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Occurrence of 2-Keto-3-Deoxy-D-*manno*-Octonic Acid in Lipopolysaccharides Isolated from *Vibrio parahaemolyticus*†

TZYY-JAN HAN* AND TUU-JYI CHAI

Horn Point Environmental Laboratory, Center for Environmental and Estuarine Studies, University of Maryland, Cambridge, Maryland 21613

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The occurrence of 2-keto-3-deoxy-D-*manno*-octonic acid (KDO) in lipopolysaccharides (LPS) of *Vibrio parahaemolyticus* was demonstrated for the first time by gas chromatography-mass spectrometry after dephosphorylation, reduction, and methylation. KDO was virtually completely phosphorylated, since no KDO was detected by either gas chromatography or thiobarbituric acid assay before dephosphorylation. The level of KDO in all six strains of *V. parahaemolyticus* investigated ranged from 0.37 to 0.69%, which was considerably lower than that in enterobacterial LPS.

2-Keto-3-deoxy-D-*manno*-octonic acid (KDO) is a unique eight-carbon sugar commonly found in the inner core region of gram-negative bacterial lipopolysaccharides (LPS) (14), but it was considered virtually missing in vibrios (9, 10). However, the observed KDO-specific antigenicity of *Vibrio cholerae* LPS led to the finding of phosphorylated KDO by gas chromatography-mass spectrometry (GC-MS) (1). By using a thiobarbituric acid (TBA) assay alone, KDO has been recognized in thiobarbiturate-negative LPS prepared from *Pseudomonas* spp., *Bacteroides* spp., and *Aeromonas* spp. after dephosphorylation (4). No conclusive evidence for the general existence of KDO in *Vibrio parahaemolyticus* LPS has been reported. The purpose of this study was to demonstrate the existence of KDO in *V. parahaemolyticus* LPS by GC-MS and chemical methods.

Six strains of *V. parahaemolyticus* were studied. Their serotypes and Kanagawa phenomena were as follows: 38C1, O4:K11 and K^+; P7, O4:K4 and K^+; P26, O2:K3 and K^+; 38C6, O3:K30 and K^-; P68, O4:K34 and K^-; and P6, O2:K30 and K^-. Kanagawa-positive (K^+) strains are able to lyse red blood cells on blood agar medium containing 7% NaCl (16). Virtually all pathogenic *V. parahaemolyticus* strains are Kanagawa positive (17). Three strains of either K^+ or K^- *V. parahaemolyticus* were used for this study to determine whether there is any correlation between the presence of KDO and Kanagawa phenomenon. Bacteria were grown in PPBE broth (1% proteose peptone, 0.2% beef extract, 2.5% NaCl) at 35°C. Cells were harvested at an optical density at 660 nm of 1.0 to 1.2 and then lyophilized. LPS was extracted by the phenol-water method (18) and purified by repeated high-speed centrifugation (10,000 × *g*, 30 min) and ultracentrifugation (105,000 × *g*, 3 h) (7). LPS was dephosphorylated in 48% HF at 4°C for 72 h in a polyethylene centricone. HF was removed either by evaporation under a vacuum (13) or by dialysis (12). Mild acid hydrolysis with 5% acetic acid at 100°C for 5 h was used to break the glycosidic linkage between polysaccharide and lipid A in control (untreated) and dephosphorylated LPS. KDO in degraded polysaccharide was derivatized by the procedure described by Waeghe et al. (15), with modifications. Degraded polysaccharide underwent the following derivatization steps: (i) prereduction of the keto group by $NaBD_4$; (ii) methylation of the hydroxyl groups of sugars by methylsulfinyl carbanion ($CH_3 \cdot SO \cdot CH_2^-$, Na^+) and CH_3I by the Hakomori method (6) (methylsulfinyl carbanion was prepared as described by Carpita and Shea [5]); (iii) carboxyl reduction by $NaBD_4$; (iv) hydrolysis of glycosidic linkages by trifluoroacetic acid; (v) methylation as in step ii; and (vi) extraction with a $CHCl_3$-methanol mixture (2:1) and then dissolution in $CHCl_3$ for GC or GC-MS analysis. Authentic KDO (Sigma, St. Louis, Mo.) was used to prepare the standard derivative, 1,2,4,5,6,7,8-hepta-*O*-methyl-3-deoxy-octitol-*1,1,d*$_2$-*2,d*. Gas chromatography was performed with a Hewlett-Packard model 5890 gas chromatograph equipped with a fused silica capillary column (SPB-5, 30 m by 0.53 mm, 15-μm film thickness). The oven was programmed for 170°C for 3 min, and the temperature was then increased at a rate of 3°C per min to 260°C. The chromatogram and retention time were recorded with a Hewlett-Packard integrator (model 3393A). GC-MS was carried out with a Hewlett-Packard system consisting of a gas chromatograph (model 5890, series II) and a mass spectrometer (5971A). The gas chromatograph had a fused silica capillary column (HP-5, 50 m by 0.2 mm, 0.33-μm film thickness). The temperature program was the same as that described above. Electron-impact mass spectra were recorded at 70 eV. The ion source temperature was 180°C. A modified TBA assay, described by Karkhanis et al. (11), was used to determine KDO in control and dephosphorylated LPS.

GC and GC-MS of authentic KDO and of KDO released from LPS. Figure 1 shows the gas chromatograms of the derivatives of authentic KDO and of dephosphorylated LPS and control LPS. The doublet peaks (retention times of 20.97 and 21.26 min) have been identified by GC-MS as the enantiomers of the deutero-reduced and permethylated KDO derivative, 1,2,4,5,6,7,8-hepta-*O*-methyl-3-deoxyoctitol-*1,1,d*$_2$-*2,d*. The fragmentation pattern and electron-impact mass spectrum of the KDO derivative are shown in Fig. 2. The doublet peaks had identical mass spectra. They were characterized by the fragments at m/z = 150 (C1-C4) and 118 (C1-C4 moiety with the loss of methanol); at m/z = 92 (C1-C2) and 59 (C1-C2 moiety with the loss of deuterated methanol); and, although with low intensity, at m/z = 194 (C1-C5), 162 (194-32), and 129 (162-33); at m/z = 47 (C1); and at m/z = 216 (C2-C8 moiety with subsequent loss of two

* Corresponding author.

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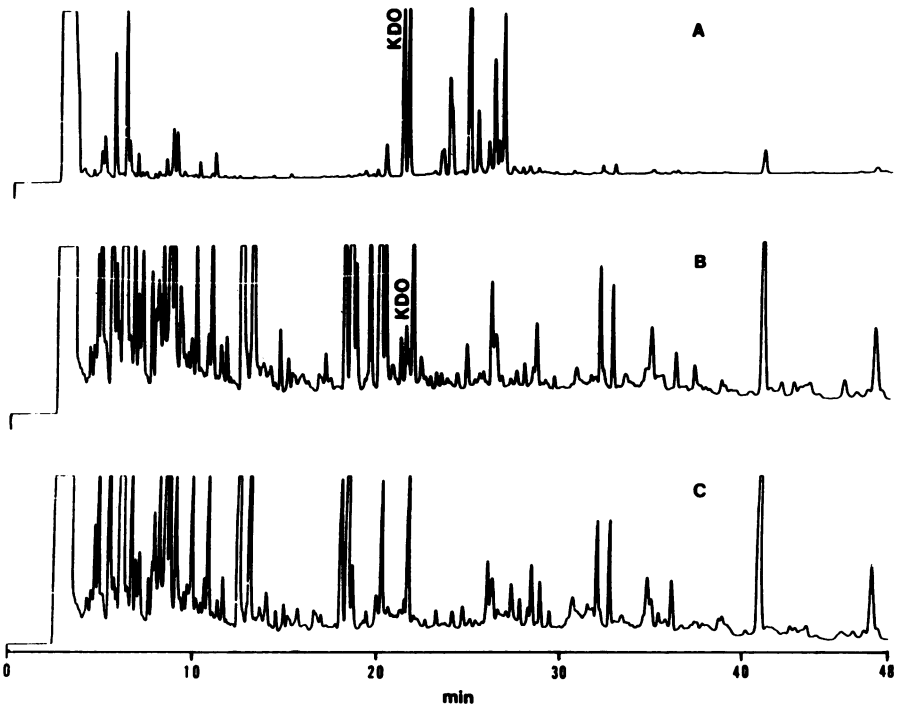

FIG. 1. Gas chromatograms of authentic KDO (A), dephosphorylated LPS (B), and control LPS (C) of *V. parahaemolyticus* 38C1 after deutero-reduction, depolymerization, and permethylation. All three derivatizations were started with 1.0 mg of material. The final derivatives were dissolved in 100 μl of $CHCl_3$. Two microliters was injected for GC analysis. The attenuation of the KDO profile was 4, and that of the dephosphorylated and control LPS profiles was 1.

methanols) and 184 (216-32). Fragments at *m/z* = 177 (C5-C8) and 133 (C6-C8) and the corresponding subfragments at *m/z* = 145 and 101 (base peak) (obtained by loss of methanol) were not characteristic, since they were found in all permethylated alditol derivatives, as were the fragments at *m/z* = 89 and 45. Among all the peaks of the gas chromatogram of authentic KDO, the doublet peaks were the only major peaks containing the characteristic fragment *m/z* = 92 (data not shown). The other peaks were probably the side products of the derivatization procedure.

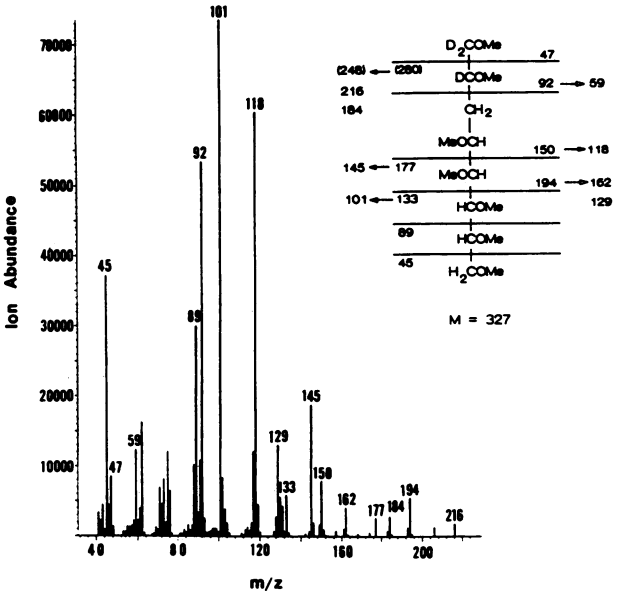

FIG. 2. Fragmentation pattern and electron-impact mass spectrum of deutero-reduced and permethylated KDO.

The doublet peaks of KDO derivative appeared in the gas chromatogram of dephosphorylated 38C1 LPS (Fig. 1). The mass spectra of these two peaks were identical to one another and to those of the authentic KDO (data not shown). However, no such peaks appeared in the gas chromatogram of control 38C1 LPS (Fig. 1). This suggested that the KDO molecules in the LPS of *V. parahaemolyticus* 38C1 were mostly phosphorylated.

TBA assay of dephosphorylated and control LPS. The occurrence of phosphorylated KDO in *V. parahaemolyticus* LPS was further demonstrated by a modified TBA assay (11). Figure 3 shows the wavelength scans of the TBA assay products of authentic KDO and of dephosphorylated and control LPS of three representative strains of *V. parahaemolyticus*: 38C1, P68, and P6. Authentic KDO had a maximal absorbance at 552 nm. No substantial absorbance at this wavelength was observed in the control LPS of any of the six strains studied. However, an absorption peak at 552 nm was clearly seen in the dephosphorylated LPS isolated from all six strains (Fig. 3).

The relative levels of KDO in the LPS of the six strains of *V. parahaemolyticus* measured by TBA assay after dephosphorylation, along with the levels of polysaccharide and lipid A in LPS after mild acid hydrolysis, are listed in Table 1. Since lipid A is the most conservative portion of an LPS molecule (2), the ratio of polysaccharide to lipid A by weight indicates the abundance of polysaccharide in the LPS molecules of a specific strain. Strains 38C1, P7, and P68 had slightly more polysaccharide in their LPS molecules than

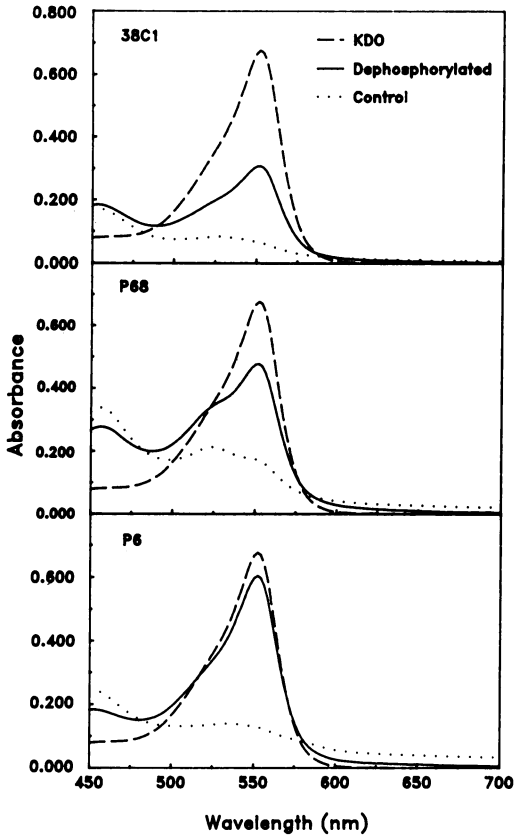

FIG. 3. Wavelength scans of TBA assay products of authentic KDO, dephosphorylated LPS, and control LPS of *V. parahaemolyticus* 38C1, P68, and P6.

strains P26, 38C6, and P6, as determined by the polysaccharide/lipid A ratio. This was supported by the mobility of these LPS on a sodium dodecyl sulfate-polyacrylamide gel. LPS of strains 38C1, P7, and P68 had similar mobilities and moved slightly slower than those of strains P26, 38C6, and P6, which also had similar mobilities, indicating that LPS of the former three strains had higher molecular weights than those of the latter three strains (data not shown). The percentage of KDO in strains containing more polysaccharide was lower than in strains containing less polysaccharide (Table 1). This suggested that the LPS molecules of all six strains of *V. parahaemolyticus* might have the same number of KDO residues and that the existence of KDO was not dependent on their Kanagawa phenomenon.

TABLE 1. The polysaccharide, lipid A, and KDO content of *V. parahaemolyticus* LPS[a]

Strain	PS (%)[b]	LA (%)[b]	PS/LA	KDO (%)[c]	KDO/LA
38C1	49.5	41.0	1.21	0.43 ± 0.01	0.010
P7	46.2	41.8	1.10	0.37 ± 0.02	0.009
P26	43.8	46.3	0.95	0.69 ± 0.04	0.015
38C6	43.0	45.0	0.96	0.57 ± 0.03	0.013
P68	53.0	39.5	1.34	0.49 ± 0.01	0.012
P6	46.5	44.5	1.04	0.68 ± 0.07	0.015

[a] Abbreviations: PS, polysaccharide; LA, lipid A.
[b] Percentage was calculated by weight after mild acid hydrolysis.
[c] Percentage was calculated on the basis of the TBA assay. Data are the means ± standard deviations of three experiments in duplicate.

The ratio of KDO to lipid A in *V. parahaemolyticus* LPS ranged from 0.010 to 0.015 (Table 1). However, the KDO/lipid A ratio of an *Escherichia coli* strain, JM103, is 0.118 (3). This indicated that fewer KDO residues exist in the *V. parahaemolyticus* LPS. Only 1 mol of KDO is believed to be present for each mole of *V. cholerae* LPS (12). However, enterobacterial LPS may contain three KDO residues (14).

The results of this study have not only demonstrated the occurrence of KDO in the *V. parahaemolyticus* LPS investigated in this study, but they also imply the general existence of KDO in *V. parahaemolyticus* LPS of all O serotypes. The failure to find KDO in LPS isolated from any O serotypes of *V. parahaemolyticus*, except for serotype O6 as described by Hisatsune et al. (8), was apparently due to the methodology used, which did not include dephosphorylation.

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