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Structural characterization of the O-specific polysaccharide from the lipopolysaccharide of the fish pathogen *Aeromonas bestiarum* strain P1S

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ABSTRACT

The O-specific polysaccharide obtained by mild-acid degradation of lipopolysaccharide of *Aeromonas bestiarum* P1S was studied by sugar and methylation analyses along with ^{1}H and ^{13}C NMR spectroscopy. The sequence of the sugar residues was determined using $^{1}H,^{1}H$ NOESY and $^{1}H,^{13}C$ HMBC experiments. The O-specific polysaccharide was found to be a high-molecular-mass polysaccharide composed of tetrasaccharide repeating units of the structure

→2)-β-D-Qui*p*3NAcyl-(1→2)-β-D-Rib*f*-(1→4)-β-D-Gal*p*-(1→3)-α-D-Fuc*p*NAc-(1→

Since small amounts of a terminal Qui*p*3N residue were identified in methylation analysis, it was assumed that the elucidated structure also represented the biological repeating unit of the O-specific polysaccharide.

1. Introduction

Gram-negative bacteria of the genus *Aeromonas* are widespread in various habitats. They have been isolated from sources such as food, drinking water, sewage, environmental water, and human clinical specimens.[1] Strains of the species *Aeromonas hydrophila* and *Aeromonas caviae* have been recognized as causative agents of sporadic diarrhea, dysentery, and life-threatening extra-intestinal infections in immuno-compromised patients and children.[2–4] The varied clinical picture of *Aeromonas* infections indicates a multifactor model of pathogenesis.[5] Cell-surface components such as outer membrane proteins, lipopolysaccharide (LPS),[6] the S-layer,[7] polar flagella, and pili[4] have been identified as putative virulence factors, which play an important role in adhesion of the bacteria to epithelial cells, determining virulence and serum resistance as well as biofilm formation.[2,4] LPS, which is a highly immunoreactive cell surface compound, has been implicated in the pathogenesis of *Aeromonas* strains.[8] Studies of antigen expression in biofilm cells of *A. hydrophila* have revealed that a complete LPS structure is important for the ability of *Aeromonas* species to maintain tetragonal S-layer on the cell surface. On the other hand, changes in the LPS molecule can result in the loss of appropriate conformation of the protein structure.[9] *Aeromonas bestiarum* and other species belonging to the motile aeromonad group, such as *A. hydrophila*, *Aeromonas sobria*, *Aeromonas veronii*, *Aeromonas allosaccharophila*, and *Aeromonas jandaei*, have been reported as fish pathogens.[10–12] The clinical manifestations of fish diseases range from dermal and ophthalmic ulcerations to more severe symptoms including soft tissue infections, hemorrhagic septicemia, motile aeromonad septicemia (MAS), or motile aeromonad infection (MAI).[2–6,10–12] Outbreaks usually occur when a fish's immune system is depressed due to an environmental stress such as overcrowding, poor water quality, organic pollution, and hypoxia in conjunction with other diseases.[13,14] *Aeromonas salmonicida* subsp. *salmonicida*, a non-motile aeromonad, is the etiological agent of a bacterial septicemia in salmonid fish, called furunculosis. Furunculosis is an important disease in wild and cultured stocks of salmonid and other fish species and can have significant negative economic impacts on aquaculture operations. Most *Aeromonas* species are opportunistic pathogens, entering through wounds or affecting only stressed fish. *A. salmonicida* subsp. *salmonicida*, however, is a specific pathogen capable of causing diseases in healthy salmonid fish at a very low level of infection.[15,16]

A comparative analysis of sequenced genomes of *A. hydrophila* strain ATCC 7966 and *A. salmonicida* strain A449 provided an opportunity to identify genes involved in host invasion and virulence. The studies revealed that genes determining the synthesis

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of LPS are located adjacent to clusters of genes encoding several types of adhesins (e.g., the surface layer protein VapA, flagella, and pili), which are important colonization factors.[16] Moreover, a structural analysis of O-specific polysaccharides (OPSs) of *A. hydrophila*, *A. salmonicida*, and *A. caviae* strains,[17–20] supported by serological studies could provide an explanation of the host–pathogen interactions contributing to the disease state.[8,9] The recently determined OPS structure of *A. bestiarum* strain 207, which consists of branched pentasaccharide-repeating units containing four L-rhamnose and one D-glucosamine residues,[21] complements the current knowledge of the compositional diversity of O-antigens among *Aeromonas* strains.

This study reports the structural characterization of the OPS of *A. bestiarum* strain P1S isolated in the course of motile aeromonad septicemia[11] in a Polish carp farm. Results of some taxonomic studies have revealed that diseases, and thus losses, in commercial aquacultures have mostly been caused by strains within this genomospecies.[11,12]

2. Results and discussion

The LPS of *A. bestiarum* P1S was isolated by the hot phenol–water procedure[22] from enzymatically-digested bacterial cells. Its SDS–PAGE profile (Fig. 1) revealed that the most prominent bands appeared in the region corresponding to the rough Ra chemotype of *Salmonella* LPS and were accompanied by much less intense slow-moving bands representing smooth (S-form) LPS. The OPS was released by mild-acid degradation of the LPS and isolated, in the void volume, by gel-permeation chromatography (GPC). The low yield of the OPS, making up only ~8% of the total amount of the material eluted from the column, confirmed that the R-type LPS dominated in *A. bestiarum* P1S cells, and only a minor part of the material was of the S-type. GLC–MS analyses of alditol acetates obtained after complete acid hydrolysis of the OPS revealed ribose (Rib), 3-amino-3,6-dideoxyglucose (Qui3N), 2-amino-2,6-dideoxygalactose (FucN), and galactose (Gal) residues in a relative peak area ratio of 1:1.4:1.1:2. Qui3N and FucN were identified by comparing their retention times and mass spectra with those of authentic samples isolated from the O-specific polysaccharides of *Escherichia coli* O5[23] (a strain obtained from the Institute of Immunology and Experimental Therapy, Wroclaw, Poland) and *Acinetobacter baumannii* strain 34,[24] respectively. The GLC–MS analysis also showed the presence of a small peak corresponding to a product of 3,6-dideoxy-3-(3-hydroxybutyramido)-glucose (Qui3NAcyl). The substitution of Qui3N with 3-hydroxybutyrate was further confirmed by NMR analysis. The absolute configurations of the monosaccharides[25] determined by GLC of the acetylated (*S*)-2-butyl glycosides showed that Rib, Gal, Qui3N, and FucN had the D-configuration, and a GLC–MS analysis of trimethylsilylated (*R*)- and (*S*)-2-octyl esters obtained by a modified method[26] demonstrated the presence of (*R*)-3-hydroxybutyrate. The D-configuration of Qui3N was confirmed by GLC coinjection experiments, an analysis of glycosylation effects in a ^{13}C NMR spectrum of the studied polysaccharide (see below), and by comparison with other published data.[23,27,28]

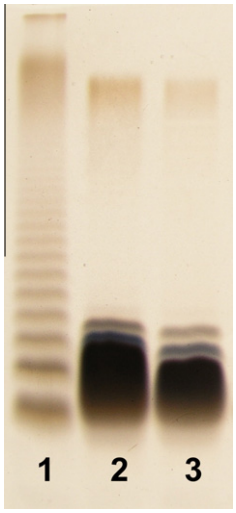

Figure 1. Silver-stained SDS–Tricine PAGE of the LPSs of *A. bestiarum* strain P1S (lane 2, 4 μg; lane 3, 2 μg) and *Salmonella enterica* sv. Typhimurium (Sigma) as reference (lane 1, 2 μg).

Methylation analysis of the OPS identified 1,2,4-tri-*O*-acetyl-3,5-di-*O*-methyl-ribitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-galactitol, 1,2,5-tri-*O*-acetyl-4-*O*-methyl-3-(*N*-methylacetamido)-3,6-dideoxyglucitol, and 1,3,5-tri-*O*-acetyl-4-*O*-methyl-2-(*N*-methylacetamido)-2,6-dideoxygalactitol, elucidating the glycosidic substitutions of ribofuranose (Rib*f*) at C-2, Gal*p* at C-4, Qui*p*3N at C-2, and Fuc*p*N at C-3. Moreover, among the methylation analysis products a small amount of 1,5-di-*O*-acetyl-2,4-di-*O*-methyl-3-(*N*-methylacetamido)-3,6-dideoxyglucitol was detected, identifying a terminal Qui*p*3N residue.

The ^{13}C NMR spectrum of the *A. bestiarum* P1S OPS (Fig. 2a) demonstrated that the polymer had a regular structure composed of tetrasaccharide repeating units. It contained signals for four anomeric carbons at δ 107.65, 105.83, 104.78, and 96.85 (labeled **A**, **D**, **C**, and **B**, respectively) with an integral intensity ratio of 1.1:1:1:1. Signals were also found for two nitrogen-bearing carbons (FucN C-2 and Qui3N C-3) at δ 48.42 and 56.35, two methyl groups of 6-deoxysugars (FucN and Qui3N) at δ 16.19 and 17.85, one *N*-acetyl group (CH_3 at δ 23.16 and CO at δ 175.06), one *N*-(3-hydroxybutyryl) group (CH_3 at δ 22.87, CO at δ 175.19, as well as CH–OH and CH_2 at δ 65.6 and 45.94, respectively), and 15 other non-anomeric sugar ring carbons in the region δ 61.76–84.28, some of which overlapped. The presence of a signal at δ 83.02, which is typical of C-4 of a furanose ring, confirmed the occurrence of a ribofuranose residue.[29,30]

Consistent with this, the ^{1}H NMR spectrum of the OPS (Fig. 2b) contained, inter alia, signals for four anomeric protons at δ 5.62, 5.53, 4.89, and 4.42, labeled **A** through **D**, respectively. In the high field region of the spectrum, there were also signals originating from the methyl groups of FucN and Qui3N at δ 1.25 and 1.31, respectively, one signal of an *N*-acetyl group at δ 2.05 (singlet), one at δ 1.23 (CH_3), two at δ 2.35 and 2.48 (CH_2, both m), and one at δ 4.21 (CH–OH) of *N*-(3-hydroxybutyryl), the chemical shifts of which were consistent with published data.[28,31]

The ^{1}H and ^{13}C NMR spectra of the OPS were assigned using ^{1}H,^{1}H, DQF-COSY, TOCSY, NOESY, ^{1}H,^{13}C HSQC, and ^{1}H,^{13}C HMBC experiments. All chemical shifts are summarized in Table 1.

The TOCSY spectrum of the OPS revealed spin systems of β-Rib (**A**), α-FucN (**B**), β-Qui3N (**C**), and β-Gal (**D**), and the data of the COSY and NOESY (Fig. 3a) experiments enabled a differentiation among protons within each spin system. *N*-Acetamido sugars were identified by correlations of the protons H-2 at δ 4.32 (for FucN) and H-3 at δ 3.96 (for Qui3N) to the corresponding carbon-bearing nitrogen at δ 48.42 and 56.35, respectively, as revealed by a ^{1}H,^{13}C HSQC experiment (Fig. 3b). The same spectrum also allowed the complete assignment of the chemical shifts of all corresponding proton–carbon resonances (Table 1). In particular, the characteristic downfield shift of the anomeric proton signal of residue **A** at δ_H 5.62 (s, $J_{1,2}$ ~1 Hz) and the corresponding carbon resonance at δ_C 107.65 identified β-D-Rib*f*.[29] In support of this, a correlation between H-1 and C-4 at δ_H/δ_C 5.62/83.02 was found in the ^{1}H,^{13}C HMBC spectrum, a finding previously observed for the furanosidic ring form.[23,30] The relatively small $J_{1,2}$ coupling constant value of