J. Biochem. **83**, 1213–1216 (1978)

Isolation of a Novel Sphingoglycolipid Containing Glucuronic Acid and 2-Hydroxy Fatty Acid from *Flavobacterium devorans* ATCC 10829

Akihiro YAMAMOTO,* Ikuya YANO,* Masamiki MASUI,* and Eiko YABUUCHI**

*Department of Bacteriology, Osaka City University Medical School, Asahimachi, Abeno-ku, Osaka, Osaka 545, and **Department of Microbiology, Kansai Medical University, Fumizono-cho, Moriguchi, Osaka 570

Received for publication, January 20, 1978

A new acidic sphingoglycolipid has been isolated from a Gram-negative, glucose-non-fermentative (obligatory aerobic) bacterium, *Flavobacterium devorans* ATCC 10829, by thin-layer chromatography on silica gel after mild alkaline hydrolysis of the cellular lipids. Chemical degradation studies, thin-layer chromatographic behavior, IR and mass-spectrometric analysis of the original and reduced glycolipid with $LiAlH_4$ revealed that the lipid contained glucuronic acid, long-chain bases, and fatty acids in a molar ratio of approximately 1:1:1. The major long-chain bases were identified by gas chromatography-mass spectrometry as dihydrosphingosine (d-18 : 0) and longer homologues, while the N-acyl group was exclusively 2-hydroxy myristic acid. The most probable structure of this glycolipid appeared to be a ceramide glucuronic acid (N-acyl dihydrosphingosine 1-glucuronic acid).

Sphingolipids are one of the most important components of biological membranes in higher animals and plants. In contrast, the occurrence of sphingolipids in bacteria is exceedingly rare (*1–7*), and hitherto, the existence of sphingoglycolipid has not been reported in bacteria yet. During a recent survey of cellular lipids of opportunistic pathogens, we found a significant amount of alkaline stable glycolipid(s) was present in several strains of Gram-negative non-fermentative (obligatory aerobic) bacteria. The present paper reports the isolation and tentative characterization of a very unusual sphingoglycolipid containing hexuronic acid from *F. devorans* and the clinical isolation of related strains.

F. devorans ATCC 10829 KM 1367 and clinically isolated strains KM 2135, KM 2266, KM 2137, KM 2406, and KM 2256 were grown aerobically in a medium containing 1% glucose, 0.5% polypeptone, and 0.2% yeast extract, with an initial pH of 7.0 on a shaker at 30°C for 20 h. Cells were harvested by centrifugation and lipids extracted with 20 vol. of chloroform-methanol (2:1, by vol) mixture. After the non-lipid materials were removed, the total extractable lipids were condensed and separated on a thin-layer plate of Silica Gel G (Merck) with solvent system (A), chloroform-methanol-water (65:25:4, by vol) or (B), chloroform-methanol-acetic acid-water (100:20:12:5, by vol.). A part of the total lipids was hydrolyzed

with 0.5 N-KOH in methanol for 5 to 6 h at 30°C, followed by neutralization with 1 N-HCl and extracted again with chloroform-methanol (2:1, by vol.). As shown in Fig. 1, the original (total) lipids gave at least 4 spots on TLC, while the alkaline stable polar lipid gave a single major spot with the two solvent systems.

On the other hand, alkaline stable lipids were separated by DEAE-cellulose (acetate form) column chromatography (*8*) and acidic lipids were eluted with a solvent of chloroform-methanol (2:1, v/v) saturated with ammonium hydroxide. The alkaline stable glycolipid thus obtained was purified until a single spot was obtained. This lipid reacted with anthrone reagents to give a brownish-purple color, but did not react with ninhydrin or molybdenum blue reagent. In a neutral solvent system (A), this lipid showed an elongated spot resembling anionic phospholipids, while in an acidic solvent system (b), the spot was round, suggesting the compound had an anionic charge. As shown in Fig. 2, the IR spectra of this lipid showed deep absorption bands at 3400-3500 cm^{-1}, due to the hydroxyl group, 1600 and 1440 cm^{-1} associated with the carboxylate ion, and a strong absorption band at 1650 and 1560 cm^{-1} due to an amide group(s). After acid methanolysis with conc. HCl-methanol (1:5, by vol.) at 85°C for 3 h, the fatty acid methyl esters and a ninhydrin positive long-chain compound possessing the same R_f value as dihydrosphingosine on TLC were obtained. Thin-layer chromatography of the fatty acid methyl

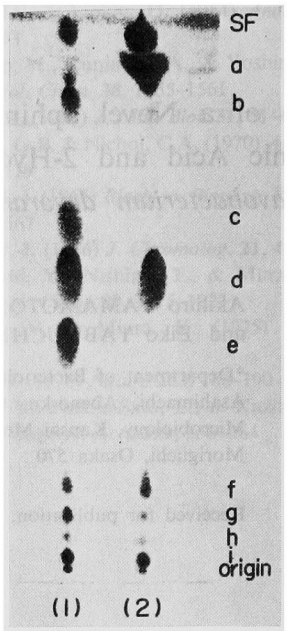

Fig. 1. Thin-layer chromatography of original and alkaline stable lipid from *F. devorans* ATCC 10829. (1) Original lipid. (2) Lipid obtained after alkaline hydrolysis (0.5 N-KOH methanol–chloroform (1 : 1, by vol.) for 6 h at room temperature). a: fatty acids, b: unknown lipid, c: unknown phospholipid, d: unknown glycolipid (alkaline stable), e: unknown phospholipid, f, g, h, i: unknown. TLC solvent: chloroform–methanol–acetic acid–water (100 : 20 : 12 : 5, by vol.).

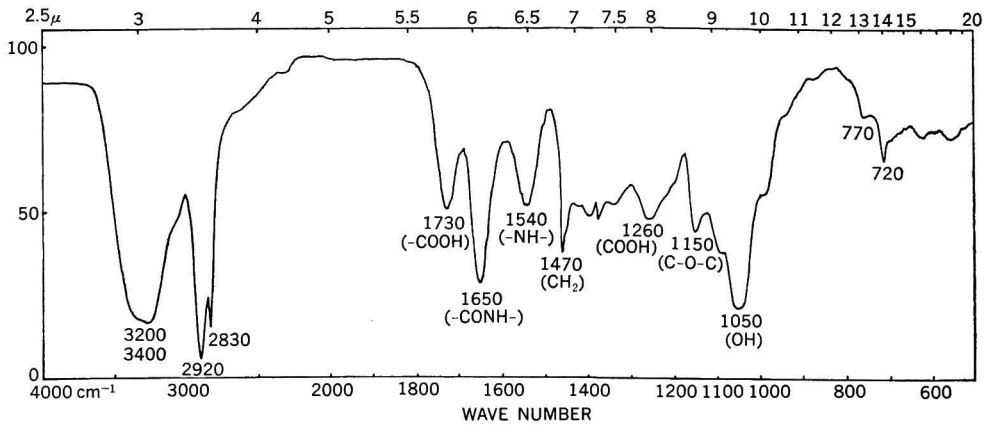

Fig. 2. Infrared spectrum of the sphingoglycolipid containing glucuronic acid from *F. devorans* ATCC 10829, pressed in KBr.

esters with a solvent system of hexane-ether (4:1, by vol.) gave almost only a single spot corresponding to 2-hydroxy fatty acid methyl ester. Furthermore, gas-chromatography of the fatty acid methyl esters gave again almost only a single peak with the same retention time as 2-hydroxy myristic acid methyl ester. This was confirmed by gas chromatographic and mass spectrometric analysis (JMS D-100 apparatus). On the other hand, the gas chromatogram of the trimethylsilyl ether derivatives of long-chain bases showed 2 or more peaks on a 1% OV-1 column, one of which, the most prominent peak coincided completely with that of dihydrosphingosine. The mass spectra of both compounds were identical with each other. Furthermore, periodate oxidation of the long-chain bases obtained from the glycolipid yielded aldehydes having an identical retention time as hexadecanal obtained from standard dihydrosphingosine. Therefore, the major long-chain base components were identified as dihydrosphingosine and a lesser amount of long-chain homologues. On the other hand, the hexane insoluble residues of the acid methanolysate were analyzed for the presence of sugars. As shown in Fig. 3, gas chromatography on a 2% OV-1 column of trimethylsilylether derivatives of the methanolysate showed the presence of peaks coinciding with the authentic trimethylsilylether derivative of methyl (D) glucuronide methyl ester. This was confirmed by gas chromatography-mass spectrometry. These results accorded with the initial observation on TLC, that the original lipid may possess an anionic charge in the molecule. To confirm this surmise, purified glycolipid was reduced with $LiALH_4$ in diethylether. After complete reduction, the glycolipid was extracted with chloroform-methanol (2:1, by vol.) and developed on TLC. In both solvent systems (A) and (B), the spot of original alkaline stable glycolipid disappeared and instead, a spot migrating close to the authentic ceramide monohexoside appeared. The reduced glycolipid reacted with anthrone reagent to give the typical reddish-purple color for neutral glycolipids. The reduced glycolipid was also methanolyzed with HCl-methanol and the resultant methyl glycoside(s) was trimethylsilylated. Gas chromatography of TMS-methylglycoside from reduced glycolipid showed the presence of TMS-methylglucoside with the concomitant disappearance of TMS-methyl glucuronide methylester. Therefore, the carbohydrate moiety of the original glycolipid was confirmed to be glucuronic acid. The lipid nitrogen contents were determined by the method of Sloane-Stanley (*9*) and uronic acids were estimated using the modified carbazole reaction (*10*). The concentrations of fatty acids were measured by gas-liquid chromatography using methyl 2-hydroxy palmitate as internal standard. The results showed a molar ratio of glucuronic acid: fatty acid: long-chain bases of 1:1:1, for this glycolipid. It was also suggested from the IR spectrum and the tendency of the parent lipid to run on TLC as a streak, that the carboxyl group of the glucuronic acid was not involved in a covalent linkage.

From the results obtained above, the alkaline stable glycolipid obtained from *F. devorans* appeared to most likely be a ceramide glucuronic acid (N-2′-hydroxy myristoyl dihydrosphingosine 1-glucuronic acid).

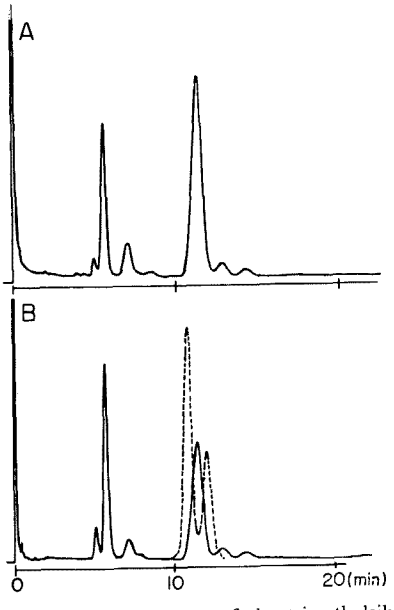

Fig. 3. Gas chromatograms of the trimethylsilylether derivatives of methyl glucuronide methylester from alkaline stable glycolipid in *F. devorans* ATCC 10829. (A) Trimethylsilylether derivative of methylglycoside from *F. devorans* ATCC 10829. (B) Trimethylsilylether derivative of authentic methyl glucuronide methylester (dotted line: trimethylsilylether derivative of authentic methylglucoside).

In gram-negative bacteria, the presence of diacyl glycerol-type glycolipids containing hexuronic acid has already been reported (*11–13*). However, the occurrence of sphingoglycolipid containing glucuronic acid as the carbohydrate moiety has not been reported so far for procaryotic and eucaryotic cells. To our knowledge, this is the first report describing the occurrence of such a type of acidic sphingoglycolipids. Detailed structural analysis and biosynthetical studies involving α-oxidation of fatty acids are now in progress.

REFERENCES

1. LaBach, J.P. & White, D.C. (1969) *J. Lipid Res.* **10**, 528–534
2. Rizza, V., Tucker, A.N., & White, D.C. (1970) *J. Bacteriol.* **101**, 84–91
3. Kunsman, J.E. (1973) *J. Bacteriol.* **113**, 1121–1126
4. Tourtellote, M.E., Jensen, R.G., Gander, G.W., & Morowitz, H.J. (1963) *J. Bacteriol.* **86**, 370–379
5. Plackett, P., Smith, P.F., & Mayberry, W.R. (1970) *J. Bacteriol.* **104**, 798–807
6. Steiner, S., Conti, S.F., & Lester, R.L. (1973) *J. Bacteriol.* **116**, 1199–1211
7. Stoffel, W., Dittmar, K., & Wilmes, R. (1975) *Hoppe-Seyler's Z. Physiol. Chem.* **356**, 715–725
8. Rouser, G., Kritchevsky, G., & Yamamoto, A. (1967) *Lipid Chromatographic Analysis* Vol. 1, pp. 99–162, Marcell Dekker, New York
9. Sloane-Stanley, G.H. (1967) *Biochem. J.* **104**, 293–295
10. Bitter, T. & Muir, H.M. (1962) *Anal. Biochem.* **4**, 330–334
11. Wilkinson, S.G. (1968) *Biochim. Biophys. Acta* **152**, 227–229
12. Wilkinson, S.G. (1968) *Biochim. Biophys. Acta* **164**, 148–156
13. Wilkinson, S.G. (1969) *Biochim. Biophys. Acta* **187**, 492–500