A Comparative Study of the Sugar Composition of Lipopolysaccharides Isolated from *Vibrio cholerae*, '*Vibrio albensis*' and *Vibrio metschnikovii*

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A comparative study was made of the quantitative sugar composition of lipopolysaccharides (LPS) isolated from *Vibrio cholerae* (O1 and non O1 groups), '*V. albensis*', '*V. proteus*' and *V. metschnikovii*. The amino sugars 4-amino-4,6-dideoxy-D-mannose (perosamine) and 2-amino-2,6-dideoxy-D-glucose (quinovosamine) were present exclusively in LPS isolated from S-form O1 group of *V. cholerae* regardless of serotype (i.e. Ogawa or Inaba) and biotype (i.e. classical or eltor). Classical O1 group *V. cholerae* was distinguishable from eltor O1 group *V. cholerae* on the basis of the fructose content of the LPS: $>3\%$ and $\leq 1\%$, respectively. Distinct differences in the sugar composition of LPS were observed between *V. cholerae* and '*V. albensis*', '*V. proteus*' and *V. metschnikovii*.

INTRODUCTION

On the basis of heat-stable somatic antigens (O-antigens), *Vibrio cholerae* is divided into the O1 group *V. cholerae* (cholera vibrios) and the non-O1 group *V. cholerae* (NAG vibrios); the former group is further subdivided into two major serological types, Ogawa and Inaba (Finkelstein, 1973; Sakazaki & Tamura, 1971). In addition, the O1 group is divided into two biotypes according to haemolytic activity on sheep blood cells, and other biological and biochemical properties. These biotypes have been named *V. cholerae* biotype 'classical' (or *cholerae*: Shewan & Véron, 1974) for classical cholera vibrios and *V. cholerae* biotype eltor for El Tor cholera vibrios (Baumann *et al.*, 1984). Shewan & Véron (1974) included two additional biotypes, *albensis* and *proteus* in the species *V. cholerae*. The biotype *proteus* is now recognized as a separate species, *V. metschnikovii* (Baumann *et al.*, 1984). '*V. proteus*' is an illegitimate synonym of *V. metschnikovii*; '*V. albensis*', formerly recognized as a separate species, is now included in *V. cholerae* (Baumann *et al.*, 1984).

A comparative study of the sugar composition of lipopolysaccharide (LPS) isolated from representatives of *Aeromonas*, *Photobacterium*, *Plesiomonas* and *Vibrio* demonstrated that, with the exception of *V. parahaemolyticus* O6 and the genus *Plesiomonas*, 2-keto-3-deoxyoctonate (KDO), a regular sugar constituent of the core region of the usual Gram-negative bacterial LPS, was undetectable in representatives of the family Vibrionaceae (Hisatsune *et al.*, 1983). Indeed, the inability to detect KDO in LPS by the routine colour reaction under conventional hydrolysis conditions has become one of the taxonomic characteristics of the family Vibrionaceae. In addition, two rare amino sugars, 4-amino-4,6-dideoxy-D-mannose (perosamine) and 2-amino-2,6-dideoxy-D-glucose (quinovosamine) were found exclusively in LPS from O1 group *V. cholerae* regardless of either the biotype (classical or eltor) or the serotype (Inaba or Ogawa) (Hisatsune *et al.*, 1983). This pair of amino sugars is not present either in non-O1 group *V.*

Abbreviation: KDO, 2-keto-3-deoxyoctonate (3-deoxy-D-manno-2-octulosonic acid).

cholerae or in the other Vibrionaceae so far examined. Hisatsune *et al.* (1983) presented only qualitative data on the composition of Vibrionaceae LPS. In the present study, a more detailed analysis of LPS isolated from *V. cholerae* (O1 and non-O1 group) was carried out.

METHODS

Bacterial strains. The source of the strains used in this study is included in Table 1.

Growth of cells. The bacteria were grown in the following media with vigorous shaking for 16–32 h at 37 °C: broth medium at pH 8.0 for *V. cholerae* (O1 and non-O1 groups); broth medium at pH 7.2–7.4 for '*V. proteus*' (NCTC 8563), *V. metschnikovii* (NCTC 2443) and '*V. albensis*' (NCMB 41). The cultures were inactivated with phenol (0.5%, w/v) at room temperature for 2 h before the cells were washed three times with distilled water, and acetone-dried. Since the dilute phenol treatment of the cultures at room temperature was considered to be much milder than heat treatment and, therefore, more appropriate for preventing or minimizing probable loss or destruction of cells, phenol-inactivated cultures were used for the extraction of LPS in this study.

Preparation of LPS. LPS was isolated from the acetone-dried cells by the phenol/water technique of Westphal *et al.* (1952) and purified by repeated (six times) ultracentrifugation (105000 *g* for 3 h) and RNAase treatment (Westphal & Jann, 1966).

Analytical methods. Fructose was determined after hydrolysis in 0.2 M-acetic acid at 100 °C for 2 h by gas-liquid chromatography (GLC) as the *O*-acetylated-*O*-methyloxime derivative prepared according to the method of Mawhinney *et al.* (1979) on an ECNSS-M column (Gasukuro Kogyo Co.). Perosamine, used as a standard for the quantitative analysis, was isolated from *V. cholerae* P1418 (Ogawa) LPS according to the method of Redmond (1975); the quinoxaline standard was isolated from *V. cholerae* tox 101-TI-N4 LPS (Hisatsune *et al.*, 1980*b*). KDO was estimated by the conventional Weissbach's periodate/thiobarbituric acid reaction under conventional hydrolysis conditions (0.01 M-H₂SO₄ at 100 °C, 20 min) (Weissbach & Hurwitz, 1959). KDO (ammonium salt) and alkaline phosphatase (type III-R) were purchased from Sigma. KDO was also determined by GLC as the peracetylated derivative on a TABSORB column (Regis Chemical Co.).

High-voltage paper electrophoresis was performed on Whatman no. 1 paper with a solvent system of pyridine/acetic acid/water (10:4:86, by vol.) pH 5.3 at 45 V cm⁻¹ for 40 min. KDO and 5-*O*-phosphoryl-KDO were detected with a thiobarbituric acid reagent (Warren, 1960). Other colorimetric and GLC analyses were done as described previously (Hisatsune *et al.*, 1980*a*, 1983). Analytical data presented are mean values of triplicate experiments.

Isolation of the unknown substance X, positive in the periodate/thiobarbituric acid reaction. LPS (50 mg) was heated in 4 M-HCl (20 ml) at 100 °C for 45 min, then the hydrolysates were rotary-evaporated under reduced pressure to dryness. The residues were dissolved in distilled water and passed through a Dowex 50 (H⁺ form) column (1.5 × 20 cm) in order to remove any basic components. The eluates, together with water washings, were applied to a Dowex 1 (acetate form) column (1.5 × 20 cm). After being washed with distilled water, the column was eluted with 2 M-HCl; the eluates which were positive for periodate/thiobarbituric acid test were collected, and then rotary-evaporated under reduced pressure to dryness to isolate the unknown substance X.

RESULTS AND DISCUSSION

Extraction yields and overall chemical composition

The extraction yields of LPS from the acetone-dried cells ranged from 0.6% to 5.9% (Table 1). [Fuller versions of Tables 1 and 2, with data for 21 strains, have been deposited with the British Library Document Supply Centre, Boston Spa, Yorkshire LS23 7BQ, UK, as Supplementary Publication no. SUP 28025 (7 pages). (Copies may be obtained from the BLDSC on demand; wherever possible, requests should be accompanied by prepaid coupons, held by many university and technical libraries and by the British Council.)] The overall chemical composition of the LPS was not very different from that of the usual Gram-negative bacterial LPS (Table 1).

Neutral and amino sugar composition

Representative results of the compositional sugar analysis of LPS are presented in Table 2. Glucose, L-glycero-D-mannoheptose and glucosamine were found as component sugars in LPS of all the strains examined. The first two sugars are regular sugar constituents of the core region of Gram-negative bacterial LPS, whereas glucosamine is a basic structural unit constituting the

Table 1. Chemical composition (% w/w) of LPS isolated from representative strains of *V. cholerae**

	Yield†	Protein	Total carbohydrate	Reducing sugar	Amino sugar	Total lipid	Total phosphorus	Source of culture‡
<i>V. cholerae</i>								
Biotype classical								
Inaba 35A3	3.4	2.6	31.3	17.5	12.0	22.5	1.9	(1)
Biotype eltor								
SLH 22	1.6	4.5	26.5	10.3	8.5	20.0	1.9	(2)
Non-O1 <i>V. cholerae</i>								
O3 4715	2.3	2.1	29.6	36.9	15.0	26.6	3.4	(3)
‘ <i>V. albensis</i> ’ NCMB 41	2.3	2.8	38.3	21.7	4.8	29.5	2.8	(3)
‘ <i>V. proteus</i> ’ NCTC 8563	2.7	2.3	51.8	21.4	3.6	26.9	3.3	(3)
<i>V. metschnikovii</i> NCTC 8443	5.9	1.9	36.7	27.4	3.1	18.7	2.5	(3)

* Data for 21 strains (including those listed above) are presented in the table deposited in the British Library Document Supply Centre as Supplementary Publication no. SUP 28025.

† From acetone-dried cells (% w/w).

‡ (1) *V. cholerae* 35A3 (Inaba) strain was obtained from Dr N. Ohtomo, Chemo-Sero-Therapeutic Institute, Kumamoto, Japan. (2) *V. cholerae* SLH22 strain was obtained from Dr S. Shimodori and Dr K. Takeya, Department of Bacteriology, School of Medicine, Kyushu University, Fukuoka, Japan. (3) Other strains were obtained from Dr T. Shimada and Dr R. Sakazaki, National Institute of Health, Tokyo, Japan. *Salmonella minnesota* R595 as a source of authentic 4-amino-4-deoxy-D-manno-arabinose was provided by Dr N. Kasai, Department of Microbiology, School of Pharmaceutical Sciences, Showa University, Tokyo, Japan. *Bordetella pertussis* phase I cells as a source of authentic 5-O-phosphoryl-KDO were provided by Dr N. Ohtomo.

lipid A disaccharide backbone of LPS (Rietschel *et al.*, 1982, 1984). With respect to the glucose content of LPS, O1 *V. cholerae* was different from either ‘biotype proteus’ (*V. metschnikovii* and ‘*V. proteus*’) or ‘biotype albensis’ (*V. albensis*): the glucose content ranged from 2.0 to 5.6% in the former, and from 11.6 to 34.7% in the latter. Galactose was found in the LPS of ‘biotype albensis’ (*V. albensis*) and some members of non-O1 *V. cholerae*, viz. O3, O6 and O7 serotypes, but O1 *V. cholerae*, and ‘biotype proteus’ (*V. metschnikovii* and ‘*V. proteus*’) lacked this sugar. Mannose was absent from all the *Vibrio* strains examined. Fructose was found in LPS from all members of O1 *V. cholerae* and most of the non-O1 *V. cholerae*, i.e. 4 of 5 serotypes. Previously, fructose was found in the LPS of all non-O1 *V. cholerae* (serotypes O3, O5, O7 and O8) (Hisatsune *et al.*, 1983). However, in the present study, fructose was not detected in the LPS of an additional strain of the non-O1 group, i.e. serotype O6. Moreover, it was further shown in a recent chemotaxonomic study on Vibrionaceae LPS (Hisatsune *et al.*, 1988) that, of 44 serotypes of non-O1 *V. cholerae* examined, as many as 9 did not contain fructose in the polysaccharide portion of their LPS. Thus, among the vibrios studied, O1 *V. cholerae* was the only group for which all strains contained fructose in their LPS.

It was particularly relevant that, within O1 *V. cholerae*, the classical biotype was distinguished from the eltor biotype by the fructose content of LPS. With the former, the LPS contained more than 3% fructose whereas the latter contained only 1% fructose. D-Glycero-D-mannoheptose, which has rarely been reported as a component of Gram-negative bacterial LPS (Wilkinson, 1977), was found in considerable amounts (4.9–5.9%) in LPS of the two strains belonging to O3 *V. cholerae* and in trace amounts in the ‘biotype proteus’ LPS.

With regard to amino sugars other than glucosamine, a pair of rarely occurring amino sugars, perosamine (4-amino-4,6-dideoxy-D-mannose) and quinovosamine (2-amino-2,6-dideoxy-D-glucose) was found in LPS from O1 *V. cholerae* regardless of either the biotype (classical or eltor) or the serotype (Inaba or Ogawa), whereas this pair was not present in non-O1 *V. cholerae*. This feature was confirmed with LPS from more than 30 additional strains of O1 group *V. cholerae* isolated from patients. In contrast, as reported previously (Hisatsune & Kondo, 1980), neither perosamine nor quinovosamine was present in LPS from R-mutants of O1 *V. cholerae* regardless of either biotype (classical or eltor) or serotype (Inaba or Ogawa), although they contained fructose. It was also previously observed that S–R mutation of O1 *V. cholerae* involves total

Table 2. Sugar composition (% w/w) of LPS isolated from representative strains of *V. cholerae**

	Glucose	Galactose	Rhamnose	Fructose	L-Glycero-D-manno-heptose	D-Glycero-D-manno-heptose	Glucosamine	Quinovosamine	Perosamine	Unknown amino sugar Y†	KDO	X1 (O-P-KDO‡) } X§	X2	Release of KDO from X
<i>V. cholerae</i>														
Biotype classical														
Inaba 35A3	5.4	—	—	4.0	10.0	—	5.4	1.2	8.6	—	ND	+	+	+
Biotype eltor														
Original-Celebes type														
SLH 22	4.8	—	—	1.0	4.4	—	1.6	1.3	8.4	—	ND	+	+	+
Non-O1 <i>V. cholerae</i>														
O3 4715	18.8	2.0	—	3.4	5.3	4.9	4.7	—	—	3.6	ND	+	+	+
‘ <i>V. albensis</i> ’ NCMB 41	11.6	3.0	—	—	23.1	—	10.2	—	—	—	ND	+	—	+
‘ <i>V. proteus</i> ’ NCTC 8563	22.3	—	—	—	6.5	tr.	3.5	+	—	—	ND	+	—	+
<i>V. metschnikovii</i> NCTC 8443	34.7	—	—	—	4.4	tr.	6.1	+	—	—	ND	+	—	+

* Data for 21 strains (including those listed above) are presented in the table deposited in the British Library Document Supply Centre Division as Supplementary Publication No. SUP 28025.

† Unknown amino sugar Y was estimated as glucosamine.

‡ O-P-KDO, O-phosphoryl-KDO.

§ X, Substance positive in the periodate/thiobarbituric acid test present in the hydrolysate of *V. cholerae* LPS.

|| Release of KDO from X1 and X2 after treatment with alkaline phosphatase.

ND, Not detected by Weissbach's periodate/thiobarbituric acid reaction (Weissbach & Hurwitz, 1959) under conventional hydrolysis conditions; tr., trace amount (less than 0.1%).

elimination of these two component amino sugars of S-form LPS, and this elimination results in the loss of O1 specificity of S-form LPS and the concomitant appearance of strong serological cross-reactivity among R-form LPS regardless of the serotypes (Inaba or Ogawa) of their S-form parent strains (Hisatsune & Kondo, 1980). From these data, it is considered that the pair perosamine and quinovosamine is substantially involved in the O1 specificity of S-form O1 *V. cholerae*. An unknown amino sugar, Y, was found in LPS from O3 *V. cholerae*. Analysis by GLC demonstrated that none of the amino sugars glucosamine, galactosamine, mannosamine, quinovosamine and fucosamine (2-amino-2,6-dideoxy-galactose) was identical to Y. In GC/MS analysis the *N*-acetylated-alditol acetate derivative of Y gave mass spectra which included peaks at *m/e* values of 144, 153, 168, 201, 242, 260, 288 and 302; these peaks are, in general, found in mass spectra for 2-amino-2,6-dideoxy-hexose. Quinovosamine was found in LPS of ‘*V. proteus*’ and *V. metschnikovii*.

There was virtually no difference between the Inaba and Ogawa serotypes of O1 *V. cholerae* in the sugar composition of their LPS, including the substances X1 and X2 that were positive in the periodate/thiobarbituric acid test (see below). Also, virtually no difference with respect to the sugar composition of LPS was observed either among the strains of the biotype eltor, including Original Celebes, cured Celebes and Classical-Ubon types (Takeya & Shimodori, 1963; Takeya *et al.*, 1965, 1968; Takeya, 1974), or (except for the fructose content) between the biotypes classical and eltor.

Apparent undetectability of KDO by conventional colour test under conventional hydrolysis conditions and the presence of O-phosphorylated KDO in strong-acid hydrolysates

No positive reaction for KDO was obtained in the Weissbach's periodate/thiobarbituric acid test with any of the LPS isolated from the vibrio strains. Although a very weak pinkish colour

Fig. 1. Absorption spectra of the coloured products produced by KDO ($50 \mu\text{g ml}^{-1}$) and by LPS isolated from *Salmonella minnesota* R595 (1 mg ml^{-1}), *Vibrio cholerae* NIH 41 (Ogawa) (5 mg ml^{-1}) and *V. cholerae* O3 (4715) (5 mg ml^{-1}) in Weissbach's periodate/thiobarbituric acid reaction. LPS from *V. cholerae* NIH 41 (Ogawa) and O3 (4715) was treated with 4 M-HCl at 100°C for 45 min before being subjected to the reaction. *S. minnesota* R595 LPS was used as standard LPS which releases KDO on mild hydrolysis in weak acid.

was occasionally obtained, it seemed nonspecific because the absorption spectra of the reaction mixtures did not show any characteristic maximal absorption.

LPS were also examined for the presence of KDO by GLC analysis on a TABSORB column. In general, KDO is released from LPS of the usual Gram-negative bacteria by heating in dilute acetic acid (0.1 M, 100°C for 2 h). However, no evidence for the presence of KDO was obtained by this technique in the acetic acid hydrolysates of LPS isolated from all the vibrios examined in this study.

Although it appeared that KDO was absent from LPS of the vibrios examined, heating the LPS of, for instance, O3 *V. cholerae* strain 4715 in 4 M-HCl at 100°C resulted in the release of a substance (X) positive in the periodate/thiobarbituric acid test, yielding a colour with maximal absorption at 549 nm, which is identical to that yielded by standard KDO (Fig. 1). This finding was confirmed with LPS of all the vibrios used. The maximal release of X was observed after 45 min heating in this hydrolysis condition, whereas free-form KDO was rapidly decomposed by this strong acid treatment.

Substance X was isolated from the hydrolysates (4 M-HCl, 100°C for 45 min) of O3 *V. cholerae* 4715 LPS by Dowex 50 (H^+) and Dowex 1 (acetate form) ion-exchange chromatography. Although the absorption spectrum of the reaction mixture of the partially purified X in the periodate/thiobarbituric acid test was the same as that of standard KDO, neither KDO nor the KDO-like substances that were found by the authors in LPS of *V. parahaemolyticus* O7 and O12 (Hisatsune *et al.*, 1980a) were identical to X with respect to stability to strong acid hydrolysis and behaviour in high-voltage paper electrophoresis. In high-voltage paper electrophoresis, X was separated into two substances; the slow-moving substance with an R_{KDO} (mobility relative to KDO) value of 1.54 is referred to as X1 and the fast-moving substance (R_{KDO} 1.80) as X2. In contrast, neither X1 nor X2 was detected in the strong-acid hydrolysates of LPS from *Salmonella typhimurium* LT2, *Salmonella minnesota* R595 or *Escherichia coli*, or of KDO itself as standard;

only KDO was detected in these hydrolysates. These results indicate that X1 and X2 are not degradation products derived from KDO as a result of the strong acid treatment.

Chaby & Szabo (1975) reported that the 5-*O*-phosphorylated derivative of KDO is present in LPS isolated from *Bordetella pertussis* and that this derivative of KDO is stable to strong acid hydrolysis (4 M-HCl at 100 °C). We investigated the possibility that X1 and X2 of O3 *V. cholerae* strain 4715 are phosphorylated derivatives of KDO, with LPS from *B. pertussis* used as a control. High-voltage paper electrophoresis showed that X1 present in the strong-acid hydrolysates of LPS from strain 4715 had the same mobility as that of the 5-*O*-phosphoryl-KDO from *B. pertussis* LPS. X1 and X2, each isolated from X on high-voltage paper electrophoresis, and 5-*O*-phosphoryl-KDO derived from *B. pertussis* were treated with alkaline phosphatase (8 units ml⁻¹ in 0.05 M-ammonium bicarbonate buffer, pH 9.6) at 37 °C for 16 h, and then subjected to high-voltage paper electrophoresis. The spots corresponding to X1, X2 and 5-*O*-phosphoryl-KDO derived from *B. pertussis* disappeared, and a spot with the same mobility as that of standard KDO was observed. These results suggested that either X1 or X2 is some kind of phosphorylated derivative of KDO, and the results of high-voltage paper electrophoresis indicate that X1 is probably 5-*O*-phosphoryl KDO. In this respect, it is relevant that the occurrence of 5-*O*-phosphoryl-KDO in LPS of O1 *V. cholerae* 95R (a rough mutant of strain Ogawa 162) and *V. cholerae* 569B (Inaba) was recently reported by Brade (1985).

Vibrios other than O3 *V. cholerae* 4715 were examined for the presence of X1 and X2 in the strong-acid hydrolysates of their LPS. As seen in Table 2, X1 was found in all the vibrios tested, while the pair X1 and X2 was found only in *V. cholerae* (both O1 and non-O1 groups) regardless of either the serotype (Inaba or Ogawa) or the biotype (classical or eltor). Thus both O1 and non-O1 group *V. cholerae* differed from *V. metschnikovii*, '*V. proteus*' and '*V. albensis*' in respect to the occurrence of the pair X1 and X2 in the strong-acid hydrolysates of LPS.

The most characteristic feature of chemotaxonomic interest demonstrated in this study is the presence of the pair perosamine and quinovosamine in LPS of O1 *V. cholerae*, including both classical and eltor biotypes and both Inaba and Ogawa serotypes. Neither non-O1 *V. cholerae* nor the other three vibrios studied (*V. metschnikovii*, '*V. proteus*' and '*V. albensis*') contain this pair. It is also important that classical O1 group *V. cholerae* is distinguished from eltor O1 group *V. cholerae* on the basis of the fructose contents of their LPS.

In the present study, the common occurrence of *O*-phosphorylated KDO was for the first time demonstrated in the strong-acid hydrolysates of LPS from *V. cholerae* (both O1 and non-O1 groups), *V. metschnikovii*, '*V. proteus*' and '*V. albensis*'. Further chemotaxonomic study on Vibrionaceae LPS, in particular with respect to the occurrence of *O*-phosphorylated KDO, is under way in our laboratory.

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