Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 340 (2005) 1509–1513
Carbohydrate RESEARCH

The structure of the antigenic O-polysaccharide of the lipopolysaccharide of *Edwardsiella ictaluri* strain MT104

Evgeny Vinogradov,[a] Ludmila Nossova,[a] Malcolm B. Perry[a,*] and William W. Kay[b]

[a]*Institute for Biological Sciences, National Research Council, Ottawa, ON, Canada K1A 0R6*
[b]*Department of Bacteriology and Biochemistry, University of Victoria, Victoria, BC, Canada V8W 2T2*

Received 7 March 2005; accepted 25 March 2005

Abstract—The structural characterization of the antigenic O-polysaccharide component of the lipopolysaccharide produced by the fish pathogenic bacterium *Edwardsiella ictaluri* MT104 was undertaken by the application of NMR spectroscopy and chemical analysis. The O-chain was found to be a linear polymer of a repeating tetrasaccharide unit composed of D-glucose, 2-acetamido-2-deoxy-D-galactose, and D-galactose in a 1:2:1 ratio having the structure:

$$[\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Gal}p\text{NAc-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\rightarrow 4)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow]_n$$

Keywords: *Edwardsiella ictaluri*; LPS; O-chain; Structure; NMR spectroscopy

1. Introduction

Channel catfish (*Ictalurus punctatus*) aquaculture contributes more than $2.5 billion (USD) annually to the US gross national product and about $300 million to the state of Mississippi alone. However, commercial catfish production is heavily impacted by a severe disease known as enteric septicemia of catfish (ESC) caused by a Gram negative bacterium *Edwardsiella ictaluri*.[1,2] *E. ictaluri* is often found commensally in the intestines of cold-blooded animals and in fresh warm water fish within temperature range from 18 to 28 °C. *E. ictaluri* was first described as a new species in 1979 in connection with disease of farm-raised channel catfish and currently represents up to 85% of diagnostic isolates from catfish farms. Consequently ESC is considered the most economically important disease of farm-raised channel catfish. The only treatments currently available are oxytetracycline and sulfadimethoxine/ormethoprim delivered orally in feed; however, since the earliest clinical signs associated with ESC is anorexia, antibiotic use is only effective in limiting the spread of disease outbreaks.

E. ictaluri isolates from ESC outbreaks at different locations were found to be biochemically and serotypically homogeneous[3–6] making feasible the prevention of the disease by vaccination using a common immunogen.[7] Unfortunately, cost-effective disease protection using killed whole bacterial vaccines have not been particularly successful in channel catfish, and although protection can be afforded with attenuated mutants there are accompanying difficulties such as consumer resistance and regulatory restrictions associated with their application.[8,9]

Consequently there have been consistent efforts to elucidate antigenic factors associated with *E. ictaluri* in the hope of providing broadly applicable, cost-effective vaccines and diagnostics.[10] The characterization of *E. ictaluri* antigenic surface macromolecules such as lipopolysaccharide (LPS) and outer-membrane proteins can assist researchers in the design of modified vaccines against ESC. Indeed, recently whole outer membrane

Abbreviation: LPS, lipopolysaccharide.

* Corresponding author. Tel.: +1 613 990 0837; fax: +1 613 941 1327; e-mail: malcolm.perry@nrc-cnrc.gc.ca

0008-6215/$ - see front matter
doi:10.1016/j.carres.2005.03.008

preparations of *E. ictaluri* have been shown to produce a protective immune response in catfish.[11] Specific LPS O-polysaccharide (O-PS) is often found to be an important bacterial surface virulence antigen, which can in principle, be used as a vaccine component and also as a diagnostic marker.[12] Recently a partial chemical composition of *E. ictaluri* O-PS was determined,[13,14] and herein we present the results of a complete structural characterization of the O-PS isolated from the LPS produced by *E. ictaluri* MT104.

2. Results and discussion

E. ictaluri MT104 originally designated as ugA 6077 by Shotts and Waltman,[15] was one strain of twelve strains isolated from moribund catfish from several catfish farms in the South-Eastern USA. Strain MT104 was designated as 'typical' of several isolates and was differentiated from other *Edwardsiella* sp. on the basis of Gram stain reaction, microscopic morphology, physical properties of colonies on Tryptose Soy Agar and Essential Iron Medium, biochemical properties [indole –ve, triple sugar iron reaction, H_2S –ve, Gas +ve] and motility +ve.[16] Fermenter grown *E. ictaluri* MT104 were harvested at late log phase by tangential flow filtration and LPS was extracted by the hot phenol–water procedure[17] and purified by ultracentrifuging gel precipitations. The aqueous and phenol phase LPSs (isolated in 5% and 1% yield) were identical in composition. The O-PS was isolated from the LPS by conventional mild hydrolysis with 2% AcOH (100 °C, 2 h) followed by column Sephadex G-50 gel chromatography of the water soluble products to yield a lipid-free high molecular mass O-PS and a low molecular mass core oligosaccharide. GLC analysis of alditol acetates prepared from the polysaccharide showed the presence of glucose, galactose, and 2-amino-2-deoxygalactose.

Quantitative analysis of monosaccharides was restricted due to the presence (ca. 10%) of a high molecular mass bacterial glycogen. The absolute configuration of the O-PS monosaccharides was determined by GLC of acetylated glycosides prepared using an optically active alcohol, for example, (*R*)-2-butanol, according to the method described by Gerwig et al.[18] The monosaccharides were established as having the D-configuration by comparing the results of GLC analysis with those of reference samples. The experimentally obtained values of ^{13}C and ^{1}H chemical shifts (Table 1) were in good agreement with the calculated values confirming the identification of the O-PS component glycoses as D-Gal, D-Glc, and D-GalN in a 1:1:2 ratio in an enzyme digested glycogen free O-PS sample.

2D NMR spectroscopic analyses of the O-PS were performed using COSY, TOCSY, NOESY, HSQC, and g-HMBC experiments (Fig. 1). The complete assignment of spectral signals and observed correlations were performed (Fig. 2, Table 1) using the Pronto program.[19] Spin systems of five monosaccharides were identified, one of which belonged to 1,4-substituted glucopyranosyl residues of a bacterial glycogen impurity and is not be discussed herein. The four systems of the O-PS components were labeled **A**–**D** in order of their decreasing anomeric proton chemical shifts. Monosaccharides were identified on the basis of vicinal proton coupling constants and ^{13}C NMR chemical shifts. Thus residues **A**, and **C** were identified as α- and β-D-Gal*p*NAc, respectively. The residue **B** was identified as α-D-Glc*p*, and the residue **D** as β-D-Gal*p*. Anomeric configurations followed from measured $J_{1,2}$ coupling constants and chemical shifts of H-1 and C-1 signals were supported by NOESY data, which showed the intraresidual NOE correlations between H-1 and H-3, H-5 for residues **C** and **D**.

The monosaccharide sequence in the polysaccharide chain was inferred using interresidual NOE correlations and long-range scalar correlations measured by HMBC. The NOE contacts were observed between protons **A**1 and **C**3, **B**1 and **A**4, **C**1 and **D**4, **D**1 and **B**4, as well as corresponding HMBC correlations. The signals of the carbon atoms at substitution sites, that is, **A**4, **B**4, **C**3, and **D**4 showed strong downfield shifts, further confirming the determined linkage position assignments. Taken together these data led to the O-PS being characterized

Table 1. ^{1}H and ^{13}C NMR chemical shift data for the O-PS of *Edwardsiella ictulari* MT104

Glycose residue	Chemical shift (ppm)						
	Nucleus	1	2	3	4	5	6,6′
[**A**] →4)-α-D-Gal*p*NAc-(1→	^{1}H	5.11	4.31	3.92	4.12	3.93	3.89
	^{13}C	95.2	50.9	68.5	79.6	71.8	61.5
[**B**] →4)-β-D-Glc*p*-(1→	^{1}H	4.99	3.63	3.94	3.68	4.19	3.88
	^{13}C	101.2	72.6	72.4	79.2	71.9	60.6
[**C**] →3)-β-D-Gal*p*NAc-(1→	^{1}H	4.75	4.06	3.84	4.09	3.62	3.79
	^{13}C	103.2	52.3	76.5	65.1	75.9	60.7
[**D**] →4)-β-D-Gal*p*-(1→	^{1}H	4.46	3.44	3.79	4.15	3.73	3.82
	^{13}C	103.9	72.2	73.7	77.3	75.6	61.9

Acetate signals: C=O: 175.8, 175.7 ppm; Me ($^{1}H/^{13}C$): 20.5/23.2, 2.08/23.7 ppm. Measurements at 40 °C.

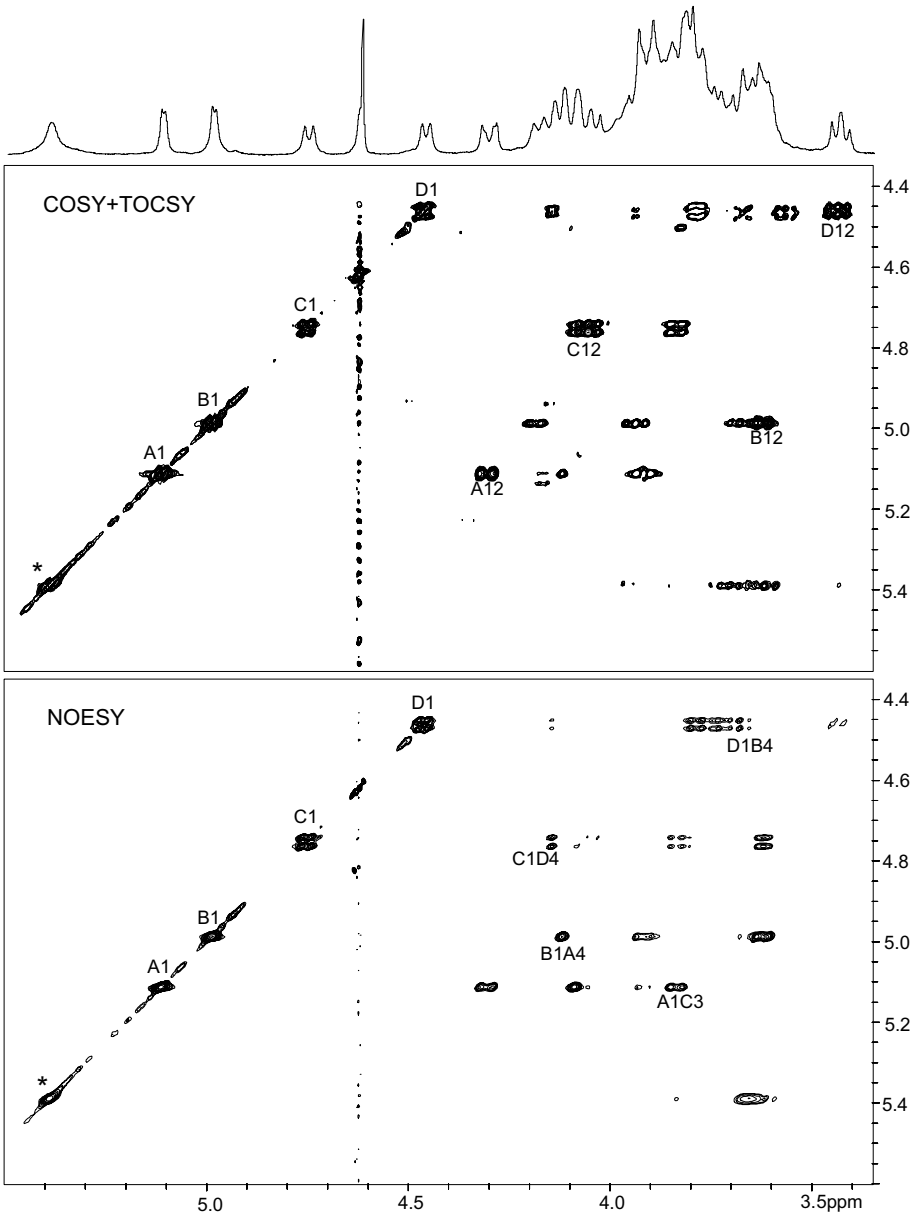

Figure 1. 1D ^{1}H NMR, COSY + TOCSY, and NOESY spectra of the LPS O-chain produced by *Edwardsiella ictulari* MT 104.

as an unbranched polymer of a repeating tetrasaccharide unit having the structure:

[B] [A] [C] [D]

$$[\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Gal}p\text{NAc-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\rightarrow 4)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow]_n$$

Substitution positions were confirmed by methylation analysis that revealed the presence of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol-1-*d*, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol-1*d*, 1,4,5-tri-*O*-acetyl-2-(*N*-methylacetamido)-2-deoxy-3,6-di-*O*-methylgalactitol-1*d*, and 1,3,5-tri-*O*-acetyl-2-(*N*-methylacetamido)-2-deoxy-4,6-di-*O*-methylgalactitol-1*d* in a 1.0:0.81:0.72:0.67 ratio consistent with the respective O-4 position of substitution of the D-Glc*p* and D-Gal*p* residues and the O-4 and O-3 glycosyl substitutions of the D-Gal*p*NAc residues.

Serological analyses using monoclonal antibodies have indicated that different isolates of *E. ictaluri* appear to be serologically homogeneous.[15] However, there is evidence that distinct strains are emerging in channel catfish aquaculture areas[20,21] suggesting that significant antigenic structural differences between *E. ictaluri* strains may exist. If new serotypes of *E. ictaluri* prove to be virulent in challenge experiments with channel fish,

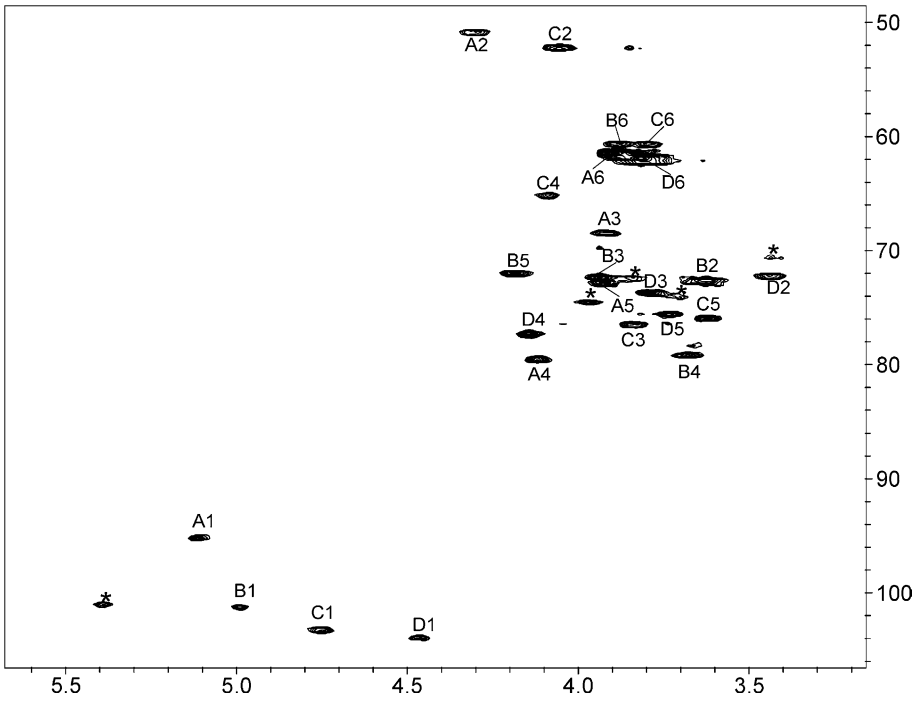

Figure 2. Fragment of the HSQC spectrum of *Edwardsiella ictaluri* MT 104 O-specific polysaccharide. Signals of starch are marked as *.

these strains may become of economic concern in aquaculture, and importance will be attached to future O-PS related serology and the structural characterization of associated LPS antigens. Clearly, LPS is an important pathogenesis factor in *E. ictaluri* since effectively attenuated mutants harboring LPS compositional alterations have been arrived at through serial subculturing. This important pathogenesis factor has been the subject of recent studies and the genes encoding its synthesis have been identified.[14]

3. Experimental

3.1. Bacterial cell production

E. ictaluri MT104 (original designation ugA 6077[15]) was stored as stocks at −80 °C in Brain Heart Infusion (BHI) liquid medium containing 15% glycerol. The growth medium was optimized partly based on the literature[22] and was composed of: Vegetable Peptone Broth (Oxoid), supplemented with D-glucose (4 g/L), and $MgSO_4$ (240 mg/L), both sterilized separately, and L-cysteine (50 mg/L sterilized separately by filtration). For large scale production of *E. ictaluri*, using the above medium, cultures were sequentially scaled up by serial log culture on supplemented VP agar to 25 mL, 250 mL, and two 1 L cultures. These were used to inoculate a 100 L, stainless steel fermenter vessel (LH) fitted with an overhead spinning disk foam breaker and bottom drive agitation. Additional D-glucose was sterilized separately and added automatically using a pH 8.0 set point controller and a maintained 28 °C. Cells were harvested at late log phase by tangential flow filtration and frozen at −80 °C.

3.2. NMR spectroscopy

^1H and ^{13}C NMR spectra were recorded using a Varian Inova 500 spectrometer in D_2O solns at 40 °C for the polysaccharide with acetone standard (2.225 ppm for ^1H and 31.5 ppm for ^{13}C) using standard pulse sequences COSY, TOCSY (mixing time 120 ms), NOESY (mixing time 200 ms), HSQC, and HMBC (optimized for a 5 Hz coupling constant).

3.2.1. Glycose analysis. For monosaccharide analysis, the polysaccharide (0.5 mg) was hydrolyzed (0.2 mL of 3 M TFA, 100 °C, 2 h), followed by evaporation to dryness under a stream of air. The residue was dissolved in water (0.5 mL), reduced with $NaBH_4$ (~5 mg, 1 h), neutralized with AcOH (0.3 mL), dried, and MeOH (1 mL) was added. The mixture was dried twice with the addition of MeOH, and the residue was acetylated with Ac_2O (0.5 mL, 100 °C, 30 min), dried, and analyzed by GLC on a HP1 capillary column (30 m × 0.25 mm) with a flame ionization detector (Agilent 6850 chromatograph) in a temperature gradient of 170 (4 min) to 260 °C at 4 °C/min.

Gel chromatography was carried out on Sephadex G-50 (2.5 m × 95 cm column) using the 0.05 M pyridinium acetate buffer (pH 4.5) as the eluent and monitor-

ing of collected fractions using a refractive index detector.

For the determination of the absolute configuration of the monosaccharides, the O-PS (1 mg) was treated with 10:1 (*R*)-2-butanol/AcCl (0.25 mL, 2 h, 85 °C), dried under a stream of air, acetylated, and analyzed by GC in comparison with authentic standards prepared from respective commercial monosaccharides with (*S*)- and (*R*)-2-butanol.[18]

3.2.2. Methylation analysis. For methylation analysis, samples of PS (2–3 mg) dissolved in Me_2SO (1.0 mL) were methylated by the Ciucanu–Kerek procedure[23] and were hydrolyzed (3 M TFA, 100 °C, 1 h), reduced ($NaBD_4$), acetylated, and analyzed using a Varian Saturn 2000 ion-trap GS-MS instrument.

Acknowledgements

We thank the Canadian Bacterial Diseases Network Centers of Excellence for their support of this work and J. Barlow and A. Bell for technical assistance.

References

1. Hawke, J. P. *J. Fish. Res. Board Can.* **1979**, *36*, 1508–1512.
2. Hawke, J. P.; McWhorter, A. C.; Steigerwalt, A. G.; Brenner, D. J. *Int. J. Syst. Bacteriol.* **1981**, *31*, 396–400.
3. Bertolini, J. M.; Cipriano, R. C.; Pyle, S. W.; McLaughlin, J. J. *J. Wildlife Dis.* **1990**, *26*, 246–252.
4. Newton, J. C.; Triche, P. L. *J. Aquat. Anim. Health* **1993**, *5*, 246–253.
5. Waltman, W. D.; Shotts, E. B.; Hsu, T. C. *Appl. Environ. Microbiol.* **1986**, *51*, 101–104.
6. Plumb, J. A.; Vinitnantharat, S. *J. Aquat. Anim. Health* **1989**, *1*, 51–56.
7. Thune, R. L.; Hawke, J. P.; Fernandez, D. H.; Lawrence, M. L.; Moore, M. M. *Dev. Biol. Stand.* **1997**, *90*, 125–134.
8. Lawrence, M. L.; Cooper, R. K.; Thune, R. L. *Infect. Immun.* **1997**, *65*, 4642–4651.
9. Thune, R. L.; Fernanzez, D. H.; Battista, J. R. *J. Aquat. Anim. Health* **1999**, *11*, 358–372.
10. Moore, M. M.; Fernandez, D. L.; Thune, R. L. *Dis. Aquat. Organ.* **2002**, *52*, 93–107.
11. Bader, J. A.; Shoemaker, C. A.; Klesius, P. H. *Fish Shell Immunol.* **2004**, *16*, 415–428.
12. Crump, E. M.; Perry, M. B.; Gale, S.; Crawford, E.; Kay, W. W. *J. Mol. Microbiol. Biotechnol.* **2003**, *6*, 182–190.
13. Weete, J. D.; Blevins, W. T.; Chitrakorn, S.; Saeed, M. O.; Plumb, J. A. *Can. J. Microbiol.* **1988**, *34*, 1224–1229.
14. Lawrence, M. L.; Banes, M. M.; Azadi, P.; Reeks, B. Y. *Microbiology* **2003**, *149*, 1409–1421.
15. Shotts, E. B.; Waltman, W. D., 2nd. *J. Wildlife Dis.* **1990**, *6*, 214–218.
16. Shotts, E. B.; Teska, J. D. In *Methods for the Microbiological Examination of Fish and Shellfish*; Huston, B., Austin, B. A., Eds.; E. Horwood Ltd: Chichester, UK, 1989, pp 146–186.
17. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 83–91.
18. Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. *Carbohydr. Res.* **1978**, *62*, 349–357.
19. Kjaer, M.; Andersen, K. V.; Poulsen, F. M. *Methods Enzymol.* **1994**, *239*, 288–308.
20. Plumb, J. A.; Klesius, P. *J. Fish Dis.* **1988**, *11*, 499–509.
21. Lobb, C. J.; Ghaffari, S. H.; Hayman, J. R.; Thompson, D. T. *Appl. Environ. Microbiol.* **1993**, *59*, 2830–2836.
22. Collins, L. A.; Thune, R. L. *Appl. Environ. Microbiol.* **1996**, *62*, 848–852.
23. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.