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Structural studies of the core region of *Aeromonas salmonicida* subsp. *salmonicida* lipopolysaccharide

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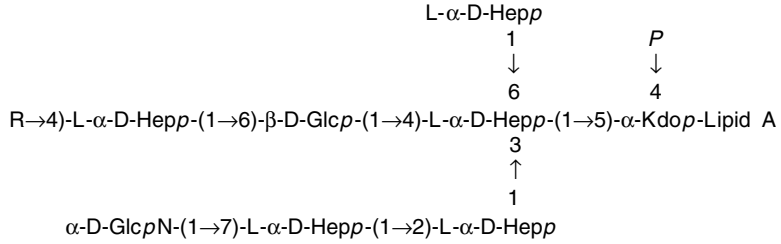
Abstract—The core oligosaccharide structure of the in vivo derived rough phenotype of *Aeromonas salmonicida* subsp. *salmonicida* was investigated by a combination of compositional, methylation, CE-MS and one- and two-dimensional NMR analyses and established as the following:



where R = α-D-Gal*p*-(1→4)-β-D-Gal*p*NAc-(1→ or α-D-Gal*p*-(1→ (approx. ratio 4:3).
Comparative CE-MS analysis of *A. salmonicida* subsp. *salmonicida* core oligosaccharides from strains A449, 80204-1 and an in vivo rough isolate confirmed that the structure of the core oligosaccharide was conserved among different isolates of *A. salmonicida*.

Keywords: *Aeromonas salmonicida*; Lipopolysaccharide; Core oligosaccharide; NMR; CE-MS

1. Introduction

Aeromonas salmonicida subsp. *salmonicida* has been recognized as a pathogen of fish for over 100 years and its geographical distribution is almost worldwide. It is the causative agent of furunculosis, an infectious disease principally in salmonid fish, and has also been associated with clinical or covert disease in a variety of non-salmonid species.[1] Since the annual worldwide losses of farmed fish to diseases involve millions of dollars, this pathogen has been subjected to considerable investigation.[2,3] Lipopolysaccharide (LPS), an integral component of the outer membrane of Gram-negative bacteria, is the most immunoreactive cell surface antigen[4] and is considered to be an important virulence factor involved in the pathogenesis of bacterial infections.[5] In order to understand the interactions between pathogen and host factors contributing to the disease state, the complete and detailed structure of LPS should be defined. Previous studies have characterized structures of O-chain polysaccharide and the core oligosaccharide region of LPS from *A. salmonicida* strain SJ-15.[6–8] Recently, we have established structures of the capsular polysaccharide and the O-chain polysaccharide from *A. salmonicida*.[9,10] Here we describe the structural elucidation of the core oligosaccharide region of the *A. salmonicida* subsp. *salmonicida* LPS from the in vivo rough isolate and isolate of strains A449 and 80204-1.

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2. Experimental

2.1. Bacterial culture and growth conditions

The in vivo derived rough phenotype of *A. salmonicida* subsp. *salmonicida* and strains A449 and 80204-1 were obtained from the Institute for Marine Biosciences, National Research Council of Canada (Halifax, Nova Scotia). The bacteria were cultured in Tryptic Soy Broth (TSB) at 18 °C for 48–72 h. The cells were killed with 1% (w/v) phenol soln (22 °C, 4 h), washed with 0.01 M phosphate buffered saline (PBS, pH 7.4) and harvested by low-speed centrifugation (3000*g*, 25 min).

2.2. LPS isolation

Bacterial cells were washed successively with 2.5% saline (w/v), EtOH, acetone and ethyl ether (anhyd), and the cells were recovered by centrifugation. LPS from the in vivo rough isolate of *A. salmonicida* subsp. *salmonicida* was extracted from dried cells with 5:5:8 phenol–$CHCl_3$–petroleum ether at 60–95 °C and precipitated as reported previously,[11,12] while LPS from strains A449 and 80204-1 was extracted by the method of Westphal and Jann.[13]

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed with a mini-slab gel apparatus (Bio-Rad) using the method of Laemmli.[14] LPS samples were prepared at a concentration of 0.1% (w/v) in a sample buffer (62.5 mM Tris/HCl, pH 6.8, 20% glycerol, 2% SDS, 5% 2-mercaptoethanol), equivalent amounts loaded in each lane and stained according to Tsai and Frasch.[15]

2.4. Mild acid hydrolysis of LPS and preparation of the N-acetylated core oligosaccharide

The purified LPS from the in vivo rough phenotype of *A. salmonicida* subsp. *salmonicida* (60 mg) was hydrolyzed with 2% AcOH (60 mL) at 100 °C for 2 h. The soln was cooled down on ice, and the precipitated lipid A removed by low-speed centrifugation. The supernatant was lyophilized and water-soluble components were fractionated by gel permeation chromatography on a Sephadex G-25 column (1.6 cm × 95 cm) (Sigma–Aldrich, St. Louis, Mo, USA) and the core oligosaccharide fraction eluted with distilled water (OS-1). LPS from *A. salmonicida* strains A449 and 80204-1 were purified and delipidated as described previously[10] using Sephadex G-25 and the resultant core oligosaccharide fractions subjected to CE-MS analysis.

For N-reacetylation, the core oligosaccharide was treated with Ac_2O (0.1 mL) in aq satd soln of sodium bicarbonate (1 mL) (22 °C, 30 min)[16] and the products purified on a Sephadex G-25 column (Sigma–Aldrich, St. Louis, Mo, USA) to give the N-acetylated core oligosaccharide (OS-2).

2.5. Deacylation of LPS and preparation of backbone oligosaccharides

LPS from the in vivo rough isolate of *A. salmonicida* subsp. *salmonicida* (80 mg) was treated with 4 M KOH (4 mL) at 125 °C for 20 h according to Holst.[17] Following neutralization and purification on a Sephadex G-25 column, the oligosaccharide fraction was further fractionated by high-performance anion-exchange chromatography (HPAEC) using a linear gradient of 0.1–0.8 M sodium acetate in 0.1 M NaOH, followed by desalting on a Sephadex G-25 column to give two fractions, F 1 (5 mg) and F 2 (14 mg). F 1 was pure and contained a backbone oligosaccharide (LPS-OH-1), while F 2 was shown to be a mixture of two backbone oligosaccharides, LPS-OH-1 and LPS-OH-2.

2.6. Compositional analysis

Core oligosaccharide samples (0.5 mg) were hydrolyzed with 2 M trifluoroacetic acid at 100 °C for 18 h and analyzed as their alditol acetates as previously described.[18]

The identity of each glycose derivative was established by comparison of its GLC retention time and mass spectrum with that of an authentic reference sample. Peracetylated heptitol derivative was found to have the L-*glycero*-D-*manno* (or D-*glycero*-L-*manno*) configuration by comparison of its GLC retention time with that of an authentic standard. The L-*glycero*-D-*manno* absolute stereochemistry was assumed on biosynthetic grounds.[19] The hexose residues were determined to have the D-configurations by capillary GLC of their acetylated (−)-2-butyl glycosides, according to the method of Leontein et al.[20]

2.7. Methylation analysis

The core oligosaccharide samples were methylated according to the method of Ciucanu and Kerek.[21] The permethylated polysaccharide was subjected to hydrolysis as described by Stellner et al.[22] and analyzed according to previously reported conditions for partially methylated alditol acetates.[23]

2.8. NMR spectroscopy

NMR spectra were performed on Varian INOVA 500 MHz spectrometer using standard software. All NMR experiments were performed at 25 °C using a 5 mm indirect detection probe with the ^1H coil nearest to the sample. The methyl resonance of acetone was