

## Fatty Acid Composition of Lipopolysaccharides of *Vibrio cholerae* 35A3 (Inaba), NIH 90 (Ogawa), and 4715 (Nag)

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Considerable amounts of odd-numbered fatty acids, such as non-hydroxy C<sub>15</sub> and C<sub>17</sub> and 3-hydroxy C<sub>11</sub> and C<sub>13</sub> acids, were found in lipopolysaccharides from *Vibrio cholerae* 35A3 (Inaba).

The fatty acid composition of lipopolysaccharides (LPS) of many gram-negative bacteria has been well investigated (11), but relatively little work has been done with *Vibrio cholerae*. Armstrong and Redmond (1) studied *V. cholerae* 569B (Inaba), and Raziuddin and Kawasaki (13) and Raziuddin (12) used *V. cholerae* E1 Tor (Inaba) as well as *V. cholerae* 569B (Inaba). Rietschel et al. (17) have studied the nature and linkages of the fatty acids present in LPS from *Vibrio metchnikovii* and *Vibrio parahaemolyticus*.

Using gas-liquid chromatography, we made a comparable study of the fatty acid composition of LPS isolated from the cell walls of selected strains of *V. cholerae*, strains 35A3 (Inaba), NIH 90 (Ogawa), and 4715 (Nag). *V. cholerae* 35A3 (Inaba), NIH 90 (Ogawa), and 4715 (Nag) (type 03 according to the classification of Sakazaki and Tamura [19]) were cultured in 1% glucose-peptone medium, pH 8.0, at 30°C for 16 h. The cells, except strain 4715 (Nag), were treated with 1% phenol for 12 h at room temperature and then washed with cold distilled water.

The cell walls were prepared, as described previously (6), by shaking the cells in a Bühler cell homogenizer (Edmund Bühler, Tübingen, West Germany) with glass beads (diameter, 0.11 to 0.12 mm).

LPS were isolated and purified from either acetone-dried crude cell walls or lyophilized crude cell walls, essentially by the phenol-water technique of Westphal et al. (20) and repeated ultracentrifugation (105,000 × *g*) and treatment with RNase (20 µg/ml) in 0.025 M Tris-hydrochloride buffer, pH 7.4.

About 3 mg of LPS was hydrolyzed with 0.5 ml of 4 N HCl for 4 h at 100°C and extracted with ether. The extract was evaporated to dryness under a flow of nitrogen, yielding residue 1 (total lipid fraction). Fatty acids present in res-

idue 1 were esterified at room temperature for 1 h by the method of Hoshi et al. (7) in a solution containing 0.2 ml of 20 mM methanolic Cu(OAc)<sub>2</sub>, 0.2 ml of CHCl<sub>3</sub>, and 1 ml of 0.5 N methanolic HCl. Fatty acid methyl esters were extracted with hexane, and the extract was washed with water and then evaporated to dryness under nitrogen, yielding residue 2 (total fatty acid methyl ester fraction).

Examination by infrared spectrometry of residue 2 confirmed that the 3-hydroxy fatty acids present in residue 1 were completely esterified. Residue 2 was therefore subjected to preparative thin-layer chromatography to isolate normal fatty acid methyl esters and 3-hydroxy fatty acid methyl esters (Fig. 1). In addition to these two fractions, which were well separated from each other, some unknown materials (Fig. 1, bands A, B, A', and B') were detected in residue 2 from both strain 35A3 (Inaba) and strain 4715 (Nag). There was one additional unknown band (Fig. 1, band C) in residue 2 from strain NIH 90 (Ogawa). The ratio of the amount of nonhydroxy fatty acid esters to the amount of 3-hydroxy fatty acid esters was estimated to be approximately 1:1 in each strain. Each of the two fractions was extracted with hexane.

Total lipid and fatty acid content of LPS from the cell walls of the three strains are shown in Table 1. The total lipid content was between 25 and 34% of the LPS. The total fatty acid content recovered as methyl esters was unexpectedly low (7%) in NIH 90 (Ogawa) LPS, approximately ½ that of both strain 35A3 (Inaba) and strain 4715 (Nag) LPS.

The results of the analysis of the nonhydroxy and 3-hydroxy fatty acid fractions by gas-liquid chromatography are presented in Table 2. The major nonhydroxy fatty acid components of all three strains were found to be C<sub>14:0</sub> and C<sub>16:0</sub>. In addition, small but significant amounts of C<sub>18:0</sub>,

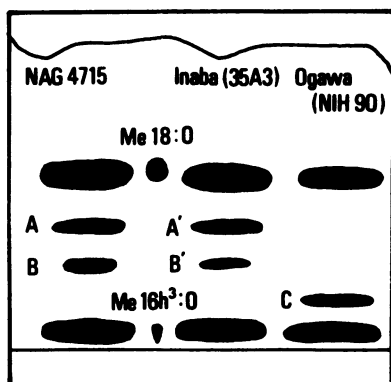


FIG. 1. Thin-layer chromatogram of the methyl esters of the fatty acids (residue 2) released by acid hydrolysis (4 N HCl) of LPS from the cell walls of *V. cholerae* 35A3 (Inaba), NIH 90 (Ogawa), and 4715 (Nag). A silica gel plate was used with the solvent system hexane-ethyl ether-acetic acid (70:30:1, vol/vol/vol). The nonhydroxy fatty acid ester and the 3-hydroxy fatty acid ester fractions were extracted with hexane from the areas on the chromatogram corresponding to those of octadecanoic acid methyl ester (Me 18:0) and 3-hydroxy-hexadecanoic acid methyl ester (Me 16h<sup>3</sup>:0), respectively.

TABLE 1. Lipid fractions of LPS of *V. cholerae*

Fraction	Amt (mg) in the following strains:		
	35A3 (Inaba)	NIH 90 (Ogawa)	4715 (Nag)
LPS	3.47	3.67	3.38
Residue 1 (total lipid)	1.06 (31) <sup>a</sup>	0.93 (25)	1.15 (34)
Residue 2 (esterified fatty acid)	0.61 (18)	0.23 (7)	0.61 (18)

<sup>a</sup> Numbers in parentheses are percentages of total LPS.

C<sub>16:1</sub>, and C<sub>18:1</sub> were observed in the LPS of all three strains. It is of particular interest that considerable amounts of odd-numbered fatty acids were found in the LPS of only strain 35A3 (Inaba); these were C<sub>15:0</sub> and C<sub>17:0</sub>.

With regard to 3-hydroxy fatty acid composition, C<sub>12h:0</sub> and C<sub>14h:0</sub> were found to be present in all three strains. Furthermore, substantial amounts of odd-numbered fatty acids were also observed, particularly in the LPS of strain 35A3 (Inaba); they were C<sub>11h:0</sub> and C<sub>13h:0</sub>. Gas-liquid chromatography-mass spectrometry data, which were obtained with an LKB model 9000S instrument (LKB Instruments Inc., Uppsala, Sweden), confirmed the presence of the above-mentioned fatty acid methyl esters. Thus, fatty acid compositions of the LPS from the three selected strains were relatively similar, with the exceptions of (i) a minor deviation in the relative

proportions of the component fatty acids, and (ii) the presence of odd-numbered normal and 3-hydroxy fatty acids, particularly in LPS from strain 35A3 (Inaba). Although C<sub>12h:0</sub> was previously reported to be the most abundant 3-hydroxy fatty acid in LPS from both 569B (Inaba) and E1 Tor (Inaba) (1, 13), the quantity of C<sub>14h:0</sub> was slightly greater than that of C<sub>12h:0</sub> in the three strains studied here.

Of the two O serotypes of cholera vibrios, Inaba and Ogawa, only the Inaba type has been investigated with regard to the fatty acid composition of endotoxic LPS; almost identical results have been obtained by Armstrong and Redmond (1) and Raziuddin and Kawasaki (13), with the exception of a minor quantitative deviation. They reported that in the LPS from both 569B (Inaba) and E1 Tor (Inaba), C<sub>14:0</sub> and C<sub>16:0</sub> are the main nonhydroxy fatty acid components and C<sub>12h:0</sub> and C<sub>14h:0</sub> are the main 3-hydroxy fatty acid components. Our results with

TABLE 2. Fatty acid composition of lipid A of LPS of *V. cholerae*<sup>a</sup>

Fatty acid	% of total in:		
	35A3 (Inaba)	NIH 90 (Ogawa)	4715 (Nag)
<b>Normal acids<sup>b</sup></b>			
?1	Trace		0.9
14:0	27.0	21.5	36.9
?2	2.3	1.1	3.0
15:0	12.2	1.1	1.3
16:1	1.9	10.9	6.0
16:0	49.9	49.4	46.6
17:0	6.3	Trace	0.5
18:1	0.3	2.7	2.5
18:0	0.8	3.3	2.3
<b>3-Hydroxy acids<sup>c</sup></b>			
10h:0	Trace	2.4	0.2
11h:0	3.1	Trace	0.3
12h:0	37.0	42.9	48.0
13h:0	13.6	Trace	1.3
14h:0	46.3	54.7	50.2

<sup>a</sup> An F and M model 7624 A gas-liquid chromatograph equipped with flame ionization detectors and a digital integrator (model 3370B, Hewlett-Packard) was used for the analytical gas-liquid chromatography of fatty acid methyl esters. A glass column (2 m by 4 mm), packed with 3% Silicone OV-1 coated on Chromosorb W with 25% diethylene glycol succinate on the same support, was used with temperature programming from 150°C at 1°C/min. The identities of the fatty acid methyl esters were based on retention times compared with those of authentic standards. The fatty acids were recovered as methyl esters. Values are the averages of duplicate determinations.

<sup>b</sup> Values for normal acids are percentages of the total normal fatty acid fraction.

<sup>c</sup> Values for 3-hydroxy acids are percentages of the total 3-hydroxy acid fraction.

Ogawa- and Nag-type strains, as well as with Inaba-type strain, coincided with their observation.

The amino groups of the glucosamine residues of lipid A of LPS are generally substituted by D-3-hydroxy fatty acids through amide linkages (4, 14, 16). This amide-linked 3-hydroxy acid is present in the LPS of all gram-negative bacteria so far investigated, with the exception of *Brucella* (2, 9-11, 16). If several 3-hydroxy fatty acids do occur, the one with the longest chain is amide linked (11, 16). It has been shown by Rietschel (16) that in LPS from *V. cholerae* strain 569B (Inaba) C<sub>14h:0</sub> is an amide-bound fatty acid. This has also been observed by Raziuddin with LPS from *V. cholerae* E1 Tor (Inaba) as well as 569B (Inaba) (12). In this study, we have shown that C<sub>14h:0</sub>, which is the most usual amide-bound fatty acid in many gram-negative bacteria (15, 16), is the longest-chain 3-hydroxy fatty acid in the LPS from all of the three selected strains of *V. cholerae*, 35A3 (Inaba), NIH 90 (Ogawa), and 4715 (Nag). Of particular interest is the finding that unexpectedly high amounts of both odd-numbered nonhydroxy and 3-hydroxy fatty acids, i.e., C<sub>15:0</sub>, C<sub>17:0</sub>, C<sub>11h:0</sub>, and C<sub>13h:0</sub>, are detected in the LPS of 35A3 (Inaba), whereas very little of these was found in the LPS of the other two strains. The occurrence of the odd-numbered fatty acids C<sub>13</sub> and C<sub>15</sub> in LPS has been reported in *Veillonella* (3, 5), C<sub>17</sub> has been reported in *Brucella* (2, 9), C<sub>15</sub> and C<sub>17</sub> have been reported in *Myxobacter* (18), and C<sub>13</sub> has been reported in *Selenomonas ruminantium* (8). It would be of interest to know whether the odd-numbered 3-hydroxy fatty acids C<sub>11h:0</sub> and C<sub>13h:0</sub> found in LPS from strain 35A3 (Inaba) are involved in amide linkage, as is LPS from *Veillonella*, where C<sub>13h:0</sub> and C<sub>15h:0</sub> are amide linked (3, 5). This question is at present under detailed investigation.

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