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# ISOLATION OF OBLIGATELY ANAEROBIC PSYCHROPHILIC BACTERIA

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## Abstract

Sinclair, N. A. (Washington State University, Pullman), and J. L. Stokes. Isolation of obligately anaerobic psychrophilic bacteria. J. Bacteriol. **87**:562–565. 1964.—A total of 11 strains of strictly anaerobic psychrophilic bacteria have been isolated from soil, mud, and sewage. The organisms grow well at 0 C in liquid and on solid media, and grow only in the complete absence of oxygen. On the basis of shape, sporulation, flagellation, and strictly anaerobic growth, all of the organisms were classified as strains of *Clostridium*. Some of the biochemical properties of the strains and the effect of temperature on growth are described.

For many years, it appeared that psychrophilic bacteria, i.e., organisms which grow well at 0 C, were restricted to only a few genera, primarily *Pseudomonas*, *Achromobacter*, and *Flavobacterium* (Ingraham and Stokes, 1959). Recent investigations, however, indicated that psychrophilic types can be found in many genera and include most of the known morphological forms: gram-positive and gram-negative rods and cocci, diphtheroids, and sporeformers (Witter, 1961; Stokes, 1963). Also, both aerobic and facultative types have been commonly isolated. There appears to be no recorded instance in the literature, however, of the isolation of strictly anaerobic psychrophiles. A recent attempt to isolate such bacteria from a variety of foods, soil, sewage, and other habitats by means of anaerobic enrichments at 0 C was unsuccessful (Upadhyay and Stokes, 1962). Although 83 pure cultures were obtained which grew well anaerobically at 0 C, all of them also grew well aerobically at the same temperature. In a new series of investigations, however, 11 strains of strictly anaerobic psychrophiles belonging to the genus *Clostridium* were obtained from river mud, soil, and sewage. The isolation and properties of these strains are described in the present paper.

## Materials and Methods

Selective enrichment was used in the attempt to isolate strictly anaerobic psychrophiles. Portions of ground meat, raw milk, river mud, soil, and sewage were placed, in duplicate, in sterile 60-ml glass-stoppered bottles. The bottles were completely filled with freshly prepared, sterile Trypticase Soy Broth (BBL). One of the duplicate sets of bottles was heated at 80 C for 15 to 20 min to destroy vegetative cells, to facilitate isolation of sporeformers. Both the heated and unheated cultures were incubated at 0 C for 2 weeks.

To isolate pure cultures, material from the enrichment cultures was streaked at 2-week intervals on Trypticase Soy Agar (BBL) plates. The plates were incubated anaerobically at 0 C in sealed jars under nitrogen. A small dish containing pyrogallic acid and 35% $K_2CO_3$ was placed in each jar just prior to gassing to absorb residual oxygen. After 2 weeks of incubation, colonies were picked from the incubated plates and inoculated in duplicate on slants of Trypticase Soy Agar. One slant was incubated aerobically and the second anaerobically under a pyrogallic acid-$K_2CO_3$ seal at 15 C. The latter temperature was used, because the rate of growth of these organisms is faster at 15 C than at 0 C and less time is required, therefore, for maximal growth. A total of 118 pure cultures of bacteria were isolated. Of the cultures, 107 were facultatively anaerobic, since they grew well with or without oxygen. However, 11 cultures were strict anaerobes, since they grew only in the complete absence of $O_2$. These strictly anaerobic psychrophilic bacteria were isolated only from samples which had been heated to 80 C and, therefore, presumably from spores.

The 11 cultures were examined for morphology and Gram stain reaction. Spores were stained by Bartholomew and Mittwer's modification of Wirtz's method, and flagella were stained by

Gray's method (Society of American Bacteriologists, 1957).

Determinations of the optimal and maximal growth temperatures were made from 0 to 35 C at intervals of 5 C in tubes of Trypticase Soy Broth which contained 0.2% yeast extract, 0.05% sodium thioglycolate, and 0.075% agar. Although the cultures were exposed to air, the thioglycolate and agar maintained anaerobic conditions. Growth always began about 0.5 in. below the surface of the medium. A detailed study was made of the kinetics of growth of strain 61, in the range of 0 to 30 C at 5-degree intervals. For this purpose, the bacteria were grown in 35-ml glass-stoppered bottles completely filled with Trypticase Soy Broth containing 0.2% yeast extract and 0.05% sodium thioglycolate. Growth was followed turbidimetrically with a Klett-Summerson colorimeter (red filter).

Sugar fermentations were carried out in tubes containing the following medium: Trypticase, 2.0%; NaCl, 0.25%; $K_2HPO_4$, 0.15%; sodium thioglycolate, 0.05%; phenol red, 0.002%; specified carbohydrate, 0.5%; and agar, 0.075%. Carbohydrates tested included glycerol, erythritol, xylose, arabinose, glucose, fructose, galactose, mannitol, sucrose, maltose, lactose, raffinose, salicin, and cellulose.

The cultures were tested for proteolysis by growing them in media containing litmus iron milk, cubes of coagulated egg white, ground beef heart, and gelatin (Society of American Bacteriologists, 1957). For nitrate reduction and indole formation, indole-nitrate medium was used. In some cases, the medium was enriched with 0.01% DL-tryptophan to increase indole formation. For $H_2S$ production, peptone-ferric ammonium citrate medium was used. All fermentation and other biochemical tests were carried out with cultures incubated at 0, 15, and 25 C.

## RESULTS

A total of 11 strains of obligately anaerobic psychrophilic bacteria were isolated by the described techniques. All of the strains grew well at 0 C within 2 weeks and only in the complete absence of oxygen. The sources from which the bacteria were isolated are as follows: strains 61, 70, and 103 from river mud; strains 69, 82, 83, and 105 from garden soil; strains 95, 97, and 99 from septic tanks maintained at 0, 4, and 10 C, respectively; and strain 100 from an anaerobic sewage lagoon. None was isolated from raw milk or ground meat.

The general properties of the 11 strains were as follows: growth at 0 C, positive; relation to oxygen, strictly anaerobic; morphology, rods (2.5 to 4.5 $\mu$ by 0.5 to 1.0 $\mu$); Gram reaction, gram-positive; sporulation, subterminal to terminal oval spores produced at 0 and 15 C; flagellation, peritrichous (many flagella); motility, positive at 0 and 15 C. Six strains sporulated readily in media containing coagulated egg white or gelatin, whereas five strains sporulated extensively in media containing soluble starch.

As indicated above, spore formation occurs at 0 C. Since the cultures were isolated at 0 C from heated samples, it would appear also that spore germination can occur at 0 C. This was confirmed with the isolated pure cultures. These were allowed to sporulate, and were then heated and transferred to fresh medium. Growth in the latter at 0 C was evidence of spore germination. Also, spores of mesophilic bacteria can germinate at low temperatures. Mundt, Mayhew, and Stewart (1954) observed germination of 95% of the spores of *C. sporogenes* at 4.4 C. Williams, Clegg, and Wolf (1957) obtained spore germination with 11 of 54 strains of *Bacillus subtilis* at 4 C. Halvorson (1962) reported germination of spores of *B. cereus* at subzero temperatures, −3 and −6 C. In no case, however, was growth observed at these low temperatures.

Motility was observed in cultures grown in motility medium at 0 C and at 15 C and in cultures maintained at these same temperatures in hanging-drop preparations. Rubentschik (1925) noted that his psychrophilic urea-decomposing bacteria were motile at 20 and 24 C but not at −1.25 to −2.5 C. In contrast, Hess (1934) found that *P. fluorescens* grown at temperatures ranging from 20 to −6.5 C was motile at all temperatures. Our data are in agreement with those of Hess and emphasize that psychrophiles are motile at low temperatures.

On the basis of shape, sporulation, flagellation, and strictly anaerobic growth, we have placed these organisms in the genus *Clostridium.*

*Cardinal growth temperatures.* The optimal and maximal growth temperatures were determined for all strains. The rate and extent of growth was measured visually. Representative data for three strains are shown in Table 1. The optimal temperature ranged from 20 to 25 C, and the maximal temperature was from 25 to 30 C. None of the strains grew at 35 C even when the incubation period was extended to 10 days.

*Kinetics of growth of strain 61.* A detailed investigation was made of the kinetics of growth of strain 61, in the range of 0 to 30 C at 5-degree intervals. The organism was grown in enriched Trypticase Soy Broth in 35-ml glass-stoppered bottles completely filled with medium (Fig. 1). Generation times were inserted in Fig. 1 for comparative purposes. It is apparent that the lag phase increases with a decrease in temperature. This is true also of the exponential phase. The rate of growth is highest at 20 C, as indicated both by the curves and the generation times. It is of interest to note that maximal

TABLE 1. *Effect of temperature on the growth of psychrophilic Clostridium strains*

| Strain | Temp (C) | | | | |
|---|---|---|---|---|---|
| | 0 | 10 | 20 | 25 | 30 |
| 83 | +7* | +3 | +2 | +2 | −9 |
| 95 | +7 | +3 | +2 | +2 | ±9 |
| 99 | +7 | +3 | +2 | +2 | −9 |

* Symbols: + = growth; − = no growth; the number indicates the days required for maximal growth or when incubation was discontinued.

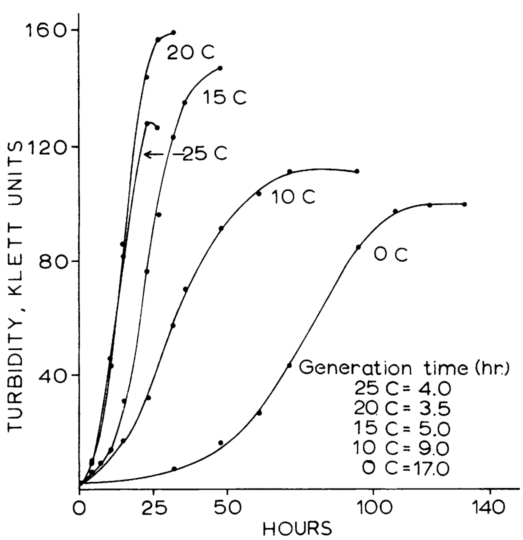


FIG. 1. *Effect of temperature on the growth of psychrophilic Clostridium strain 61.*

TABLE 2. *Effect of temperature on the fermentation of sugars by psychrophilic Clostridium strains*

| Strain | 15 C (7 days) | | | 0 C (16 days) | | |
|---|---|---|---|---|---|---|
| | Xylose | Glucose | Sucrose | Xylose | Glucose | Sucrose |
| 69 | A* | AG | AG | AG | AG | AG |
| 82 | A | A | A | — | A | — |
| 100 | — | AG | AG | — | A | AG |

* A = acid; G = gas.

growth occurs at the optimal growth temperature. This has been observed previously with facultative bacteria grown anaerobically, and is in contrast to aerobic growth of bacteria in stationary cultures where oxygen becomes limiting and maximal growth is obtained at temperatures considerably below the optimal growth temperature (Sinclair and Stokes, 1963).

*Biochemical properties.* The strains were examined for their ability to ferment carbohydrates and related compounds at 0, 15, and 25 C. The following representative data at 15 C for five strains and six sugars were obtained. Glycerol and sucrose each were fermented by three strains. Glucose and salicin were fermented by all five strains. Xylose was fermented by one strain and lactose and raffinose by another strain. Fermentation always gave rise to both acid and gas. Cellulose was not fermented. In general, identical results were obtained at 0, 15, and 25 C, although biochemical changes occurred much more slowly at 0 C due to the much slower growth at that temperature. Some variations, however, were observed (Table 2). With the exception of one strain and one sugar, sugars fermented at 15 C with the production of acid or acid and gas were either not fermented at 0 C or fermented with the production only of acid. Similar changes in fermentation patterns at different temperatures have been obtained also with facultative psychrophiles (Upadhyay and Stokes, 1962).

None of the strains produced indole or reduced nitrates. Gelatin was liquefied by three strains, and starch was hydrolyzed by four strains. Slow acid formation or reduction occurred in litmus milk with all strains, but neither coagulation nor proteolysis was evident. Small amounts of gas were formed in chopped beef heart medium, but digestion and blackening did not occur. Hydrogen sulfide was produced by three strains. In general, the results obtained at 25 and 0 C were similar to those at 15 C.

## Discussion

The major significance of these investigations lies in the demonstration that strictly anaerobic psychrophilic bacteria exist in nature. Thus, all types of oxygen-requiring bacteria, with the possible exception of microaerophiles, have been shown to occur in the psychrophilic form, i.e., obligate aerobes, facultative anaerobes, and obligate anaerobes. This, together with the increasing number of generic types which are being isolated and the existence of psychrophilic yeasts, molds, and actinomycetes, suggests that psychrophiles are of considerable importance in the cycles of matter in nature. Quantitative data, however, on the occurrence and changes in the psychrophile population in different habitats and in the same habitat under varying conditions are virtually nonexistent. Such data are needed to fully evaluate the role of psychrophiles in nature.

## Acknowledgment

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