Short Communication

J. Gen. Appl. Microbiol., **17**, 329–334 (1971)

OCCURRENCE OF ACYLATED TREHALOSES IN *NOCARDIA*

IKUYA YANO, YOSHIYA FURUKAWA AND MASAMICHI KUSUNOSE

Laboratory of Biochemistry, Toneyama Hospital, National Sanatorium, Toyonaka, and Toneyama Institute for Tuberculosis Research, Osaka City University Medical School, Toyonaka, Osaka 560

(Received April 16, 1971)

"Cord factor", a toxic glycolipid of *Mycobacterium tuberculosis* discovered by BLOCH (*1*) in 1950, was identified as trehalose 6, 6′-dimycolate by NOLL *et al.* (*2*). This glycolipid was found to be present in many strains of mycobacteria and some strains of corynebacteria (*3*). We have recently found that the solvent-extractable lipids from nocardia also contained some anthrone-positive glycolipids consisting of trehalose and fatty acids, one of which was very similar to the cord factor (*4*). The present paper describes that cultivation of nocardia in glucose-rich media produced a great increase in the content of trehalose-containing lipids. During this work was in progress, IONEDA *et al.* (*5*) isolated the cord factor from *Nocardia asteroides* and *N. rhodochrous*, and established its structure mainly by mass spectrometry.

Several strains of nocardia were kindly supplied by Dr. M. Mayama, Shionogi Research Laboratories, Osaka, and Prof. S. Fukui, Department of Industrial Chemistry, Kyoto University, Kyoto. The medium contained 1% "Polypeptone" (Daigo-eiyo Chemical Co., Osaka), 0.5% yeast extract (Nakarai Chemical Co., Kyoto), and various amounts of glucose or glycerol; the pH was adjusted to 7.0. The cells were incubated at 30° for 24–110 hr on a rotary shaker. After the cells were harvested, lipids were extracted with 10 volume of chloroform-methanol (2 : 1, by vol.) and washed by the procedure of FOLCH *et al.* (*6*). The lipid extracts were applied on a thin-layer plate (0.5 mm thick) of Silica Gel H (Merck). The plate was developed with a solvent system of chloroform-methanol (90 : 10, by vol.) (Solvent A) or chloroform-methanol-acetone (90 : 10 : 5, by vol.) (Solvent B). For the two-dimensional development, chloroform-methanol-28% ammonia (65 : 35 : 5, by vol.) was employed as the first solvent, and chloroform-acetone-methanol-acetic acid-water (200 : 30 : 15 : 15 : 7, by vol.), as the second solvent. For the purpose of preparation, glycolipids were located by spraying with anthrone

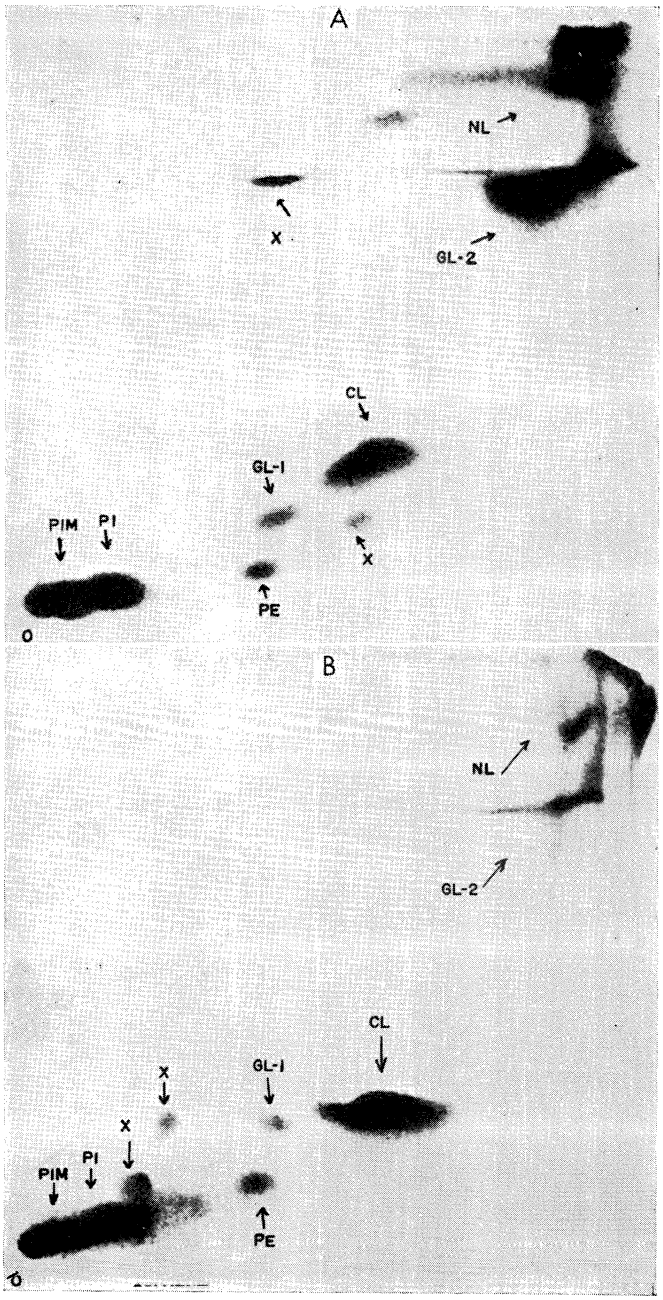

Fig. 1

reagent, and individual components were recovered from the plate, and purified by rechromatography. After alkaline hydrolysis, deacylated products of glycolipids were analyzed by paper chromatography in a solvent system of butanol-pyridine-water (6 : 4 : 3, by vol.) or by thin-layer chromatography in a solvent of methyl ethyl ketone-acetic acid-water (6 : 2 : 2, by vol.). Acid hydrolysis of glycolipids was achieved with 5% HCl in dry methanol at a reflux temperature overnight, and the products were separated into two phases. The water-soluble fraction was analyzed by paper, thin-layer, or gas-liquid chromatography. For gas-liquid chromatography, trimethylsilyl derivatives were prepared (*7*), and the column packed with 1% OV-17 or 3% SE-30 was operated at 150° with a flow rate of 30 ml/min. The ether-soluble fraction was analyzed by thin-layer chromatography, mass spectrometry, or gas-liquid chromatography after pyrolysis.

The lipids of the cells of *N. asteroides* grown with glucose or glycerol were separated by two-dimensional thin-layer chromatography as shown in Fig. 1. Both cells had in common three major phospholipids consisting of cardiolipin, phosphatidylinositol, and phosphatidylinositol monomannoside with phosphatidylethanolamine as a minor phospholipid component. Such pattern of phospholipids was consistent with that of the phospholipids in *N. polychromogenes* as reported earlier (*8*). On the other hand, there was a striking difference in the content of glycolipids between the two kinds of cells. The cells grown with glucose contained a large amount of compound GL-2, the Rf value of which coincided with that of the cord factor from *Mycobacterium tuberculosis*. This was in marked contrast to the glycolipids in the cells grown with glycerol. In the latter cells, glycolipids were detected only as a minor lipid component. Table 1 shows the quantitative comparison of the lipids in the two kinds of cells. In the cells grown with glucose, the glycolipids accounted for 20% of the total lipids. Similar findings were observed in *N. polychromogenes*, *N. erythropolis*, *N. corallina*, *N. rubra*, and *N. eppingerii*.

Mild alkaline hydrolysis of compound GL-2 gave one product with the same Rf value as trehalose on paper and thin-layer chromatograms. Acid

Fig. 1. Two-dimensional thin-layer chromatograms of the solvent-extractable lipids from *N. asteroides*.

Developed with chloroform-methanol-ammonia (35 : 35 : 5, by vol.) in the first dimension (from left to right) and with chloroform-acetone-methanol-acetic acid-water (200 : 30 : 15 : 15 : 7, by vol.) in the second dimension (vertical dimension). (A) The cells grown on a medium containing 1% polypeptone, 0.5% yeast extract, and 3% glucose for 3 days. (B) The cells grown on a medium containing 1% "Polypeptone," 0.5% yeast extract, and 3% glycerol for 3 days. PI, phosphatidylinositol; PIM, phosphatidylinositol monomannoside; X, unidentified compounds; PE, phosphatidylethanolamine; GL-1, compound GL-1; CL, cardiolipin; GL-2, compound GL-2; NL, neutral lipids (pigments, glycerides, free mycolic acids and free non-polar fatty acids).

Table 1. Lipid composition of *N. asteroides*.

Cells were grown on a medium containing 1% "Polypeptone," 0.5% yeast extract, and 3% glucose or 3% glycerol for 4 days. Lipid extracts were separated on a thin-layer plate with Solvent B. Total phospholipid contained cardiolipin and more polar phospholipid components, total glycolipid consisted largely of compound GL-2, and total neutral lipid contained pigments, glycerides, free mycolic acids, and free nonpolar fatty acids. Values are expressed as weight percentages of total lipid.

	Medium containing	
	3% glucose	3% glycerol
Total phospholipid (%)	58.2	92.4
Total glycolipid (%)	20.1	1.2
Total neutral lipid (%)	21.7	6.4

hydrolysis produced one reducing sugar agreeing with D-glucose. Gas-liquid chromatography of its trimethylsilyl derivative also showed the peaks coinciding with those of glucose derivative. The ether-soluble fraction from acid hydrolysis exhibited a chromatographic behavior very similar to that of mycolic acid. The molar ratio of acyl group to trehalose was approximately 2. Moreover, a preliminary work revealed that this compound was very toxic when it was injected to mice. These results indicated that compound GL-2 was trehalose dimycolate, possibly identical with the cord factor. In addition, compound GL-1, which was present in a lesser but significant amount, behaved in a similar fashion. The molar ratio of acyl group to trehalose for this compound was found to be approximately one.

To test the biosynthetic activity of such glycolipids, the washed cells of *N. asteroides* grown in a medium containing 3% glucose were incubated with 2-^{14}C-acetate or uniformly ^{14}C-labeled glucose for 1 hr, and the lipids were extracted and separated on a thin-layer plate. Figure 2 shows that radioactivity from labeled acetate was uniformly incorporated into different lipid components, whereas that from labeled glucose was incorporated into compound GL-2 and to a lesser extent into compound GL-1, together with phosphatidylinositol and phosphatidylinositol monomannoside. When compound GL-2 incorporated from labeled glucose was recovered from the plate and subjected to alkaline hydrolysis, radioactivity was found exclusively in the water-soluble portion. Paper chromatography of this fraction revealed that the peaks of radioactivity agreed with that of authentic trehalose. This demonstrated that the cells of *N. asteroides* were very active in synthesizing trehalose-containing lipids.

Recently, BRENNAN *et al.* (*9*) reported that acylated glucoses were present in mycobacteria and corynebacteria when they were grown in the presence of glucose. The data in this paper provide another example showing

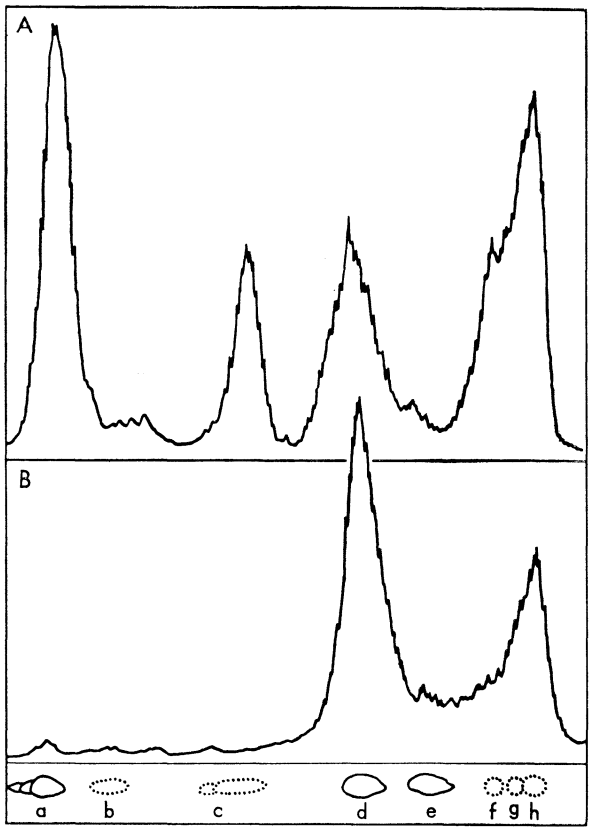

Fig. 2. Radioactive scan of the lipids from the washed cells of *N. asteroides* after incubation with 2-^{14}C-acetate (A) and uniformly ^{14}C-labeled glucose (B) for 1 hr.

Flasks (final 5 ml) contained 2 μCi of the radioactive precursors, 0.2 M phosphate buffer (pH 7.0), and the cell suspension (5 g wet cells) of *N. asteroides* grown in 3% glucose-containing medium for 3 days. Lipids were separated on a thin-layer plate with Solvent B. a, Neutral lipids and pigments; b, free nonpolar fatty acids; c, free mycolic acids; d, compound GL-2; e, cardiolipin; f, compound GL-1; g, phosphatidylethanolamine; h, origin (phosphatidylinositol and phosphatidylinositol monomannoside).

that the presence of a high concentration of glucose in growth media results in a significant increase of acylated sugars.

The authors are very grateful to Dr. J. Maeda in this hospital for providing them with a sample of the cord factor from *M. tuberculosis*, H37Rv strain.

REFERENCES

1) H. BLOCH, *J. Exp. Med.*, **91**, 197 (1950).
2) H. NOLL, H. BLOCH, J. ASSELINEAU and E. LEDERER, *Biochim. Biophys. Acta*, **20**, 299 (1956).
3) J. ASSELINEAU, "The Bacterial Lipids," Hermann, Paris, p. 173 (1966).
4) I. YANO, Y. FURUKAWA and M. KUSUNOSE, *Proc. 25th Meeting, Experimental Research of Tuberculosis*, 1970, p. 1.
5) T. IONEDA, E. LEDERER and J. ROZANIS, *Chem. Phys. Lipids*, **4**, 375 (1970).
6) J. FOLCH, M. LEES and G.H. SLOANE-STANLEY, *J. Biol. Chem.*, **226**, 497 (1957).
7) C.C. SWEELEY, R. BENTLEY, M. MAKITA and W.W. WELLS, *J. Am. Chem. Soc.*, **85**, 2497 (1963).
8) I. YANO, Y. FURUKAWA and M. KUSUNOSE, *J. Biochem.* (Tokyo), **63**, 133 (1968).
9) P.J. BRENNAN, D.P. LEHANE and D.W. THOMAS, *European J. Biochem.*, **13**, 117 (1970).