JOURNAL OF BACTERIOLOGY, Oct. 1969, p. 528-530
Vol. 100, No. 1

Differentiation Between *Clostridium sordellii* and *Clostridium bifermentans* by Gas Chromatography

J. B. BROOKS, C. W. MOSS, AND V. R. DOWELL

U.S. Department of Health, Education, and Welfare, National Communicable Disease Center, Atlanta, Georgia 30333

Received for publication 18 July 1969

Clostridium bifermentans can be differentiated from *Clostridium sordellii* by gas chromatography on the basis of amines detected after growth for 6 hr in cooked-meat medium. Products detected after exposure of resting cells to amino acids gave evidence for the probable origin of the amines.

The taxonomy of *Clostridium bifermentans* and *C. sordellii* is an unsettled issue. Because of the similarity of biochemical, cultural, morphological, and serological characteristics of these organisms, *C. sordellii* has been considered a toxigenic variant of *C. bifermentans* (1). This viewpoint has been challenged by those who believe that the two species are distinct and that they can be differentiated primarily on the basis of toxin and urease production by *C. sordellii* (3-6). J. B. Brooks (Ph.D. Thesis, Virginia Polytechnic Institute, 1969) found that the two organisms could be distinguished by the presence of relatively large amounts of amines in growing cultures of *C. bifermentans*. In this study, data on amine production by additional strains of these two species are presented. The potential use of gas chromatographic tests for amines as a rapid diagnostic aid to distinguish these organisms is discussed.

Ten strains of *C. bifermentans* and nine strains of *C. sordellii* obtained from stock cultures at the National Communicable Disease Center (NCDC) were examined. The organisms tested and their NCDC strain numbers are as follows: *C. bifermentans*, 1718B, 409A, 462, 1720A, 1628, 1668, 4483, 5064, 4511, and 4665; *C. sordellii*, 1720C, 383, 1718A, 1766, 1717, 5297, 4798, 5298, and 5257.

The cultural and differential biochemical tests were performed as previously described (2). All strains of *C. sordellii* were urease-positive; *C. bifermentans* strains were urease-negative.

To test for amines, 0.1 ml of an actively growing culture was inoculated into 8 ml of cooked-meat medium (CM), prepared as described (2); the medium was adjusted to *p*H 6.8 with 4 N HCl and incubated under anaerobic conditions for 6 or 24 hr at 37 C. The cultures were then acidified to about *p*H 2 with 50% (v/v) H_2SO_4 and were extracted with 5 ml of spectrograde ethyl ether (Eastman Chemical Products, Inc., Kingsport, Tenn.). The ether layer was discarded. The residual aqueous layer was then made basic (about *p*H 10) with 8 N NaOH and was extracted with 5 ml of pesticidequality chloroform (Matheson Co., Inc., East Rutherford, N.J.). The chloroform layer was removed and concentrated by a gentle stream of air to about 0.09 ml. The amine standards were added to water and processed in the same manner as the cultures. The amines were converted to their trifluoroaceticanhydride (TFA) derivatives as described by J. B. Brooks (Ph.D. Thesis, Virginia Polytechnic Institute, 1969).

The amine TFA derivatives were analyzed by injecting 0.8 ml of sample into a Barber-Colman gas chromatograph (GC) equipped with flame ionization detectors and dual glass columns (0.38 cm inside diameter by 7.3 m in length) packed with chromosorb W (AW-DMCS, H.P.) coated with 3% OV-1 (Applied Science Laboratories, Inc., State College, Pa.). The instrument was operated isothermally for 1 min at 80 C, and then the temperature was programmed for a linear increase of 7.5 C per min to 265 C. The detector temperature was 295 C; the injector inlet temperature, 250 C. The electrometer attenuation was 10^2; the helium flow was 38 cc/min. A recorder input signal of 1 mv was used with a chart speed of 30 inches per hr.

Figure 1 shows representative amine patterns for the two organisms after growth for 6 hr in CM medium. The *C. bifermentans* profile (curve B) is markedly different from *C. sordellii* (curve A) and is characterized by the presence of two major components (peaks 2 and 9). These two components were not present either in *C. sordellii*

(curve A) or in uninoculated CM medium. Peak 2 was tentatively identified as β-phenylethylamine and peak 9 as tryptamine on the basis of identical GC retention times compared to known standards of these compounds. Strains of each of the two species gave results similar to those shown in Fig. 1, with the exception of *C. bifermentans* strain 4483. The amine pattern for this culture was typical of *C. sordellii*, but the culture was negative for urease. No explanation for this variation can be given at this time. The culture may possibly be a varient of *C. sordellii*, which failed to produce urease.

A GC standard curve of known amounts of β-phenylethylamine, ranging from 0.8 to 200 μmoles, was constructed. Most strains of *C. bifermentans* produced approximately 25 μmoles of β-phenylethylamine after incubation for 6 hr and approximately 50 μmoles after 24 hr. None of the *C. sordellii* strains produced as much as 0.8 μmole of β-phenylethylamine after either time period. Approximately 50 μmoles of tryptamine was produced by cultures of *C. bifermentans* after 6 hr; none was detected in *C. sordellii* after either 6 or 24 hr. The presence of 12.5 μmoles of tryptamine could easily have been detected with these procedures.

It was assumed that the presence of amines in growing cultures was due to decarboxylation of amino acids. Therefore, resting-cell studies were made to determine the enzymatic production of β-phenylethylamine and tryptamine from L-phenylalanine and L-tryptophan, respectively. Cells were grown overnight in Trypticase Soy Broth (BBL); they were washed with 0.85% NaCl solution and then with phosphate buffer (*p*H 6.8) solution which was supplemented with 0.0001% pyridoxal phosphate (PBP buffer). A thick cell suspension was made in PBP buffer, and 0.5 ml of the suspension was added to 4 ml of PBP buffer, which contained 200 μmoles of either phenylalanine or tryptophan, and also to 4 ml of PBP buffer containing 200 μmoles of each of both amino acids. The reaction mixture was incubated both aerobically and anaerobically at 37 C for various periods of time up to 3 days. Control samples consisted of amino acid mixtures without cells and heat-treated cell suspensions (4 min at 100 C) which were added to the amino acid mixtures as described above for nonheated cells. For analysis, the cells were removed by centrifugation, and the supernatant fluid was tested for amines in the same manner as for growing cultures.

Resting cells of *C. bifermentans* strain 1718B exposed to L-phenylalanine produced a compound with a GC retention time identical to that of β-phenylethylamine, and when exposed to L-tryptophan they produced a compound with a retention time identical to tryptamine. These data indicate the probable origin of these two amines in growing cultures. When *C. bifermentans* resting cells were incubated with the two amino acids, both β-phenylethylamine and tryptamine were produced (Fig. 2, curve B). The heat-treated cell mixtures and uninoculated amino

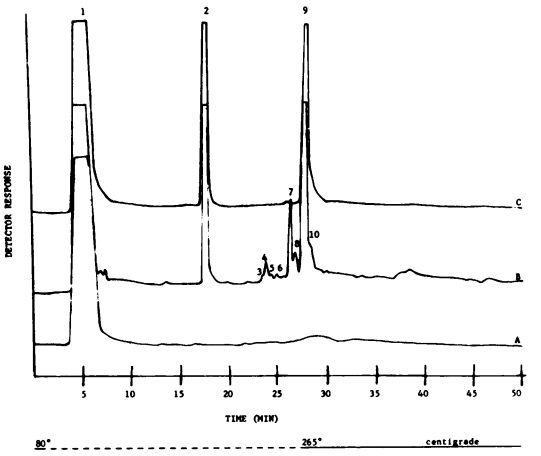

FIG. 1. *Gas-liquid chromatography of amines produced by growing cultures (6 hr) of C. sordellii (curve A) and C. bifermentans (curve B) in cooked-meat medium. Peaks 2 and 9 in curve C are TFA derivative standards of β-phenylethylamine and tryptamine, respectively. Peak 1 is chloroform and reagents, and peaks 3–8 and 10 are unidentified compounds extractable with chloroform only under basic conditions.*

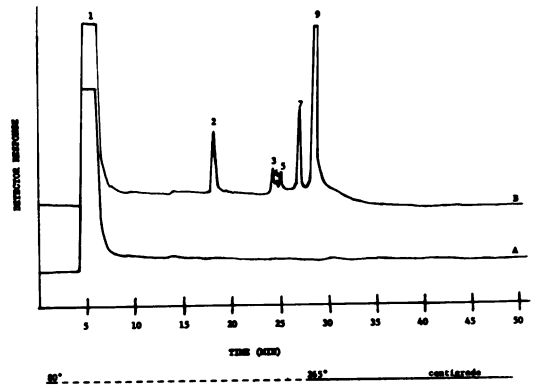

FIG. 2. *Gas-liquid chromatography of amines produced by resting cell suspensions of C. bifermentans exposed to L-phenylalanine and L-tryptophan (curve B). Curve A represents heat-treated C. bifermentans cells. Peak 1 is chloroform and reagents; peak 2, β-phenylethylamine. Peak 9 is tryptamine and peaks 3–7 are unidentified basic extractable compounds.*

acid controls contained no detectable amines (Fig. 2, curve A). A number of unidentified basic extractable compounds were detected when resting cells were incubated with a mixture of the two amino acids (Fig. 2), but they were not detected from resting cells incubated with individual amino acids. This suggests the possibility that these unidentified compounds may arise through enzymatic action on phenylalanine and tryptophan in combination. Likewise, the origin of the unidentified compounds observed in growing cultures (Fig. 1, peaks 3–8) may be the enzymatic transformation of mixtures of amino acids. No amines were detected in heated or unheated resting-cell suspensions of *C. sordellii* strain 1717. Both β-phenylethylamine and tryptamine were produced by incubating resting cells of *C. bifermentans* under either aerobic or anaerobic conditions.

The above results indicate that gas chromatography is a valuable aid in distinguishing between *C. bifermentans* and *C. sordellii*. The ability to detect β-phenylethylamine and tryptamine production from growing cultures after only 6 hr of incubation provides a rapid means of distinguishing these organisms. The procedures used are rapid and reproducible, and can be easily adapted to other GC equipment.

We thank Mary Ann Lambert for capable technical assistance.

LITERATURE CITED

1. Breed, R. S., E. G. D. Murray, and N. R. Smith. 1957. Bergey's manual of determinative bacteriology. 7th ed., p. 665. The Williams & Wilkins Co., Baltimore.
2. Dowell, V. R., Jr., and T. M. Hawkins. 1968. Laboratory methods in anaerobic bacteriology. U.S. Public Health Service Publication no. 1803. U.S. Government Printing Office, Washington, D.C.
3. Meisel, H., and A. Switalska. 1966. The problem of taxonomy of *Clostridium sordellii* and *Clostridium bifermentans*. Exp. Med. Microbiol. 18:31–38.
4. Moore, W. E. C., E. P. Cato, and L. V. Holdeman. 1966. Fermentation patterns of some *Clostridium* species. Intern. J. Syst. Bacteriol. 16:383–415.
5. Prévot, A. V. 1966. Manual for the classification and determination of the anaerobic bacteria. 3rd ed., p. 276–278. Lea and Febiger, Philadelphia.
6. Smith, L. D-S., and L. V. Holdeman. 1968. The pathogenic anaerobic bacteria, p. 374–382. Charles C Thomas, Publisher, Springfield, Ill.