Characterization of *Flavobacterium* Species by Analysis of Volatile Fatty Acid Production

By D. RASOAMANANJARA,¹ F. PELADAN,¹ J. C. TURLOT,²
H. MONTEIL^{1*} AND C. RICHARD²

¹*Institut de Bactériologie de la Faculté de Médecine, Université Louis Pasteur, 3, rue Koeberlé,
67000 Strasbourg, France*

²*Centre d'Études Statistiques, IRMA, Université Louis Pasteur, 7, rue Descartes,
67000 Strasbourg, France*

³*Service des Entérobactéries, Unité INSERM 199, Institut Pasteur,
75724 Paris Cedex 15, France*

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Seventy-four *Flavobacterium* strains were characterized by gas–liquid chromatographic analysis of volatile fatty acids produced in the culture medium. Principal components analysis permitted the graphic representation of the relative positions of the different strains, and aggregation according to the variance enabled a hierarchical classification to be established. The study revealed three subgroups each for *F. meningosepticum* and *F. odoratum*. Our *F. breve*, *Flavobacterium* sp. group IIb and *F. multivorum* strains appeared to be homogeneous. These results tallied with those of previous studies on DNA base composition and reassociation, electrophoretic protein profiles and cellular fatty acid composition.

INTRODUCTION

The genus *Flavobacterium* (Holmes *et al.*, 1984) includes pigmented, strictly aerobic, non-motile bacteria, with a DNA G + C content ranging from 30 to 42 mol %. Seven species are described by Holmes *et al.* (1984): *F. aquatile*, *F. breve*, *F. balustinum*, *F. meningosepticum*, *F. odoratum*, *F. multivorum* and *F. spiritivorum*. Flavobacteria are common in soil and water and are frequently isolated in hospitals. In human pathology, the role of *F. meningosepticum* is well known and the unnamed *Flavobacterium* sp. group IIb of King (1959) is sometimes involved. Other species, such as *F. odoratum*, *F. breve* and *F. multivorum*, have also been implicated in nosocomial infections and have been isolated in liquids for hospital use and in wounds with secondary infections. Marked drug-resistance patterns of these bacteria have increased their clinical interest (Von Graevenitz, 1981). The identification of *Flavobacterium* by biochemical characters may be difficult. The present study was intended to develop a rapid method for characterizing some species of this genus by analysis of volatile fatty acids (VFA) produced in culture supernatants, a method that has proved useful for pseudomonads (Peladan & Monteil, 1984a; Peladan *et al.*, 1984).

METHODS

Bacterial strains. Seventy-four strains were tested (Table 1): 31 *F. meningosepticum*, 9 *F. breve*, 13 *Flavobacterium* sp. group IIb, 15 *F. odoratum* and 6 *F. multivorum*. Ten were reference strains. Fifty-three were isolated at Strasbourg University Hospital and 11 were from Dr M. J. Pickett (Department of Microbiology, University of California, Los Angeles, Calif., USA). Table 1 gives the identities of strains as determined by Richard & Monteil (1983). All strains were kept at –80 °C in a medium containing brain heart Infusion (Difco), horse serum and glycerol (10:1:1, by vol.).

Abbreviations: HAC, hierarchical ascendant clustering; PCA, principal components analysis; VFA, volatile fatty acids.

Table 1. *Origin of strains and groups after classification*

Strain	Source	Serotype	Group after classification (see Fig. 1)
<i>F. meningosepticum</i>			
NCTC 10016 (= ATCC 13253)	Reference strain	A	B ₁
NCTC 10585 (= ATCC 13254)	Reference strain	B	D
NCTC 10586 (= ATCC 13255)	Reference strain	C	D
NCTC 10587	Reference strain	D	D
NCTC 10588	Reference strain	E	D
NCTC 10589	Reference strain	F	D
B 40470	Bronchial secretion		B ₂
B 40705	Sputum	G	B ₁
B 41693	Sputum	G	B ₁
B 41972	Bronchial secretion	G	B ₂
B 46136	Sputum		B ₁
B 41667	Bronchial secretion	G	B ₂
B 37566	Sputum	G	B ₁
B 38611	Sputum	G	B ₁
B 14430	Catheter tip	G	B ₂
B 26972	Sputum	G	B ₂
B 25649	Aspiration fluid	G	B ₂
B 23643	Bronchial secretion	G	B ₂
B 26724	Bronchial secretion	G	B ₂
B 26345	Bronchial secretion	G	B ₁
B 46356	Bronchial secretion		B ₁
B 38594	Sputum	G	B ₂
B 37907	Blood		B ₁
B 35810	Bronchial secretion	G	B ₂
B 30560	Bronchial secretion	G	B ₂
B 40192	Bronchial secretion		B ₂
B 41047	Bronchial secretion	G	B ₂
B 39452	Bronchial secretion	G	B ₂
B 68800	Blood	F	B ₂
B 41312	Bronchial secretion	G	B ₂
B 40185	Bronchial secretion	G	B ₂
<i>F. odoratum</i>			
A 16987	Urine: Strasbourg		X
A 66955	Urine: Strasbourg		F
A 67866	Urine: Strasbourg		C
A 75632	Urine: Strasbourg		F
B 77131	Wound: Strasbourg		A
B 78400	Bronchial secretion: Strasbourg		A
B 83305	Ulcer: Strasbourg		A
B 91164	Abcess: Strasbourg		A
K 1031	St Mary's Hospital, Long Beach, Calif., 1975		C
K 1518	API 76-2669, 1979		F
K 1689	Clinical Laboratories, UCLA, Calif., 1980		F
K 1778	Public Health Laboratories, Orange Country, Calif., 1981		C
K 1830	Huttington Memorial Hospital, Pasadena, Calif., 1982		F
K 1899	Wadsworth Veterans Administration Hospital, Los Angeles, Calif., 1983		A
K 1961	Biopathology Clinical Laboratories, North Hollywood, Calif., 1983		C
<i>Flavobacterium</i> sp. group IIb			
B 32502	Sputum		E
B 37750	Sputum		E
B 47466	Unknown		E
B 47812	Unknown		E
B 48260	Bronchial secretion		E
B 49427	Unknown		E
B 49942	Unknown		E

Table 1. (continued)

B 69546	Sputum	E
B 72648	Sputum	E
B 77095	Sputum	E
B 77833	Sputum	E
B 82341	Sputum	E
B 82423	Sputum	E
<i>F. multivorum</i>		
NCTC 11033	Reference strain	H
NCTC 11034	Reference strain	H
K 1210	API 76797, 1977 (Gilardi 2311)	H
K 1213	API 76796, 1977 (Gilardi 2312)	H
K 1222	API 76799, 1977 (Gilardi 2302)	H
K 1231	California Department of Health, 13527, Berkeley, Calif., 1977	H
<i>F. breve</i>		
CL 200/75	Reference strain	G
CL 666/76	Reference strain	X
A 54615	Urine	G
B 33892	Vaginal swab	G
B 38674	Vaginal swab	G
B 44444	Unknown	G
B 49835	Unknown	G
B 54566	Vaginal and anal swab	G
B 79158	Unknown	G

Culture conditions. Starter cultures were prepared in 5 ml trypticase soy broth (Diagnostics Pasteur, Paris) with shaking for 6 h at 30 °C; 1 ml of this starter culture was added to 20 ml of a culture medium comprising (per litre of medium): CaCl₂, 15 mg; MgSO₄ · 7H₂O, 123 mg; KH₂PO₄, 680 mg; K₂HPO₄, 2610 mg; Casamino acids, 20 g; L-tryptophan, 10 mg; NaCl, 1 g; carbon source (glucose, sodium acetate and ethanol), each 1 g; alcohols solution (0.2 g l⁻¹ of each of the following: *n*-propyl, *n*-butyl, isobutyl, *n*-pentyl and isopentyl alcohol), 1 ml; thiamin, 5 µg (thiamin was the sole growth factor required for growth and optimal VFA production by all strains; the addition of other factors had no effect, or was inhibitory). The pH was adjusted, with NaOH, to 7.5. Cultures were grown for 18 h at 30 °C with shaking.

Extraction, identification and titration of VFA produced in culture supernatants. The methods of Peladan & Monteil (1984b) were used. Peaks on the chromatograms were identified by comparing their retention times with those of chromatographically pure standards.

Data gathering. The values of the sterile control sample were subtracted from the chromatographic peak areas of the samples and the resulting values were normalized by expressing each acid produced as a percentage of the total acid output of the strain under examination.

Reproducibility of the method. Reproducibility was tested on six reference strains: two strains of *F. meningosepticum* (NCTC 10016, NCTC 10585), two of *F. multivorum* (NCTC 11033, NCTC 11034) and two of *F. breve* (CL 200/75, CL 666/76). Each strain was grown for two different periods in 20 ml of two sets of culture medium inoculated with 1 ml of the starter culture. Two extractions were performed on each culture supernatant and chromatographed three times, giving 24 chromatograms for each species tested. Variations were studied by computation of mean value and standard error for each acid and for each species.

Statistical analysis. Each strain is supposed to have a point representation on the set of variables constituting a multidimensional space. Representation of all information obtained was achieved by means of the first factors of principal components analysis (PCA) (Harman, 1968; Hope, 1968; see Alderson, 1985, for a review). This representation was followed by a hierarchical ascendant clustering (HAC) classification based upon the same distances as for PCA, so the results of both methods could be compared.

Hierarchical classification. The distance between two strains in PCA is given by the usual Euclidian distance where the common variance of the variables is equal to 1. HAC uses the same distance (similarity) to build the most homogeneous classes. The within-class variance defines the homogeneity of the classes. At each step, a new class is formed by amalgamation of the nearest two classes (Hartigan, 1975). The variance of the new class is greater than the sum of the variances of the two classes before grouping and the difference between these two quantities is called the level of clustering. This level reveals the similarity of the two classes. The results of the HAC are represented by a dendrogram. The selected partition can be interpreted on the graphs by the first principal components.

Computing of results. SPAD programs edited by CESIA (Paris, France) were used for PCA and aggregation according to the variance and were run on UNIVAC 1110 computer at the Centre de Calcul du CNRS, Strasbourg, France.

RESULTS

We had originally intended to use data on the production of eight VFA for the analysis, but three of the VFA could not be used. The results for acetate were too variable for use; none of the strains produced any *n*-valeric acid, and the amounts of isocaproic acid produced were too small to be computed. The analysis was therefore based on the production of propionic, isobutyric, *n*-butyric, isovaleric and *n*-caproic acids.

Growth and VFA production kinetics of *F. meningosepticum* ATCC 10588 are shown in Fig. 1 (the other species gave similar results). An 18 h incubation was selected as the shortest time for enough growth and VFA production. Reproducibility of the methods is summarized in Table 2, showing mean acid percentages and 95% confidence limits for six reference strains. Variation was < 10% for each acid for all six strains. Typical chromatograms of the species are shown in Fig. 2. All six strains of *F. multivorum* produced a high proportion of isovaleric acid (> 80%) whereas strains of all the other species produced < 60%.

The first three principal components accounted for 93% of the total variance, which was sufficient for the sample to be described. The correlations between variables and principal components are summarized in Table 3 and represented in Fig. 3. The first component, F_1 , was characterized by isobutyric and isovaleric acids [correlation coefficients: $r(F_1, V_2) = +0.92$, $r(F_1, V_4) = -0.99$]. Using these two variables, clusters H and C could be opposed to clusters A, D, E and F (Fig. 3a). F_2 gave the opposition between *n*-butyric and *n*-caproic acids (these two variables were uncorrelated with the others) and allowed differentiation of cluster G from B_1 and B_2 . Component F_3 was roughly defined by propionic acid and separated clusters A, D and E from F. The separation of the groups can be seen in Fig. 3. No variable distinguished groups B_1 and B_2 correctly.

The results of the aggregation according to variance are summarized in Fig. 4. The hierarchical level at which clusters are delineated (0.12) was arbitrarily selected to give clusters corresponding approximately to phenetic species. Eight clusters were obtained: groups A to H. Group A (7 strains) was composed of two subgroups: the first comprised 5 strains of *F. odoratum* (Table 1) and the second comprised *F. odoratum* A 16987 and *F. breve* CL 666/76. The latter subgroup (named group X) could not be considered as characteristic because of the small number of strains: this will be discussed below. Group B was composed of two *F. meningosepticum* subgroups: B_1 (9 strains) and B_2 (17 strains). However, these subgroups agglomerated at a low level. Group C comprised 4 strains of *F. odoratum*, group D comprised 5 strains of *F. meningosepticum*, group E comprised 13 strains of *Flavobacterium* sp. group IIb, group F comprised 5 strains of *F. odoratum*, group G comprised 8 strains of *F. breve*, and group H comprised 6 strains of *F. multivorum*.

DISCUSSION

This study sought to establish a simple and rapid method for characterizing *Flavobacterium*. It should be borne in mind that the species we studied are represented by relatively few strains, and so the classification provided cannot be considered as definitive. Moreover, the level of cluster delineation in the dendrogram was arbitrarily selected so as to assign the strains to their original species. This level was not necessarily the best one, as the original species could have been split in unexpected ways. The representation of the strains on the first three principal axes of the PCA showed a good separation of the eight groups obtained by hierarchical clustering, and it also showed the variables that characterized these groups, especially isovaleric acid for group H and *n*-butyric acid for group G.

Flavobacterium sp. group IIb and *F. multivorum* were the most distinct taxa. As regards VFA production, *Flavobacterium* sp. group IIb (cluster group E) formed a single cluster, quite distinct from the others, especially from *F. meningosepticum*. This is valuable, since Richard & Monteil

Fig. 1. Growth (■) and VFA production kinetics (□) of *F. meningosepticum* strain ATCC 10588.

Table 2. Mean values (percentage of total VFA) $\pm$ 95% confidence limits for VFA produced by six reference strains

Strain	Propionic acid	Isobutyric acid	n-Butyric acid	Isovaleric acid	n-Caproic acid
<i>F. meningosepticum</i> NCTC 10016	8.9 $\pm$ 0.72	66.1 $\pm$ 4.8	0	22.5 $\pm$ 1.2	2.8 $\pm$ 0.17
<i>F. meningosepticum</i> NCTC 10585	22.5 $\pm$ 1.7	55.5 $\pm$ 4.21	0.48 $\pm$ 0.03	22.12 $\pm$ 0.18	1.41 $\pm$ 0.09
<i>F. multivorum</i> NCTC 11033	1.7 $\pm$ 0.09	9.91 $\pm$ 0.52	1.61 $\pm$ 0.12	84.3 $\pm$ 5.2	0.33 $\pm$ 0.02
<i>F. multivorum</i> NCTC 11034	1.72 $\pm$ 0.1	6.0 $\pm$ 0.32	1.51 $\pm$ 0.1	92.21 $\pm$ 6.31	0.53 $\pm$ 0.04
<i>F. breve</i> CL 200/75	20.2 $\pm$ 2	48.2 $\pm$ 4	13.7 $\pm$ 0.05	16 $\pm$ 1.3	0.1 $\pm$ 0.01
<i>F. breve</i> CL 666/76	18.9 $\pm$ 0.52	47.2 $\pm$ 3.5	6.7 $\pm$ 0.6	23.82 $\pm$ 1.51	0.43 $\pm$ 0.02

(1983) found that the only biochemical characters which separated *F. meningosepticum* from *Flavobacterium* sp. group IIb were amylase production and the intense pigmentation which became red after alkalization. Owen & Lapage (1974) found a similar DNA base composition and no consistent phenotypic differences between these two species, and their cellular fatty acid compositions are similar (Moss & Dees, 1978). Our results do not indicate that *Flavobacterium* sp. group IIb is heterogeneous, as suggested by Owen & Snell (1976) and Owen & Holmes (1981), perhaps because of the small size of our sample. The study of more strains from various origins, and including reference strains, might have led to another conclusion.

VFA analysis showed that *F. multivorum* (group H) was distinct from all other flavobacteria. Yabuuchi *et al.* (1983) reached the same conclusion, based on the presence of high concentrations of sphingolipids in the cellular lipid fraction. They placed *F. multivorum* in a new genus, *Sphingobacterium*, comprising *S. spiritivorum*, *S. multivorum* and *S. mizutae*.

In our study, *F. odoratum*, *F. meningosepticum* and *F. breve* showed differences from the accepted classification. The VFA analysis indicated three subgroups of *F. odoratum*. Although they considered this species as phenetically homogeneous, Owen & Holmes (1978) showed that there were two DNA homology groups of *F. odoratum* which could be distinguished phenotypically only by their colony morphology. Furthermore, Owen & Jackman (1983) showed, by means of electrophoretic protein patterns, that there were three distinct subgroups within *F. odoratum*, two of them corresponding to the DNA homology groups and a third

Fig. 2. Typical chromatographic profiles of five *Flavobacterium* species and the control (uninoculated medium). VFA: 1, acetic; 2, propionic; 3, isobutyric; 4, *n*-butyric; 5, isovaleric; 6, *n*-valeric; 7, isocaproic; 8, *n*-caproic.

intermediate group. It is not clear at present if the VFA groups correspond to the DNA homology groups.

Owen & Lapage (1974) described *F. meningosepticum* as a biochemically homogeneous taxon, whereas Sottile *et al.* (1973) considered it heterogeneous on the basis of DNA reassociation, and suggested that most strains did not belong to the same species. This was confirmed by Holmes (1983) who reported some heterogeneity within *F. meningosepticum*. Our results appear to corroborate this: *F. meningosepticum* strains fell into two separate groups, B (consisting of subgroups B₁ and B₂) and D. Group D included all the reference strains except the type strain NCTC 10016. Owen & Snell (1976) demonstrated that this strain was atypical on the basis of DNA reassociation. In our study, it clustered in the subgroup B₁ with lower similarity than all the other strains of this subgroup. All strains of group B except NCTC 10016 (serogroup A) and B 68800 (serogroup F) belonged to serogroup G. No biochemical tests allow differentiation of these groups.

Group G, comprising 8 of the 9 *F. breve* strains studied, appeared to be rather homogeneous. The reference strain CL 666/76 clustered with *F. odoratum* A 16987 in group X, though it has been described as closely related to *F. breve* by DNA reassociation (Owen & Holmes, 1980). We suggest two hypotheses to explain this. (1) This strain belonged to group G, which would imply that it is very widely scattered. This hypothesis would also be valid for *F. odoratum* group A which would be very scattered as well, there being no clear boundaries between the two groups. (2) This reference strain did not belong to group G, and although it was an authentic strain of *F. breve*, it belonged to another *F. breve* subgroup not examined in our study.

As regards group X, Fig. 3 shows that the centre of group G is the closest to strain CL 666/76 and that of group A to strain A 16987.

In conclusion, this study has demonstrated that satisfactory characterization of the five *Flavobacterium* species studied can be achieved by analysis of five VFA. Quantitative results for VFA analyses are usually highly variable, but by normalizing the values, a maximum of 10% variability for each acid was obtained. Our results corroborate those of other studies, especially those based on DNA analysis. An important advantage of VFA analysis is its simplicity and

Fig. 3. Two-dimensional representations on the first 3 principal axes of PCA: (a) on the 1st and 2nd principal axes, (b) on the 1st and 3rd principal axes. ○, *F. meningosepticum*; □, *F. odoratum*; ■, *F. breve*; ▲, *F. multivorum*; ●, centre of groups. Strains marked with an asterisk are reference strains.

Fig. 4. Dendrogram of hierarchical classification of the *Flavobacterium* strains. The strains in each cluster are shown in Table 1. *, NCTC 10016; †, B 49835.

speed. Once an organism has been assigned to the genus *Flavobacterium*, identification takes only 24 h (6 h starter culture, 18 h culture, 1 h extraction, 8 min chromatography), whereas

Table 3. Correlation coefficients, *r*, between variables and principal components

Variable	Principal component:		
	F ₁	F ₂	F ₃
Propionic acid	0.51	0.19	-0.84
Isobutyric acid	0.92	-0.06	0.40
<i>n</i> -Butyric acid	-0.09	0.90	0.13
Isovaleric acid	-0.99	-0.10	-0.09
<i>n</i> -Caproic acid	0.07	-0.90	-0.06

characterizing the various species by biochemical features takes 2 to 6 d. We are currently developing a new method for identifying unknown strains, which does not involve PCA.

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