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The first description of a (1→6)-β-*D*-glucan in prokaryotes: (1→6)-β-*D*-glucan is a common component of *Actinobacillus suis* and is the basis for a serotyping system

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Abstract

The chemical and antigenic properties of the cell-surface lipopolysaccharides (LPSs) and capsular polysaccharides (CPSs) of seven representative strains of *Actinobacillus suis* from healthy and diseased pigs were investigated. Four strains produced a linear (1→6)-β-D-glucan homopolymer, β-D-Glc*p*-(1-[→6)-β-D-Glc*p*-(1-]$_n$→, as a LPS-O-chain (O1) and as a CPS (K1). Polyclonal antisera prepared against a (1→6)-β-D-glucan-containing strain showed a positive reaction against both LPSs and CPSs derived from the above strains (designated serotype O1/K1). One strain carried the (1→6)-β-D-glucan solely as a LPS-O-chain (serotype O1) and two strains did not express the (1→6)-β-D-glucan, but, instead, produced a different O-chain (designated serotype O2); these three strains expressed their own characteristic CPSs. (1→6)-β-D-Glucan structures are common cell wall components of yeast, fungi and lichens, but, to our knowledge, this is the first time a (1→6)-β-D-glucan has been described in a prokaryotic organism. Conformational and nuclear magnetic resonance analyses showed that the β-D-Glc*p*-(1→6)-β-D-Glc*p* linkage was flexible and two distinct glycosidic conformers are described. Cross-reactive antibodies to the *A. suis* (1→6)-β-D-glucan could be detected in sera from a variety of species and in sera from specific pathogen free pigs. This cross-reactivity may arise from immuno-stimulation of organisms present in the surrounding environment that contain (1→6)-β-D-glucan, which may also explain the high incidence of false positive results in previous serological tests for *A. suis*. In addition, these (1→6)-β-D-glucan background antibodies may be protective against *A. suis* infection. The characterization herein of (1→6)-β-D-glucan is the foundation for the development of a serotyping system for *A. suis*.

Keywords: *Actinobacillus suis*; Lipopolysaccharide; Capsule; Serotyping system; (1→6)-β-D-Glucan

1. Introduction

In recent years, the Gram-negative bacterium *Actinobacillus suis* has emerged as an important pathogen of high health status pigs [1]. In very young animals, *A. suis* infection can cause an acute and rapidly fatal septicemia, whereas in older animals it is associated with a wider variety of diseases, such as meningitis, metritis, pneumonia erysipelas-like lesions, and sometimes abortion [2]. Little is

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Table 1
Approximate molar ratios (± 0.1) of the sugar linkage types present in *A. suis* strains ATCC 15557, B49, H93-0055, SO4 and C84 as observed by detection of permethylated alditol acetates in GLCMS

Linkage type	CPS ATCC	PS-1 ATCC	OS-1 ATCC	OS-2 ATCC	PS-1 B49	PS-1 H93	PS-1 SO4	PS-1 C84
Glc-(1→	1	1	1	1	1	1	1	1
Gal-(1→		1	1	1	1	1	1	1
DDHep-(1→		1	1	1	1	1	1	1
LDHep-(1→		1	1	1	1	1	1	1
→6)-Glc-(1→	35	25		2	18	20	22	14
→6)-Gal-(1→		0.5	0.3		0.5	0.5	0.5	0.5
→2)-LDHep-(1→		1	1	1	1	1	1	1
→4/6)-LDHep-(1→		1	1	1	1	1	1	1
→3/4/6)-LDHep-(1→		1	1	1	1	1	1	1

known about the pathogenesis of *A. suis*, but some lines of evidence suggest that this organism has several virulence factors (e.g., Apx toxins) that are very similar to those of *A. pleuropneumoniae* [3,4]. To date, attempts to develop a serodiagnostic test for the detection of *A. suis*-infected animals have not been successful due to pervasive non-specific cross-reactivity [5]. Furthermore, it is not known whether all *A. suis* isolates have the same pathogenic potential. On the basis of biotyping, restriction endonuclease fingerprinting and slide agglutination, the population structure of *A. suis* was thought initially to be very homogeneous [4]. Recently, however, immunobloting experiments with crude lipopolysaccharide (LPS) preparations revealed that there were some noticeable differences in the O-antigen chains (O-chains) of *A. suis*. In view of this, this study was undertaken in order to characterize the cell surface carbohydrate molecules of representative *A. suis* strains and hopefully obtain some insights into the pathogenesis of this organism.

The cell surface capsular polysaccharides {CPSs, {[K-chain polysaccharide]→[lipid]∼cell} and lipopolysaccharides {LPSs, [O-chain polysaccharide]→[core oligosaccharide]→[lipid A]∼cell} of Gram-negative bacteria play key roles in bacteria–host interactions [6]. Previous investigations that dealt with the fine chemical structures, and their relation to specific serogroups, of another primary swine pathogen, *A. pleuropneumoniae*, revealed a wide array of CPS and LPS structures [7]. The chemical structures and antigenic properties of CPSs and LPSs from seven strains of *A. suis* are the focus of this investigation.

2. Results and discussion

*Characterization of (1→6)-β-*D*-glucan in the CPS and LPS of* A. suis *strain ATCC 15557.*—Sugar composition analyses by the alditol acetate [8] and but-2-yl glycoside [9] methods revealed that the CPS isolated from the *A. suis* reference strain ATCC 15557 was composed solely of D-glucose (Glc). Two linkage types were determined to be present in the CPS of this strain by methylation analysis, those being, 6-substituted Glc*p* giving 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol and terminal Glc*p* giving 1,5,-di-*O*-acetyl-2,3,4,6-

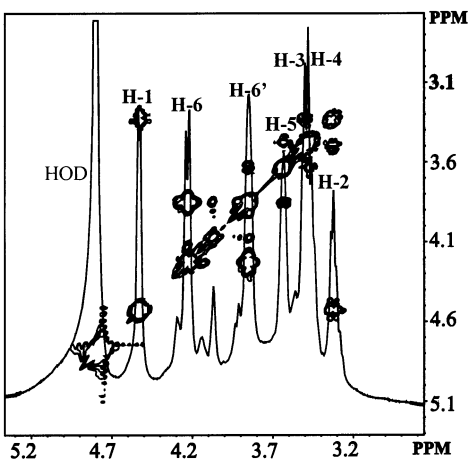

Fig. 1. 1-D ^{1}H NMR and 2-D ^{1}H–^{1}H COSY spectra of the CPS from *A. suis* reference strain ATCC 15557. The spectra show the β-Glc unit proton assignments (H-1, -2, -3, -4, 5, -6 and 6′) of the linear (1→6)-β-D-glucan.

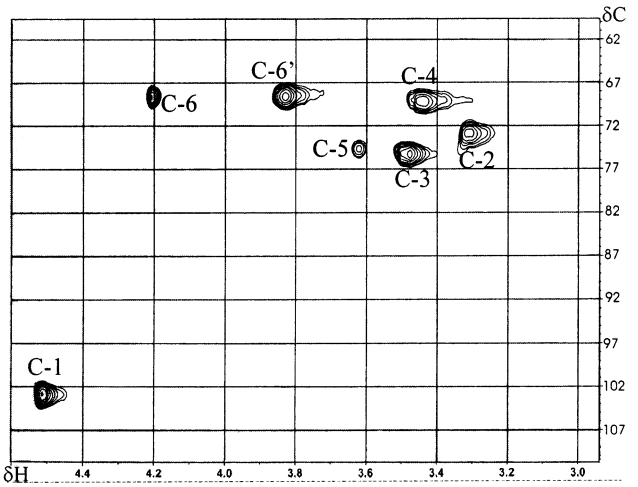

Fig. 2. 2-D ^{1}H-^{13}C HMQC spectra of the CPS from *A. suis* reference strain ATCC 15557 showing carbon assignments for the 6-linked β-Glc unit.

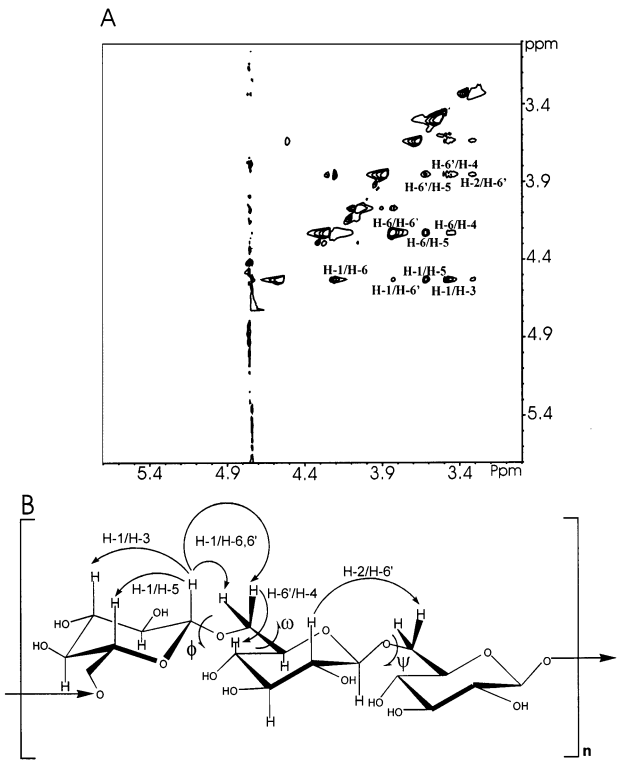

Fig. 3. (A) 2-D ^{1}H–^{1}H NOESY spectrum of *A. suis* reference strain ATCC 15557 CPS showing the inter-space nOe connectivities between the glycosidic anomeric proton (H-1) and the aglyconic H-6,6′ protons confirming the -[→6)-β-Glc-(1→6)-β-Glc-(1-]→ sequence, and the intra-nOe connectivities between H-1 and H-3/H-5; (B) a schematic representation of the (1→6)-β-D-glucan where the arrows indicate the inter- and intra-space nOe connectivities observed in the ^{1}H–^{1}H NOESY spectrum. Two potential glycosidic conformers are shown.

tetra-*O*-methylglucitol (Table 1). The ^{1}H NMR spectrum (Fig. 1) of the CPS showed one β anomeric (H-1) resonance at $\delta_{H\text{-}1}$ 4.51 ($J_{1,2}$ 7.2 Hz). Two-dimensional (2-D) ^{1}H–^{1}H correlation (COSY) (Fig. 1) and total correlation spectroscopic (TOCSY) experiments defined this anomeric signal as belonging to a β-Glc*p* unit by virtue of the ~10 Hz coupling constants between all vicinal ring protons (Fig. 1) [$\delta_{H\text{-}2}$ 3.21, $\delta_{H\text{-}3}$ 3.52, $\delta_{H\text{-}4}$ 3.47, $\delta_{H\text{-}5}$ 3.64 (m), and $\delta_{H\text{-}6}$ 4.21 (m) and $\delta_{H\text{-}6'}$ 3.88 (m)] indicative of the axial orientation of all sugar ring hydrogens in line with a β-glucopyranose configuration, presumably the 6-linked Glc*p* unit (Table 1). One-dimensional (1-D) ^{13}C NMR and a 2-D ^{1}H–^{13}C homonuclear quantum correlation (HMQC) (Fig. 2) experiments performed on ATCC 15557 CPS also showed a single β anomeric carbon resonance at δ_{C} 103 ($J_{H\text{-}1,C\text{-}1}$ 163 Hz) and aided in confirming the location of the H-6,6′ protons in the ^{1}H NMR spectrum, through the detection of the secondary carbon C-6 (δ 68.5) in a ^{13}C NMR *J*-modulated spin-echo experiment. The carbon chemical shifts of the CPSs 6-substituted Glc homopolymer unit were observed at $\delta_{C\text{-}1}$ 103, $\delta_{C\text{-}2}$ 73, $\delta_{C\text{-}3}$ 76, $\delta_{C\text{-}4}$ 70, $\delta_{C\text{-}5}$ 75, and $\delta_{C\text{-}6}$ 68.5 (Fig. 2). The fact that these 6-substituted Glc residues were in fact glycosidically linked to each other was shown by the detection of ^{1}H–^{1}H through-space connectivities in a 2-D ^{1}H–^{1}H nuclear Overhauser spectroscopy (NOESY) experiment (Fig. 3(A)), which yielded strong inter-nOe connectivities between the β anomeric resonance (H-1) and H-6,6′ at δ 4.21 and 3.88 (Fig. 3(A)). Both glycosidic inter-nOe connectivities were observed between H-1 and H-6, and H-1 and H-6, but the connectivity between H-1 and H-6 at δ 4.21 was clearly stronger than its counterpart at δ 3.88. Of particular interest, this ^{1}H–^{1}H NOESY spectrum also revealed an inter-nOe connectivity between H-2 (δ 3.21) and H-6′ (δ 3.88), which illustrated the possibility of different conformers (as shown later) through the rotary motion of the C-1–O–C-6 glycosidic bond (Fig. 3(B)). Also observed in the 2-D ^{1}H–^{1}H NOESY spectrum were intra-nOe connectivities between H-1 and H-3 (δ 3.52), between H-1 and H-5 (δ 3.64) and between H-4 (δ 3.47) and H-6,6′ (Fig. 3). The chemical and spectroscopic data unanimously pointed towards the presence of a (1→6)-β-D-glucan, β-D-Glc*p*-(1-[→6)-β-D-

Glc*p*-(1-]$_n \rightarrow$6)-β-D-Glc*p*-(1$\rightarrow$, as the CPS of *A. suis* ATCC 15557. No typical LPS core oligosaccharide units, such as L-*glycero*-D-*manno*-heptose (LDHep) and 3-deoxy-*manno*-D-octulosonic acid (Kdo), were observed in the chemical or spectroscopic analyses of the CPS material discussed above.

D-Glc was the overwhelming glycose unit present in the water-soluble intact LPS-1 from the *A. suis* reference strain (Fig. 4(A)). Other minor components observed, which emanated from the core region, were D-galactose (Gal), D-*glycero*-D-*manno*-heptose (DDHep), L-*glycero*-D-*manno*-heptose (LDHep), and D-glucosamine (GlcN), which was later shown to arise from the lipid A region. The major component present in LPS-1 was found to be a 6-substituted Glc*p* residue as deduced by methylation analysis (Fig. 4(B)). The GLC response ratios showed that there were approximately 25 O-chain 6-linked Glc residues for each core-related unit. These results pointed to the presence of (1$\rightarrow$6)-D-glucan as the O-chain polysaccharide of *A. suis* ATCC 15557 LPS. In addition, Gal*p* (terminal and 6-substituted), DDHep*p* (terminal), and LDHep*p* (terminal, 2-, 4/6-, and 3/4/6-substituted) permethylated alditol acetate deriva-

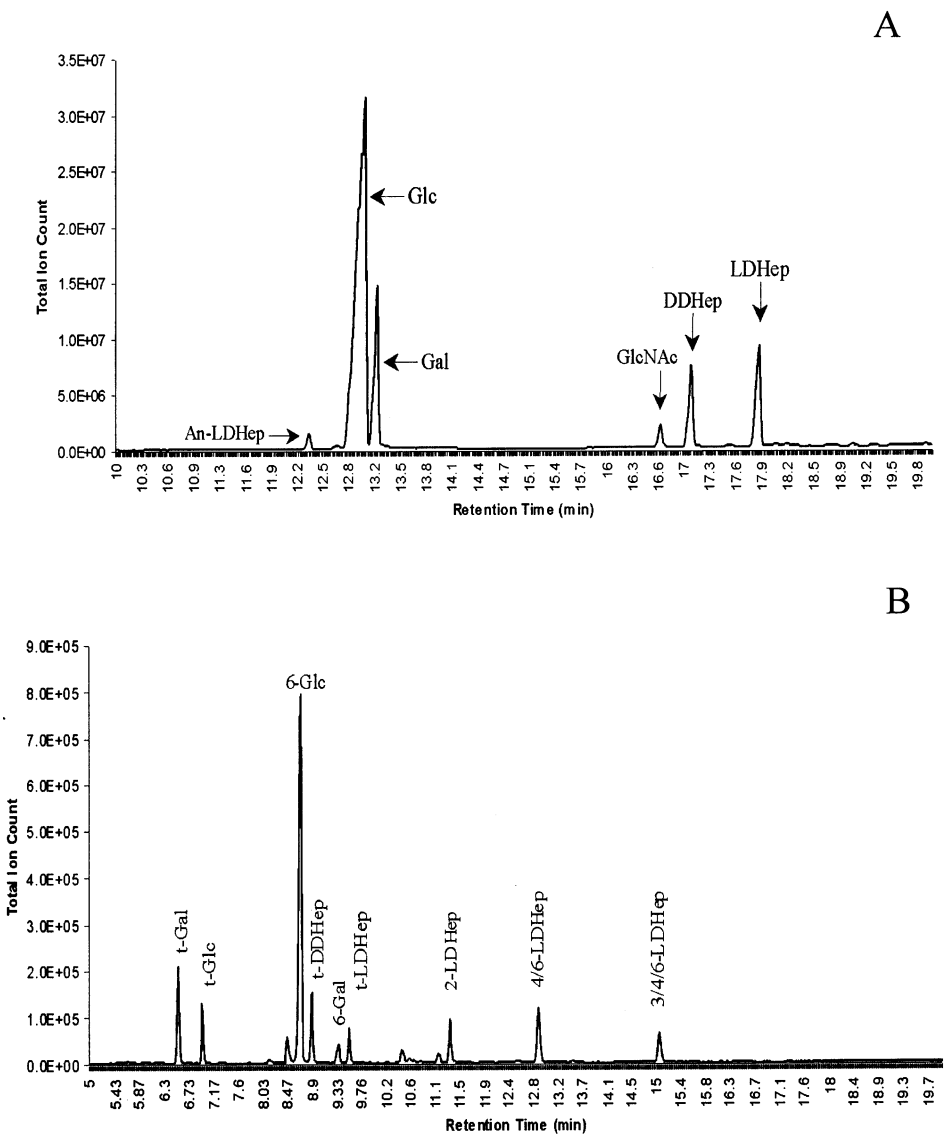

Fig. 4. GLC profiles of the (A) alditol acetate derivatives from *A. suis* ATCC 15557 intact LPS-1 showing the dominant Glc unit from the O-chain and the minor components from the core and lipid A region; (B) permethylated alditol acetate derivatives from *A. suis* ATCC 15557 intact LPS-1 showing the O-chain residue, 6-substituted Glc, and the other linkage types from the inner regions of the LPS.

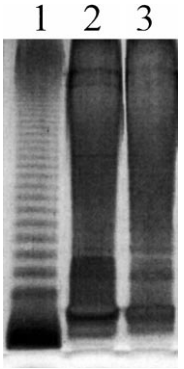

Fig. 5. Silver-stained SDS-PAGE of *Salmonella enterica* sv. Enteriditis LPS (lane 1), *A. suis* ATCC 15557 LPS (lane 2) and *A. suis* ATCC 15557 CPS (lane 3).

tives were also detected in the linkage analysis of *A. suis* ATCC 15557 LPS-1 (Fig. 4(B) and Table 1). The same results were obtained when the lipid A-free *A. suis* ATCC 15557 PS-1 was analyzed, except for the GlcN derivatives, which were no longer present in the composition analysis, indicating that these units were members of the lipid A moiety (typically present as O- and N-acylated GlcN units). The ^{1}H NMR spectrum of the lipid-free PS-1 showed one dominant β anomeric (H-1) resonance at δ 4.51 ($J_{1,2}$ 7.2 Hz), similar to that in Fig. 1, that belongs to the O-chain 6-substituted Glc units (Fig. 4(B)), and other anomeric resonances of lesser intensity emanating from the core oligosaccharide sugars. Indeed, as with the related CPS (Fig. 1), a 2-D ^{1}H–^{1}H COSY experiment defined the major β anomeric signal as belonging to a β-Glc*p* residue, whilst 1- and 2-D ^{13}C NMR experiments ($\delta_{C\text{-}6}$ 68.5) help confirm the location of the H-6,6′ protons in the ^{1}H NMR spectrum of PS-1. Final confirmation for the presence of the (1→6)-β-glucan LPS-O-chain was obtained through a 2-D ^{1}H–^{1}H NOESY experiment, which yielded inter-nOe connectivities between the β anomeric resonance and H-6,6′ ($\delta_{H\text{-}6}$ 3.86, $\delta_{H\text{-}6'}$ 4.20) resonances. Collectively, the data suggested that the O-chain polysaccharide of the LPS from *A. suis* ATCC 15557 was a (1→6)-β-D-glucan similar to that representing the CPS of the same strain.

As the SDS-PAGE profile of the *A. suis* reference strain ATCC 15557 LPS suggested (Fig. 5), dispersity of molecular weight was also detected in the Bio-Gel P-2 elution profile of the delipidated LPS. The high M_r broad band furnished several molecules containing O-chains of variable sizes. These molecules differed only in the degree of O-chain extension and indeed several glycan molecules were isolated (PS-2, -3, -4, and -5) containing shorter (1→6)-β-D-glucan O-chain polysaccharides, as shown by the diminishing intensity, in each respective ^{1}H NMR spectrum of the β anomeric resonance ($\delta_{H\text{-}1}$ 4.51) belonging to the 6-substituted Glc, and also by composition and linkage analysis where the quantity (in brackets) of 6-substituted Glc units gradually decreased for each PS molecule [PS-1(25), PS-2(19), PS-3(15), PS-4(10), PS-5 (6)]. A low M_r oligosaccharide fraction (OS-1) was also isolated with baseline separation from the high M_r broadband fraction, which expressed the same core-related sugar units (Table 1), but no 6-substituted Glc units were detected. Molecule OS-1 was thus a core oligosaccharide devoid of O-chain. The presence of these PSs molecules carrying shorter (1→6)-β-D-glucans provided evidence that this glucan homopolymer was an O-chain of variable sizes covalently attached to a core oligosaccharide, which, within the limits of detection, was shown to carry the same structural units regardless of O-chain extension.

To substantiate the fact that (1→6)-β-D-glucan in LPS was an O-chain polysaccharide attached to a core similar to core-OS-1, the molecule with the longest O-chain, PS-1, was subjected to acetolysis, a hydrolytic process selective for (1→6)-glycosidically linked sugars [10]. As expected, acetolysis degraded the (1→6)-β-D-glucan and yielded, after fractionation on Bio-Gel P-2, an oligosaccharide molecule (OS-2) that contained, as seen in the linkage analyses (Table 1), a residual core oligosaccharide still carrying an average of two O-chain-related 6-substituted Glc units. The absence of the elongated (1→6)-β-D-glucan O-chain in OS-2 was also observed in the ^{1}H NMR spectrum of this final acetolytic product in that the anomeric resonances of the core units could still be observed, but the overriding β anomeric resonance (δ_H 4.51), that belonged to the O-chain 6-linked Glc residue, was no longer present. Later eluting

low M_r glycan molecules, from degradation of the O-chain, were also detected, but were solely composed of Glc units, as shown by composition analysis. The core-associated units found in OS-2 (Table 1) were analogous to those of core OS-1.

Characterization of (1→6)-β-D-glucan in the CPSs and LPSs from A. suis *strains B49, H93-0055, SO4 and C84.*—In addition to the CPS and LPS from the *A. suis* reference strain ATCC 15557, CPSs and LPSs from six additional *A. suis* strains, B49, H93-0055, C84, SO4, H89-1173, and H91-0380, were also investigated by chemical and spectroscopic analyses. The ^{1}H NMR spectra of the CPSs and PSs-1 (delipidated LPSs) isolated from strains B49, H93-0055, SO4, and C84 (PS-1 only) clearly showed a dominant β anomeric signal at δ 4.51 ($J_{1,2}$ 7.1 Hz), as observed earlier in the ^{1}H NMR spectra of (1→6)-β-D-glucan PS-1 and CPS homopolymer from *A. suis* reference strain ATCC 15557 (Fig. 1). Methylation analyses performed on the CPS and PS-1 materials mentioned above (Table 1) showed the presence of major amounts of 6-substituted Glc residues in each molecule. The presence of the (1→6)-β-D-glucan in these strains was also illustrated by the inter-nOe connectivities observed in each independent 2D ^{1}H–^{1}H NOESY experiment between the β anomeric resonance and the H-6,6′ resonances ($\delta_{H\text{-}6}$ 3.86, $\delta_{H\text{-}6'}$ 4.20). Sugar linkage analyses (Table 1) and ^{1}H NMR spectroscopy data indicated that a (1→6)-β-D-glucan was present as CPSs and LPS-O-chains of strains B49, H93-0055, and SO4 and as the LPS-O-chain of strain C84. Similar chemical analyses performed on the LPSs and CPSs from strains H89-1173 and H91-0380 and on the CPS of C84 showed that (1→6)-β-D-glucan was not a constituent of these molecules. Preliminary studies have shown that the LPS-O-chains of strains H89-1173 and H91-0380 are identical, and that they were composed of a tetrasaccharide repeating block comprised of terminal and 3-substituted Gal, 4,6-substituted Glc, and 4-substituted GlcNAc. However, strains C84, H89-1173 and H91-0380 each produced their own characteristic CPSs, the complete structures of which will be published at a later date.

Core oligosaccharide components of A. suis.—The linkage analysis performed on *A. suis* ATCC 15557 LPS-1 revealed, in addition to the 6-substituted units of the O-chain homopolymer, derivatives from the core oligosaccharide region, those being, terminal Gal, DDHep and LDHep, 6-substituted Gal, 2-, 4/6- and 3/4/6-substituted LDHep (Table 1 and Fig. 4(B)). These sugar components were also found in the core-related molecules OS-1 (native core devoid of O-chain) and OS-2 (core derived from O-chain degradation). The most interesting feature was the simultaneous presence of DDHep and LDHep end-group units, a feature not frequently found in LPSs. The core glycose units described above were detected by methylation analysis (Table 1) in all the *A. suis* strains examined in this study, which demonstrated the presence of common structural features in the core regions of these strains. Akin to *A. suis*, the core oligosaccharide of several strains of *A. pleuropneumoniae* have also been observed to possess DDHep as a terminal unit (M.A. Monteiro, unpublished results).

Conformational analysis of the β-D-Glcp-(1→6)-β-D-Glcp glycosidic linkage using the Metropolis algorithm.—The glycosidic linkage present in the (1→6)-β-D-glucan homopolymer of CPSs and LPS-O-chains of *A. suis* strains, β-D-Glc*p*-(1→6)-β-D-Glc*p*, was investigated by the Metropolis Monte Carlo (MMC) method for conformational analysis [11], and to determine the relative ^{1}H–^{1}H nOe values for comparison with nOe connectivities obtained experimentally by NMR spectroscopy carried out on *A. suis* (1→6)-β-D-glucan CPSs and LPS-O-chains. Dihedral angles ϕ (O-5–C-1–O-1–C-6) and ψ (C-1–O-1–C-6–C-5) at the glycosidic linkage, and ω (O-6–C-6–C-5–O-5) were investigated. The potential energy surfaces for the β-D-Glc*p*-(1→6)-β-D-Glc*p* linkage are shown in Fig. 6(A), with $\omega = -60$, $\omega = +60$. The potential energy maps had similar local minima. The parts of the energy surfaces of the disaccharide covered during the simulations are shown in Fig. 6(B) as scatter plots. The plots are complementary to the energy surfaces in Fig. 6(A). The observed central values for the ϕ and ψ angles of the two observed global min-

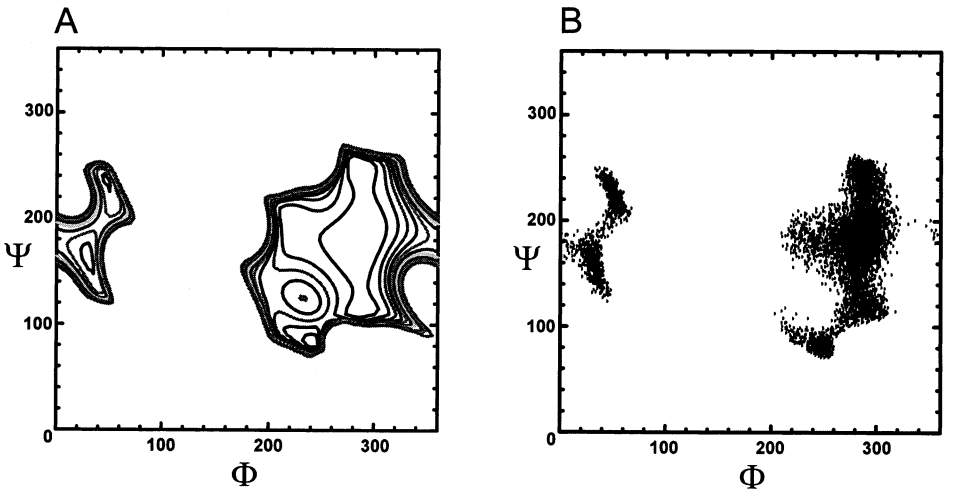

Fig. 6. (A) Potential energy surfaces as a function of ϕ and ψ for β-D-Glc*p*-(1→6)-β-D-Glc*p*; (B) scatter plots resulting from 250,000 macro step MMC simulations for β-D-Glc*p*-(1→6)-β-D-Glc*p* with the temperature set at 300 K.

ima, as derived from the geometry of saccharides (GESA) by routine calculations, were (ϕ, ψ) = (30, 170) and (ϕ, ψ) = (280, 170). The MMC calculations performed here on the β-D-Glc*p*-(1→6)-β-D-Glc*p* glycosidic linkage showed that this disaccharide possessed a considerable flexible character (Fig. 3(B)). On the basis of the energy surfaces (Fig. 6), $^1H-^1H$ nOe parameters were calculated and corroborated the observed $^1H-^1H$ nOe connectivities in the 2D NOESY experiment (Fig. 3(A)). The inter-nOe H-1/H-6,6′ connectivities seen in Fig. 3 were calculated to be present in the conformer at (ϕ, ψ) = (280, 170), whereas the glycosidic conformer that yielded the interesting inter-nOe between H-2 and H-6 was shown to be in the (ϕ, ψ) = (30, 170) minima pool. Hence, hard experimental data obtained by NMR and theoretical data from MMC calculations were complementary and showed the presence of at least two distinct glycosidic conformers (Fig. 3(B)) in the flexible (1→6)-β-D-glucan.

*Antigenic properties of (1→6)-β-*D*-glucan from* A. suis.—When immunoblots of LPSs were probed with hyperimmune convalescent antisera to *A. suis* strain SO4 (a strain expressing (1→6)-β-D-glucan in the LPS and CPS), a broad high-molecular-weight band was strongly recognized in the LPSs of ATCC 15557, SO4, B49, and H93-0055 (Fig. 7(A), lanes 2–5), but not with those of strains H91-0380 or H89-1173 (Fig. 7(A), lanes 6 and 7). Identical reactions were seen with CPSs from

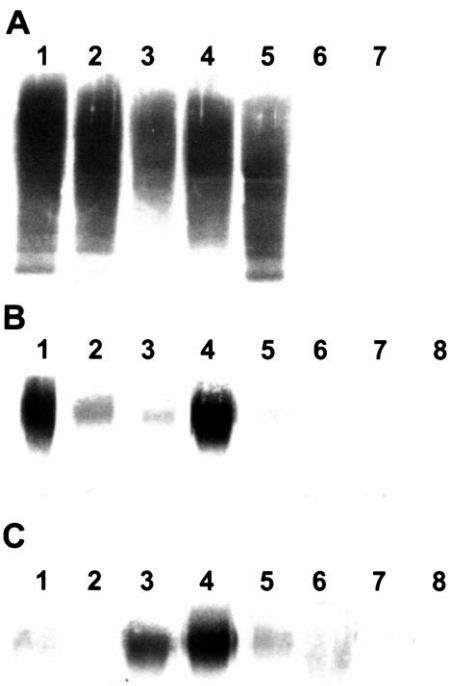

Fig. 7. Immunoblots of (A) various *A. suis* LPS and CPS probed with rabbit antisera to O1/K1 *A. suis* SO4: ATCC 15557 CPS (lane 1), ATCC 15557 LPS (lane 2), SO4 LPS (lane 3), B49 LPS (lane 4), H93-0055 LPS (lane 5), H91-0380 LPS (lane 6), and H89-1173 (lane 7); (B) ATCC 15557 LPS probed with various antisera. Preimmune rabbit (lanes 1, 2, and 3), equine (lane 4), bovine (lanes 5 and 6), fetal bovine serum (lane 7), and mouse monoclonal antibody isotypeIgG2 (lane 8) and (C) ATCC 15557 LPS probed with antisera from pigs infected with various pathogens. Preimmune (lane 1–3), *A. suis*-infected (lane 4–6); *A. pleuropneumoniae* serotype 1-infected (lane 7); *A. pleuropneumoniae* serotype 7-infected (lane 7) and *A. pleuropneumoniae* serotype 5-infected (lane 8).

ATCC 15557 (Fig. 7(A), lane 1), B49, SO4, and H93-055, but not with the CPSs from H89-1173, H91-0380 or C84. Therefore, strains that contained the (1→6)-β-D-glucan in the LPSs and CPSs were recognized by the SO4/(1→6)-β-D-glucan antisera and were serologically designated serotype O1/K1.

A variable, but considerable reaction to the purified *A. suis* O1 antigen was seen when immunoblots were probed with normal rabbit, bovine, and equine sera (Fig. 7(B), lanes 1–6). However, no reaction was observed with fetal bovine serum (Fig. 7(B), lane 7) or with an irrelevant mouse monoclonal antibody to a bovine cell surface marker (Fig. 7(B), lane 8). Some inconsistent cross-reactivity could also be detected in preimmune sera from healthy pathogen free pigs (Fig. 7(C), lanes 1 to 2), but a strong reactivity was detected with antisera from *A. suis*-infected pigs (Fig. 7C, lanes 3–5). A weak and irregular reactivity was also detected with sera from specific pathogen-free pigs infected with *A. pleuropneumoniae* serotype O1 (Fig. 7(C), lane 6), O7 (Fig. 7(C), lane 7), and O5 (Fig. 7(C), lane 8).

This investigation delving into the chemical and antigenic properties of cell surface glycan molecules from seven representative strains of *A. suis* revealed that four strains possessed a linear (1→6)-β-D-glucan as a LPS-O-chain (O1 antigen) and as a CPS (K1 antigen). These strains were assigned to the O1/K1 serotype group. One *A. suis* strain also possessed an LPS O1 antigen (serotype O1), but had a unique CPS (non-K1). This is the first reported account of a prokaryotic organism being able to produce a (1→6)-β-D-glucan. In nature, fungi, lichens and yeast also elaborate (1→6)-β-D-glucans, typically as branched molecules, as components of their cell building blocks [12–16]. The other two *A. suis* strains had a different O-antigen ('O2') and unique CPSs (non-K1). Immunoblot analysis of the O-antigen types with a large number of *A. suis* isolates (151) from both healthy and diseased pigs suggested that serotype O2 strains are more likely to be associated with severe disease than O1 strains (D. Slavic, unpublished).

The core oligosaccharide region of the *A. suis* strains investigated here possessed similar sugar linkage types with the noteworthy characteristic of carrying DDHep and LDHep as end-group moieties (Table 1). A terminal DDHep unit has also been observed in the core oligosaccharides of some *A. pleuropneumoniae* serogroups (M.A. Monteiro, unpublished results). The *A. suis* cores also carried LDHep glycose units as 2-, 3/4-, and 3/4/6-substituted residues.

Immunoblots with homologous sera, raised against an *A. suis* strain containing the (1→6)-β-D-glucan, and LPSs and CPSs from several strains containing the same antigen, clearly showed that this antisera (O1/K1 specific) strongly recognized the cell surface (1→6)-β-D-glucan molecules. A considerable cross-reactivity against (1→6)-β-D-glucan from *A. suis* was observed with many sera (Fig. 7(B) and (C)), for example, antibodies recognizing the *A. suis* O1 antigen were detected in preimmune sera from several species (Fig. 7(B)). This observed reactivity was not due to nonspecific binding of antibodies or serum proteins, as shown by the lack of reaction between LPS and fetal bovine serum, and between LPS and an irrelevant mouse monoclonal antibody (Fig. 7(B), lane 7 and 8, respectively). This cross-reactivity is most likely due to exposure to common environmental organisms that contain (1→6)-β-D-glucans [12–16].

The presence of (1→6)-β-D-glucans in *A. suis* strains and in common environmental fungal organisms may have a number of important consequences. Firstly, as a result of exposure to these ubiquitous microorganisms that express (1→6)-β-D-glucan, some pigs may have low levels of antibody to (1→6)-β-D-glucan that could confer some protection when they are first challenged with *A. suis* strains that contain the (1→6)-β-D-glucan as CPS and/or LPS. Indeed, serological analysis using O1 and O2 specific antisera have suggested that O2 strains are associated with more severe diseases than O1 strains. These same background antibodies may also explain, at least in part, why researchers have found a very high incidence of false positive results when they have tried to develop a serotyping system for *A. suis* by using LPS/CPS based ELISAs [1]. Finally, the fact that (1→6)-β-D-glucan has been shown to have an immuno-

stimmulatory effect in mice [17] further supports the notion that serotype O1 strains may be less virulent than O2 strains.

This study has described the presence of (1→6)-β-D-glucan in the LPSs and CPSs of *A. suis*. The *A. suis* strains expressing (1→6)-β-D-glucan as LPS-O-chains and CPSs were designated serotype O1/K1. As noted above, this finding may have important implications for the control and diagnosis of *A. suis* infections. Additional investigations into the precise chemical structures of the O2 and non-K1 antigens and production of specific antisera to these molecules are currently underway and should aid in creating a defined serotyping system for *A. suis*.

3. Experimental

Bacterial strains and growth conditions.—The origins, biotype, and restriction endonuclease fingerprinting type of the *A. suis* strains used in this work have been described previously [4]. Strain *A. suis* ATCC 15557 was recovered from a healthy pig following exposure to an atomic blast, strains B49 and C84 were slaughterhouse isolates from presumably healthy animals, strain SO4 was isolated from an animal in a herd with endemic *A. suis*, and strains H93-0055, H89-1173 and H91-0380 are clinical isolates from cases of enteritis, pneumonia and septicemia, respectively. Bacteria were routinely grown overnight at 37 °C in an atmosphere of 5% CO_2 on blood agar. Large-scale (2 L) cultures were grown in trypticase soy both in a fermenter with aeration for preparation of cell surface carbohydrates [4].

Isolation and preparation of CPSs, LPSs, PSs and OS-1.—The CPSs were isolated by EtOH precipitation from the paste obtained by the hot water–phenol [18] treatment of bacterial cells. The water-soluble CPSs were then purified on a column of Bio-Gel P-6. In each case, one broad high M_r glycan fraction was detected by the phenol–H_2SO_4 assay [19]. These high M_r fractions (CPSs) were used for structural analyses. The LPSs were collected as pellets by high-speed centrifugation (26000*g*, 2 h, 4 °C). In all cases, some of the LPS were re-dissolved in water and purified through a column of Bio-Gel P-2, which yielded a broad fraction at the void-volume. The high M_r region of this broad peak (LPS-1) was used for analyses. To rid the LPSs of lipid A, the crude LPSs were subjected to 2% AcOH acid treatment for 2 h at 100 °C, followed by centrifugation at 1500*g* to collect the water-insoluble lipid A. The supernatants, after purification through Bio-Gel P-2, gave a broad high M_r fraction from which PS-1, -2, -3, -4, and-5 were collected in succession [one PS per tube (3 mL)], and a distinct slower moving fraction (with base-line separation) from which an oligosaccharide, OS-1, was collected.

Sugar composition and methylation linkage analyses.—Sugar composition analysis was performed by the alditol acetate method [8]. The hydrolysis was performed in 4 M trifluoroacetic acid for 4 h at 100 °C, followed by reduction (overnight) in water with $NaBD_4$. Acetylation was performed with Ac_2O using residual NaOAc as the catalyst for 1 h at 100 °C. The alditol acetate derivatives were analyzed by gas–liquid chromatography–mass spectrometry (GLCMS) using a Hewlett–Packard chromatograph equipped with a 30 m DB-17 capillary column (210 °C (30 min), 240 °C at 2 °C/min) and MS in the electron impact (EI) mode was recorded using a Varion Saturn II mass spectrometer. The sugars absolute configurations were assigned by characterization of the but-2-yl glycosides in GLCMS [9]. Methylation analysis was carried out by the NaOH/DMSO/CH_3I procedure [20] and with characterization of the permethylated alditol acetate derivatives by GLCMS in the EI mode (DB-17 column, isothermally at 190 °C for 60 min).

Acetolysis of A. suis *ATCC 15557 PS-1.*—Delipidated polysaccharide, *A. suis* PS-1, was treated with Ac_2O, AcOH and H_2SO_4 in a 10:10:1 (v/v) mixture for 12 h at 35 °C (7). After O-de-acetylation with NaOMe, the resulting acetolyzate glycans were passed through a Bio-Gel P-2 column where the eluting fractions were assayed for carbohydrate by the phenol–H_2SO_4 method [18].

Nuclear magnetic resonance (NMR) and conformational analysis.—1-Dimensional (1-D) and 2-D NMR experiments carried out on

the CPS and PS-1 molecules were recorded on a Bruker AMX 500 spectrometer at 300 K using standard Bruker software. Prior to performing NMR experiments, the samples were lyophilized three times with D_2O (99.9%). Acetone was used as the internal reference at δ_H 2.225 and δ_C 31.7. The mixing times for the 2-D $^1H-^1H$ total correlation spectroscopy (TOCSY) and $^1H-^1H$ nuclear Overhauser enhancement spectroscopy (NOESY) experiments were 25 and 140 ms, respectively.

Conformational analysis employing the MMC algorithm was used to explore conformational spaces spanned by the exocyclic dihedral angles of the β-D-Glc*p*-(1→6)-β-D-Glc*p* linkage. All computational methods used in this study have been described in detail by Peters et al. [11].

Immunoblotting and silver staining.—For immunoblotting experiments, hyperimmune rabbit antiserum was prepared against *A. suis* SO4 by intravenous injection of formalin-fixed bacteria as described previously [21,22] and used at a dilution of 1:100. Water–phenol purified LPS of *Salmonella enterica* sv. Enteritidis was purchased from Sigma Chemical Co., St. Louis, MO. Purified LPS or CPS (8 μg) was suspended in sample buffer (2% sodium-dodecyl sulfate (SDS), 4% 2-mercaptoethanol, 10% glycerol, 1 M Tris pH 6.8, and 0.05% Bromophenol Blue), separated by SDS-polyacrylamide gel electrophoresis (PAGE) in a 12% gel, and transferred to nitrocellulose (Bio-Rad Laboratories, Richmond, CA). Immunoblots were visualized using protein A-alkaline phosphatase conjugate (Bio-Rad) and Nitro Blue Tetrazolium with 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co., St. Louis, MO) as per the manufacturer's instructions. Some gels were also stained using the Silver Stain Plus kit from Bio-Rad. Antibodies used in the studies on cross-reactivity were the generous gift of P. Shewen, University of Guelph (irrelevant monoclonal antibody) and B. Fenwick, University of Kansas (SPF swine sera).

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References

[1] J.I. MacInnes, R. Desrosier, *Can. J. Vet. Res.*, 63 (1999) 83–89.
[2] S.E. Sanford, in A.D. Leman, B.E. Straw, W.L. Mengeling, S. d'Allaire, D.J. Taylor (Eds.), *Diseases of Swine*, seventh ed., Iowa State University Press, Ames, 1992, pp. 633–636.
[3] R. Bada, K.R. Mittal, R. Higgins, *Vet. Microbiol.*, 51 (1996) 393–396.
[4] J. van Ostaaijen, J. Frey, S. Rosendal, J.I. MacInnes, *J. Clin. Microbiol.*, 35 (1997) 1131–1137.
[5] B. Fenwick, An overview of Actinobacillus suis as an emerging disease. *In Proceedings of American Association of Swine Practitioners, 28th annual meeting*, Quebec City, 1997, pp. 467–470.
[6] E.T. Rietschel, H. Brade, O. Holst, L. Brade, S. Muller-Loennies, U. Mamat, U. Zahringer, F. Beckmann, U. Seydel, K. Brandenburg, A.J. Ulmer, T. Mattern, H. Heine, J. Schletter, H. Loppnow, U. Schonbeck, H.D. Flad, S. Hauschildt, U.F. Schade, F. Di Padova, S. Kusumoto, R.R. Schumann, *Curr. Top. Microbiol. Immunol.*, 216 (1996) 39–81.
[7] M.B. Perry, E. Altman, J.-R. Brisson, L.M. Beynon, J.C. Richards, *Serodiagn. Immunother. Infect. Dis.*, 4 (1990) 299–308.
[8] J.S. Sawardeker, J.H. Sloneker, A. Jeanes, *Anal. Chem.*, 37 (1965) 1602–1604.
[9] K. Leontein, B. Lindberg, J. Lonngren, *Carbohydr. Res.*, 62 (1978) 359–362.
[10] J. Kocourek, C.E. Ballou, *J. Bacteriol.*, 100 (1969) 1175–1181.
[11] T. Peters, B. Meyer, R. Stuike-Prill, R. Somorjai, J.-R. Brisson, *Carbohydr. Res.*, 238 (1993) 49–73.
[12] V.J. Cid, A. Duran, F. del Rey, M.P. Snyder, C. Nombela, M. Sanchez, *Microbiol. Rev.*, 59 (1995) 345–386.
[13] R.C. Goldman, A. Branstrom, *Curr. Pharm. Des.*, 5 (1999) 473–501.
[14] B. Lindberg, J. McPherson, *Acta Chem. Scand.*, 8 (1954) 985–988.
[15] M. Lussier, A.-M. Sdicu, S. Shahinian, H. Bussey, *Proc. Natl. Acad. Sci. USA*, 95 (1998) 9825–9830.
[16] S. Shahinian, H. Bussey, *Mol. Microbiol.*, 35 (2000) 477–489.
[17] T. Watanabe, *Kansenshogaku Zasshi*, 70 (1996) 574–583.
[18] O. Westphal, K. Jann, *Methods Carbohydr. Chem.*, 5 (1965) 83–91.
[19] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
[20] I. Ciucanu, F. Kerek, *Carbohydr. Res.*, 131 (1984) 209–217.
[21] S. Rosendal, L. Lombin, J. DeMoor, *Can. J. Comp. Med.*, 45 (1981) 271–274.
[22] S. Rosendal, K.R. Mittal, *Can. J. Comp. Med.*, 49 (1985) 164–170.