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Characterization of Clostridia by Gas Chromatography: Differentiation of Species by Trimethylsilyl Derivatives of Whole-Cell Hydrolysates

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Trimethylsilyl (TMS) derivatives prepared from whole-cell hydrolysates of 36 strains, representing 10 species of *Clostridium* were examined by gas-liquid chromatography (GLC). The TMS profile of each species contained a group of peaks which characterized the species. Variation among strains within a species was much lower than variation between species. Some of the closely related clostridia could be differentiated by comparing their TMS profiles. Strains of *Clostridium botulinum* were distinguished from *C. sporogenes* on the basis of the ratio of two GLC peaks which corresponded to arabinose and glucose. A peak with a retention time identical to that of mannose was present in all *C. bifermentans* strains but was absent in those of *C. sordellii*.

The genus *Clostridium* is composed of a very heterogeneous group of organisms which are gram-positive, anaerobic, catalase-negative, spore-forming rods. Some species of this genus fix atmospheric nitrogen, some are thermophilic, and others are pathogenic for man and animals.

Various approaches have been used to study this diverse group of bacteria. Biochemical tests have been used by some workers (5, 11), serological and immunological studies by some (3, 6, 7, 13, 18, 21), and enzymatic studies by others (12, 19).

In recent years, gas, column, and thin-layer chromatography have yielded impressive amounts of new data on the clostridia. Volatile fatty acids of clostridia have been studied (1, 15). Moss and Lewis (16) studied cellular fatty acids of 13 species of clostridia by gas-liquid chromatography (GLC) and divided these into four general groups. One group, however, contained 10 species which were not further subdivided.

In a previous report from this laboratory (8), we observed differences in genus and species profiles of trimethylsilyl (TMS) derivatives of whole-cell hydrolysates of various bacteria. This report describes our study to evaluate the applicability of the TMS technique for the analysis of whole-cell hydrolysates of various species and strains of clostridia.

MATERIALS AND METHODS

Cultures and procedures. The cultures used in this study were identified by the Anaerobic Bacteriology Laboratory of the National Communicable Disease Center, Atlanta, Ga., as outlined elsewhere (4). Thirty-six strains representing the following 10 *Clostridium* species were tested: *C. septicum*, *C. tertium*, *C. capitovale*, *C. difficile*, *C. subterminale*, *C. botulinum*, *C. perfringens*, *C. sporogenes*, *C. sordellii*, and *C. bifermentans*. All organisms were grown in 125 ml of Trypticase Soy Broth (BBL) in 250-ml flasks and incubated anaerobically for 24 hr at 37 C, as described previously (8). The lecithinase test was performed on modified egg yolk medium (4).

Sample preparation. Twenty milligrams of lyophilized cells was placed in screw-capped test tubes fitted with Teflon-lined caps. Then the test tubes were filled with 1 N HCl in methanol (20 ml) and were hydrolyzed for 8 hr at 100 C. The samples were cooled and dried on a flash-evaporator (Buchler Instruments, Fort Lee, N.Y.). The dried sample was resuspended in anhydrous methanol and again evaporated. This procedure was repeated several times until the hydrolysate was neutral (*p*H 7.0). The dry residue was dissolved in 1 ml of dry pyridine (stored over KOH pellets) and transferred into a stoppered glass conical centrifuge tube. Then 0.3 ml of hexamethyldisilazane and 0.15 ml of trimethylchlorosilane (Applied Science Laboratories, Inc., State College, Pa.) were added. After shaking for 30 sec, the mixture was left overnight at room temperature. The reaction mixture was centrifuged. The supernatant fluid was transferred to another centrifuge tube and evaporated to dryness at 55 C under a gentle stream of nitrogen. The residue was dissolved in 10 ml of hexane, and the volume was reduced to 0.25 ml under nitrogen. Reference sugar samples obtained from Sigma Chemical Co., St. Louis, Mo., were processed by the same procedure.

GLC analysis. The samples were analyzed with a gas chromatograph (model 5000; Barber-Colman Co., Rockford, Ill.) equipped with a hydrogen flame ionization detector. Nitrogen was used as the carrier gas. Nitrogen inlet pressure was adjusted to 24 psi. Samples were analyzed on both polar and nonpolar columns. The nonpolar column was an 8-ft (2.44 m) U-tube glass column packed with 3% OV-1 coated on 60 to 80 mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, Pa.). The column was temperature-programmed from 130 to 220 C at a rate of 3 C per min; the detector temperature was 275 C, and the injector temperature was 300 C. The polar column was a 6-ft (1.8 m) U-tube glass column packed with 1% OV-17 on 80 to 100 mesh high performance Chromosorb G (Regis Chemical Co., Chicago, Ill.). This column was temperature-programmed from 120 to 200 C at a rate of 5 C per min. The injector and detector temperatures were 300 and 275 C, respectively.

All samples were run routinely on the OV-1 column, as described above, for 30 min. After this time, the column temperature was raised to 250 C and maintained at this temperature for 10 min. Then the column was cooled for 5 min and left for 5 min to allow the temperature to stabilize at the initial program setting (130 C). This procedure was found to give reproducible retention time values. The polar column (OV-17) was used for identification and verification purposes. The quantitative data were obtained as described before (8).

RESULTS

In a previous report we found that when pure crystalline monosaccharides were used to prepare TMS derivatives, only a single GLC peak for each compound was obtained (9). Results obtained during the present study showed that, when standard carbohydrates were hydrolyzed and processed like other samples, more than one GLC peak was occasionally obtained for each compound. This result has also been reported by other workers and is probably due to anomerization (20). However, with each known standard, there was always a major peak in the chromatogram, and identification was based on this peak.

Examination of the GLC chromatograms (TMS profile) revealed variations among strains within species as well as variations among species. However, in spite of this variation among the strains, each species examined could be differentiated. This ability to distinguish each species was due to the presence of certain peaks (common peaks) in each strain tested. Moreover, some species could be distinguished from others by comparing the ratio of one peak to another.

Strain variation within species. Eight strains of *C. sordellii* were studied. Four of these strains were lecithinase-positive, and the other four were lecithinase-negative. Each of the eight strains tested shared 11 common peaks, which accounted for 75 to 92% of the total area of all peaks. The area of the remaining peaks on the chromatograms ranged from 8 to 25%, with an average of 12.9% for the eight strains. The lecithinase-positive and lecithinase-negative strains could not be differentiated from each other. Strain 2474 had a relatively high percentage of uncommon peaks but shared all of the common peaks of *C. sordellii*. The TMS profile of this strain and of the other *C. sordellii* strains could easily be differentiated from the profiles of *C. bifermentans* by the absence of a peak with the retention time of 17.9 min (Table 1).

The strains of *C. bifermentans* had 11 common peaks. The total area of the other peaks ranged from 2.5 to 27%, with an average of 12.8% for the five strains tested (Table 2).

All strains of *C. perfringens* used in this study were type A, four of which were Hobbs food-poisoning strains (10). Hobbs strains could not be distinguished from other type A strains. The six cultures of *C. perfringens* had 17 common peaks present in their chromatograms. The area of the peaks not shared by all strains averaged 7.2% of the total area of all peaks (Table 3).

Variation among the strains of *C. botulinum* and *C. sporogenes* was less than that observed with the other species used in this study. The average percentages of the area of peaks not shared by strains of these two species were 6.0% for *C. botulinum* and 2.0% for *C. sporogenes* (Tables 4 and 5). With this method, *C. botulinum* type A cultures could not be differentiated from type B cultures.

Relationship between species. A comparison of all common peaks in one species with the common peaks of another cannot be used for differentiating between species. This is due to the fact that some of the common peaks in one species are present in some strains of the other species but not in all of them. Thus, only those common peaks which are present in all strains of a given species and absent in all strains of the other species are useful for differentiation.

The TMS profiles of *C. bifermentans* strains differed from those of *C. sordellii*, as can be seen by comparing chromatograms G and H in Fig. 1. The major difference between these two species was the presence of peak no. 8 in *C. bifermentans* and its absence in *C. sordellii*. This peak had a retention time of 17.9 min, the same as that for mannose on both the polar and nonpolar columns. The area of this peak varied among the five strains of *C. bifermentans* and ranged from 12 to 25% of the total area (Table 2). Even though peak no. 7 in *C. sordellii* (Fig. 1H) had a retention time close to that of peak no. 8 of *C. bifermentans* (Fig. 1G), they have different retention times

(18.2 and 17.9 min in Tables 1 and 2, respectively). Another difference between the two species was the presence of two peaks with retention times of 17.1 and 18.2 min (peaks 6 and 7 in Fig. 1H) in *C. sordellii*. These peaks were absent in *C. bifermentans*.

Strains of *C. perfringens* could easily be distinguished from *C. sordellii* and *C. bifermentans*. All *C. perfringens* strains had peaks with retention times of 6.3 and 8.2 min. These peaks were absent in *C. sordellii* strains. Strains of *C. perfringens* had peaks with retention times of 8.2, 13.9, 16.0, 17.1, 19.1, 20.1, and 26.4 which were absent in all *C. bifermentans* strains. An addi-

TABLE 1. *Relative percentage of common and other peaks obtained from GLC chromatograms of TMS derivatives prepared from whole-cell hydrolysates of Clostridium sordellii*

C. sordellii strain	Lecithinase	Retention time (min) of common peaks[a]											Per cent of other peaks[b]
		8.6	10.9	11.3	17.1	18.2	19.1	20.8	21.7	24.6	25.6	26.9	
5456	+	0.6[c]	5.3	11.1	8.9	3.8	29.2	14.2	5.3	1.0	1.2	2.7	16.7
2474	+	1.8	2.5	9.1	3.1	3.7	8.0	27.3	11.9	3.3	1.5	2.9	24.9
3869	+	1.5	3.4	12.5	1.1	4.4	13.9	32.4	13.2	1.5	0.7	1.7	13.7
383	+	1.2	3.7	13.3	8.5	2.1	35.4	15.5	4.4	0.9	1.4	4.2	9.4
3015	−	1.6	4.6	14.0	10.9	1.6	26.5	16.9	4.4	0.7	1.8	4.1	12.9
6024	−	1.2	3.8	11.9	11.6	2.7	25.2	20.4	8.1	1.0	1.4	3.5	9.2
5677	−	0.9	3.2	0.9	13.1	3.6	26.0	27.5	9.7	1.2	1.4	3.8	8.7
1720C	−	1.5	7.4	18.1	11.2	1.3	32.0	12.5	2.9	0.5	1.9	2.7	8.0

[a] The greatest variation in retention time was less than ± 0.1 min.
[b] Average, 12.9.
[c] Percentage of total area of all peaks.

TABLE 2. *Relative percentage of common and other peaks obtained from GLC chromatograms of TMS derivatives prepared from whole-cell hydrolysates of Clostridium bifermentans*

C. bifermentans strain	Retention time (min) of common peaks[a]											Relative per cent of other peaks[b]
	10.9	11.3	17.9	18.9	20.8	21.7	23.0	23.7	24.6	25.8	26.8	
3299	2.5[c]	13.9	14.0	2.5	20.3	7.3	1.9	3.6	0.8	2.9	3.3	27.0
4572	1.2	23.2	19.4	5.3	15.9	4.9	0.9	1.3	0.8	0.6	1.1	25.4
4612	1.7	20.6	12.9	9.5	32.3	10.7	1.1	1.4	1.4	1.2	4.7	2.5
3790	1.2	18.1	25.6	3.7	27.1	11.6	1.0	1.5	1.1	1.0	1.5	6.6
409A	2.2	17.0	21.5	12.4	22.6	13.2	0.7	0.9	2.0	0.5	4.2	2.8

[a] *See* footnote *a* of Table 1.
[b] Average, 12.8.
[c] *See* footnote *c* of Table 1.

TABLE 3. *Relative percentage of common and other peaks obtained from GLC chromatograms of TMS derivatives prepared from whole-cell hydrolysates of Clostridium perfringens*

C. perfringens strain	Retention time (min) of common peaks[a]																Relative per cent of other peaks[b]
	6.3	8.2	8.7	10.9	11.3	13.9	16.0	17.1	19.1	20.1	20.8	21.7	22.3	26.4	26.7	28.2	29.3	
A28	3.9[c]	1.4	1.0	9.9	28.5	T[d]	1.7	2.4	9.6	2.3	12.3	1.5	1.1	3.7	2.7	0.8	10.4	6.8
A37	3.3	1.4	1.1	9.4	22.8	0.8	2.5	5.5	14.8	2.6	11.4	1.3	2.7	2.3	2.1	1.5	6.2	8.3
Hobb's 8	0.7	1.8	0.6	9.8	32.4	0.9	1.7	2.5	15.7	2.8	7.5	0.9	1.3	4.5	3.6	1.7	3.6	8.0
Hobb's 11	1.1	0.9	0.5	6.8	24.3	1.4	2.1	3.6	20.0	3.0	13.2	4.2	1.6	T	5.7	1.0	3.8	6.8
Hobb's 13	1.3	0.6	T	6.8	22.5	1.5	1.4	2.6	21.0	2.7	13.5	3.4	1.9	3.9	3.9	1.3	6.3	5.4
Hobb's 7	1.3	1.6	0.9	15.9	25.2	4.0	0.5	1.9	17.6	2.3	7.4	1.8	2.7	1.6	2.4	1.6	3.4	7.9

[a] *See* footnote *a* of Table 1.
[b] Average, 7.2.
[c] *See* footnote *c* of Table 1.
[d] T designates trace amount (less than 0.5%).

TABLE 4. *Relative percentage of common and other peaks obtained from GLC chromatograms of TMS derivatives prepared from whole-cell hydrolysates of Clostridium sporogenes*

C. sporogenes strain	Retention time (min) of common peaks[a]																	Per cent of other peaks[b]
	10.4	10.9	11.3	13.9	14.5	16.1	17.1	17.6	18.8	20.8	21.7	22.4	24.8	25.5	25.9	26.8	29.3	
9	2.0[c]	17.0	27.8	1.3	1.1	T[d]	1.3	1.4	T	23.7	12.2	1.1	1.8	T	0.5	1.7	1.8	5.3
96	2.7	7.5	28.5	2.1	2.7	0.8	2.4	2.8	5.5	28.3	5.2	0.8	1.3	0.5	1.2	3.6	1.9	2.2
1783	4.6	7.3	29.1	6.1	2.5	1.4	2.0	1.9	7.3	21.3	2.4	1.2	1.9	0.5	0.8	5.1	3.7	0.9
81	T	16.0	29.0	6.0	1.5	1.2	2.2	1.2	7.0	21.0	2.4	1.3	T	0.6	0.9	5.7	3.0	1.0
1831	4.0	13.8	25.0	7.3	2.2	0.8	2.5	1.4	2.7	23.5	5.4	0.9	1.6	0.5	0.8	3.4	2.0	2.2
11	1.7	20.0	30.1	4.3	1.5	T	3.4	0.8	8.8	13.3	2.4	1.3	1.4	0.6	1.0	6.5	2.4	0.5

[a] *See* footnote *a* of Table 1.
[b] Average, 2.0.
[c] *See* footnote *c* of Table 1.
[d] *See* footnote *d* of Table 3.

TABLE 5. *Relative percentage of common and other peaks obtained from GLC chromatograms of TMS derivatives prepared from whole-cell hydrolysates of Clostridium botulinum*

C. botulinum strain	Toxin type	Retention time (min) of common peaks[a]											Per cent of other peaks[b]
		11.0	11.3	13.8	16.1	17.2	18.9	20.8	21.7	24.7	26.8	29.3	
30	A	2.1[c]	24.7	0.7	2.3	T[d]	12.9	29.4	11.6	1.5	6.9	1.8	6.1
27	A	1.7	12.1	1.8	2.5	1.8	7.1	40.8	17.4	3.8	3.0	1.9	6.1
16	A	1.9	13.8	T	2.2	3.5	8.6	38.7	14.0	1.6	4.4	2.8	8.5
28	A	1.3	9.4	T	1.7	1.8	6.2	36.6	23.3	4.2	6.5	4.0	5.0
2	B	2.1	18.1	T	T	0.9	3.0	49.7	13.7	1.6	1.5	2.3	7.1
17	B	2.2	15.7	T	2.5	1.8	6.8	42.8	13.7	2.1	5.0	4.0	3.4

[a] *See* footnote *a* of Table 1.
[b] Average, 6.0.
[c] *See* footnote *c* of Table 1.
[d] *See* footnote *d* of Table 3.

tional difference between these two species was the presence of a peak at 23.0 min in *C. bifermentans* which was absent in *C. perfringens* cultures (Tables 2 and 3).

In addition to differentiation on the basis of some minor qualitative differences, *C. sporogenes* could be distinguished from *C. botulinum* on the basis of the area of two major peaks at retention times of 11.3 and 20.8 min which occurred in each species. The area of the 11.3-min peak was always higher than the 20.8-min peak in *C. sporogenes* (Table 4); the reverse was true in all cultures of *C. botulinum* (Table 5). These differences between the two species can be seen by comparing the chromatograms of *C. sporogenes* (Fig. 1A, B, and C) with those of *C. botulinum* (Fig. 1D, E, and F). The 11.3-min peak was tentatively identified as arabinose and the 20.8-min peak was identified as glucose on the basis of identical retention times on both polar and nonpolar columns compared to known standards of these two sugars.

The single strains of five species listed in Table 6 could easily be separated from each other by the presence or absence of various peaks. For example, *C. tertium* had 15 peaks that *C. subterminale* did not have (Fig. 1J and L, respectively), and *C. subterminale* had 4 peaks that were not present in *C. tertium*. *C. difficile* had 13 peaks that *C. subterminale* did not have in its chromatogram (Fig. 1K and L). There were six peaks in *C. tertium* that were absent in *C. difficile*, and *C. difficile* had five peaks that *C. tertium* did not have (Fig. 1J and K). There were 13 and 9 peaks in *C. capitovale* and *C. septicum*, respectively, that were absent in *C. subterminale* (Table 6). There were four peaks present in *C. capitovale* that were absent in *C. difficile*, and the former had five peaks which were absent in the latter. Ten peaks were present in *C. capitovale* which were absent in *C. septicum*, and 11 peaks were present in *C. tertium* that were absent in *C. septicum*. It was also easy to distinguish the species listed in Table 6 from strains of the other species discussed above. For example, *C. capitovale* contained 5 peaks which were absent in

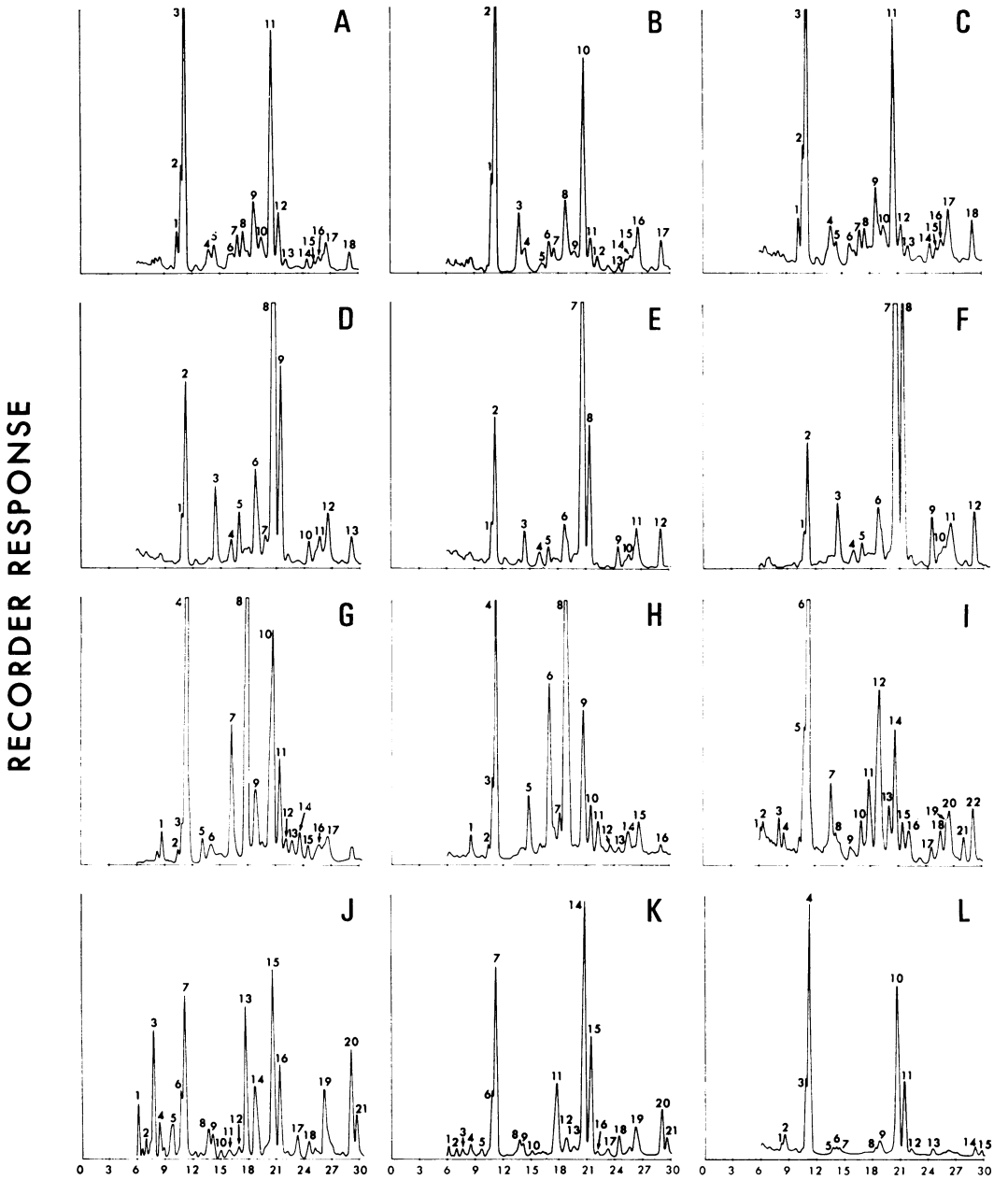

FIG. 1. *Gas-liquid chromatograms of TMS profile of the whole-cell hydrolysates obtained from the OV-1 column. (A) C. sporogenes 96, (B) C. sporogenes 81, (C) C. sporogenes, 1783, (D) C. botulinum 16 type A, (E) C. botulinum 17 type B, (F) C. botulinum 28 type A, (G) C. bifermentans 4572, (H) C. sordellii 1720C, (I) C. perfringens Hobbs 7, (J) C. tertium 481, (K) C. difficile 362, (L) C. subterminale 529A.*

TABLE 6. *Relative percentage of common and other peaks obtained from GLC chromatograms of TMS derivatives prepared from whole-cell hydrolysates of various Clostridium species*

Common peaks retention time (min)	*C. capitovale*		*C. tertium*		*C. difficile*		*C. septicum*		*C. subterminale*	
	Other peaks retention time (min)[a]	Relative per cent	Other peaks retention time (min)	Relative per cent	Other peaks retention time (min)	Relative per cent	Other peaks retention time (min)	Relative per cent	Other peaks retention time (min)	Relative per cent
10.9		4.7[b]		3.8		7.2		4.9		10.3
11.3		22.0		11.2		17.7		21.2		35.8
13.9		1.2		1.8		2.1		0.9		1.1
19.1		13.4		8.1		2.0		5.5		2.9
20.8		20.5		15.2		27.3		26.7		27.8
21.7		8.6		7.4		12.9		10.9		11.8
24.7		1.4		1.4		2.2		0.6		0.9
29.3		2.2		8.7		4.9		1.2		1.3
	6.2	0.6	6.2	3.1	6.2	0.7	10.4	3.3	8.3	0.9
	7.1	0.5	6.6	0.5	7.0	0.6	13.3	0.7	8.6	3.2
	8.2	1.3	7.0	1.0	7.7	0.8	14.6	2.6	14.3	1.3
	8.7	1.0	8.0	7.4	8.6	0.9	15.4	1.0	14.6	1.0
	14.5	1.5	8.6	2.1	9.8	0.6	16.0	1.0	18.6	0.8
	14.9	0.8	10.0	3.7	14.3	1.5	16.7	0.9	22.4	0.9
	15.9	0.5	12.5	T[c]	15.2	0.5	17.9	6.8		
	16.3	0.6	13.1	T	16.0	T	20.1	2.5		
	17.8	2.9	14.4	1.6	16.4	T	25.9	4.8		
	18.2	4.4	15.2	T	17.9	9.7	26.6	4.5		
	20.1	4.9	16.1	T	19.8	0.7				
	22.2	1.3	17.2	T	22.3	0.5				
	23.4	0.6	18.0	12.1	23.4	1.0				
	25.7	0.8	23.6	1.9	25.8	0.8				
	26.5	1.9	25.5	T	26.5	3.6				
	26.8	1.4	26.5	5.4	29.9	1.8				
	29.9	1.0	29.9	3.4						
		26.0[d]		42.2		23.7		28.1		8.1

[a] *See* footnote *a* of Table 1.
[b] *See* footnote *c* of Table 1.
[c] *See* footnote *d* of Table 3.
[d] Total per cent of other peaks.

strains of *C. sordellii* and 10 peaks which were absent in *C. bifermentans* strains. There were 11 peaks in *C. difficile* which were absent in all strains of *C. botulinum*. There were nine peaks in *C. capitovale* which were not present in either of the *C. sporogenes* cultures.

The average per cent of the total of the other peaks (uncommon peaks) among strains of the same species (Tables 1–5) was much lower than the average per cent of the total of the other peaks among strains of various species (Table 6). The difference would be even greater if strains of all 10 species were compared, since they share only three peaks. Thus, it is apparent that there are fewer similarities among strains of various species than among strains of the same species.

DISCUSSION

One of the problems we encountered at the beginning of this study was changes of retention times. This problem was found to be due to slight variation in column temperature at the start of the programming run. Retention times of TMS derivatives are apparently very sensitive to column temperature, and the starting temperature had to be controlled as accurately as possible. For this purpose, we found that the cooling time and the time interval before the next sample was injected must be rigorously controlled. The standard deviation for the retention times of individual peaks was reported in an earlier study to be 4.9 sec (8). However, with the procedures used in this study, the standard deviation for retention times was less than 3.0 sec.

The results from this and an earlier study (8) indicate that GLC of TMS derivatives of whole-cell hydrolysates is a useful technique for characterizing microorganisms. The methods employed for forming TMS derivatives of whole-cell hydrolysates were developed with the ultimate

goal of providing a simple, rapid procedure for distinguishing various bacteria. Because of the complexity of the chromatograms, this method is best used to compare only a few species at a time. It is particularly valuable where differentiation of species by conventional biochemical tests is difficult.

The taxonomy of *C. sordellii* and *C. bifermentans* has been a problem (14). The urease test has been shown to be unreliable for differentiating *C. sordellii* from *C. bifermentans*. Novotny (17), testing 16 strains of *C. sordellii*, found 14 to be ureolytic and 2 to be nonureolytic. In our study, we found variation in lecithinase production among *C. sordellii* strains. To clarify the taxonomy of *C. sordellii* and *C. bifermentans*, Novotny (17) studied the chemical composition of the cell walls of 16 strains of each species. Paper chromatography data showed mannose to be present in 13 of 16 strains of *C. bifermentans* but absent in each of the *C. sordellii* cultures. Our results with the whole-cell hydrolysates are consistent with those of Novotny (17) and indicate that mannose is characteristically absent in *C. sordellii*. Thus, analysis of the carbohydrate composition of whole cells appears to be useful for distinguishing these two organisms. Our results lend support to the recent study of Brooks et al. (2), who found that the two organisms could be distinguished on the basis of amine production.

Conventionally, *C. botulinum* is separated from *C. sporogenes* only by the toxicity of the former for mice, a procedure requiring 3 days. With the TMS techniques described here, differentiation can be made within 24 hr. Further studies are needed to test additional strains of each species for conformity with the results of the present study. The TMS procedure reported here can possibly be refined to produce a more rapid test for diagnostic purposes. The results from this and other laboratories indicate that gas chromatography provides a potentially valuable tool to assist in the characterization of clostridia.

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