JOURNAL OF BACTERIOLOGY, Feb. 1985, p. 795–798
0021-9193/85/020795-04$02.00/0

Vol. 161, No. 2

Occurrence of 2-Keto-Deoxyoctonic Acid 5-Phosphate in Lipopolysaccharides of *Vibrio cholerae* Ogawa and Inaba

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Received 30 July 1984/Accepted 24 October 1984

A phosphorylated 2-keto-3-deoxyoctonic acid (KDO) was released from the lipopolysaccharides of *Vibrio cholerae* Ogawa and Inaba after strong acid hydrolysis. The phosphorylated KDO was identified by gas-liquid chromatography and mass spectrometry after reduction and permethylation as KDO-5-phosphate and an isomer of it being phosphorylated at position 7 or 8. After treatment with alkaline phosphatase, KDO was detected by gas-liquid chromatography and mass spectrometry. It was indistinguishable from authentic 2-keto-3-deoxy-D-*manno*-octonic acid.

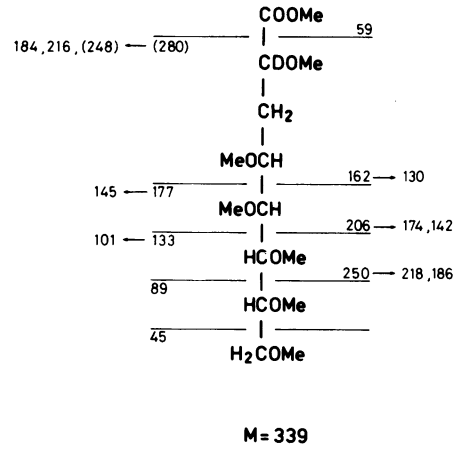
Recently, a new antigen, designated common lipopolysaccharide (LPS) specificity, has been described which occurs in the LPS of many gram-negative bacteria, e.g., *Enterobacteriaceae*, *Neisseriaceae*, and *Pseudomonadaceae*, and resides in the inner core region (3, 4). Since it was shown that the antigenic determinant contains 2-keto-3-deoxyoctonic acid (KDO) and at least one neutral sugar, it was expected that the LPS of *Vibrio cholerae*, which has been reported by several authors to lack KDO (11, 13, 14), does not express the new antigenic specificity. The unexpected observation that the LPSs from two strains of *V. cholerae* were equally active inhibitors of the serological test system specific for the common LPS specificity prompted investigations for the presence of KDO in these LPSs.

LPS was extracted by the phenol-chloroform-petroleum ether method (8) from *V. cholerae* 95R (a rough mutant of strain Ogawa 162), and by the phenol-water procedure from *V. cholerae* Inaba 569B (18), and was purified as described in the respective references. The ammonium salt of KDO was kindly provided by F. M. Unger (Vienna, Austria). The lithium salt of KDO-5-phosphate (a mixture of D-*gluco*- and D-*manno*- epimers) was a generous gift from L. Szabó (Orsay, France). Methylation was done according to Hakomori (9) as modified by Waeghe et al. (17). Combined gas-liquid chromatography–mass spectrometry was performed on a Hewlett-Packard instrument (model 5985) equipped with a fused-silica capillary column (25 m) with chemically bonded phase SE54 Weeke (Mühlheim, Federal Republic of Germany). The temperature program was 170°C for 3 min with an increase of 3°C/min to a final temperature of 260°C. Electron impact spectra were recorded at 70 eV; chemical ionization was achieved with methane or ammonia as a reactant gas. The ion source temperature was 200°C.

Release and isolation of KDO phosphate from LPS. LPS (500 mg) of both *Vibrio* strains was hydrolyzed in 4 M hydrochloric acid (10 ml) at 100°C for 1 h with constant stirring. The dark brown hydrolysate was diluted with water (30 ml) and filtered through glass wool; the direct thiobarbituric acid assay (5) was positive in the filtrate (λ_{max} = 549 nm). Anion exchanger resin (CH_3COO^- form) was added (ca. 50 ml) until the thiobarbituric acid assay became negative in the supernatant. The ion exchanger was transferred to a column (3 by 25 cm), washed with water, and eluted with 1 M acetic acid (fractions of 5 ml were collected and tested by the thiobarbituric acid assay). Positive fractions were combined and taken to dryness under reduced pressure by several additions of water to remove the acid completely. The dry residue was dissolved in a small volume of water (2 ml) and divided into two aliquots. To one sample, 1 M Tris-hydrochloride (pH 8.6) (120 μl), alkaline phosphatase (5 μl), and water (75 μl) were added, and the mixture was incubated at 37°C for 2 h. The second sample, containing all reagents but phosphatase (5 μl of 1 M ammonium sulfate was added instead), was treated in the same way. Both samples (10 μl) were subjected to high-voltage paper electrophoresis and stained with Ag^+-OH^- (16), the thiobarbituric acid (2), and the molybdate reagent (10). A similar pattern was obtained with the LPSs of *V. cholerae* Inaba and Ogawa 95R. One main and two minor spots were visualized in the hydrolysate without phosphatase treatment, one of which migrated like authentic KDO (M_{KDO} = 1.0) and stained with Ag^+OH^- and the thiobarbiturate reagent, but not with the phosphate reagent. The other minor spot was not further investigated. The main spot migrated to the same position as authentic KDO-5-phosphate (M_{KDO} = 2.2) and stained with all three aforementioned reagents. After phosphatase treatment, the faster-migrating spot (M_{KDO} = 2.2) was no longer detectable, and the spot comigrating with KDO became more intense.

Gas-liquid chromatography–mass spectrometry of synthetic KDO, KDO-5-phosphate, and KDO phosphate after release from LPS. The two reference compounds and the hydrolysates before and after phosphatase treatment were analyzed after reduction and permethylation. Figure 1 shows the fragmentation pattern and the mass spectrum after electron impact ionization of deutero-reduced and permethylated authentic KDO. The base peak at m/z = 130 (162 − 32) derives from a fission between C4 and C5 (m/z = 162) and subsequent loss of methanol therefrom. Other characteristic fragments are the C1–C5 and the C1–C6 moiety at m/z = 206, 174 (206 − 32), and 250, 218 (250 − 32), respectively. Fragments at m/z = 177 (C5–C8), 133 (C6–C8), and the corresponding subfragments at m/z = 145 and 101 (obtained by loss of methanol) are not characteristic since they are found in all permethylated alditol derivatives, as are the fragments at m/z = 59, 71, 75, and 89.

Figure 2 shows the proposed fragmentation pattern for authentic KDO-5-phosphate (reduced and permethylated) and its mass spectrum after electron impact ionization. It is characterized by the fragments at m/z = 161 and 129 (base



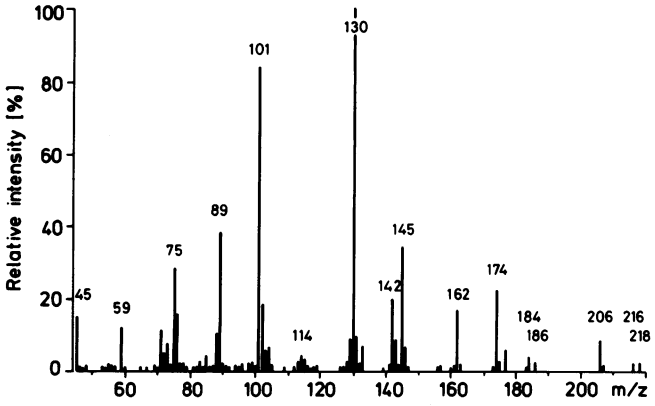
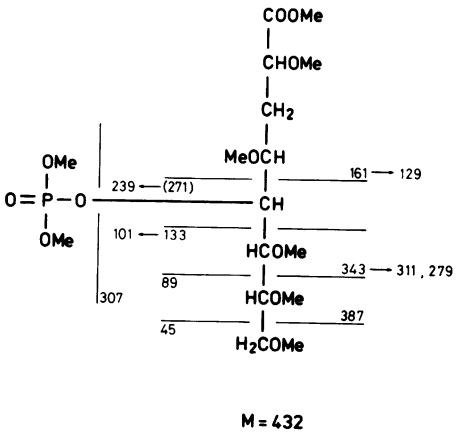


FIG. 1. Fragmentation pattern and mass spectrum after electron impact ionization of deutero-reduced and permethylated KDO.

peak, representing the C1–C4 moiety), at m/z = 343, 311, and 279 (C1–C6), at m/z = 387 (C1–C7), and, although with low intensity, at m/z = 271 and 239 (C5–C8). Chemical ionization with either methane or ammonia as the reactant gas yielded the expected pseudomolecular ion peaks at m/z = 433 (M+1) and m/z = 450 (M+18), respectively (spectra not shown).

The samples obtained from the hydrolyzed LPS were analyzed as deutero-reduced and permethylated derivatives before and after treatment with alkaline phosphatase. The untreated samples were subjected to selected ion monitoring after chemical ionization with methane and after electron impact ionization. In the former case, the spectra were selected for the pseudomolecular ion peak (m/z = 434, M+1) of a deutero-reduced and permethylated KDO phosphate and for the fragment ions at m/z = 130 and 174, which are obtained with high intensities in KDO derivatives that are not substituted in positions 4 and 5 (compare Fig. 1). For both bacterial strains the results were similar. After chemical ionization with methane, two main peaks were observed by selected ion monitoring for m/z = 434, both peaks appearing as double peaks, which is expected for reduced keto sugars. Selected ion monitoring for the fragment ions at m/z = 130 and 174 after electron impact ionization revealed that both fragments were present in the later-eluting doublet, whereas the fragment ion at m/z = 174 was missing in the first, suggesting a substitution at C5. The first doublet of peaks had retention times of 18.12 and 18.20 min (identical to those of authentic KDO-5-phosphate), and the electron impact spectrum (not shown) was comparable to the one shown in Fig. 2. This indicates that the compound is deutero-reduced and permethylated KDO-5-phosphate. The second doublet of peaks (retention times of 20.32 and 20.44 min) exhibited a fivefold greater area compared with the aforementioned peaks and an electron impact spectrum that is shown in Fig. 3A. The spectrum shows pairs of fragments at m/z = 162 and 130, 206 and 174, and 250 and 218, indicating that carbon atoms 1 through 6 do not carry the phosphate group. The spectrum after chemical ionization with methane (Fig. 3B) is characterized by the pseudomolecular ion peak at m/z = 434 (M+1) and by the fragment ions at m/z = 402 (M+1−32) and 308 (loss of the phosphate group). The fragment ions observed at m/z = 294, 262, and 230 could be formally derived from the non-phosphorylated C1–C7 moiety; however, the same fragments were also seen with the KDO-5-phosphate. Therefore, it cannot be decided whether the phosphate group is substituting position 7 or 8. The phosphatase-treated samples exhibited all characteristics of a deutero-reduced and permethylated KDO residue when analyzed by gas-liquid chromatography–mass spectrometry. A doublet of peaks was obtained (retention times of 8.20 and 8.28 min) which in



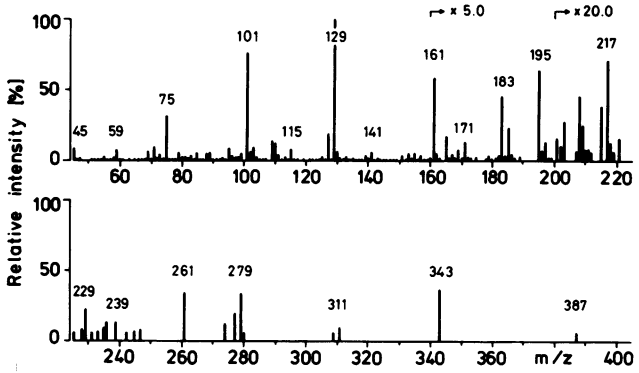


FIG. 2. Fragmentation pattern and mass spectrum after electron impact ionization of reduced and permethylated KDO-5-phosphate.

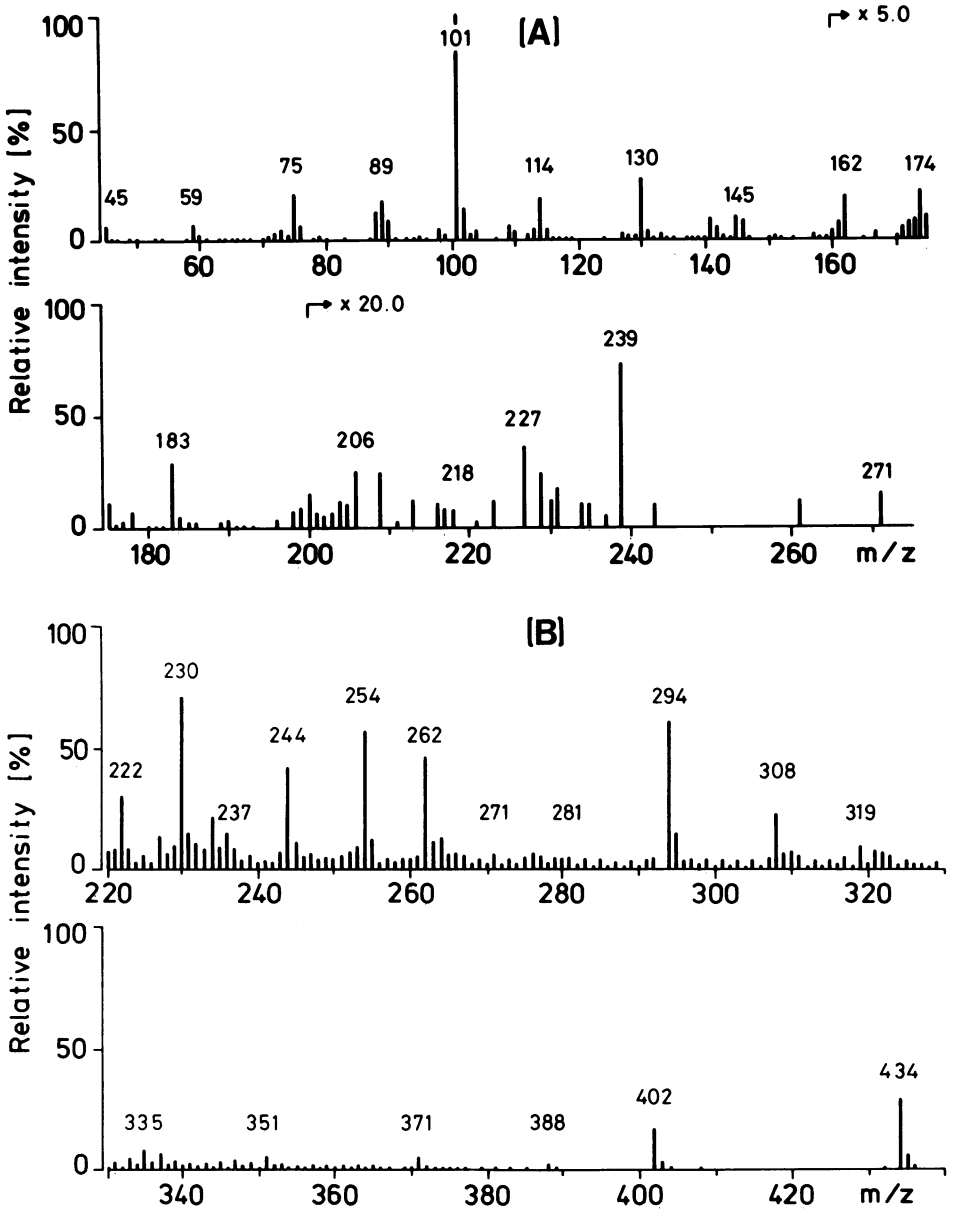


FIG. 3. Mass spectra of deutero-reduced and permethylated KDO phosphate released from *V. cholerae* LPS. (A) Electron impact ionization; (B) chemical ionization with methane.

comparison with similarly derivatized authentic KDO had (i) the same retention time, (ii) the same molecular weight (M = 339), and (iii) an electron impact spectrum superimposible to the one shown in Fig. 1. From these results, it is concluded that the analyzed substance is deutero-reduced and permethylated KDO.

It was shown in the present study that a KDO phosphate is a constituent of the LPS of *V. cholerae* Ogawa and Inaba. This compound is liberated under harsh hydrolytic conditions and can be dephosphorylated subsequently by the action of alkaline phosphatase. The identification was achieved (i) by high-voltage paper electrophoresis of the hydrolysates, whereby a thiobarbituric acid-positive, phosphate-containing substance was detected that comigrated with authentic KDO-5-phosphate, and, after treatment with alkaline phosphatase, was converted to a thiobarbituric acid-positive, phosphate-free substance that migrated to the same position as authentic KDO, and (ii) by combined gas-liquid chromatography–mass spectrometry of the deutero-reduced and permethylated derivatives in comparison with appropriate standards.

The results indicate that at least two different KDO phosphates are present in the hydrolysate; one of them was identified as KDO-5-phosphate, the other as the 7- or 8-phosphate. Whether the latter compound represents a constituent of the LPS remains to be studied. It may have been formed by phosphate migration, although Chaby and Szabó (6) and Danan et al. (7) considered phosphate migration an unlikely mechanism in the case of KDO-5-phosphate. In their studies, however, the acidic conditions were much milder than those employed in the present investigation. The occurrence of KDO-5-phosphate has been reported for the LPS of

Bordetella pertussis, in which a polysaccharide with KDO-5-phosphate as the reducing end was obtained (6, 15). From this polysaccharide, the phosphorylated sugar could be liberated by further hydrolysis in 2 M hydrochloric acid.

These results show that the lack of KDO is not a taxonomical criterion for members of the *Vibrionaceae* as proposed earlier (11). This is also suggested by previous reports on the presence of KDO or "KDO-like material" in other genera of this family (1, 12). Finally, the data further support our hypothesis that the recently described common LPS specificity requires the presence of KDO (4).

I thank E. T. Rietschel and O. Lüderitz for discussion and fruitful criticism, and S. Schlecht (Freiburg, Federal Republic of Germany) for cultivating the bacteria. The excellent technical assistance of V. Susott is gratefully acknowledged as well as the experienced help of H. Moll in mass spectrometry.

This work was supported by the Deutsche Forschungsgemeinschaft (Br 731/3).

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