3-Deoxy-D-manno-2-octulosonic acid in the lipopolysaccharide of various strains of *Pseudomonas cepacia*

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Summary. Six clinical isolates of *Pseudomonas cepacia* (representing the five serotypes of the organism) were examined for the presence of 3-deoxy-D-manno-2-octulosonic acid (KDO) in their lipopolysaccharide (LPS). Purified LPS was examined for the presence of KDO by the thiobarbituric acid (TBA) assay and by gas chromatography. All strains possessed KDO. One strain possessed KDO that was detectable by the TBA assay after mild acid hydrolysis with 0.04 M H₂SO₄ at 100°C for 20 min. The other strains also possessed KDO but it was only demonstrable by the TBA assay after strong acid hydrolysis (4 M HCl for 60 min at 100°C). All six purified LPS preparations were shown to possess KDO by two separate gas chromatography procedures. LPS isolated from the six strains of *P. cepacia* was toxic for mice.

Introduction

The consequences of *Pseudomonas cepacia* infection in cystic fibrosis (CF) patients are receiving much attention,¹ because CF patients are currently the largest group of individuals infected with this bacterium.^{2–4}

Because so little is known about the virulence factors of *P. cepacia*, we decided to examine one aspect of a virulence factor about which controversy presently exists—the presence or absence of 3-deoxy-D-manno-2-octulosonic acid (KDO) in the lipopolysaccharide (LPS). Maniello *et al.*⁵ were the first to state that they could find no detectable KDO in LPS extracted from two strains of *P. cepacia* when they employed the procedure of Osborn.⁶ Anwar *et al.*⁷ were also unable to detect any KDO in the outer membrane of one strain of *P. cepacia*. However, Straus *et al.*⁸ reported the isolation of KDO from the culture supernate of 2 of 10 strains of *P. cepacia* they examined. Their material gave a positive thiobarbituric acid (TBA) reaction product,⁶ which absorbed strongly at 549 nm, and appeared to be spectrally identical to that produced by the purified KDO used as the standard. In the present study we examined the LPS of six clinical isolates of *P. cepacia* for the presence of KDO by TBA assay and by gas chromatography.

Materials and methods

Bacteria and media

The six strains of *P. cepacia* employed in this study were isolated from sputum samples from patients with CF; their origins and serotypes were as follows: strains Pc61g (serotype D), Pc527i (D) and Pc99bb (B) from Dr J. D. Klinger of Integrated Genetics, Framington, MA; strains K63-2 (E) and K19-2 (A) from Dr C. L. Prober, The Hospital for Sick Children, Toronto, Ontario; and strain 5530pk (C) was isolated at Alberta Children's Hospital, Calgary, Alberta, Canada. The serotyping of these strains has been described;⁹ storage and handling of the organisms were as previously described.¹⁰

Determination of KDO in purified LPS

Cultures were grown in 10 L of M-9 medium¹¹ containing glucose 0.2% with shaking (180 revolutions per min) at 37°C for 24 h. The samples were immediately chilled on ice and centrifuged at 17 700 *g* for 30 min at 4°C. LPS was then extracted by the procedure of Darveau and Hancock.¹² Purified LPS preparations were examined for protein content by the procedure of Lowry *et al.*¹³ and for nucleic acid content by the method of Kabat and Mayer.¹⁴

Purified LPS preparations were assayed for KDO by the procedure described by Osborn⁶ with KDO (Sigma) as the standard. This assay with a 0.04 M H₂SO₄ hydrolysis step for 20 min at 100°C was considered to be mild hydrolysis; strong acid hydrolysis involved exposure to

4 M HCl at 100°C for 60 min. The presence of KDO in these hydrolysed preparations was determined by spectral analysis in the range 500–600 nm in a Beckman DU-70 spectrophotometer. Samples from which a curve similar to that of purified KDO was obtained, with an optimum absorbance at 550 nm, were considered probably to contain KDO.

KDO in purified LPS was analysed by gas chromatography on two different columns as the methyl glycoside trifluoroacetate as follows. Purified LPS preparations (5–10 mg) were dried under a stream of nitrogen at 40–50°C and kept under vacuum overnight. Methanolysis was accomplished by heating each sample in 2.0 ml of 1.5 M HCl in dry methanol (Supelco) at 80°C for 18 h under nitrogen. The methanol was removed under a stream of nitrogen at 40°C. Methanol was added and evaporation was repeated once to remove residual HCl. The samples were then kept under vacuum for at least 1 h. Acetonitrile (200 μ l) and then trifluoroacetic anhydride (200 μ l) were added and the samples were heated to 80°C for 30 min. The volume of each sample was reduced to c. 50 μ l under a stream of nitrogen at 25°C. Samples were chromatographed on a 12-m capillary column, with an internal diameter of 0.32 μ m coated with SE-30 (Supelco) in a Varian Model 3300 Gas Chromatograph equipped with a flame-ionisation detector. The carrier gas was nitrogen with stream-splitting at 1 in 100. The initial temperature was held at 70°C for 1 min and then increased to 150°C at 4°C/min. KDO (Sigma) was used as a standard. In the second procedure, the methyl glycoside trifluoroacetates were chromatographed on a 15-m capillary column with an internal diameter of 0.32 μ m, coated with SP-2250 (Supelco) at an initial temperature of 100°C. The temperature was raised to 160°C at 4°C/min.

The LPS preparations from each of the six strains were examined for their toxicity in experimental animals. The LD₅₀ values of the purified, UV sterilised LPS preparations were determined by intraperitoneal (i.p.) injection into Swiss-Webster mice. After 72 h, the number of dead mice in each group was determined and LD₅₀ values were calculated by the method of Reed and Muench.¹⁵

Results

Occurrence of KDO in P. cepacia

Strain Pc61g, serotype D, contained KDO in a form that could be detected by the TBA assay after mild acid hydrolysis⁶ (fig. 1). Purified LPS of strain Pc61g gave a distinctly positive TBA reaction, which absorbed maximally at 550 nm and was spectrally identical to the purified KDO used as a standard. With the other five strains, material giving this absorbance was detected only after strong hydrolysis which did, however, destroy much of the KDO.

Proof that the purified LPS from strain Pc99bb possessed KDO was afforded by gas chromatogra-

Fig. 1. Absorption spectrum of (A) 5.0 μ g of KDO standard and (B) 50 μ l of purified LPS from strain Pc61g.

phy. Purified KDO in SE-30 gas chromatography gave a retention profile with four peaks at approximately 13.2 min (minor), 14.4 min (minor), 16.3 min and 17.0 min (data not shown). These peaks at 16.3 min and 17.0 min were seen in the profile of the purified LPS from strain Pc99bb (fig. 2) as well as in the SE-30 profiles of the other five purified LPS preparations (data not shown). The peaks at 13.2 min and 14.4 min occur in regions of the chromatogram containing numerous peaks, which makes their use in identification of KDO questionable.

Fig. 3 shows the retention times of KDO in gas chromatography on SP-2250 columns. Again, four peaks were observed in standards with retention times as 7.1 min (minor), 8.4 min (minor), 9.4 min and 12.3 min. The peaks at 7.1 and 8.4 min were not useful for KDO analysis. On the basis of

Fig. 2. Retention time profile of O-methyl glycoside trifluoroacetates prepared from the purified LPS from strain Pc99bb by gas chromatography with SE-30 as the immobile phase. The arrows indicate the two KDO peaks (A and B) that were found in all LPS samples.

differences in peak areas, we believe that the peaks at 9.4 and 12.3 min (SP-2250) are derived from the same substances that give rise to peaks at 17.0 and 16.3 min respectively on SE-30 chromatography. These peaks are labelled "A" and "B" in figs 2 and 3 to indicate this. The presence of both peaks is indicative of KDO. Multiple peaks are believed to result from α and β anomers of pyranoside and furanoside ring structures of KDO methyl glycoside trifluoroacetates.

Fig. 4 shows the absorption spectrum of TBA-reactive material in purified LPS from strain Pc99bb after mild and strong acid hydrolysis. LPS from strain Pc99bb produced a chromogen with a

Fig. 3. Retention time profile of O-methyl glycoside trifluoroacetates prepared from the purified LPS from strain Pc99bb by gas chromatography with SP-2250 as the immobile phase. Peaks A and B in the profile are believed to be identical to peaks A and B, respectively, in the profile in fig. 2.

maximum absorbance at 536 nm, which is not indicative of KDO (fig. 4A); however, after strong acid hydrolysis (fig. 4B), the same material produced a TBA-reactive compound with a maximum absorbance at 550 nm typical of KDO.

Characterisation and toxicity of LPS preparations from P. cepacia

All six LPS preparations contained <0.6% protein and <1% nucleic acid. The table shows the absorbance maxima of purified LPS obtained from the six *P. cepacia* isolates after strong hydrolysis and the LD₅₀ values of these substances for mice. All six LPS preparations were more or less equally toxic for mice when injected i.p. There was no

Fig. 4. Visible absorption spectrum of 50 μ l of purified LPS from *P. cepacia* strain 99bb after (A) mild and (B) strong acid hydrolysis followed by the thiobarbituric acid assay.

Table. KDO determinations and LD50 values in mice of purified LPS from clinical isolates of *P. cepacia*

Strain	Serotype	LPS LD50 (μ g)	Absorbance maxi- mum after strong hydrolysis
K19-2	A	1000	550
Pc99bb	B	1500	550
5530pk	C	1250	550
Pc527i	D	1100	550
Pc61g	D	1000	550
K63-2	E	1250	550

correlation between the toxicity of the preparation and the severity of the hydrolysis conditions required for the detection of KDO.

Discussion

The detection of KDO in gram-negative bacterial LPS is usually achieved by the TBA assay.^{6-8,10} In this assay, KDO reacts as a free unsubstituted monosaccharide, and thus has to be liberated from LPS by hydrolysis.

KDO-deficient endotoxins have been reported in several bacterial species.¹⁶ This conclusion is usually based upon the lack of reactivity of LPS in the TBA assay. *P. cepacia* is one of the gram-negative organisms whose LPS was originally reported not to contain KDO.⁵ This finding was confirmed by Anwar *et al.*⁷ in their examination of the outer membrane of one strain of *P. cepacia*. However, Straus *et al.*¹⁰ reported that they could detect extracellular KDO in approximately 20% of the *P. cepacia* strains they examined. In another study, Straus *et al.*⁸ confirmed the presence of KDO in approximately 20% of the *P. cepacia* strains; sweet potato acid phosphatase had no effect on the amount of KDO detected.

By employing capillary gas chromatography, we have now demonstrated that KDO is present in the purified LPS of all six strains of *P. cepacia* examined. It appears that approximately 80% of *P. cepacia* strains produce an LPS that does not react with the TBA reagent after mild acid hydrolysis. For KDO to be reactive in the TBA assay, the -OH groups in the C-4 and C-5 positions must be unsubstituted; however, if the -OH group in the C-5 position is substituted, the C-4, C-6 and C-7 positions must be free.¹⁷ The majority of *P. cepacia* strains contain

KDO that is detectable only after strong acid hydrolysis which indicates some form of substitution in one of the appropriate carbon positions.

The purified LPS from strain Pc61g after mild acid hydrolysis gives a maximum absorbance at 550 nm. This indicates that this strain possesses KDO that is unsubstituted in one of the carbon positions that would block this reactivity. Gas chromatography on SE-30 demonstrated KDO in our six LPS preparations. Purified KDO yielded characteristic peaks at 16.3 and 17.0 min; these peaks were seen in chromatograms of all six LPS preparations. Chromatography on a SP-2250 column confirmed the presence of KDO.

Endotoxins lacking KDO have been described in *Bacteroides* spp.¹⁸ *Pseudomonas* spp.¹⁹ and *Aeromonas* spp.²⁰ These observations were usually based on the absence of TBA-reactive material after mild acid hydrolysis which liberates the fragment (OHC-CH₂-CO-COOH); reactive material cannot be thus generated if KDO is substituted at certain carbon positions. Theoretically, each -OH group found in a KDO molecule may contain a substitution. KDO²¹ and phosphate²² have been found attached to KDO at carbon position 4. Rietschel *et al.*²² have reported that carbon position 5 is substituted with such neutral sugars as L,D-heptose or D-mannose; that phosphoethanolamine can be present at the 7 carbon position of some bacterial KDO molecules; and that KDO or 4-amino-4-deoxy-L-arabinopyranose may be bound to the primary -OH in carbon position 8 of some KDO molecules. Strong acid hydrolysis enables the detection of KDO molecules substituted as described above, presumably by hydrolysis of the substituting group.^{16,21,23} We are currently trying to determine, by nuclear magnetic resonance, the nature and location of the substitutions on the KDO molecules that are only detectable by strong acid hydrolysis.

Finally, all *P. cepacia* LPS molecules that we tested were toxic for mice when injected i.p. We may conclude that substitutions do not influence toxicity of the LPS preparations of the various strains.

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