------|
| 9               | 25                       | 52                   | 6.4                                    | 3.3                                 |
| 10              | 25                       | 60                   | 4.6                                    | 2.8                                 |
| 11              | 63                       | 57                   | 2.3                                    | 1.3                                 |
| 12              | 35                       | 57                   | 0.78                                   | 0.44                                |
| 13              | 13                       | 85                   | 0.0035                                 | 0.0030                              |
| 14              | 40                       | 13                   | 0.16                                   | 0.021                               |
| 15              | 21                       | 31                   | 0.45*                                  | 0.14                                |
| 16              | 21                       | 9.5                  | 0.092                                  | 0.0087                              |
| 17              | 21                       | 14                   | 0.092                                  | 0.013                               |
| 18              | 37                       | 14                   | 0.24                                   | 0.035                               |
| 19              | 34                       | 24                   | 0.043                                  | 0.010                               |
| 20              | 26                       | 38                   | 1.6                                    | 0.61                                |
| 21              | 37                       | 8.1                  | 0.17                                   | 0.01                                |
| 22              | 15                       | 73                   | 0.023                                  | 0.017                               |
| 23              | 19                       | 42                   | 0.000064*                              | 0.00027                             |
| 24              | 51                       | 80                   | 1.6                                    | 1.28                                |
| 25              | 18                       | 5.6                  | 4.9                                    | 0.27                                |
| 26              | 29                       | 0                    | 0.22 (approx.)                         | <0.007                              |
| A               | 26                       | 27                   | 0.24                                   | 0.065                               |
| B               | 34                       | 62                   | 1.6                                    | 0.44                                |
| C               | 17                       | 29                   | 0.11                                   | 0.032                               |

*Mean of two determinations.

### Table 4. Clostridia isolated from 21 soil samples

| Species* | Isolated from samples |
|----------|-----------------------|
| C. subterminale | 9, 11, 12, 15, 20, 24, B |
| C. sordellii  | 10, 13, 18, 22, B, C  |
| C. sporogenes | 11, 15, 24, A, B      |
| C. indolis   | 11, 14, 23, B         |
| C. bifermantans | 9, 10, 24, C       |
| C. manganoti | 11, 12, 19, 24       |
| C. perfringens | 14, 23, B           |
| C. butyricum  | 11, 23, A             |
| C. malenominatum | 13, 24               |
| C. paraperfringens | B, C                 |

* C. aurantibutyricum, C. botulinum type C (non-toxic), C. botulinum E, C. cadiaveris, C. cochlearium, C. felisineum, C. paraputricium, C. perenne, C. plagarum, C. scatologenes, C. septicum, C. sphenoides, C. sticklandii, C. tertium, and C. tetani were each isolated from one soil sample.
of marine sediments of Puget Sound, for these workers found three-fourths of their isolates to fall into three species, *C. perfringens*, *C. bifermantans*, and *C. novyi*. Because the methods used for isolation and identification in that study and this were much alike, the difference in the organisms found probably reflects a real difference in the anaerobic flora of the two sites.

Type A strains of *C. botulinum* as determined by antitoxin neutralization tests were demonstrated in 6 of 21 soil samples, a proteolytic strain of type B in one, and types E and F in one. All type A strains were from samples taken west of the Missouri River, a finding in accord with those of Meyer and Dubovsky (10) who found type A strains predominantly in soils from the western United States. All the soil samples in which *C. botulinum* types A and B were demonstrated in this study were neutral or alkaline in reaction (average pH 7.9 compared with an over-all average of 6.3), were low in organic matter (average 1.4% compared with 4.09), and had low anaerobic MPN counts (0.83 \( \times 10^6 \) compared to 1.22 \( \times 10^6 \) per g). This association between the occurrence of *C. botulinum* types A and proteolytic B and neutral to alkaline soils was statistically significant at the \( P = 0.001 \) level; the association of these organisms with soil of low organic matter content was also significant (\( P = 0.005 \)), but that with low anaerobic MPN counts was not. *C. botulinum* types E and F were demonstrated only in one sample, an acid soil from the Olympic rain forest which had a pH of 4.5, 13.7% organic matter, and an anaerobic MPN count of 0.13 \( \times 10^6 \).

The two soil specimens in which *C. tetani* was demonstrated had intermediate pH values, 5.5 and 6.3, with organic matter contents of 7.5 and 5.3% and anaerobic MPN counts of 0.24 and 1.6 \( \times 10^6 \) (Table 5). Dubovsky and Meyer (3) demonstrated *C. tetani* in 19 of 2,379 samples of soil and vegetables that they examined. They found this organism in 17 soil samples from the United States; 16 were from 397 specimens taken east of the Mississippi and one from 991 specimens from the western United States. They concluded that this organism was to be found in virgin and uncultivated forest soil where plant material is undergoing fermentation and decay. Neither *C. botulinum* nor *C. tetani* was demonstrated in or isolated from six soil specimens from Virginia or three from Costa Rica.

Most of the other clostridial species did not seem to be regionally distributed, except that *C. bifermantans* and *C. sordellii* were isolated much more frequently from eastern soils than from western, and *C. mangenotii* was isolated only from soils of pH 5.7 or lower.

The reasons for the association of certain clostridia, such as *C. botulinum* type A and *C. mangenotii*, with soil of certain characteristics are not known. Although the association with alkaline and acid soils, respectively, seems clear, this may be more than the simple effect of the level of hydrogen ion concentration. The complexity of soil structure allows the establishment of a multitude of different environments in a small volume and, consequently, the existence of a complex microbial population. Nevertheless, the restriction of these two species to soils of different levels of acidity indicates that some simple parameters may be of importance in determining the make-up of the microbial population, at least for the anaerobes.

### Table 5. Demonstration of *C. botulinum* and *C. tetani* in soil samples

| Organism               | Soil no. | Number of 1-g specimens containing *C. botulinum* or *C. tetani* of 10 examined |
|------------------------|----------|----------------------------------------------------------------------------------|
| *C. botulinum* type A  | 14       | 8                                                                                 |
| *C. botulinum* type A  | 15       | 4                                                                                 |
| *C. botulinum* type A  | 16       | 1                                                                                 |
| *C. botulinum* type A  | 17       | 1                                                                                 |
| *C. botulinum* type A  | 20       | 1                                                                                 |
| *C. botulinum* type A  | 22       | 9                                                                                 |
| *C. botulinum* type B  | 26       | 1                                                                                 |
| *C. botulinum* types E, F | 21     | 1                                                                                 |
| *C. tetani*            | 18       | 1                                                                                 |
| *C. tetani*            | 24       | 2                                                                                 |

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