*J. Gen. Appl. Microbiol.*, **34**, 57–66 (1988)

# TAXONOMIC SIGNIFICANCE OF HYDROXY FATTY ACIDS IN MYXOBACTERIA WITH SPECIAL REFERENCE TO 2-HYDROXY FATTY ACIDS IN PHOSPHOLIPIDS

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(Received November 30, 1987)

The cellular hydroxy fatty acid composition of myxobacteria was investigated. Strains tested included 7 strains of *Myxococcus* species and one strain of *Archangium*, together with other gliding bacteria, 2 strains of *Cytophaga* species, as reference bacteria. Myxobacteria commonly had 2-hydroxy and 3-hydroxy fatty acids as the hydroxy fatty acid components. The hydroxy fatty acid profile of myxobacteria was characterized as follows: 2-hydroxy-15-methylhexadecanoic acid (2-OH iso $C_{17:0}$) is the major hydroxy fatty acid component. In lipids extractable with chloroform/methanol (extractable lipids), 2-hydroxy fatty acids were the major hydroxy fatty acid components. In lipids non-extractable with chloroform/methanol (bound lipids), both 2-hydroxy and 3-hydroxy fatty acids appeared. This hydroxy fatty acid profile was different from that of *Cytophaga* species which commonly had 3-hydroxy-13-methyltetradecanoic acid (3-OH iso $C_{15:0}$) and 3-hydroxy-15-methylhexadecanoic acid (3-OH iso $C_{17:0}$) as the major hydroxy fatty acids, and these 3-hydroxy fatty acids occurred in both extractable and bound lipids. The myxobacteria were characterized also by phospholipid components in the extractable lipids. Phosphatidylethanolamine was the major phospholipid. It consisted of two types, one containing only non-hydroxy fatty acids and the other containing approximately 50% hydroxy fatty acids in the total fatty acids. The myxobacteria were characterized by the above hydroxy fatty acid profile.

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Myxobacteria are unique in producing well-differentiated forms like fruiting bodies (*1–3*). The vegetative cells are gram negative, and move by gliding. Due to the lack of identified cultures and difficulty in cultivation, microbiological and taxonomical studies have not been adequate.

In a previous paper (*4*), the isolation and classification of myxobacteria were reported, and 30 strains of myxobacteria were taxonomically characterized. Further, the genus *Myxococcus* was chemotaxonomically characterized as a rather homologous group on the basis of cellular fatty acid composition, quinone system and DNA base composition.

A hydroxy fatty acid profile has proven to be useful for differentiating certain gram negative bacteria (*5–10*). As hydroxy fatty acids, both 2-hydroxy and 3-hydroxy fatty acids were found as constituents of cells and their distribution varied depending on the types of microorganisms (*5, 6*). Therefore, such a profile has been shown to be also a tool for grouping of bacteria. For myxobacteria, cellular fatty acid profiles including hydroxy fatty acids have been described for only a small number of myxobacteria (*8,9,11*).

This communication deals with the determination of hydroxy fatty acids and their distribution in cells and phospholipids in characterizing myxobacteria.

## MATERIALS AND METHODS

*Microorganisms.* The microorganisms used were: *Myxococcus fulvus* No. 2, *Myxococcus stipitatus* No. 18, *Myxococcus flavescens* No. 38 (IAM 13189, JCM 6245$^T$) (*4*), *Myxococcus coralloides* No.44, *Archangium gephyra* No. 65, *Myxococcus virescens* ATCC 25203$^T$, *Myxococcus macrosporus* ATCC 29619, *Myxococcus xanthus* IFO 13542$^T$ (ATCC 25232), *Cytophaga johnsonae* KS 0404 (NCIB 10150), *Cytophaga flevensis* AJ 11632 (ATCC 27994). The two strains in the genus *Cytophaga* were used for comparison, because they are gliding bacteria like myxobacteria.

Abbreviations for culture collections: AJ, Central Research Laboratories, Ajinomoto Co., Kawasaki, Japan; ATCC, American Type Culture Collection, Rockville, Maryland, USA; IAM, Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan; IFO, Institute for Fermentation, Osaka, Japan; JCM, Japan Collection of Microorganisms, Wako, Japan; NCIB, National Collection of Industrial Bacteria, Aberdeen, U.K.

*Cultivation of microorganisms.* The medium used contained 20 g of Casitone (Difco Laboratories, Detroit, Michigan, USA), 1 g of $MgSO_4 \cdot 7H_2O$ and 1 *l* of distilled water (pH 7.2). Microorganisms were cultivated at 30°C for 7 days with shaking in 100 ml of the above medium n 500 ml flask.

*Extraction and analysis of lipids.* Lipids were extracted according to the BLIGH-DYER method (*12*). The lipids prepared by this method are called extractable lipids, and the non-extractable lipids are called bound lipids.

The extractable lipids were applied on the DEAE-Toyopearl (Toyo Soda

MFG. Co., Tokyo, Japan) column chromatography (1 cm × 5 cm). Phosphatidylethanolamine (PE) was eluted from the column using chloroform–methanol (1 : 4, v/v) as described by MURATA et al. (*13*). Standard phospholipids used for identification were purchased from Serdary Research Laboratories, Inc. (Ontario, Canada). PE was further separated on a silica gel G plate (Merck Co., 5721) using chloroform–acetone–methanol–acetic acid–water (10 : 4 : 2 : 2 : 1, v/v) as a developing solvent. Phospholipids were scraped off the silica gel plate with a spatula and fatty acids in the phospholipids were liberated and methylated with 5% HCl-methanol for fatty acid analysis.

Phospholipids on a silica gel plate were detected by various coloring reagents such as DITTMER's (*14*) and ninhydrin reagents, and were tentatively identified by *Rf* values. The PEs were further identified by the method of mild alkaline hydrolysis described by DAWSON (*15*). The deacylated products, glycerylphosphorylethanolamines, were identified by paper chromatography on Whatman No. 1 paper (Whatman, Ltd., England) in a solvent system of butanol–acetic acid–water (5 : 3 : 1, v/v) and visualized by the coloring reagents described above.

*Analysis of fatty acids.* The fatty acid composition of dried cells and lipids was analyzed according to the method described previously with a slight modification (*4*). Methyl esters of non-hydroxy fatty acids were analyzed with a Shimadzu gas chromatograph GC-9A on a fused silica capillary column (25 m × 0.33 mm) coated with Hi Cap CBP1 (Shimadzu Co., Kyoto, Japan). The column temperature was programmed from 170 to 230°C at 3°C/min. Methyl esters of hydroxy fatty acids were analyzed on a glass column packed with 5% Thermon 3000 coated on Shimalite W (AW-DMCS) (Shimadzu) at a column temperature of 180°C.

Hydroxy fatty acids were analyzed after separation by TLC on silica gel G plates (Merck 5721) using petroleum ether–ether (85 : 15, v/v) as a developing solvent. Fatty acid methyl esters were identified using GC-MS QP 1000 (Shimadzu). To analyze unsaturated fatty acids and branched fatty acids, pyrrolidide derivatives were prepared to determine the position of the double bond and methyl group, respectively (*16*, *17*).

Abbreviations for fatty acids are as follows: In the shorthand numbering systems used to identify fatty acids, the figures preceding the colon indicates the number of carbon atoms in the fatty acids, while that following the colon represents the number of double bonds present. The prefix OH indicates a hydroxy group at the position indicated.

## RESULTS

### *Total cellular fatty acid composition*

Table 1 shows total cellular fatty acids of 8 strains (each belonging to the different species) of myxobacteria (*Myxococcus* and *Archangium*) and two strains of *Cytophaga*. As a whole, non-hydroxy fatty acids constituted more than 90% and hydroxy fatty acids less than 10%. As is characteristic of the myxobacteria, the

Table 1. Cellular fatty acid composition of myxobacteria

| Microorganism | Fatty Non-hydroxy acid | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | iso $C_{13:0}$ | iso $C_{14:0}$ | $C_{14:0}$ | iso $C_{15:0}$ | anteiso $C_{15:0}$ | $C_{15:0}$ | iso $C_{16:0}$ | $C_{16:1}$ | $C_{16:0}$ |
| *M. fulvus* No. 2 | — | 3.6[a] | — | 21.4 | — | 1.0 | 2.2 | 38.5 | 10.6 |
| *M. stipitatus* No. 18 | — | 4.3 | 1.1 | 26.2 | — | 1.1 | 2.9 | 29.3 | 3.6 |
| *M. flavescens* No. 38 | — | 3.2 | 1.1 | 35.6 | — | 5.7 | 3.5 | 18.1 | 3.2 |
| *M. coralloides* No. 44 | — | 1.3 | 3.4 | 29.7 | — | 1.1 | 1.1 | 19.6 | 13.0 |
| *A. gephyra* No. 65 | — | 2.3 | 0.9 | 10.0 | — | 1.4 | 1.7 | 21.2 | 20.4 |
| *M. virescens* ATCC 25203 | 2.6 | 4.8 | 3.8 | 19.0 | — | 3.8 | 3.1 | 21.5 | 14.2 |
| *M. macrosporus* ATCC 29619 | — | 4.3 | — | 21.9 | — | — | 2.8 | 31.3 | 9.6 |
| *M. xanthus* IFO 13542 | 0.5 | 7.0 | 1.9 | 45.6 | — | 0.9 | 4.4 | 16.8 | 3.6 |
| *C. johnsonae* KS 0404 | — | — | 1.3 | 32.4 | 16.2 | 12.2 | — | 13.5 | 8.5 |
| *C. flevensis* AJ 11632 | — | — | 1.5 | 15.2 | 18.0 | 7.3 | — | 34.7 | 6.3 |

[a] The number refers to the acid as a percentage of the total fatty acids.

principal fatty acids of *Myxococcus* were 13-methyltetradecanoic acid (iso $C_{15:0}$), 15-methylhexadecanoic acid (iso $C_{17:0}$) and *n*-hexadecenoic acid ($C_{16:1}$, palmitoleic acid), and *Archangium gephyra* had in addition a large amount of 10-methyloctadecanoic acid (10-methyl $C_{19:0}$).

Of the hydroxy fatty acids, both 2-hydroxy and 3-hydroxy fatty acids were detected. The mass spectra of 2-hydroxy-15-methylhexadecanoic acid methyl ester (2-OH iso $C_{17:0}$) and 3-hydroxy-15-methylhexadecanoic acid methyl ester (3-OH iso $C_{17:0}$) are shown in Figs. 1 and 2. The molecular weight of 2-hydroxy fatty acid was determined by a molecular peak ($M^+ = 300$) and 3-hydroxy fatty acid was deduced from fragment peaks at $M^+ - 18$, $M^+ - 50$ (each corresponding to the loss of water and methanol-water). The high intensity mass fragment of the 2-hydroxy fatty acid at $m/z = 241$ ($M^+ - 59$), due to 1,2-cleavage, is typical for 2-hydroxy fatty acids compared with a low intensity of mass peak at $m/z = 103$, due to 3,4-cleavage. A characteristic fragment peak in the 3-hydroxy fatty acid at $m/z = 103$ appeared as a base peak. The detailed structures of these fatty acids with regards to the branching were determined by comparing their mass spectra with reported spectral data (*18*, *19*).

The 2-hydroxy fatty acids were the major hydroxy fatty acids and the 3-hydroxy fatty acids comprised less than about 1% of the total fatty acids. 2-OH iso $C_{17:0}$ was predominant in all of the strains tested. In *Cytophaga* the major fatty acids detected were iso $C_{15:0}$, $C_{16:1}$ and 12-methyl-tetradecanoic acid (anteiso $C_{15:0}$), and 3-hydroxy fatty acids were found as the major hydroxy fatty acids. Myxobacteria were differentiated from *Cytophaga* by the absence of anteiso $C_{15:0}$ and the presence of 2-OH iso $C_{17:0}$.

tested (Including hydroxy fatty acid).

| acid | | | | | | 2-OH | | | 3-OH | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| iso $C_{17:1}$ | $C_{17:1}$ | iso $C_{17:0}$ | $C_{17:0}$ | $C_{18:0}$ | 10-methyl $C_{19:0}$ | iso $C_{15:0}$ | $C_{16:0}$ | iso $C_{17:0}$ | $C_{14:0}$ | iso $C_{15:0}$ | $C_{16:0}$ | iso $C_{17:0}$ |
| — | 7.6 | 9.5 | — | — | — | 0.2 | 0.3 | 4.6 | — | 0.4 | — | 0.2 |
| — | 15.3 | 8.5 | — | 0.9 | — | 0.2 | 0.1 | 5.0 | — | 0.4 | — | 0.4 |
| — | 8.4 | 16.2 | 0.6 | — | — | — | 0.2 | 1.8 | — | 0.6 | — | 0.6 |
| — | 5.6 | 18.9 | — | 0.9 | — | — | — | 5.0 | — | — | — | 0.2 |
| — | 3.1 | 4.3 | 1.6 | 1.2 | 29.5 | — | — | 1.3 | — | 0.7 | — | — |
| — | 3.5 | 21.7 | — | — | — | 0.2 | — | 1.3 | — | 0.2 | — | 0.2 |
| — | 6.2 | 15.4 | — | — | — | — | — | 9.8 | — | — | — | — |
| — | 4.6 | 10.8 | — | 1.7 | — | — | — | 1.6 | — | — | — | — |
| 1.6 | 2.2 | — | — | — | — | 1.1 | — | — | — | 3.7 | 2.6 | 4.7 |
| 3.3 | 6.0 | — | — | — | — | — | — | — | — | 3.0 | 1.6 | 3.1 |

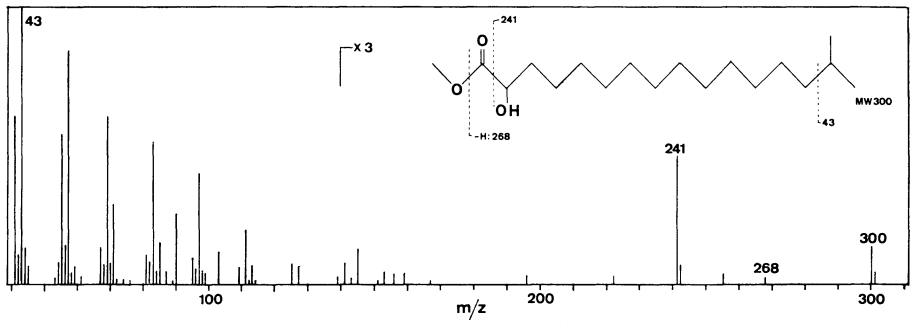


Fig. 1. Mass spectrum of methyl 2-hydroxy-15-methylhexadecanoate of *Myxococcus fulvus* No. 2.

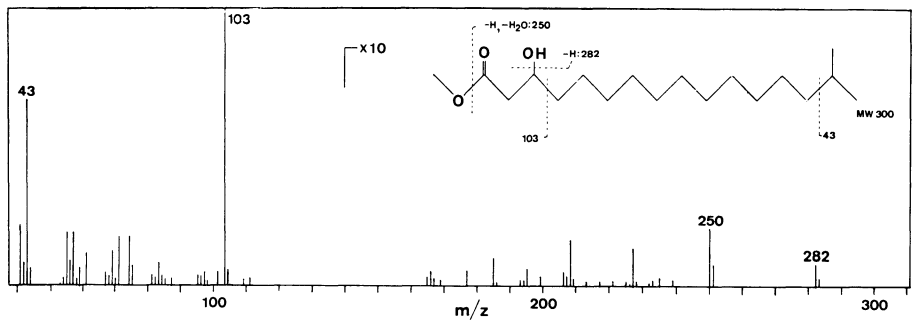


Fig. 2. Mass spectrum of methyl 3-hydroxy-15-methylhexadecanoate of *Myxococcus fulvus* No. 2.

Table 2. Distribution of hydroxy acids between extractable lipid fraction and bound lipid fraction.

| Microorganism | | Fatty acid | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 2-OH | | | 3-OH | | | |
| | | iso $C_{15:0}$ | $C_{16:0}$ | iso $C_{17:0}$ | $C_{14:0}$ | iso $C_{15:0}$ | $C_{16:0}$ | iso $C_{17:0}$ |
| *M. fulvus* No.2 | A[a] | 4.9[c] | 7.3 | 86.1 | — | — | — | 1.6 |
| | B[b] | 2.7 | 3.8 | 25.9 | 1.4 | 17.1 | 8.1 | 41.1 |
| *M. stipitatus* No. 18 | A | 4.0 | 2.4 | 92.7 | — | 0.8 | — | — |
| | B | 2.3 | 0.8 | 31.5 | — | 23.5 | 5.3 | 36.8 |
| *M. flavescens* No. 38 | A | 6.3 | 6.3 | 81.3 | — | 4.2 | — | 2.1 |
| | B | 5.5 | 4.6 | 41.3 | — | 15.6 | — | 33.0 |
| *M. coralloides* No. 44 | A | — | 47.1 | 52.9 | — | — | — | — |
| | B | — | — | 72.6 | — | — | — | 27.4 |
| *A. gephyra* No. 65 | A | — | — | 80.3 | — | 11.5 | — | 8.2 |
| | B | — | — | 16.7 | — | 38.9 | — | 44.4 |
| *M. virescens* ATCC 25203 | A | 13.6 | — | 72.7 | — | — | — | 13.6 |
| | B | 2.9 | 1.5 | 38.2 | 2.9 | 19.1 | — | 35.3 |
| *M. macrosporus* ATCC 29619 | A | 3.1 | 5.6 | 88.1 | — | 1.6 | — | 1.6 |
| | B | 3.0 | 3.2 | 59.1 | — | — | — | 34.7 |
| *M. xanthus* IFO 13542 | A | — | 14.0 | 83.7 | — | — | — | 2.3 |
| | B | — | — | 41.6 | — | 14.1 | — | 44.3 |
| *C. johnsonae* KS 0404 | A | 17.3 | — | — | — | 13.5 | 25.6 | 43.6 |
| | B | 3.6 | — | — | — | 10.4 | 19.3 | 66.8 |
| *C. flevensis* AJ11632 | A | — | — | — | 8.1 | 27.9 | 24.4 | 39.5 |
| | B | — | — | — | 2.0 | 31.9 | 14.9 | 51.2 |

[a] Extractable lipid fraction.
[b] Bound lipid fraction.
[c] The numbers refer to the acid as a percentage of the total hydroxy fatty acids in the fraction.

*Distribution of hydroxy fatty acids in extractable lipids and bound lipids*

Table 2 shows the hydroxy fatty acid profile of the myxobacteria strains. 2-OH iso $C_{17:0}$ was the predominant extractable lipid in all of the strains tested. 2-hydroxy fatty acids and 3-hydroxy fatty acids were the bound lipids detected, and 2-OH iso $C_{17:0}$, 3-OH iso $C_{15:0}$ and 3-OH iso $C_{17:0}$ were the major hydroxy fatty acids.

In *Cytophaga* 3-hydroxy fatty acids were observed in both extractable and bound lipids, and 3-OH iso $C_{17:0}$ was the most abundant.

*Analysis of phospholipids*

Of the extractable lipids, PE was the major phospholipid in all the strains of

Table 3. Fatty acid composition of two types of phosphatidylethanolamines in extractable lipids with special reference to hydroxy fatty acids.

| Microorganism | | Fatty acid | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Non-hydroxy acid[c] | 2-OH | | | 3-OH | | |
| | | | iso $C_{15:0}$ | $C_{16:0}$ | iso $C_{17:0}$ | $C_{14:0}$ | iso $C_{17:0}$ |
| *M. fulvus* No. 2 | PE-1[a] | 98.4[d] | — | — | — | — | — |
| | PE-2[b] | 31.4 | — | — | 63.7 | — | — |
| *M. stipitatus* No. 18 | PE-1 | 98.8 | — | — | — | — | — |
| | PE-2 | 42.9 | — | 3.9 | 53.3 | — | — |
| *M. flavescens* No. 38 | PE-1 | 100.0 | — | — | — | — | — |
| | PE-2 | 33.2 | — | 13.5 | 53.3 | — | — |
| *M. coralloides* No. 44 | PE-1 | 100.0 | — | — | — | — | — |
| | PE-2 | 51.3 | — | 3.8 | 44.9 | — | — |
| *A. gephyra* No. 65 | PE-1 | 97.3 | — | — | — | — | — |
| | PE-2 | 41.1 | — | 5.8 | 47.2 | 5.8 | — |
| *M. virescens* ATCC 25203 | PE-1 | 95.7 | — | — | — | — | — |
| | PE-2 | 72.5 | — | 10.2 | 12.8 | 4.5 | — |
| *M. macrosporus* ATCC 29619 | PE-1 | 96.9 | — | — | — | — | — |
| | PE-2 | 39.4 | — | 18.0 | 42.8 | — | — |
| *M. xanthus* IFO 13542 | PE-1 | 98.5 | — | — | — | — | — |
| | PE-2 | 32.6 | — | 11.5 | 55.9 | — | — |

[a,b] Two types of phosphatidylethanolamine.
[c] The sum of non-hydroxy fatty acids.
[d] The number refers to the acid as a percentage of the total fatty acids.

myxobacteria tested. Phosphatidylglycerol (PG) and cardiolipin (CL) were also detected, but only in small amounts. The PE eluted from the DEAE-Toyopearl column was further separated into two compounds on TLC as detected by DITTMER's reagent. One, PE-1, had an *Rf* value of 0.64 (corresponding to the standard PE) and the other, PE-2, 0.38. Both were stained by ninhydrin, and the alkaline hydrolyzate of these lipids gave only one spot which had the same *Rf* value (0.21) as the hydrolyzate of the standard PE. From these observations, both compounds were identified as PE.

The fatty acid composition of these PEs was investigated in detail. PE-1 consisted only of non-hydroxy fatty acids and PE-2 contained more than 50% hydroxy fatty acids (the major hydroxy fatty acid was 2-OH iso $C_{17:0}$), as shown in Table 3.

## DISCUSSION

The major fatty acids in strains of *Myxococcus* were iso $C_{15:0}$, iso $C_{17:0}$ and $C_{16:1}$. These data are in line with published data (*4*, *20*, *21*), except the report that the strains of *Myxococcus xanthus* had anteiso $C_{15:0}$, anteiso $C_{17:0}$ and $C_{16:1}$ as the major fatty acid species (*22*).

*Archangium gephyra* showed a little different fatty acid profile (Table 1) in having 10-methyl $C_{19:0}$ in a large amount. Further investigation including usage of other strains of *Archangium* is necessary for the taxonomic application of these results.

MONTEOLIVA-SANCHEZ et al. (*23*) reported that *Corallococcus coralloides* could be differentiated from the strains of *Myxococcus* by the fatty acid pattern. Fatty acids of 17:0 branched and straight chain acids, which were present in species of *Myxococcus*, were not detected in all of the strains of *Corallococcus coralloides* tested. *Corallococcus coralloides* was proposed by REICHENBACH and DWORKIN (*3*) who insisted that *Myxococcus coralloides* should be transferred from the genus *Myxococcus* to a new genus *Corallococcus* on the basis of characteristics of fruiting bodies. Our data for *Myxococcus coralloides* No. 44, however, were not in accord with these results because it also had iso $C_{17:0}$ in appreciable amounts. Further studies are needed to clarify these points.

Hydroxy fatty acids were detected in the myxobacteria tested, and 2-OH iso $C_{17:0}$ was dominant among them. The hydroxy fatty acid profiles have been proven to be useful for chemotaxonomy of bacteria such as *Pseudomonas* (*6*, *10*), *Flavobacterium-Cytophaga* complex (*7*), gliding bacteria (*8*, *9*) and various gram negative bacteria (*5*). Species of *Pseudomonas* were reported to be divided into various groups according to the fatty acid composition and the ubiquinone system, with special reference to the existence of 3-hydroxy fatty acids (*10*). YANO et al. (*5*) studied the hydroxy fatty acid profiles of the species of *Enterobacteriaceae* and *Vibrionaceae*. Also they reported those profiles in *Pseudomonas*, *Xanthomonas*, *Achromobacter*, *Alcaligenes* and *Acetobacter*. From these results they emphasized the taxonomic significance of hydroxy fatty acid composition in gram negative bacteria. In myxobacteria the data on hydroxy acid composition were fragmentary. FAUTZ et al. (*8*, *9*) reported that strains of *Myxococcus fulvus*, *Stigmatella aurantiaca*, *Cystobacter velatus* had 2-OH iso $C_{17:0}$ as the major hydroxy fatty acid, but *Nannocystis exedens* and *Sorangium cellulosum* did not contain hydroxy fatty acids.

All of the myxobacteria we tested contained 2-hydroxy and 3-hydroxy fatty acids, and 2-OH iso $C_{17:0}$ as the major hydroxy fatty acid. These results suggest that myxobacteria are uniform in this point.

The extractable lipids contain mainly membrane phospholipid, and bound lipids contain mainly lipopolysaccharide and lipoprotein from the outermembrane of gram negative bacteria (*5*). ROSENFELDER et al. (*11*) demonstrated that 3-hydroxy fatty acids are bound to lipopolysaccharide in *Myxococcus fulvus*.

Myxobacteria could be characterized by the distribution of hydroxy fatty acids

as follows: Extractable lipids were composed of 2-hydroxy fatty acids as the hydroxy fatty acid component and bound lipids were composed of both 2 and 3-hydroxy fatty acids.

In the extractable lipid of myxobacteria, two types of PE were present. PE-2 was unique in being composed of about 50% 2-OH iso $C_{17:0}$ as the fatty acid component. In *Myxococcus virescence*, the 2-OH iso $C_{17:0}$ content was much lower than that of others, probably because the PE 2 was replaced by other types of lipids (Table 3). The phospholipid type in myxobacteria is reported for the first time in this paper and myxobacteria was found to be distinguished by the presence of a particular phospholipid containing about 50% 2-OH iso $C_{17:0}$.

In gram negative bacteria PE was described as a wide spread phospholipid, while in gram positive bacteria the presence of PE was reported in strains of only a few genera such as *Corynebacterium* (*24*), *Nocardia* (*24*,*25*) and *Mycobacterium* (*24*). In *Nocardia* (*25*), two types of PE were found, one composed only of 2-hydroxy fatty acids in the 2-position and non-hydroxy fatty acids in the 1-position. The other was composed of non-hydroxy fatty acids only. The presence of such a type of phospholipid, rich in hydroxy fatty acid in myxobacteria is interesting in that a similar type has been reported only in bacteria apparently distantly related to myxobacteria.

The authors are indebted to Miss E. Kenjo for her assistance in the analysis of lipids and Dr. R. Tsugawa of the Central Research Laboratories, Ajinomoto Co., for his encouragement during this work.

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