Fatty Acid Patterns in the Classification of some Representatives of the Families *Enterobacteriaceae* and *Vibrionaceae*

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(Received 3 July 1979)

Twenty-three strains representing the families *Enterobacteriaceae* and *Vibrionaceae* were analysed for fatty acid composition of whole cells by means of glass capillary column gas chromatography. Among the several alternatives tested, cluster analysis based on data normalized to hexadecanoate and logarithmically transformed provided good separations of species, genera and families. Strains from the genera *Salmonella*, *Escherichia*, *Proteus*, *Enterobacter*, *Klebsiella*, *Vibrio* and *Aeromonas* were studied.

INTRODUCTION

In recent years gas chromatography has been applied to the analysis of microbial cells and metabolic products (Mitruka, 1975). This technique can provide a detailed description of bacteria, since the analytical parameters are variables measured on a continuous scale. In this respect gas chromatography is superior to biochemical analyses which mainly produce binary data.

Identification of Gram-negative bacteria from aquatic and food sources has proved difficult due to the unsatisfactory state of their classification. Analyses of cellular fatty acids were therefore undertaken as a means of classifying various representative strains from the families *Enterobacteriaceae* and *Vibrionaceae*.

To extract the maximum useful information from the complex fatty acid profiles, mathematical techniques must be applied; various multivariate statistical methods exist for the analysis of such data (Sneath & Sokal, 1973; Späth, 1977). In the present work, several algorithms were compared and it was found that cluster analysis based on a logarithmic transformation of data for fatty acid methyl esters (Jantzen *et al.*, 1974) provided the best results.

METHODS

Bacterial cultures. Organisms used were: *Escherichia coli* NCTC 10082; *Proteus morganii* NCTC 2818 and NCTC 2815; *Vibrio parahaemolyticus* ATCC 17802; *Enterobacter cloacae* 20894/73, from the Culture Collection, Department of Microbiology, University of Bergen, Norway; *Vibrio ichthyoderms* (now called *V. anguillarum*), *V. anguillarum* V962, V767, V768, V873 and A272 and *Aeromonas salmonicida* A367/76 and A567/76, from the Culture Collection, National Veterinary Institute, Oslo, Norway; *Salmonella typhimurium* FSK1, *Salmonella dublin* FSK2, *Enterobacter cloacae* FSK20, *Klebsiella pneumoniae* FSK25, *Vibrio anguillarum* FSK14, FSK30, FSK31 and FSK45, *Vibrio parahaemolyticus* FSK43 and FSK44, isolated at the Directorate of Fisheries, Central Laboratory, Bergen, Norway, and identified using the appropriate keys and diagnostic tables of Cowan *et al.* (1974).

Growth of bacteria and derivative formation. All cultures were grown on Plate Count Agar (Difco no. 0479-01) and incubated for 18 to 24 h; *V. anguillarum* and *A. salmonicida* were grown at 25 °C, and the other strains at 37 °C. For vibrio cultures the medium was supplemented with NaCl (2%, w/v).

After incubation, cells were carefully removed from the plate surface and transferred to a test tube containing 5 ml of 5% (w/v) NaOH in 50% (v/v) aqueous methanol. The fatty acids liberated by saponification were methylated with BCl₃/methanol, according to Moss *et al.* (1974).

Gas-liquid chromatography (g.l.c.). A Perkin-Elmer F22 gas chromatograph equipped with a flame ionization detector and 25 m $\times$ 0.25 mm i.d. glass capillary column coated with OV-101 (Perkin-Elmer) was used, with the injector at 230 °C, the detector at 270 °C, and the oven held at 150 °C for 2 min and then increased by 4 °C min⁻¹ to 225 °C. Samples (1 μ l) were injected on the column by the splitless technique (Grob & Grob, 1972, 1974). Results were quantified by electronic integration (Spectra Physics SP 4000 data system). Tentative identifications were established by retention times relative to methyl hexadecanoate, compared with a standard mixture, containing 23 fatty acid methyl esters (FAMES), obtained from Supelco Inc. (no. 4-5436).

G.l.c./mass spectrometry. Mass spectra were obtained using an HP 5992 g.l.c./mass spectrometer equipped with a 27 m $\times$ 0.25 mm i.d. OV-101 glass column. Spectra of FAMES were compared with spectra of the 23 reference compounds. For FAMES that could not be identified as belonging to the set of reference compounds, structures were tentatively assigned by comparing their mass spectra with those given by Ryhage & Stenhagen (1960).

Numerical analysis. The data were evaluated by a multivariate technique based on relative peak areas for 17 FAMES measured for each of the bacteria. The necessary programs were written for a Hewlett Packard 9825 desktop calculator having a 32 K RAM memory to perform the following general functions: raw data input and output including editing; transformation of raw data; calculation of similarity coefficients; and cluster analysis. Four data sets were used.

Set no. 0: g.l.c. raw data normalized to the peak area for hexadecanoate. The complete raw data set consisting of relative peak areas for 17 FAMES was used (Table 1) and, in addition, reduced sets consisting of only those FAMES having relative peak areas > 10% were employed.

Set no. 1: set 0 transformed according to $x = 1 + 9(x - x_{\min})/(x_{\max} - x_{\min})$.

Set no. 2: set 0 logarithmically transformed, $x = \ln(1 + x)$.

Set no. 3: set 0 autoscaled, $x = (x - x_m)/S_x$, where x_m is the mean value and S_x the standard deviation (N weighting).

Two similarity coefficients were used for these data sets, namely, the correlation coefficient R (Sokal & Michener, 1958) and the coefficient based on euclidean distance D between pairs of bacteria, $1 - D/D_{\max}$ (Harper *et al.*, 1977). Clustering of strains was achieved by the unweighted pair group method (Sokal & Michener, 1958; Sneath & Sokal, 1973) and results are presented graphically as phenograms.

RESULTS AND DISCUSSION

Methyl esters of fatty acids containing from 12 to 20 carbon atoms were identified in the extracts of whole bacteria. A typical chromatogram is shown in Fig. 1. Up to 15 FAMES were identified in each extract by comparing their mass spectra and relative retention times with those of the standards. The branched C_{16:0} and C_{18:0} acids were also included in the construction of the classification although standards were not available (Table 1). Compounds eluting earlier than C_{12:0} were not studied further. Hydroxy acids were detected but were not included in the construction of the classification because of poor reproducibility of the relative peak areas. Two acids whose identity was only tentatively assigned, namely, branched C_{14:0} and C_{14:1}, and which were found only in *V. parahaemolyticus* species were also not studied further.

In addition to the FAMES described above, each chromatogram showed 10 to 20 unidentified components in the 1 to 10% relative peak area range. Analysis of the extracts from 10 subcultures of *V. anguillarum* FSK14 revealed that eight of these small components showed good reproducibility and therefore provide data of value for classification. However, since none of these components were identified as fatty acid methyl esters, they were not included in the construction of the classification. The classification was based on only one type of compound in order to minimize problems of poor reproducibility caused by changing experimental parameters (e.g. g.l.c. columns).

By expressing the peak areas as percentages relative to hexadecanoate (Table 1) rather than to the total fatty acid methyl esters, interclass discrimination is favoured while intra-class relations are very little affected.

Fig. 1. Gas chromatogram of fatty acid methyl esters from *E. coli* NCTC 10082. The FAME designations corresponding to the numbers on the chromatogram are given in Table 1.

Few studies have been reported on the analysis of fatty acids isolated from bacteria belonging to the families *Enterobacteriaceae* and *Vibrionaceae*. The profile of the major fatty acids of *E. coli* was described by Ingram (1977), and was similar to that found in the present study. Distinguishing features of the family *Enterobacteriaceae* are the prominent hexadecanoate ($C_{16:0}$) peak, and the presence of hexadecenoate ($C_{16:1}$) and DL-*cis*-9,10-methylene hexadecanoate ($C_{17:0\Delta}$) in comparable amounts at approximately 40% of the hexadecanoate peak. These characteristics have been reported previously for other members of the family *Enterobacteriaceae* (Machtiger & O'Leary, 1973). Fatty acids isolated from *V. cholerae* were described by Brian & Gardner (1968) who found a fatty acid pattern similar to that obtained from strains of *Vibrio* listed in Table 1.

The reproducibility of the FAME pattern was studied by analyses of 10 subcultures of *V. anguillarum* FSK14 (Table 2). Although the overall pattern was reproducible, the results varied for individual FAMES, notably for those having relative peak areas below 10%. For this reason and other reasons discussed below, we decided to study the behaviour of several algorithms applied to the full data set of 17 FAMES, and also to sets comprising only FAMES of relative peak area > 10%.

The similarity matrix obtained by using algorithm R2 with 11 FAMES, i.e. by using the logarithmic transformation 2 combined with correlation coefficients R , is shown in Table 3. For each of the 10 subcultures of *V. anguillarum*, the maximum value of the similarity index was found, and the difference between the highest and lowest pairs was obtained. It can be seen from Table 3 that the highest pair is 1 and 7, with $R = 100$, and the lowest pair is 1 and 9, with $R = 97$. The complete results for the eight algorithms used with 17 and 11 FAMES are given in Table 4. Irrespective of the number of FAMES included in the analysis, the raw data combined with correlation coefficients provide a treatment which is insensitive to variations in FAME pattern. Thus, this treatment cannot be expected to detect small but significant differences between bacteria. The algorithm R2 provides differences between pairs of bacteria of 6 and 3% for 17 and 11 FAMES, respectively. These differences are considered to be appropriate considering that the test set consists of subcultures of one

Table 1. *Fatty acid methyl esters isolated from bacteria.*

Strain	FAME* No.	Peak area (%) relative to hexadecanoate																
		12:0	13:0	14:0	15:0	16:0	16:1	16:2	a- 17:0	a- 17:0Δ	17:0	17:0Δ	18:0	18:1	18:2	19:0	19:0Δ	20:0
1. <i>Salmonella typhimurium</i> FSK1	26	1	25	2	4	0	45	100	1	46	7	0	44	3	10	0	2	
2. <i>S. dublin</i> FSK2	17	1	24	0	3	0	40	100	2	41	2	0	42	2	9	1	1	
3. <i>Escherichia coli</i> NCTC 10082	17	1	29	0	4	0	43	100	0	33	4	0	29	4	3	0	1	
4. <i>Enterobacter cloacae</i> FSK20	8	1	28	0	6	0	14	100	0	57	6	0	28	9	6	0	1	
5. <i>Ent. cloacae</i> 20894/73	7	1	37	1	4	0	23	100	0	62	4	1	30	3	5	1	0	
6. <i>Proteus morganii</i> NCTC 2818	12	1	26	1	4	0	36	100	0	40	2	0	8	1	1	1	1	
7. <i>P. morganii</i> NCTC 2815	16	2	24	0	5	0	56	100	1	34	2	0	12	1	1	0	1	
8. <i>Klebsiella pneumoniae</i> FSK25	4	1	38	0	5	0	10	100	0	61	1	0	24	2	19	0	1	
9. <i>Aeromonas salmonicida</i> A367/76	27	2	10	1	8	2	170	100	8	4	5	4	41	8	1	1	2	
10. <i>A. salmonicida</i> A567/76	31	2	23	2	10	2	210	100	9	12	6	7	40	21	1	1	9	
11. <i>Vibrio anguillarum</i> FSK30	14	1	36	1	2	34	175	100	2	1	1	0	54	6	1	1	1	
12. <i>V. anguillarum</i> FSK31	10	1	32	1	2	53	227	100	1	1	1	2	52	3	1	1	1	
13. <i>V. parahaemolyticus</i> FSK43	17	0	42	0	3	79	166	100	2	2	1	7	25	6	0	0	2	
14. <i>V. parahaemolyticus</i> FSK44	15	1	41	2	2	71	168	100	2	0	1	6	31	7	0	0	1	
15. <i>V. anguillarum</i> V962	14	1	23	1	2	42	183	100	4	1	3	9	91	6	1	0	0	
16. <i>V. anguillarum</i> V767	10	1	24	1	3	38	168	100	3	1	2	3	69	6	1	0	0	
17. <i>V. anguillarum</i> V768	23	1	29	2	4	14	210	100	8	2	5	5	114	8	1	1	0	
18. <i>V. anguillarum</i> V873	19	1	30	1	3	34	193	100	4	1	3	2	76	7	1	0	0	
19. <i>V. anguillarum</i> FSK45	15	0	20	0	2	23	180	100	3	2	3	7	65	10	0	0	0	
20. <i>V. anguillarum</i> A272	24	1	31	2	3	40	183	100	4	2	3	10	67	8	0	0	0	
21. <i>V. ichthyodermis</i> (tentative)	20	1	31	2	3	97	237	100	3	0	2	14	96	8	1	0	0	

* In FAME designations, the first number gives the number of carbon atoms in the fatty acid, and the second, the number of double bonds: a designates anteiso branched; br, a branched chain, for which no standards were available (equivalent chain lengths were 15.6 for FAME no. 6 and 17.6 for FAME no. 12); Δ, the presence of a cyclopropane ring.

Table 2. Fatty acid methyl esters isolated from 10 subcultures of *Vibrio anguillarum* FSK14

FAME* No.	Peak area (%) relative to hexadecanoate																	
	12:0 1	13:0 2	14:0 3	a-15:0 4	15:0 5	br-16:0 6	16:1 7	16:0 8	a-17:0 9	17:0Δ 10	17:0 11	br-18:0 12	18:1 13	18:0 14	19:0Δ 15	19:0 16	20:0 17	
Subculture																		
1	15	0	32	3	9	27	250	100	8	3	8	7	57	7	0	0	8	
2	22	1	34	2	8	27	256	100	8	3	8	7	57	8	0	0	5	
3	19	0	30	2	8	27	237	100	7	4	11	6	77	17	3	2	15	
4	20	0	34	1	9	22	253	100	8	4	10	11	64	12	0	2	6	
5	15	1	29	2	7	23	244	100	7	3	7	4	60	10	1	2	9	
6	19	0	32	3	8	27	230	100	8	3	8	13	80	7	0	0	12	
7	17	1	32	2	8	34	254	100	7	3	8	6	60	7	2	3	7	
8	9	1	31	1	10	37	261	100	8	3	9	4	59	7	6	2	5	
9	21	1	38	2	11	39	284	100	8	4	8	4	55	7	0	0	17	
10	8	1	29	1	8	26	241	100	8	1	9	8	56	9	5	1	8	
Mean	16.5	0.6	32.1	1.9	8.6	28.9	251	100	7.7	3.1	8.6	7.0	62.5	9.1	1.7	3.0	9.2	
Standard deviation	4.6	0.8	2.6	0.9	1.2	5.5	14.2	—	0.6	0.9	1.2	3.0	8.4	3.2	2.2	5.8	4.0	

* For definition of FAME designations, see Table 1.

Table 3. *Similarity matrix for 10 subcultures of Vibrio anguillarum FSK14 based on 11 FAMEs and algorithm R2*

Based on the data from Table 2, FAMEs 2, 4, 9, 10, 15 and 16 being excluded.
Correlation coefficients $R(\times 100)$; raw data transformed according to $x = \ln(1+x)$.

Sub-culture	1	2	3	4	5	6	7	8	9
2	99								
3	97	95							
4	98	99	95						
5	99	98	99	97					
6	98	97	95	97	96				
7	100	99	97	98	99	98			
8	98	97	95	96	97	94	98		
9	97	95	96	92	97	94	97	95	
10	98	96	96	97	97	97	98	98	94

Table 4. *Minimum and maximum values of similarity indexes ($\times 100$) for pairs in the test set of 10 subcultures from Vibrio anguillarum FSK14*

Transformation*	Similarity index	Code	Using 17 FAME			Using 11 FAME		
			Highest	Lowest	Difference	Highest	Lowest	Difference
0	R	R0	100	100	0	100	100	0
0	$1-D/D_{\max}$	D0	84	46	38	85	52	33
1	R	R1	81	33	48	86	46	40
1	$1-D/D_{\max}$	D1	70	29	41	74	32	42
2	R	R2	99	93	6	100	97	3
2	$1-D/D_{\max}$	D2	80	49	31	79	38	41
3	R	R3	89	29	60	82	49	33
3	$1-D/D_{\max}$	D3	67	34	33	77	32	45

* 0, None; 1, $x = 1+9(x-x_{\min})/(x_{\max}-x_{\min})$; 2, $x = \ln(1+x)$; 3, $x = (x-x_m)/S_x$.

strain. Transformations 1 and 3 tend to exaggerate the differences between the FAME patterns and may therefore be of value in studying the fine details of the phenograms. However, other studies (described below) indicate that these two transformations are of limited value.

Cluster analysis based on algorithm R2 and FAMEs with relative peak areas $> 10\%$ combines data derived from the 10 analyses of *V. anguillarum* FSK14 into one tight cluster with $R = 0.973$. A similar cluster was found for 10 subcultures of *V. parahaemolyticus* ATCC 17802 ($R = 0.969$). The long-term reproducibility of the g.l.c. analysis was evaluated by analyses of the standard mixture over a 12 month period using two different columns. Cluster analysis revealed one tight cluster with $R = 0.969$ based on algorithm R2 and 15 of the FAMEs listed in Table 1, numbers 6 and 12 being excluded. Thus the raw g.l.c. data are of sufficiently high quality to permit further mathematical treatment.

The families *Enterobacteriaceae* and *Vibrionaceae* were easily separated by their different FAME patterns. Visual examination of the eight similarity matrices D,R-0,3 based on 17 FAMEs revealed that, with one exception, the two families were clearly separated. A typical similarity matrix is given in Table 5. Algorithm R3 combining the transformation $x = (x-x_m)/S_x$ with correlation coefficients did not separate the two families. Transformation 3 weights all FAMEs equally and the poor result obtained for the two families may be connected with the great variance already noted for FAMEs with relative peak areas $< 10\%$. However, as shown below, even in the case of using only FAMEs with relative peak areas $> 10\%$, transformation 3 is unsatisfactory.

Table 5. *Similarity matrix for bacteria no. 1 to 21 (see Table 1) based on 17 FAMES and algorithm R0*

Correlation coefficients R(×100); values greater than 80.																				
No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
2	100																			
3	98	99																		
4	93	94	93																	
5	94	95	94	99																
6	94	95	98	94	95															
7	94	95	98	0	0	98														
8	0	91	0	98	98	92	0													
9	0	0	0	0	0	0	0	0												
10	0	0	0	0	0	0	0	0	99											
11	0	0	0	0	0	0	0	0	97	96										
12	0	0	0	0	0	0	0	0	96	96	99									
13	0	0	0	0	0	0	0	0	0	0	96	96								
14	0	0	0	0	0	0	0	0	91	91	97	98	100							
15	0	0	0	0	0	0	0	0	95	93	98	97	92	94						
16	0	0	0	0	0	0	0	0	96	95	99	98	94	96	100					
17	0	0	0	0	0	0	0	0	95	94	96	95	0	0	99	98				
18	0	0	0	0	0	0	0	0	97	96	99	98	93	95	98	100	98			
19	0	0	0	0	0	0	0	0	98	97	99	98	92	94	99	99	98	100		
20	0	0	0	0	0	0	0	0	97	96	100	99	95	96	99	100	97	100	99	
21	0	0	0	0	0	0	0	0	90	0	96	98	95	96	98	97	94	97	96	97

Table 6. *Levels of similarity coefficients for Enterobacteriaceae*

Algorithm	Based on 17 FAME		Based on 7 FAME*	
	Between species	Between genera	Between species	Between genera
R0	99, 98, 99	98, 98	99, 97, 99	97, 97
D0	78, 64, 74	66, 66	80, 64, 77	66, 68
R1	—	—	93, 78, 89	64, 79
D1	—	—	77, 74, 66	66, 54
R2	96, 96, 97	97, 94	98, 98, 98	95, 84
D2	46, 46, 58	45, 48	86, 82, 80	65, 57
D3	—	—	75, 73, 69	66, 52

* Having relative peak areas > 10%.

Bacteria 1 to 8 (see Table 1) represent five different genera within the *Enterobacteriaceae*. A successful cluster analysis must be able to group together strains belonging to the same genus and must also connect the constituent genera at a lower level of similarity. Examination of the eight phenograms based on 17 FAMES revealed that algorithms R0, D0, R2 and D2 showed the expected connections between species and genera. However, levels of similarity values for species and genera overlapped and none of the algorithms based on the 17 FAMES was satisfactory (Table 6). Many examples of cluster analyses based on FAMES ranging from below 1 to 100% are given in the literature. These studies should be regarded critically, and especially so as in the majority of studies no indications are given concerning the reproducibility of the FAME patterns. Classifications based on seven FAMES with relative peak areas > 10% provided the correct relationships with the algorithms R2, D2 and D3 (Fig. 2).

Bacteria 9 to 21 represent two genera within the family *Vibrionaceae*, i.e. *Vibrio* and *Aeromonas*. Within the genus *Vibrio*, three species were represented, viz. *V. parahaemolyticus*, *V. anguillarum* and *V. ichthyoderms*. Four algorithms (R0, D0, R2 and D2) were used with the full data set of 17 FAMES obtained with these strains. Examination of the corresponding phenograms revealed that R0 and D0 did not separate the two genera, but successful

Fig. 2. Phenograms of *Enterobacteriaceae* strains based on seven fatty acid methyl esters and algorithms R2, D2 and D3.

Fig. 3. Phenograms of *Vibrionaceae* strains based on 10 fatty acid methyl esters and algorithms R2 and D2.

separations were achieved using algorithms R2 and D2. However, the latter did not separate the three *Vibrio* species. Thus, we conclude that with the full data set, none of the algorithms worked well.

All eight algorithms R,D-0,3 were tested with a reduced data set comprising 10 FAMES having relative peak areas > 10%. The phenograms based on R1, D1, R2 and D2 gave the correct structure, while R0, D0, R3 and D3 did not separate the two genera. Comparison of the families *Enterobacteriaceae* and *Vibrionaceae* indicated that the algorithms R2 and D2 gave satisfactory results for both families when applied to reduced data sets consisting

of FAMES having relative peak areas $> 10\%$. Phenograms for bacteria 9 to 21 are given in Fig. 3.

Autoscaling (transformation 3) of data for FAMES with relative peak areas $> 10\%$ did not give satisfactory results, irrespective of the type of similarity index used. Autoscaling is often used as a first step in data analysis, but the present results demonstrate that this transformation should be used with caution.

The following strategy for cluster analysis of bacteria is recommended as a result of the present study: families should be separated by examination of similarity matrices based on raw data for all FAMES, with peak areas normalized to a prominent peak ($C_{16:0}$), and the structure within families should be studied by using only FAMES with relative peak areas $> 10\%$ and applying the logarithmic transformation $x = \ln(x+1)$ to the area normalized raw data.

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