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Nature, Type of Linkage, and Absolute Configuration of (Hydroxy) Fatty Acids in Lipopolysaccharides from *Xanthomonas sinensis* and Related Strains

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The fatty acids present in lipopolysaccharides from *Xanthomonas sinensis* were identified as decanoic, 9-methyl-decanoic, 2-hydroxy-9-methyl-decanoic, D-3-hydroxy-decanoic, D-3-hydroxy-9-methyl-decanoic, D-3-hydroxy-dodecanoic, and D-3-hydroxy-11-methyl-dodecanoic acid. These fatty acids occur in the lipid A component where they are bound through ester and amide linkages to glucosamine residues. All types of fatty acids are ester bound; however, part of D-3-hydroxy-dodecanoic and D-3-hydroxy-11-methyl-dodecanoic acid is also involved in amide linkage. The hydroxyl groups of ester-linked 3-hydroxy fatty acids are not substituted. Similar fatty acid patterns were obtained from lipopolysaccharides of nine other *Xanthomonas* species.

The lipopolysaccharide of *Xanthomonas sinensis*, a plant pathogen, like lipopolysaccharides of other gram-negative bacteria (23) consists of a complex polysaccharide portion (38, 39, 40), to which a lipid component, termed lipid A, is covalently bound. The chemical structure of lipid A, which represents the endotoxic center of lipopolysaccharides (10, 11, 22), has been studied in many organisms. In general, lipid A is made up of phosphorylated glucosamine residues, to which long-chain (hydroxy) fatty acids are bound through ester and amide linkages (22).

As part of our investigations on the chemical structure of lipid A from *Xanthomonas* lipopolysaccharides, the present paper describes the identification of the nature and type of linkage of fatty acids and the absolute configuration of 3-hydroxy fatty acids present in lipopolysaccharides of *X. sinensis*. It will be shown that iso-branched (3-hydroxy) fatty acids predominate. In addition larger amounts of 3-hydroxy-dodecanoic acids are found. All 3-hydroxy acids were shown to possess the D configuration.

MATERIALS AND METHODS

Bacteria and lipopolysaccharides. The source and cultivation of bacteria were as previously described (38, 39).

Lipopolysaccharides were isolated from bacteria with phenol-water (43) as described (38).

Authentic fatty acids. Methyl esters of decanoic, dodecanoic, tetradecanoic, hexadecanoic, and heptadecanoic as well as 3-hydroxy-dodecanoic and 3-hydroxy-tetradecanoic acid were obtained from Analabs (North Haven). 2-Hydroxy-tetradecanoic and 2-hydroxy-hexadecanoic acid were obtained from Serva (Heidelberg). A mixture of fatty acids containing branched (iso- and ante-iso) fatty acids was obtained from Applied Science Labs (State College, Pa.).

Liberation of fatty acids. Total fatty acids were liberated from the lipopolysaccharide by alkaline hydrolysis (4 N NaOH, 5 h, 100 C). Ester-bound acids were transesterified with 0.25 N $NaOCH_3$ as described earlier (27). For identification of amide-bound acids, de-*O*-acylated preparations were hydrolyzed with alkali (4 N NaOH, 5 h, 100 C).

Modification of fatty acids. Free fatty acids were carbomethylated with diazomethane in diethyl ether (34). Hydroxy acids were converted to methoxy derivatives by diazomethane/BF_3-etherate (Fluka) as previously described (27). 3-Hydroxy fatty acids were dehydrated by heating the acids in a mixture of acetic acid anhydride/pyridine (1:1, vol/vol) at 100 C for 30 min (32). The resulting α,β-unsaturated acids were carbomethylated and reduced to the corresponding saturated acids with nascent hydrogen as described by Rosenfelder et al. (31).

Thin-layer chromatography. Polar fatty acids were separated from nonpolar fatty acids on thin-layer plates coated with silica gel H (type 60, Merck, Darmstadt) using petroleum ether/diethyl ether/acetic acid (35:15:1, vol/vol/vol) as solvent.

Configuration analysis of hydroxy acids. To determine the abolute configurations of hydroxy fatty acids a modification of the method of Karlsson and Paschar was used (18). Briefly, alkaline hydrolysates (4 N NaOH) of lipopolysaccharides containing hydroxy fatty acids were methylated with diazometh-

ane/BF_3-etherate. The resulting methoxy fatty acid methyl esters were saponified with 0.1 N KOH in methanol/water (9:1, vol/vol) for 2 h at 60 C, and the hydrolysate was submitted to thin-layer chromatography. The fraction corresponding to methoxy fatty acids was scraped off the plates, fatty acids were extracted with diethyl ether, and the acids were converted to acid chlorides with thionyl chloride. After addition of L-(−)-phenylethylamine (Merck-Schuchardt, Darmstadt), the resulting diastereomeric methoxy fatty acid-L-phenylethylamides were analyzed by gas-liquid chromatography (see below) using a stainless-steel column (0.32 by 152 cm) packed with 3% OV-1 on Gas Chrom Q (100 to 120 mesh) at 170 C with nitrogen as carrier gas. Retention times of D- or-L-methoxy-fatty acid-L-phenylethylamides were based on tetradecanoic acid-L-phenylethylamide (t_R = 1.00).

Gas-liquid chromatography. Fatty acid methyl esters were separated on two types of stainless-steel columns (0.32 by 152 cm): (i) castorwax (2.5% on Chromosorb G, 80 to 100 mesh) at 175 C, and (ii) EGSS-X (15% on Gas Chrom P, 100 to 120 mesh) at 140 C.

The columns were fitted to a Perkin-Elmer Fractometer F20 equipped with a flame ionization detector (250 C). Nitrogen (25 ml/min) served as carrier gas. Heptadecanoic acid methyl ester was used as an internal standard for the quantitative determinations of fatty acids. The amounts of individual fatty acids were calculated on spectra obtained with the castorwax column. The relative retention times obtained on castorwax (t_R[C]) and EGSS-X (t_R[E]) were based on tetradecanoic acid methyl ester (t_R[C] = 1.00; t_R[E] = 1.00).

Mass spectrometry. A Perkin-Elmer mass spectrometer, model 270 B, connected to a gas-liquid chromatograph was used. Fatty acid methyl esters were separated on castorwax and EGSS-X under the same conditions as described above. Mass spectra were taken at 70-eV ionizing potential (2,000-V acceleration voltage, 100-μA ionization current intensity, 170 C ion source temperature, 260 C manifold temperature). Mass spectra were visualized by a Honeywell visicorder, model 3508.

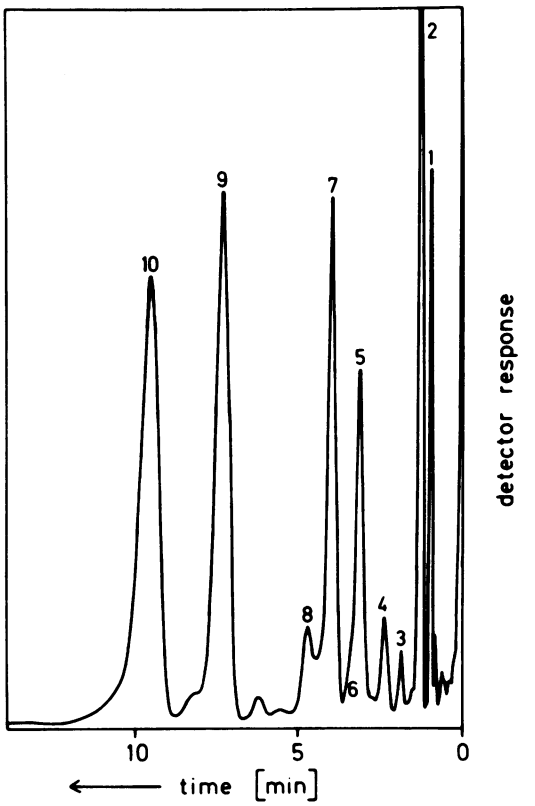

FIG. 1. *Gas-liquid chromatogram (castorwax, 175 C) of methyl esters of fatty acids released by alkaline hydrolysis (4 N NaOH) from X. sinensis lipopolysaccharides. The peaks correspond to: decanoic (1), 9-methyl-decanoic (2), Δ^2-9-methyl-decanoic (3), Δ^2-dodecanoic (4), 2-hydroxy-9-methyl-decanoic (5), D-3-hydroxy-decanoic (6), D-3-hydroxy-9-methyl-decanoic (7), D-3-hydroxy-dodecanoic (9), and D-3-hydroxy-11-methyl-dodecanoic acid (10). Peak 8 corresponds to both Δ^2-11-methyl-dodecanoic and a polar fatty acid not further characterized.*

RESULTS

Qualitative analysis of fatty acids. After liberation from lipopolysaccharides by strong alkaline hydrolysis, the fatty acids were carbomethylated and analyzed as methyl esters by gas-liquid chromatography. Spectra obtained with the castorwax column (Fig. 1) showed five major peaks (peaks 2, 5, 7, 9, 10) and five smaller peaks (peaks 1, 3, 4, 6, 8); however, peak 6 was not well separated from peak 5. A similar picture was obtained after separation of the esters on the EGSS-X column, except that peaks 5 and 6 were now well resolved. The relative retention times of the individual fatty acid methyl esters are listed in Table 1.

Polar acids were separated from nonpolar acids using silica gel thin-layer chromatography. Two major bands could be visualized with iodine. The faster moving band migrated like authentic dodecanoic acid (nonpolar fatty acids, NFA) and the other band like authentic 3-hydroxy tetradecanoic acid (polar fatty acids, PFA). The two fractions were removed from the plates, and the fatty acids were extracted with diethylether, carbomethylated, and analyzed by gas-liquid chromatography. Peaks 1 and 2 predominated on chromatograms from fraction NFA; however, trace amounts of peaks 3, 4, and 8 could also be seen. On spectra of fraction PFA, peaks 5, 6, 7, 9, and 10 were found.

NFA: peaks 1 and 2. The major NFA (peaks 1 and 2) could be tentatively identified by comparison of their relative retention times to

those of authentic standard fatty acids. Peak 1 exhibited identical retention times (t_R[C] = 0.18; t_R[E] = 0.25) to authentic decanoic acid methyl ester (t_R[C] = 0.18; t_R[E] = 0.25). Retention times of peak 2 (t_R[C] = 0.23; t_R[E] = 0.29) were identical to those of 9-methyl-decanoic acid methyl ester (t_R[C] = 0.23; t_R[E] = 0.29). The retention times of the latter iso-branched acid was obtained by extrapolation from known t_R values of authentic iso-branched fatty acid esters. Final proof of the identity of the acids corresponding to peaks 1 and 2 was obtained by mass spectrometry (35). Mass spectra of peaks 1 and 2 showed the expected molecular peaks (186 and 200, respectively) with the base peak of both spectra at *m/e* = 74 (Table 2). Furthermore, spectra of peak 2 showed fragments at *m/e* = 135 (M-65). In

TABLE 1. *Identity, retention times, and amounts of fatty acids in hydrolysates (4 N NaOH, 5 h, 100 C) of X. sinensis lipopolysaccharides*[a]

Peak no.	Fatty acid		Retention time		Amounts in hydrolysates (μmol/mg)
			Castorwax (175 C)	EGSS-X (140 C)	
1	Decanoic	(C-10:0)	0.18	0.25	0.05
2	9-Methyl-decanoic	(9-Me-C-10:0)	0.23	0.29	0.31
5	2-Hydroxy-9-methyl-decanoic	(2-OH-9-Me-C-10:0)	0.57	1.61	0.04
6	D-3-Hydroxy-decanoic	(D-3-OH-C-10:0)	0.59	2.08	0.02
9	D-3-Hydroxy-dodecanoic	(D-3-OH-C-12:0)	1.33	4.08	0.23
7	D-3-Hydroxy-9-methyl-decanoic	(D-3-OH-9-Me-C-10:0)	0.73	2.44	0.11
10	D-3-Hydroxy-11-methyl-dodecanoic	(D3-OH-11-Me-C-12:0)	1.74	4.77	0.26
4	Δ^3-Dodecanoic	(Δ^3-C-12:1)	0.42	0.69	0.03
3	Δ^2-9-Methyl-decanoic	(Δ^2-9-Me-C-10:1)	0.34	0.54	0.01
8	Δ^2-11-Methyl-dodecanoic	(Δ^2-11-Me-C-12:1)	0.85	1.08 (2.91)[b]	0.02[c]

[a] Peak numbers are as in Fig. 1. Retention time values (t_R) are based on tetradecanoic acid methyl ester, t_R = 1.00.

[b] Peak 8 corresponds in addition to a polar fatty acid, not further characterized.

[c] Calculated as Δ^2-11-Me-C-12:1.

TABLE 2. *Fragments (m/e) from mass spectrometry used for characterization of fatty acids in hydrolysates of X. sinensis lipopolysaccharides*[a]

Peak no.	Fatty acid	Molecular weight (calculated)	Peaks indicating molecular weight (M)	Base peak	Peaks characteristic for branching, hydroxyl, or methoxyl group
1	C-10:0	186	186 M	74	
2	9-Me-C-10:0	200	200 M	74	135 (M-65) 153 (M-47)
5	2-OH-9-Me-C-10:0	216	216 M 157 (M-59)	83	90 157 (M-59)
6	D-3-OH-C-10:0	202	184 (M-18) 152 (M-50)	43	103
9	D-3-OH-C-12:0	230	212 (M-18) 180 (M-50)	43	103
7	D-3-OH-9-Me-C-10:0	216	198 (M-18) 166 (M-50)	43	103
10	D-3-OH-11-Me-C-12:0	244	(226 [M-18])[b] 194 (M-50)	43	103
9 (—OCH_3—)	D-3-OCH_3-C-12:0	244	229 (M-15) 214 (M-30)	75	117
7 (—OCH_3—)	D-3-$OCH_3$9-Me-C-10:0	230	215 (M-15) 200 (M-30)	75	117
10 (—OCH_3—)	D-3-OCH_3-11-Me-C-12:0	258	243 (M-15) 228 (M-30)	75	117

[a] Peak numbers are as in Fig. 1. Molecular weights are calculated for fatty acid methyl esters.

[b] Not visible on mass spectrum.

addition, M-31 (m/e = 169) was greater than M-29 (m/e = 171), indicating that peak 2 corresponds to an iso-branched fatty acid and not to an ante-iso-branched fatty acid. From these data it is concluded that peak 1 corresponds to decanoic and peak 2 to 9-methyl-decanoic acid, respectively.

PFA: peaks 5 to 10. The nature of the PFA could be tentatively identified by chemical modifications. Final proof for the identity of fatty acids corresponding to peaks 5 to 10 was obtained by mass spectrometry.

(i) Chemical modifications. The PFA were first heated with acetic acid anhydride/pyridine. This treatment is known to convert 3-hydroxy fatty acids to the corresponding α,β-unsaturated fatty acids. The treated acids were carbomethylated, reduced with nascent hydrogen (31), and analyzed by gas-liquid chromatography. The spectra obtained with both types of columns showed three major peaks with ratios similar to peaks 7, 9, and 10 on spectra of fraction PFA. The retention times of these peaks corresponded to those of 9-methyl-decanoic acid (t_R[C] = 0.23; t_R[E] = 0.29), to dodecanoic acid (t_R[C] = 0.41; t_R[E] = 0.50), and to 11-methyl-dodecanoic acid (t_R[C] = 0.54, t_R[E] = 0.59). In addition a minor peak (t_R[C] = 0.18; t_R[E] = 0.25) was present corresponding to decanoic acid methyl ester.

The hydroxy acids in fraction PFA could be further characterized in the form of their methoxy-derivatives. After *O*-methylation of PFA, four new peaks appeared. Two of these peaks had relative retention times identical to authentic 3-methoxy-decanoic acid ester (t_R[C] = 0.31; t_R[E] = 0.62) and 3-methoxy-dodecanoic acid ester (t_R[C] = 0.74; t_R[E] = 1.26). The two other peaks (t_R[C] = 0.41; t_R[E] = 0.73 and t_R[C] = 0.98; t_R[E] = 1.47, respectively) corresponded to 3-methoxy-9-methyl-decanoic (t_R[C] = 0.40; t_R[E] = 0.73) and 3-methoxy-11-methyl-dodecanoic acid (t_R[C] = 0.99, t_R[E] = 1.47), respectively. The retention times of the two latter iso-branched methoxy acids were extrapolated from known retention times of 3-methoxy-13-methyl-tetradecanoic and 3-methoxy-15-methyl-hexadecanoic acids isolated from *Myxococcus fulvus* lipopolysaccharides (31). From these experiments it is concluded that four 3-hydroxy fatty acids are present in fraction PFA, namely, 3-hydroxy-decanoic acid (peak 6, t_R[C] = 0.53; t_R[E] = 2.08), 3-hydroxy-9-methyldecanoic acid (peak 7, t_R[C] = 0.73; t_R[E] = 2.44), 3-hydroxy-dodecanoic acid (peak 9, t_R[C] = 1.33; t_R[E] = 4.08), and 3-hydroxy-11-methyl-dodecanoic acid (peak 10, t_R[C] = 1.74; t_R[E] = 4.77).

(ii) Mass spectrometry. Mass spectra were taken after separation of the fatty acid methyl esters from fraction PFA on castorwax with the exception of peaks 5 and 6, which were characterized after elution from the EGSS-X column. Mass spectra of peaks 6, 7, 9, and 10 exhibited prominent peaks at m/e = 43 (base peak) and at m/e = 103, the latter indicating that the corresponding acids are 3-hydroxy fatty acids (Table 2). Mass peaks indicating the molecular weights (M-18 and M-50, respectively) were found on spectra of peak 6 at m/e = 184 and 152, of peak 9 at m/e = 212 and 180, of peak 7 at m/e = 198 and 166, and of peak 10 at m/e = 194. The fragment M-50 (m/e = 226) deriving from the latter peak 10 was not visible on the mass spectrum. From these fragments the molecular weights of 3-hydroxy fatty acid methyl esters could be calculated (Table 2). They were M = 202 (peak 6, 3-OH-C-10:0), M = 230 (peak 9, 3-OH-C-12:0), M = 216 (peak 7, 3-OH-9-Me-C-10:0), and M = 244 (peak 10, 3-OH-11-Me-C-12:0). The hydroxy fatty acids corresponding to peaks 7, 9, and 10 could be further characterized by mass spectrometry of the 3-methoxy acid methyl ester derivatives. The base peak on mass spectra of the modified hydroxy acids was at m/e = 75 with an additional prominent peak at m/e = 117, both mass peaks being characteristic for 3-methoxy fatty acid methyl esters (33). The molecular weights of the methoxy acids were obtained from fragments M-15 and M-30, which were at m/e = 215 and 200 (peak 7 [—OCH_3—], M = 230), at m/e = 229 and 214 (peak 9 [—OCH_3—], M = 244) and at m/e = 243 and 228 (peak 10 [—OCH_3—], M = 258), respectively (Table 2).

Mass spectrometry also allowed the identification of the PFA corresponding to peak 5 (t_R[C] = 0.57; t_R[E] = 1.61). The mass spectrum of peak 5 was identical to that published by Moss et al. (25) for 2-hydroxy-9-methyl-decanoic acid methyl ester. The base peak was at m/e = 83. Fragments characteristic for 2-hydroxy acids were found at m/e = 90 and 157 (M-59). From the latter mass peak the molecular weight was calculated as 216 which is to be expected for 2-hydroxy-9-methyl-decanoic acid methyl ester. Also, since the retention times of peak 5 were identical to those extrapolated for iso-branched 2-hydroxy-undecanoic acid methyl ester (t_R[C] = 0.56; t_R[E] = 1.60), it is concluded that peak 5 corresponds to 2-hydroxy-9-methyl-decanoic acid.

Unsaturated fatty acids: peaks 3, 4 and 8. From previous work (27, 30, 31) it is known that on treatment with alkali 3-hydroxy fatty acid esters or amides are partially converted to the

corresponding α,β- as well as β,γ-unsaturated fatty acids. It was therefore expected that unsaturated fatty acids derived from the main 3-hydroxy acids (peaks 7, 9, and 10) would occur as artifacts in alkaline hydrolysates of lipopolysaccharides. In fact, three minor peaks (peaks 3, 4, and 8) were found which corresponded according to their retention times to authentic Δ^3-dodecanoic (peak 4, t_R[C] = 0.42; t_R[E] = 0.69), Δ^2-9-methyl-decanoic (peak 3, t_R[C] = 0.34; t_R[E] = 0.54) and Δ^2-11-methyl-dodecanoic acid (peak 8, t_R[C] = 0.85; t_R[E] = 1.08). Peak 8 on spectra from the castorwax column contained an additional polar fatty acid (also found in fraction PFA), which on the EGSS-X column had a retention time of t_R[E] = 2.91. These retention times are similar to those of 3-hydroxy-undecanoic acid (t_R[C] = 0.84; t_R[E] = 2.90). Because of the small amount of the unknown polar fatty acid present in fraction PFA, no further characterization was attempted.

Configuration of hydroxy acids. For determination of the absolute configurations of hydroxy acids of *Xanthomonas* lipopolysaccharides, a minor modification of the procedure described by Karlsson and Pascher was used (18). The diastereomeric standard, D-3-methoxy-tetradecanoic acid-L-phenylethylamide was synthesized from D-3-hydroxy-tetradecanoic acid isolated from *Salmonella* lipopolysaccharides (27). In addition, diastereomeric L-phenylethylamides were made from racemates of 3-methoxy-dodecanoic and 3-methoxy-tetradecanoic acid. Table 3 shows the retention times of the diastereomeres in gas-liquid chromatographic analysis (OV-1 column). It is obvious, that the D-acid-L-amide-enantiomer elutes from the column before the L-acid-L-amide enantiomer. A mixture of iso-branched 3-methoxy fatty acid-L-phenylethylamides were prepared for reference compounds in the following way: the PFA were isolated from *Xanthomonas sinensis* lipopolysaccharides and treated with acetic acid anhydride/pyridine as described above. The resulting unsaturated acids were carbomethylated followed by treatment with methylate (0.5 N $NaOCH_3$, 12 h, 37 C). The racemic 3-methoxy ethers formed were then converted to the L-phenylethylamides and their retention times were estimated by gas-liquid chromatography (Table 3).

When 3-hydroxy acids from *X. sinensis* lipopolysaccharides were liberated by strong alkali and analyzed by gas-liquid chromatography in the form of their 3-methoxy acid-L-phenylethylamides, three major peaks were obtained with relative retention times of 0.35, 0.58, and 0.75, respectively. Comparison of these retention times with those of known standards showed that the major peaks correspond to the L-phenylethylamides of D-3-methoxy-9-methyl-decanoic (t_R = 0.35), D-3-methoxy-dodecanoic (t_R = 0.58) and D-3-methoxy-11-methyl-dodecanoic acid (t_R = 0.75), respectively. In addition to the three major peaks, two poorly resolved minor peaks were also present. One of the minor peaks exhibited an identical retention time to that calculated for the L-phenylethylamide of D-3-methoxy decanoic acid (t_R = 0.27). The other peak was not identified. Thus, all 3-hydroxy fatty acids present in *Xanthomonas* lipopolysaccharides possess the D configuration.

TABLE 3. *Retention times on gas-liquid chromatograms (OV-1, 170 C) of L-(−)-phenylethylamides of standard 3-methoxy acids and polar fatty acids from X. sinensis lipopolysaccharides*[a]

L-Phenylethylamides	Retention time of:	
	Standard	Sample
D-3-OCH_3-C-12:0	0.58	0.58
L-3-OCH_3-C-12:0	0.64	
D-3-OCH_3-C-14:0	1.23	
L-3-OCH_3-C-14:0	1.34	
D-3-OCH_3-9-Me-C-10:0	0.35	0.35
L-3-OCH_3-9-Me-C-10:0	0.39	
D-3-OCH_3-11-Me-C-12:0	0.75	0.75
L-3-OCH_3-11-Me-C-12:0	0.83	

[a] Retention time values (t_R) are based on tetradecanoic acid-L-phenylethylamide, t_R = 1.00.

Differentiation between ester- and amide-bound acids. Ester-bound fatty acids liberated from lipopolysaccharides by $NaOCH_3$ (0.25 N, 12 h, 37 C) were identified by gas-liquid chromatography. It was shown that the fatty acids corresponding to peaks 1 to 8 were exclusively involved in ester linkages. Also part of D-3-hydroxy-dodecanoic acid (peak 9, 0.06 μmol/mg = 28.4%) and D-3-hydroxy-11-methyl-dodecanoic acid (peak 10, 0.12 μmol/mg = 42%) were ester bound. In total, therefore, 69% (0.75 μmol/mg) of total fatty acids are present as *O*-acyl residues. No 3-methoxy fatty acids were found after methanolysis indicating that ester-bound 3-hydroxy acids are not substituted at the 3-hydroxyl group (27, 29).

The amide-bound acids were liberated from the de-*O*-acylated preparation (after methanolysis) by strong alkali (4 N NaOH, 5 h, 100 C) and identified. Gas-liquid chromatography showed that both D-3-hydroxy-dodecanoic acid (peak 9) and D-3-hydroxy-11-methyl-dodeca-

noic acid (peak 10) were amide bound (Fig. 2). The latter two hydroxy acids are therefore involved in ester as well as in amide linkage.

Quantitation of fatty acids. The amount of individual fatty acids present in the lipopolysaccharides from *X. sinensis* was calculated from gas-liquid chromatograms (castorwax) using heptadecanoic acid methyl ester as an internal standard. As shown in Table 1, 9-methyl-decanoic (0.31 μmol/mg), D-3-hydroxy-9-methyl-decanoic (0.11 μmol/mg), D-3-hydroxydodecanoic (0.23 μmol/mg), as well as D-3-hydroxy-11-methyl-dodecanoic acid (0.26 μmol/mg) predominate. In total, 1.08 μmol (23%) of fatty acids per mg are present in the lipopolysaccharide.

Fatty acid pattern of lipopolysaccharides of various Xanthomonas strains. In Table 4 the results of fatty acid analyses on lipopolysaccharides from 10 distinct *Xanthomonas* species are summarized. The fatty acid patterns of all preparations investigated appear to be qualitatively identical to that of *X. sinensis*. Also, the fatty acid spectra of the various *X.* strains are similar quantitatively in that 3-hydroxy fatty acids as well as 9-methyl-decanoic acid predominate.

DISCUSSION

In the present study, the constituent fatty acids of lipopolysaccharides from *X. sinensis*, a plant pathogen, were investigated and identified as 9-methyl-decanoic, D-3-hydroxy-9-methyl-decanoic, D-3-hydroxy-dodecanoic, and D-3-hydroxy-11-methyl-dodecanoic acid. In addition, smaller amounts of decanoic, D-3-hydroxydecanoic and 2-hydroxy-9-methyl-decanoic acid are present. Similar fatty acid patterns were found in lipopolysaccharides from other *Xanthomonas* species. The results presented show that the fatty acid pattern of *X. sinensis* is distinct from that found in *Enterobacteriaceae*, *Pseudomonadaceae*, *Neisser-*

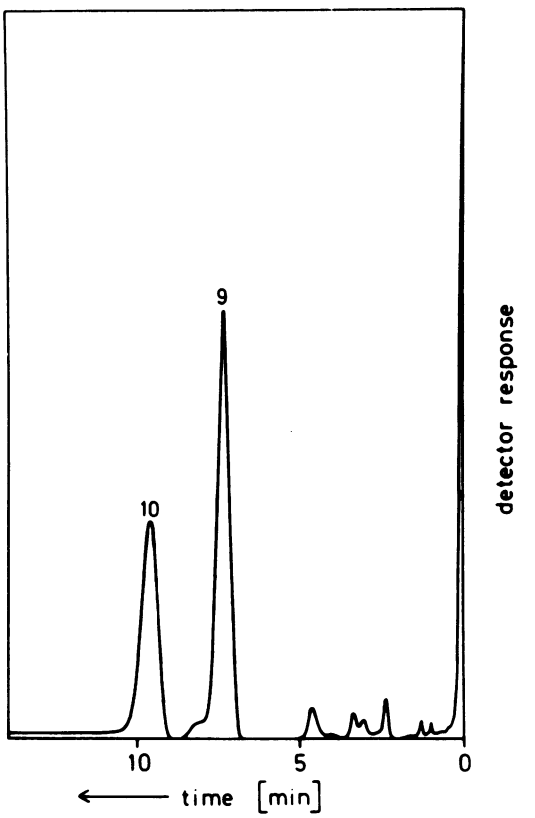

FIG. 2. *Gas-liquid chromatograms of methyl esters of the amide-bound fatty acids released by alkaline hydrolysis (4 N NaOH) from de-O-acylated preparations obtained by methanolysis (0.25 N $NaOCH_3$) of lipopolysaccharides of X. sinensis. Identity of fatty acids as in Fig. 1.*

TABLE 4. *Nature and relative amounts of fatty acids from lipopolysaccharides of various Xanthomonas strains*

Fatty acid	*Xanthomonas* species									
	X. sinensis	*X. geranii*	*X. campestris*	*X. badrii*	*X. vasculorum* (mol %)	*X. ricinicola*	*X. carotae*	*X. maculifolii-gardeniae*	*X. alfalfae*	*X. pruni*
C-10:0	3.6	5.4[a]	6.2	10.2	4.9	8.7	14.1	8.4	8.4	5.2
9-Me-C-10:0	27.5	19.4	24.5	21.0	13.9	18.7	13.6	18.5	17.4	16.1
2-OH-9-Me-C-10:0	3.9	5.1	4.2	5.0	5.3	6.2	6.3	6.2	6.8	6.3
3-OH-C-10:0	1.7	3.0	2.1	2.8	2.4	4.2	2.9	3.5	4.2	2.2
3-OH-C-12:0	25.6[b]	34.3	25.8	29.9	36.0	30.9	33.3	33.8	35.5	44.5
3-OH-9-Me-C-10:0	9.9	9.5	19.1	13.3	18.6	12.0	14.7	12.3	11.0	13.4
3-OH-11-Me-C-12:0	27.6	15.1	18.0	17.5	18.9	18.7	13.9	17.1	15.3	12.0

[a] *X. geranii* contained additional hexadecanoic acid (8%).

[b] Amounts of unsaturated fatty acids, representing artifacts after alkaline hydrolysis, were added to amounts of corresponding hydroxy acids.

iaceae, Spirillaceae, and other groups of gram-negative bacteria (22). It is similar, however, to the fatty acid spectrum of the *Myxobacteria* (31).

About 70% of total fatty acids are iso-branched fatty acids. To our knowledge, iso-branched (hydroxy) fatty acids as constituents of lipopolysaccharides have only been found in *Myxobacteria* (31) and two strains of *Pseudomonas rubescens* (44). In addition, firmly bound lipids in *Pseudomonas maltophila* were found to contain iso-branched nonhydroxylated as well as iso-branched 2- and 3-hydroxy fatty acids (25). In fact, the fatty acid spectrum of the tightly bound lipids of the latter organism strongly resembles that of *Xanthomonas*.

As in *P. maltophila* lipids, 2-hydroxy-9-methyl-decanoic acid occurs in lipopolysaccharides of all *Xanthomonas* species investigated. Although 2-hydroxy acids are known to be lipopolysaccharide constituents (9, 13, 17, 44), iso-branched 2-hydroxy acids have not been found in lipopolysaccharides. *Myxococcus fulvus* contains 2-hydroxy-15-methyl-hexadecanoic acid, but this iso-branched 2-hydroxy acid was shown to be derived from contaminating phospholipids (31). Recently, 2-hydroxy-13-methyl-tetradecanoic acid was identified as a major component of *Bdellovibrio bacteriovorus* sphingolipids (36). In *Xanthomonas*, the ester-bound, iso-branched 2-hydroxy acid was found in lipopolysaccharide preparations from all strains investigated. Nevertheless, the possibility cannot be excluded that the iso-branched 2-hydroxy acid in *Xanthomonas* may also be derived from contaminating phospholipids.

X. sinensis possesses four (or possibly five) distinct 3-hydroxy fatty acids which account for 65% of the total fatty acids. Such a range of 3-hydroxy acids has only been observed in a few cases (4, 31, 44). Only one type of 3-hydroxy fatty acid (3-hydroxy-tetradecanoic acid) is present in lipopolysaccharides from *Salmonella* (27), *Shigella* (24), *Proteus* (26), *Escherichia coli* (15), *Serratia* (3), *Aerobacter* (12, 20), and *Coxiella* (5). Two types of 3-hydroxy acids, which differ in chain length, occur in the lipopolysaccharides from *Vibrio* (16, 29), *Neisseria* (1), *Acinetobacter* (37), and *Pseudomonas* (9, 13, 19, 44). In *Moraxella* (2) and *Veillonella* (7, 14) three distinct 3-hydroxy acids were found. However, in these lipopolysaccharide preparations the percentage of 3-hydroxy acids per total fatty acids seems to be of limited variability and to be independent of the number of different 3-hydroxy acids present. Thus, in most bacterial lipopolysaccharides or lipid A preparations, as in those from *S. sinensis*, about 55 to 75% of the total fatty acids present are 3-hydroxy fatty acids. This percentage of 3-hydroxy fatty acids was found, for instance, in *Salmonella* (27), *Pseudomonas* (9, 19), *Neisseria* (1), *Rhodopseudomonas* (42), *Shigella* (24), *Vibrio* (29), *Acinetobacter* (37), *Myxobacteria* (31), *Veillonella* (14), and *Serratia* (3). In *Aerobacter* (12, 20), however, only 6% of 3-hydroxy fatty acids were found and in *Brucella* 3-hydroxy fatty acids are absent (6, 21).

In *X. sinensis*, as in lipopolysaccharides from the *Enterobacteriaceae* and most other bacterial groups, glucosamine represents the backbone of lipid A, providing hydroxyl and amino groups to which fatty acids are covalently bound. It was found that all types of fatty acids present in the lipopolysaccharide are ester bound. However, part of the 3-hydroxy-dodecanoic and 3-hydroxy-11-methyl-dodecanoic acid is also involved in amide linkage. Thus, as in other lipopolysaccharides investigated to date, the amide-bound acids are exclusively represented by 3-hydroxy acids. Unlike in other lipopolysaccharides where only one type of 3-hydroxy fatty acid is linked to amino groups, two 3-hydroxy fatty acids form amide bonds in *Xanthomonas*. An example in which a pair of 3-hydroxy fatty acids, both possessing identical chain length, one however being iso-branched, is also seen in *M. fulvus*, where 3-hydroxy-hexadecanoic as well as 3-hydroxy-15-methyl-hexadecanoic acid are amide-bound (31). It seems therefore that if two classes of 3-hydroxy fatty acids (i.e., straight chain and iso-branched) occur, that of each class the 3-hydroxy acid with the longest chain is amide linked and the others are ester bound.

In *Xanthomonas* the 3-hydroxyl group of ester linked 3-hydroxy fatty acids is not *O* substituted. This is in contrast to *Salmonella* (27) and two *Vibrio* strains (29), where ester-bound 3-myristoxymyristic acid as well as 3-lauroxylauric acid occur, respectively.

All 3-hydroxy fatty acids present in *X. sinensis* lipopolysaccharides possess the D configuration. D-3-Hydroxy acids were also found in lipopolysaccharides from *E. coli* (15), *Salmonella* (27), *Shigella* (24), and *Pseudomonas* (13). It is known that in *E. coli* D-(−)-3-hydroxy fatty acids represent natural intermediates in fatty acid biosynthesis and that L-(+)-3-hydroxy acids are formed by β-oxidation (8).

Lipopolysaccharides of *Xanthomonas* strains are highly active endotoxins. They are lethal for mice, they elict strong local Shwartzman reactions in rabbits (38), and they are potent pyrogens (E. Th. Rietschel and W. A. Volk,

unpublished observations). Thus, despite significant differences in the fatty acid moiety, the biological activity of *Xanthomonas* lipopolysaccharides is comparable to that of *Salmonella* endotoxins. In view of the unique fatty acid composition of *Xanthomonas* lipid A we are currently investigating its behavior in pyrogenic cross-tolerance (28, 41).

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