Electron Capture Gas Chromatography Study of the Acid and Alcohol Products of *Clostridium septicum* and *Clostridium chauvoei*

JOHN B. BROOKS,* MERLE J. SELIN, AND CYNTHIA C. ALLEY

Center for Disease Control, Atlanta, Georgia 30333

Received for publication 28 October 1975

The metabolic products produced by several strains of *Clostridium septicum* obtained from patients and animals, along with strains of *Clostridium chauvoei*, were studied in chopped meat glucose medium by electron capture gas-liquid chromatography (EC-GLC). The strains of *C. septicum* and *C. chauvoei* were shown to comprise five different metabolic groups. Both the EC-GLC study and the O and H antigenic study performed previously showed that strains of *C. septicum* comprise a heterogeneous group. One type of metabolic profile was found only in strains of *C. chauvoei*. The O antigen types and EC-GLC metabolic types of *C. septicum* correlated fairly well in isolates from cancer patients but not in stock culture and animal isolates.

Clostridium septicum infects both man and animals. It has been associated with numerous diseases in animals and man (M. J. Selin, Ph.D. thesis, Univ. of North Carolina, Chapel Hill, 1973). *Clostridium chauvoei*, on the other hand, causes a high percentage of deaths of range cattle (9), but probably is unable to infect man (11). A study of the metabolic products of these two organisms might reveal similarities or differences to explain the diversity in their spectra of disease production although possessing such closely related cultural and biochemical characteristics.

Several investigators have reported that microorganisms produce carcinogenic compounds in vitro (2, 3, 7, 12) and in vivo (3), and that some anaerobes have antitumor activity (6). One of the pathological conditions *C. septicum* has been associated with is underlying malignancy (1). Therefore, the metabolic products of *C. septicum* isolated from cancer patients could also be of value in studying their physiological properties and might be useful in identifying these organisms.

The serological properties of *C. septicum* and *C. chauvoei* strains from various sources have been extensively studied, and several different serological groups have been recognized (Selin, Ph.D. thesis, 1973). The object of this study was to investigate, by use of frequency pulse electron-capture gas-liquid chromatography (EC-GLC) and iodomethyltetramethylmethyldisiloxane (IMTMMDS) esters and iodomethyldimethylsilyl (IMDMS) ethers, the metabolic

profiles exhibited by several strains of *C. septicum* and *C. chauvoei*.

Both the IMTMMDS esters of acids and the IMDMS ethers of alcohols have been studied by mass spectrometry, O¹⁸-labeled acetate, and EC-GLC (4). The theoretical advantages of using frequency pulse modulation for electron capture detectors have been described (10). Practically, these are: (i) quantitative reliability, (ii) a greatly improved overload response, and (iii) a much higher tolerance to detector contamination. The high tolerance to contamination means less variance in sensitivity and longer life for the ⁶³Ni foil.

MATERIALS AND METHODS

Organisms. The source and strain numbers of the *C. septicum* and *C. chauvoei* strains are listed in Table 1. All strains of each species were identified by standard biochemical procedures used by the Center for Disease Control (5). The biochemical and serological procedures and results are presented in detail elsewhere (Selin, Ph.D. thesis, 1973).

Growth medium and cultural procedures. The chopped meat medium with glucose (CMG) was prepared as previously described (5), except that sterile glucose was added after the medium was autoclaved. Single colony isolates were inoculated into 8 ml of CMG medium, then incubated under anaerobic conditions at 35 C for 4 days.

Preparation of IMTMMDS esters of acids and IMDMS ethers of alcohols. IMTMMDS esters of acids and IMDMS ethers of alcohols were prepared essentially as described (4). Two grams (± 0.2 g) of NaCl was added to 8 ml of the spent culture medium to reduce emulsions. The sample was then placed in

TABLE 1. Stock cultures of *C. septicum* (A) and *C. chauvoei* (B)

Meta- bolic	CDC ^a no.	Source	Source identi- fication no.	Anti- gens de- tected	
				O	H
A					
III	10803	NCTC ^b	NCTC 281	B	4
IV	10805	NCTC	NCTC 284	A	1
III	10806	NCTC	NCTC 281	B	5
III	10807	NCTC	NCTC 501	A	2
II	11455	A. W. Bernheimer ^c	BX 96	B	6
III	11457		Robertson III	A	3
B					
III	10813	NCTC	NCTC 8070	C	7
V	10855	I. Batty ^d	CN 1501	C	8
III	10863		CN 3600	C	8
I	10866		CN3695	C	7
V	10870		CN 3940	C	—
III	10875		CN4945	C	8
V	10876		CN5144	C	7
III	10877		CN5196	C	7

^a CDC, Center for Disease Control.^b NCTC, National Culture Type Collection, Central Public Health Laboratory, England.^c New York University Medical Center.^d Wellcome Research Laboratory.

a 50-ml screw-cap centrifuge tube. The contents of the tube were acidified to pH 2 (pH paper) with 0.5 ml of 50% H₂SO₄ (vol/vol) and extracted with 20 ml of diethyl ether (Baker Analytical Grade stabilized with ethanol). After brief centrifugation, the ether layer (top) was decanted into a 50-ml beaker and evaporated with a gentle stream of clean dry air to about 1 ml. The aqueous bottom layer was made basic (pH 10 to 11), reextracted with chloroform, and stored at 4 C to await further analysis for amines. Next, the concentrate was transferred to a test tube (12 by 75 mm) with a disposable Pasteur pipette. Care was taken to discard any visible layer of moisture (bottom layer) that was present in the pipette. Then about 100 mg (ca. 0.1 the volume of the concentrated sample) of MgSO₄ was added. The contents of the tube were thoroughly mixed by shaking. The sample was briefly centrifuged, and the ether layer was decanted into a second test tube. One milliliter of ether was added to the sedimented MgSO₄; then the tube was shaken to mix the contents and briefly centrifuged. The ether layer was decanted and combined with the previously decanted ether layer; the MgSO₄ was discarded. At this point the dried extracts were evaporated in a tube (12 by 75 mm) by using clean dry air to a volume of less than a drop. Next, about 0.01 ml of 1 part (by volume) of diethylamine to 11 parts of nanograde chloroform was added as a catalyst; then 0.01 ml of 1 part of bromomethyltrimethylchlorosilane to 11 parts of chloroform was added as the derivatizing agent. The test tube containing the reaction mixture was shaken to mix the contents, and the test tube was corked (reaction vessel important, see reference 9), taped, and heated in an 80 C water bath for 1 h. The sample was

removed from the bath, and cooled under tap water, and the chloroform was evaporated by clean dry air. Next, 0.1 ml of a saturated solution of NaI in acetone, stored at room temperature in a screw-cap flask, was added. The test tube was shaken to mix the contents, corked, taped, and incubated for 30 min at 35 C to form IMTMMDS esters of acids and IMDMS ethers of alcohols. The sample was then removed from the incubator, and 0.9 ml of acetone was added. Next, 0.5 μ l of the sample was injected for GLC analysis or the sample was corked and stored at 4 C for future GLC analysis.

Gas chromatography analysis. A Perkin-Elmer model 900 gas chromatograph equipped with a ⁶³Ni (10 mCi) frequency pulse modulated electron capture detector, Beckman three-way and four-way switching valves, and a 10-inch (ca. 24.40-cm) potentiometric recorder were used. The switching valves allowed a comparative analysis from either column on a single detector and, in addition, permitted the column gas to be vented when analysis was not being made. The instrument was operated with two coiled glass columns (0.3 cm ID by 7.3 m long). One column (nonpolar) was packed with Chromosorb W 80/100 mesh (AW-DMCS H.P.) coated with 3% OV-1 (Applied Science Laboratories). The second column (polar) was packed with TA33 TABSORB (Regis Chemical Co.). The instrument was programmed from 100 C for the TABSORB column or from 125 C for the OV-1 column to 225 C at a linear increase of 3 C per min, then held isothermally at 225 C for 32 min. The injector temperature was 225 C; the manifold temperature, 250 C; and the detector temperature, 275 C. The electrometer was set so that 5.12 $\times$ 10⁴ H_z gave full-scale response. A mixture of argon-methane (95-5, Matheson) was used as carrier gas at a flow rate of 50 ml/min. The carrier gas line was modified between the manifold and detector to permit a flush gas to be used. Use of a flush gas improved the base line and overload characteristic of the detector and supplied carrier gas to the detector while the columns were being vented. The flush gas was regulated so that the combined flow of gas from the column and from the flushing system through the detector was 67 ml/min. The recorder was operated with an input signal of 1 mV and a chart speed of 30 inches (ca. 76.20 cm)/h. New columns were conditioned for 12 h at 245 C, and longer if necessary, to obtain a good base line. The columns were also conditioned each morning before use by heating them at 245 C for 30 min. Some of the alcohols and acids were tentatively identified by comparing retention times of known standards with the retention times of unknowns on both polar and nonpolar columns. Identification of some acids was confirmed by mass spectrometry.

Mass spectra. An LKB model 9000 gas chromatograph-mass spectrometer was used. The resolution of the instrument was about 1,000; the temperature of the ion source was 290; and the electron energy was 70 eV. Acceleration voltage was 3.5 kV; the scan (*m/e*) limits were from 0 to 500; *m/e* scan speed used was 6 (0 to 500 *m/e* in 16 s); and the ultraviolet oscillograph chart speed was 5 cm/s. The instrument

contained a coiled glass column (0.3 cm ID by 3.6 m in length), which was packed with Chromosorb W 80/100 mesh (AW-DMCS H.P.) coated with 3% OV-1. The column was heated isothermally for 5 min at 70 C and then heated at a temperature increase of 5 C per min to 225 C. Helium was used as the carrier gas at a flow rate of 36 ml/min. The recorder was operated with an input signal of 2 mV (full scale) and a chart speed of 30 inches (ca. 76.20 cm)/h.

RESULTS AND DISCUSSION

Neither the *C. septicum* nor the *C. chauvoei* strains produced homogeneous EC-GLC metabolic profiles in CMG. Five different EC-GLC metabolic profiles were detected (Fig. 1 and 2) and were designated groups I, II, III, IV, and V. Group V profile was found in 38% of the *C. chauvoei* strains listed in Table 1B. Group V C.

chauvoei cultures produced propionic, butyric, and valeric acids. Isoamyl alcohol was also tentatively identified (Fig. 2, chromatogram C). The remaining five strains of *C. chauvoei* produced EC-GLC metabolic profiles similar to those shown for *C. septicum* groups I and III (Fig. 1, chromatogram A, B, C). None of the *C. septicum* strains gave a group V profile.

Seven strains of *C. septicum* gave EC-GLC metabolic profiles like the one shown (Fig. 1, curve C) for group I. Butanol, isoamyl alcohol, acetic, propionic, isobutyric, butyric, isovaleric, valeric, and isocaproic acids were detected. Furthermore, unidentified peaks 1, 2, and 4 were found. Seven other strains of *C. septicum* gave an EC-GLC metabolic profile like the one shown in Fig. 1, chromatogram B (group II). In group II acetic acid was not detected in

FIG. 1. EC-GLC chromatograms of ethyl ether extracts of acidified (pH 2.0) medium (chopped-meat with added glucose). The extracts were treated to form electron capturing IMTMMDS esters of acids and IMDMS ethers of alcohols. The analyses were made on a 3% OV-1 glass column, 0.3 cm ID by 7.3 m long. A frequency pulse modulated ^{63}Ni electron capture detector was used. (A) Metabolic group III; (B) metabolic group II; (C) metabolic group I; and P, by-products of the derivatization procedure.

FIG. 2. EC-GLC chromatograms of ethyl ether extracts of acidified (pH 2.0) spent culture medium (chopped meat with added glucose). The extracts were treated to form electron capturing IMTMMDS esters of acids and IMDMS ethers of alcohols. The analyses were made on a 3% OV-1 glass column, 0.3 cm ID by 7.3 m long. A frequency pulse modulated ^{63}Ni electron capture detector was used. (A) Profile obtained from the control medium; (B) metabolic group IV; and (C) metabolic group V.

amounts greater than the amount found in the control medium; propionic acid was detected in trace amounts; butyric acid was the major compound detected. Isoamyl alcohol was also tentatively identified. The largest EC-GLC metabolic group of *C. septicum* was group III (Fig. 1, chromatogram A), which consisted of 18 strains. Production of large amounts of acetic and butyric acids was typical of group III. Some propionic acid and isoamyl alcohol were also found. Two strains of *C. septicum* produced an EC-GLC metabolic profile like the one shown in Fig. 2, chromatogram B (group IV). Typical of group IV strains were peak 1 and small amounts of acetic and isovaleric acids.

Once heterogeneous EC-GLC metabolic profiles were found among the strains of *C. septi-*

cum and *C. chauvoei*, a representative strain from each metabolic group was studied further. The gas chromatography and biochemical tests (5) were repeated with similar results. Furthermore, the morphological characteristics of the organism were repeatable. Since the battery of tests (5) did not show metabolic differences in the *C. septicum* and *C. chauvoei* strains, one can only conclude that the criteria specified (5) are insufficient to distinguish the metabolic differences detected by EC-GLC. The same strains of *C. septicum* have also been shown to be antigenically heterogeneous (Selin, Ph.D. thesis, 1973). Even though the strains of *C. septicum* have been shown to be heterogeneous by two different sets of criteria (EC-GLC and antigenic grouping), results from these two meth-

ods do not in many instances agree with each other (Tables 1, 2, and 3). Since the organisms were compared by EC-GLC after growth on the same lot of CMG medium the heterogeneity in metabolism is probably not due to changes in medium composition.

The largest EC-GLC metabolic group (group III) of *C. septicum* did, in general, conform to acid profiles described by using different media and different GLC methods (8) in that acetic and butyric acids were the major acid products, but even in this group propionic acid and isoamyl alcohol were also found. The differences detected in group III from those reported (8) could be due to the more sensitive EC-GLC methods used in this study, a different growth medium, strain differences, or a combination of all of these; however, the strain differences found in EC-GLC groups I, II, and IV (Fig. 1 and 2) are distinct and clearly demonstrate that

differences in metabolism exist within this species as defined (5). Furthermore, these differences cannot be explained on the basis of media or method differences.

We tentatively identified all alcohols and acids listed in Fig. 1 and 2 on both polar and nonpolar columns. In addition, we confirmed by mass spectrometry that the following acids were present in either group I or group II cultures: acetic, propionic, isobutyric, butyric, isovaleric, valeric, and isocaproic. Gas chromatographic identification of acetic and butyric acids has been reported (8).

From Tables 1, 2, and 3 a comparison can be made between the EC-GLC metabolic groups, the sources of the organism, and the stock strains of *C. septicum* consisted of 66% metabolic group III, 17% metabolic group II, and 17% metabolic group IV. The stock strains of *C. chauvoei* consisted of 12% metabolic group I, 50% EC-GLC metabolic group III, and 38% EC-GLC metabolic group V. Of the *C. septicum* strains isolated from animals (Table 2) one strain was placed in group I, one in group II, one in group IV, and three in group III. Little correlation was found between the EC-GLC metabolic groups and the Selin antigenic groups of the *C. septicum* strains isolated from animals (Table 2).

Table 3 shows the metabolic groups and antigenic types of *C. septicum* strains isolated from cancer patients. Of the *C. septicum* strains isolated from patients with leukemia, 62% were EC-GLC metabolic group III; 23%, metabolic

TABLE 2. Metabolic and serological characteristics of *C. septicum* strains isolated from animals

Metabolic group	CDC ^a no.	Isolated from	Antigens detected	
			O	H
I	247	Chicken	B	6
III	2150	Chicken	A	1
III	5482	Bovine	A	1,2,3
II	6914	Bovine	B	4
IV	6920	Bovine	A	1,2
III	6926	Bovine	B	4

^a Center for Disease Control.

TABLE 3. Metabolic and serological characteristics of *C. septicum* strains isolated from humans

Metabolic group	CDC no.	Isolated from	Clinical diagnosis	Antigens detected	
				O	H
II	322	Blood	Leukemia (lymphoblastic)	B	4,6
III	634	Blood	Leukemia (sub acute myeloblastic)	A	1
III	1394	Blood	Leukemia (granulocytic)	A	1,2,3
III	1395	Blood	Leukemia (granulocytic)	A	1,2,3
III	1659	Blood	Leukemia, performatum cecum	A	1,2,3
III	1709	Blood	Leukemia (acute lymphoblastic)	A	1,2,3
I	1840	Blood	Leukemia (chronic lymphatic)	B	6
III	2255	Blood	Leukemia	B	4,5,6
II	3767	Blood	Leukemia		
III	3768	Blood	Leukemia		
III	9479	Blood	Lymphatic leukemia	B	4,6
II	12581	Blood	Leukemia (lymphoblastic)	A	1,2,3
III	9485	Blood	Otitis media	B	4,6
II	1302	Abdomen	Cancer of the cecum	B	5
I	3157	Blood	Colloidal cancer of the cecum		
II	5133	Blood	Adenocarcinoma of the cecum	B	6
I	9564	Blood	Ovarian adenocarcinoma	A	3
III	9689	Blood	Reticulo cell sarcoma		
I	369	Bleb from leg	Unknown	A	2
I	5193	Amputated foot	Gas gangrene		
I	2702	Blood	Leukemia	A	1

group II; and 15%, EC-GLC, metabolic group I. Of the *C. septicum* isolates from the remaining eight cancer patients (Table 3), 50% produced EC-GLC metabolic profiles like group I; 25%, like group II; and 25% like group III.

Table 3 also shows the antigens detected in the strains of *C. septicum* isolated from cancer patients. Of the strains isolated from patients with leukemia, 54% contained Selin O antigen type A; 31%, O antigen type B; and 15% were nontypable. Additionally, the *C. septicum* strains isolated from cancer patients were predominately type A. The *C. septicum* isolates obtained from leukemic patients were mostly antigenic type A and were mostly group III metabolic type (Table 3). With the *C. septicum* strains isolated from cancer patients other than leukemics, the O antigens were mixed, with type B slightly predominating over type A and the nontypables. A greater percentage of the antigens in the group were nontypables. They comprised 38% of the strains isolated from non-leukemia patients, as opposed to 15% of the group isolated from leukemia patients (Table 3).

Some of the conclusions that can be drawn from these data follow. (i) Heterogeneity exists both in metabolism and antigenic composition among strains of *C. septicum* and *C. chauvoei* grouped by conventional identification. (ii) By using EC-GLC, metabolic heterogeneity can be detected at the strain level. Some interesting observations are: the organisms studied represent five metabolic groups, and *C. septicum* strains in EC-GLC metabolic group III and belonging to Selin O antigen type A predominated among those isolated from leukemic patients. (iii) The *C. septicum* isolates obtained from patients with other types of human cancer were predominately of the EC-GLC metabolic group I type, and this group consists mostly of O antigenic type B and nontypables. (iv) The stock cultures of *C. septicum* were devoid of EC-GLC metabolic group I, were predominately of the metabolic group III type, and were antigenically mixed. (v) The stock cultures of *C. chauvoei* (Table 1) were predominately of the metabolic group III type, but a significant percentage (38%) were of the EC-GLC metabolic group V type. (vi) The EC-GLC metabolic group V type appeared only in strains of *C. chauvoei* (Selin, Ph.D. thesis, 1973). In general, little correlation was observed between antigenic types and EC-GLC metabolic types. However,

better correlation existed between the metabolic types and the antigenic types found in human cancer isolates. This correlation may indicate selection for certain types of *C. septicum* strains.

Since *C. septicum* infections frequently occur in certain types of cancer patients, questions arise concerning the role of these organisms. We cannot determine the reasons these organisms are present on certain types of patients from data now available, but the study of metabolic products of these *Clostridia* will continue to be of interest from the standpoints of bacterial classification, taxonomy, and physiological activity.

EC-GLC may provide an effective tool for better grouping these organisms and for further studying their metabolic products.

LITERATURE CITED

1. Alpern, R. J., and V. R. Dowell, Jr. 1969. *Clostridium septicum* infections and malignancy. J. Am. Med. Assoc. 209:385-388.
2. Ayanaba, A., and M. Alexander. 1973. Microbial formation of nitrosamine in vitro. Appl. Microbiol. 25:862-868.
3. Brooks, J. B., W. B. Cherry, L. Thacker, and C. C. Alley. 1972. Analysis by gas chromatography of amines and nitrosamines produced in vivo and in vitro by *Proteus mirabilis*. J. Infect. Dis. 126:143-153.
4. Brooks, J. B., J. A. Liddle, and C. C. Alley. 1975. Electron capture gas chromatography and mass spectral studies of iodomethyltetramethylmethyldisiloxane esters and iodomethyldimethylsilyl ethers of some short-chain acids, hydroxy acids, and alcohols. Anal. Chem. 47:1960-1965.
5. Dowell, V. R., Jr., and T. M. Hawkins. 1968. Laboratory methods in anaerobic bacteriology. Public Health Service publication no. 1803. United States Printing Office, Washington, D.C.
6. Hattori, T., and A. Mori. 1973. Antitumor activity of anaerobic *Corynebacterium* isolated from the human bone marrow. Gann 64:15-27.
7. Hawksworth, G., and M. J. Hill. 1971. Bacteria and the N-nitrosation of secondary amines. Br. J. Cancer 25:520-526.
8. Holdeman, L. V., and W. E. C. Moore. 1972. Anaerobe laboratory manual, Southern Printing Co., Blacksburg, Va.
9. Jones, L. W., and C. E. Clifton. 1972. Metabolism and nutrition of *Clostridium chauvoei*. J. Bacteriol. 65:560-564.
10. Maggs, R. J., P. L. Joyner, A. J. Davis, and J. E. Lovelock. 1971. The electron capture detector. A new mode of operation. Anal. Chem. 43:1966-1971.
11. Smith, L. S. D., and L. V. Holdeman. 1968. The pathogenic anaerobic bacteria, p. 369. Charles C Thomas, Springfield, Ill.
12. Thacker, L., and J. B. Brooks. 1974. In vitro production of N-nitrosodimethylamine and other amines by *Proteus* species. Infect. Immun. 9:648-653.