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NOTES

Differentiation of *Bacillus anthracis* from *Bacillus cereus* by Gas Chromatographic Whole-Cell Fatty Acid Analysis

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Three strains of *Bacillus anthracis* and seven strains of *Bacillus cereus* were grown on complex medium and on synthetic medium. Gas chromatographic analysis of whole-cell fatty acids of strains grown on complex medium gave nearly identical fatty acid patterns. Fatty acid patterns of strains grown on synthetic medium showed a high content of branched-chain fatty acids. Significant differences between the fatty acid patterns of the two species were found. Odd iso/anteiso fatty acid ratios were about equal in *B. anthracis* strains, whereas in *B. cereus* strains the fractions of iso acids were at least twice as high as the fractions of anteiso acids. The method described herein is used in our diagnostic laboratory to help differentiate between these two species.

One of the specific tasks of the Institute for Tropical Animal Health is the identification of specimens of bacterial pathogens. From tropical countries we receive field samples containing mostly endospore-forming bacteria (*Bacillus* and *Clostridium* species), which are identified by gas chromatographic (GC) analysis of long-chain fatty acids or fermentation products (5, 17).

Clostridium species are easily differentiated. In contrast, *Bacillus anthracis* and *B. cereus* are very similar species that differ in very few characteristics (4). Several methods have been developed to obtain a good differentiation between these two species (1, 2, 11, 12, 15, 16, 18). However, none of these methods provides the means for an absolutely correct differentiation. We need a quick, simple, and reliable method of differentiation to use in our epizoological studies of anthrax disease in livestock and game in Africa.

The use of GC identification of bacteria is becoming widespread in many diagnostic laboratories (14). The method is similar to the method we developed for the identification of *Clostridium* species (5). It was the aim of this work to establish a computer program for differentiation of *B. anthracis* and *B. cereus* by GC whole-cell fatty acid analysis.

Kaneda (7, 8) analyzed the fatty acids of two *Bacillus thuringiensis* strains and two pathogenic *B. anthracis* strains. He found that *B. thuringiensis*, like *B. cereus*, has higher relative proportions of i13:0, a13:0, and i14:0 acids than does *B. anthracis*. However, this was not demonstrated with avirulent strains.

Microorganisms. *B. anthracis* and *B. cereus* strains used in this study were obtained from the German Collection of Microorganisms or were isolates or reference strains of the Institute for Tropical Animal Health. All strains are listed in Table 1.

Culture media. Reinforced clostridial medium (RCM) contained the following ingredients (in grams per liter): meat extract, 10.0; peptone, 10.0; yeast extract, 3.0; glucose, 5.0; starch, 1.0; NaCl, 5.0; sodium acetate, 3.0; cysteine hydrochloride, 0.5; agar, 0.5 (all from Merck, Darmstadt, Federal Republic of Germany). The pH was adjusted to 6.8, and the medium was autoclaved for 15 min at 121°C.

Chemically defined RM medium was developed by Leppla (13) to increase yields of anthrax toxin. It contained the following ingredients at the indicated final concentrations (milligrams per liter), with all amino acids being of the L configuration: tryptophan, 35; glycine, 65; tyrosine, 144; lysine hydrochloride, 230; valine, 173; leucine, 230; isoleucine, 170; threonine, 120; methionine, 73; aspartic acid, 184; sodium glutamate, 612; proline, 43; histidine hydrochloride, 55; arginine hydrochloride, 125; phenylalanine, 125; serine, 235; NaCl, 2,920; KCl, 3,700; adenine sulfate, 2.1; uracil, 1.4; thiamine hydrochloride, 1.0; cysteine, 25; KH_2PO_4, 460; Tris, 9,060; glucose, 5,000; $CaCl_2 \cdot 2H_2O$, 7.4; $MgSO_4 \cdot 7H_2O$, 9.8; $MnCl_2 \cdot 4H_2O$, 1.0; $NaHCO_3$, 8,000 (all from Sigma Chemie, Deisenhofen, Federal Republic of Germany). For sterilization, the medium was filtered after the pH was adjusted to 7.2; 50-ml samples were placed in 100-ml Erlenmeyer flasks.

Incubation. Each strain was grown for 24 h in 5 ml of RM medium at 37°C under aerobic conditions. This was done to adapt field strains to a new medium. After 24 h of growth, 5 ml of the suspension was pipetted into 50 ml of the medium and incubated for 48 h at 37°C under aerobic conditions.

Isolation of fatty acids. After 48 h of incubation, the cells were harvested by centrifugation, washed with saline to remove the medium, and then lyophilized overnight in a Beta I lyophilizer (Heraeus-Christ, Osterode, Federal Republic of Germany). Lyophilized cells (approximately 100 mg [dry weight]) were saponified in a capped tube by adding 1 ml of a solution containing 50% 7.5 M NaOH–50% methanol (high-pressure liquid chromatography grade; Merck) and heating for 30 min at 100°C in a boiling-water bath. Fatty acids were methylated by adding 2 ml of a solution containing 55% 6 N HCl–45% methanol and then incubating for 10

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TABLE 1. Origins of *Bacillus* strains[a]

Species and strain	Source	Original no.
B. anthracis		
Sterne	Wellcome	Sterne
Zambia	TAH	Field strain
18-74	TAH	CN 18-74
B. cereus		
1661	DSM	DSM 487
1664	DSM	DSM 2301
1688	DSM	DSM 351
1689	DSM	DSM 360
1690	DSM	DSM 508
1691	DSM	DSM 626
1692	DSM	DSM 2299

[a] TAH, Institute for Tropical Animal Health, Göttingen, Federal Republic of Germany; DSM, German Collection of Microorganisms, Braunschweig, Federal Republic of Germany; Wellcome, Wellcome Research Foundation Laboratories. *B. anthracis* Sterne is an avirulent strain that is used for vaccination studies at our institute. *B. anthracis* Zambia is an isolate from an elephant from Zambia, Africa.

min at 80°C. The fatty acid methyl esters were extracted with 1.25 ml of 50% hexane and 50% diethyl ether (high-pressure liquid chromatography grade; Merck). The organic layer was washed with 3 ml of 0.3 N NaOH. Approximately 250 μl of the upper layer was placed in autosampler vials for GC analysis.

GC. GC analysis of fatty acid methyl esters was carried out with a Perkin-Elmer gas chromatograph (Sigma 2000) with an autosampler (AS 8300) and a flame ionization detector on a PVMS-54 capillary column (length, 25 m; film thickness, 0.32 μm; inner diameter, 0.2 mm) (Bodenseewerk Perkin-Elmer, Überlingen, Federal Republic of Germany). The flow rate of the carrier gas (N_2) was 3 ml/min, the split ratio was 100:1, and the injection volume was 1 μl. The GC data were integrated and quantified as percent total peak area with a Perkin-Elmer LCI-100 laboratory computing integrator connected to an IBM personal computer. Whole-cell fatty acids from each strain were saponified, methylated, and chromatographed at least two times.

The temperature program of the GC oven was as follows: the temperature was initially 100°C; it was raised to 220°C with a ramp rate of 3°C/min, raised to 280°C with a ramp rate of 30°C/min, and then held for 3 min. The run time was 45 min, and the temperature of the injector and detector was 350°C. A commercial mix of 27 bacterial fatty acid methyl esters (Supelco, Bad Homburg, Federal Republic of Germany) was used as a calibration standard. Components that were not in the calibration standard were tentatively identified by calculation of the equivalent chain length (ECL) according to the following equation: $ECL_x = [(RT_x - RT_n)/(RT_{n+1} - RT_n)] + n$, where x is an unknown component, RT_x is the retention time of x, RT_n is the retention time of $C_{n:0}$ (methyl ester before unknown component), RT_{n+1} is the retention time of $C_{(n+1):0}$ (methylester after unknown component), and n is the number of C atoms.

Calculation of similarities. A computer program written in "C" on an IBM personal computer was used to evaluate GC data. The similarities between the fatty acid profiles of the strains were estimated by calculating the Bravais-Pearson coefficient of linear correlation (3) with the following equation: $r = \{[N \times \Sigma xy] - [(\Sigma x) \times (\Sigma y)]\}/\sqrt{[N \Sigma x^2 - (\Sigma x)^2][N \Sigma y^2 - (\Sigma y)^2]}$, where x and y are variables and N is the number of observations (components). x and y correspond to pairs of percentage peak areas for each peak in turn in a pair of chromatograms. An r value of +1 indicates complete positive correlation of analyses, and an r value of 0 indicates no correlation. Peaks of unknown identity were not taken into account because their total amount was never higher than about 1% of total peak area.

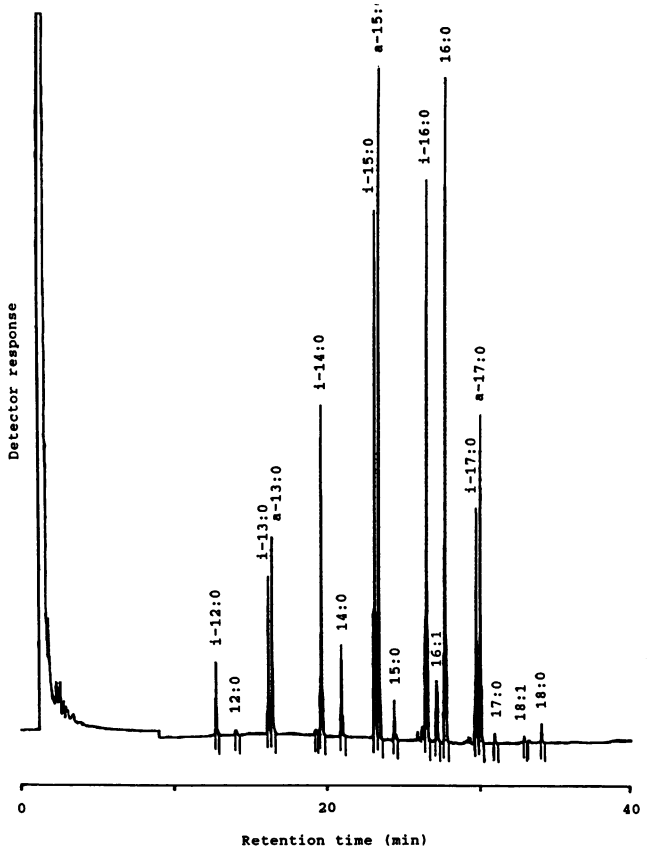

FIG. 1. Chromatogram of whole-cell fatty acids from *B. anthracis* Zambia grown on synthetic medium.

Fatty acid patterns. Two original chromatograms of strains grown on synthetic medium are shown in Fig. 1 (*B. anthracis* Zambia) and Fig. 2 (*B. cereus* 1661). The fatty acids that were detected in all examined strains are listed in Table 2. All values are the means of at least two analyses of two experiments. The fatty acid patterns of *B. anthracis* and *B. cereus* strains grown on RCM are also listed in Table 2. *B. anthracis* and *B. cereus* strains both produced nearly identical fatty acid patterns when grown on complex medium. The most predominant fatty acids were hexadecanoic acid (42.60 to 63.52% of the total) and tetradecanoic acid (8.87 to 21.06% of the total). Branched-chain fatty acids made up 11.17 to 29.73% of the total.

The fatty acid distributions in *B. anthracis* and *B. cereus* strains grown on synthetic medium are also given in Table 2. The contents of branched-chain fatty acids were much higher (67.51 to 84.30%) than those of strains grown on complex medium. The fractions of hexadecanoic and tetradecanoic acids were much lower (10.67 to 20.36% and 1.90 to 3.72%, respectively).

Similarity of strains. (i) Growth on complex medium. Similarities of the fatty acid profiles of the strains grown on complex medium (RCM) were estimated by calculation of

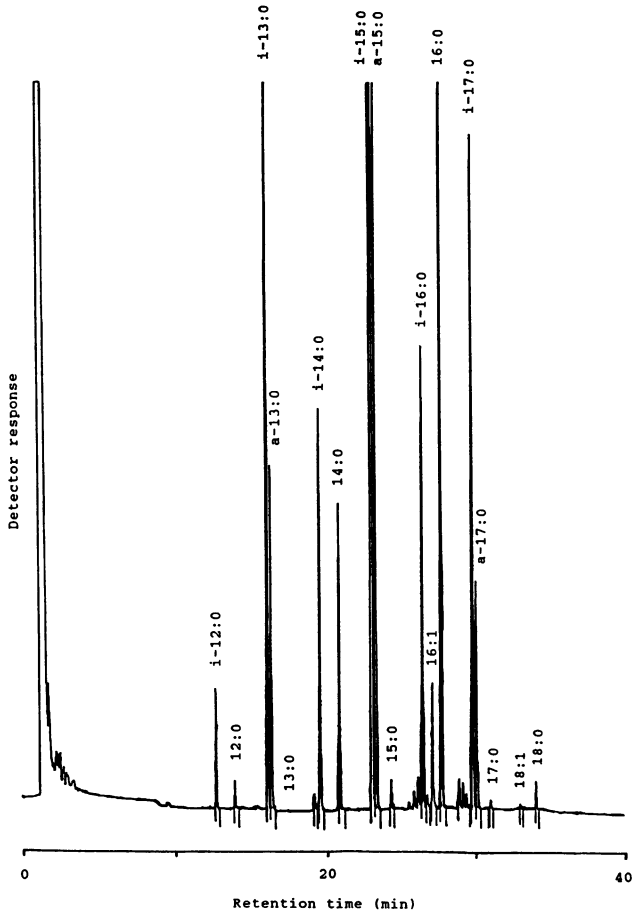

FIG. 2. Chromatogram of whole-cell fatty acids from *B. cereus* 1661 grown on synthetic medium.

the coefficient of linear correlation. The lowest correlation coefficient between all strains of the two species was 0.946. This means high similarity of the fatty acid profiles. Differentiation is not possible.

(ii) Growth on synthetic medium. Similarities of the fatty acid profiles of the strains grown on synthetic medium (RM) were also estimated by calculation of the coefficient of linear correlation (Table 3). *B. anthracis* strains had correlation coefficients of at least 0.946, and the fatty acid profiles showed high similarity. Most of the fatty acid profiles of the *B. cereus* strains showed high similarity with correlation coefficients between 0.969 and 0.993; some of the correlation coefficients (0.801 to 0.932) indicated higher diversity of the fatty acid profiles. The correlation coefficients between *B. anthracis* and *B. cereus* were never higher than 0.872.

Ratios of branched-chain fatty acids. The most significant difference between the fatty acid profiles of the two species are the ratios of the odd iso and anteiso fatty acids (Table 4).

B. anthracis strains had odd iso-branched-chain acid/corresponding anteiso acid ratios between 0.64 and 1.03. This indicates that the fractions of iso acids were almost the same as the fractions of the anteiso acids.

In *B. cereus* strains the fractions of the iso acids were at least twice as high as the fractions of the corresponding anteiso acids (ratios of 2.23 to 8.65).

Kaneda (8) found that the synthesis of the fatty acids i13:0, a13:0, and i14:0 in *B. thuringiensis* and *B. cereus* was

TABLE 2. Fatty acid distribution in *B. anthracis* and *B. cereus* strains grown on RCM and RM media[a]

Fatty acid	% of indicated fatty acid in strain:																			
	Sterne		Zambia		18-74		1661		1664		1688		1689		1690		1691		1692	
	RCM	RM	RCM	RM	RCM	RM	RCM	RM	RCM	RM	RCM	RM	RCM	RM	RCM	RM	RCM	RM	RCM	RM
i12:0	0.81	1.72	0.98	1.67	2.01	0.81	1.44	1.32	0.82	0.34	2.13	1.31	1.83	1.76	2.78	1.15	2.25	1.55	1.20	1.39
12:0	1.42	0.15	0.65	0.14	3.01	0.00	2.36	0.19	2.45	0.14	3.92	0.49	5.50	0.54	4.31	0.31	4.03	0.49	2.27	0.33
i13:0	1.62	6.00	4.69	3.73	4.29	2.88	4.84	11.96	3.55	12.02	2.64	12.59	2.10	13.13	6.49	11.35	3.68	14.05	4.82	9.35
a13:0	0.57	5.80	1.63	4.74	1.61	3.38	1.33	3.92	0.72	1.49	1.22	2.88	0.88	4.27	2.60	3.87	1.22	3.60	1.48	3.72
13:0	0.36	0.09	0.60	0.02	1.59	0.00	0.44	0.03	0.40	0.00	3.01	0.05	1.61	0.00	1.20	0.01	2.46	0.05	0.00	0.06
i14:0	2.89	8.95	4.03	8.00	4.26	6.69	3.57	4.92	2.13	1.87	3.91	4.38	2.54	4.82	4.52	4.55	3.73	4.95	3.10	6.15
14:0	8.87	1.98	10.14	2.33	9.99	1.46	11.73	3.72	13.26	1.91	18.03	3.35	21.06	2.86	13.67	2.71	16.03	3.10	11.83	3.55
i15:0	5.08	17.79	12.23	13.53	4.13	18.16	9.48	30.49	7.95	35.12	2.10	23.00	1.66	20.85	5.80	24.26	2.56	19.29	8.65	26.80
a15:0	1.74	17.91	3.85	16.80	1.99	18.94	2.89	10.67	1.69	5.63	1.45	7.57	0.78	9.13	2.63	10.76	1.15	8.19	3.05	12.00
15:0	3.17	1.16	2.71	1.04	4.56	1.56	2.34	0.39	2.91	0.15	6.92	0.52	3.55	0.20	3.17	0.57	5.55	0.63	2.60	0.69
i16:0	3.22	13.04	2.90	14.39	2.56	14.75	3.78	5.74	1.82	3.18	1.73	4.87	1.09	4.96	2.23	5.99	0.50	4.53	2.82	8.05
16:1[9]	0.47	0.86	0.99	1.76	1.64	0.88	2.70	1.63	1.76	1.11	1.23	3.38	0.79	3.24	2.63	2.41	0.66	1.45	2.66	1.88
16:0	63.52	10.67	48.27	16.63	50.72	14.58	42.60	12.74	54.48	13.33	46.23	20.17	53.27	20.36	42.90	15.63	51.67	24.54	45.39	13.20
i17:0	1.79	6.23	1.53	6.15	1.38	5.83	4.96	8.60	2.17	20.51	0.59	10.74	0.29	9.73	1.51	11.38	0.60	9.27	3.10	8.34
a17:0	0.78	6.86	0.85	8.25	0.56	9.03	1.22	2.87	0.62	2.37	0.33	2.64	0.00	2.77	0.53	3.65	0.00	2.08	1.11	3.74
17:0	0.86	0.47	0.34	0.27	2.05	0.60	0.77	0.12	0.54	0.06	2.01	0.27	0.58	0.06	0.82	0.30	1.42	0.34	0.91	0.15
18:1[9]	0.66	0.03	0.56	0.08	0.43	0.00	0.55	0.03	0.78	0.06	0.42	0.07	1.20	0.07	0.43	0.03	0.10	0.06	1.05	0.04
18:0	2.17	0.30	0.86	0.48	2.73	0.45	1.90	0.33	1.57	0.64	1.94	1.42	1.02	0.87	1.16	0.65	1.38	1.19	1.95	0.32

[a] All values are means of four determinations.

TABLE 3. Similarities of fatty acid profiles of *B. anthracis* and *B. cereus* strains grown on synthetic medium[a]

Species and strain	Linear correlation of fatty acid profile of:									
	B. anthracis			*B. cereus*						
	Sterne	Zambia	18-74	1661	1664	1688	1689	1690	1691	1692
B. anthracis										
Sterne	**1.000**	**0.946**	**0.971**	0.798	0.633	0.714	0.743	0.815	0.680	0.872
Sambia		**1.000**	**0.977**	0.683	0.527	0.701	0.738	0.753	0.714	0.783
18-74			**1.000**	0.748	0.569	0.704	0.728	0.785	0.681	0.836
B. cereus										
1661				**1.000**	**0.932**	**0.929**	**0.914**	**0.974**	0.849	**0.988**
1664					**1.000**	**0.904**	0.864	**0.934**	0.801	**0.902**
1688						**1.000**	**0.993**	**0.975**	**0.975**	**0.926**
1689							**1.000**	**0.969**	**0.986**	**0.918**
1690								**1.000**	**0.922**	**0.978**
1691									**1.000**	0.854
1692										**1.000**

[a] Numbers in boldface type indicate correlation coefficients higher than 0.900.

different from that in *B. anthracis*. The difference was small but clear. These fatty acids added up to nearly 6% of the total fatty acid contents of *B. cereus* but only made up about 0.1 to 2% of the total fatty acid content of *B. anthracis*. However, this was only tested on one virulent strain, which cannot be representative and could not be confirmed by our results. We found that the contents of these three fatty acids were very similar in all tested strains.

According to Kaneda (7–10), the content of branched-chained fatty acids can be increased by adding branched-chain amino acids to the medium as precursors for fatty acid synthesis. For example, the addition of isoleucine results in an increase of odd anteiso branched-chain fatty acids, and the content of even iso acids is increased by adding valine to the medium. Fatty acid synthesis is catalyzed by two synthetases with different specifities toward the chain initiator.

The differences in the fatty acid patterns of the two species might be explained by the different substrate specificities of the fatty acid synthetase complexes. This will be the object of further investigations. The method described here is currently being tested on further reference and field strains and other species of the genus *Bacillus*, especially *B. thuringiensis*, which is also similar to *B. cereus* (4). Early investigations with one strain of *B. thuringiensis* resulted in fatty acid patterns similar to those of *B. cereus*.

In most diagnostic laboratories working with GC methods, bacterial strains are usually grown on a standard complex medium. It was shown in this report that differentiation of *B. cereus* from *B. anthracis* is not possible under these conditions. However, growth on synthetic RM medium leads to small but significant differences in the fatty acid patterns of the two species. This characteristic might be valuable for differentiation of avirulent *B. anthracis* strains; it might also help to avoid animal tests for pathogenicity.

TABLE 4. Ratios of odd iso and anteiso fatty acids of *B. anthracis* and *B. cereus* strains grown on synthetic medium

Species and strain	i13:0/a13:0	i15:0/a15:0	i17:0/a17:0
B. anthracis			
Sterne	1.03	0.99	0.90
Sambia	0.78	0.80	0.74
18-74	0.85	0.95	0.64
B. cereus			
1661	3.05	2.86	2.99
1664	8.07	6.23	8.65
1688	4.37	3.03	4.07
1689	3.07	2.28	3.51
1690	2.93	2.25	3.12
1691	3.90	2.35	4.45
1692	2.51	2.23	2.23

The computer programs used in this study were written by Andreas Heine, and the bacteriological work was assisted by Ute Sukop. Their contributions are gratefully acknowledged.

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