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Structure determination of *Streptococcus suis* serotype 9 capsular polysaccharide and assignment of functions of the *cps* locus genes involved in its biosynthesis

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ABSTRACT

Streptococcus suis serotype 9 is the most prevalent *S. suis* serotype in several European countries. In spite of its pathogenicity for pigs and increasing zoonotic potential, limited information is available on this serotype. Here we determined for the first time the chemical composition and structure of serotype 9 capsular polysaccharide (CPS), a major bacterial virulence factor and the antigen at the origin of *S. suis* classification into serotypes. Chemical and spectroscopic data gave the repeating unit sequence: [3)Glcol-6-*P*-3-[D-Gal(α1-2)]D-Gal(β1-3)D-Sug(β1-3)L-Rha(α1-)]$_n$. Compared to previously characterized *S. suis* CPSs (serotypes 1, 1/2, 2 and 14), serotype 9 CPS does not contain sialic acid but contains a labile 4-keto sugar (2-acetamido-2,6-dideoxy-β-D-*xylo*-hexopyranos-4-ulose), one particular feature of this serotype. A correlation between *S. suis* serotype 9 CPS sequence and genes of this serotype *cps* locus encoding putative glycosyltransferases and polymerase responsible for the biosynthesis of the repeating unit was tentatively established. Knowledge of CPS structure and composition will contribute to better dissect the role of this bacterial component in the pathogenesis of *S. suis* serotype 9.

1. Introduction

Streptococcus suis ranks among the five most important health challenges of pigs worldwide. It is associated with numerous diseases, such as meningitis, septicemia, arthritis and endocarditis in both traditional and modern intensive swine operations. *S. suis* is also an emerging zoonotic agent in humans and thus considered a public health threat, especially for people working in the swine/pork industry. The natural habitat of *S. suis* is the upper respiratory tract, more particularly the tonsils and nasal cavities, but also the genital and digestive tracts of pigs of all ages. Transmission of the infection between herds usually occurs by the movement of healthy carrier animals harboring virulent strains. Sows infect their own piglets during the birth process and probably also through the respiratory route. In fact, horizontal transmission seems to be significant, especially in the presence of clinical signs, with a considerable higher number of bacteria in the environment that would increase transmission either by aerosol or direct contact. Pig carrier rate of *S. suis* is close to 100%, although the proportion of animals colonized by virulent strains is lower [1].

S. suis isolates express a “capsular polysaccharide” (CPS) which is considered one of the major bacterial virulence factor [2–4]. Originally, 35 serotypes were described and the structure of the CPS defines the serotype. Recent molecular analyses have led to the proposed reclassification of six of those serotypes as other species than *S. suis*, namely serotypes 20, 22, 26, 32, 33, and 34; and the discovery of novel *cps* loci [5–7]. Yet, most *S. suis* organisms isolated from diseased pigs belong to a limited number of serotypes. Serotype 2 has been worldwide considered the most prevalent and virulent *S. suis* type [8]. However, there is a clear geographical effect

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on the distribution of serotypes, and recent epidemiological studies indicates that serotype 9 is the most prevalent serotype in several European countries, such as Spain and the Netherlands [8].

As mentioned above, *S. suis* is also an emerging zoonotic agent, being responsible for septicemia with or without septic shock, meningitis and other less common infections usually related to generalized septicemia. Albeit well-known in veterinary medicine, its significance has been neglected in human medicine. Infections in humans were considered sporadic in people working with pigs or pork-derived products. Yet, recent epidemiological data from Asia, including important and deadly human outbreaks, has changed the perspective of the threat posed by this pathogen to human health. While in Western countries infections in humans are considered an occupational disease, in some Asian countries, the general population is at risk. Different from pigs, the main route of entry of *S. suis* in humans is through skin contact with contaminated animals, carcasses or meat. However, this situation seems to be different in some Asian countries where the oral route is taken into consideration after ingestion of contaminated raw pork products as part of traditional dishes. Serotype 2 is the most prevalent in humans, but sporadic cases with other serotypes have been also described [8]. A recent report of a human case due to serotype 9 in Thailand suggests a high zoonotic potential for this serotype as well [9].

The CPS is an important virulence factor at the interface of the bacteria and the host, contributing to the modulation of bacterial colonization, systemic dissemination and bacterial persistence. The CPS regulates the innate and the adaptive immune response of the infected host and is the target of protective antibodies. Finally, it is the antigen at the base of serological and molecular diagnostic and serotyping [8,10]. Due to its importance, ongoing research yielded complete structural determination for the CPSs of *S. suis* serotype 2 [11], serotype 14 [12], and serotypes 1 & 1/2 [13]. A sialic acid-containing side chain was found as a common feature of all these four serotypes. Besides, lectin-based studies and *cps* locus genetic analyses predicted the presence of this sugar in serotypes 6, 13, 16 and 27 as well [14,15]. Albeit serotype 9 does not belong to the *S. suis* sialylated-CPS group, it presents a high virulence and zoonotic potential. Thus, in this work, we describe for the first time the purification and the primary structure of the CPS polymer for *S. suis* serotype 9.

2. Material and methods

2.1. Capsular polysaccharide production and purification

S. suis serotype 9 field strain 1273590 was used in this study (isolated from a diseased pig in Quebec, Canada - our collection). For the CPS production, 6 L of Todd-Hewitt Broth was inoculated and grown overnight as previously described [12]. Two methods of extraction were compared. **Method A** was based in previous publications [11,12]. Briefly, bacterial cells from the 6 L culture were pelleted by centrifugation at 10 000*g* for 40 min, suspended by repeated pipetting in a buffer containing 33 mmol/L phosphate and 145 mmol/L NaCl pH 8.0, and chilled. The bacterial suspension was autoclaved at 121 °C for 15 min. The supernatant containing the crude capsule was recovered by centrifugation at 9000*g* for 50 min. Extraction with an equal volume of chloroform eliminated lipids, whereas nucleic acids were removed by precipitation by adding $CaCl_2$ to 0.1 mol/L and ethanol to 25% *v/v*, and then centrifuged at 7200*g* for 30 min at room temperature. The material was then concentrated by precipitating in ethanol 80% *v/v*. Pellets were dissolved in 50 mmol/L NH_4HCO_3, dialyzed against the same solution for 48 h with a Spectra/Por 2 membrane (Spectrum Laboratories) MWCO 12–14 kDa, and freeze dried. The CPS was further purified by gel filtration chromatography on a XK-26-100 column filled with Sephacryl S-400 HR (GE Healthcare, Uppsala, Sweden) eluted with 50 mmol/L NH_4HCO_3 at a flow rate of 1.3 mL/min, using an ÄKTA Purifier 10 system (GE Healthcare), including a UV-900 Monitor, and equipped with a Knauer Smartline 2300 RI Detector (Knauer, Berlin, Germany) connected to the system via an AD-900 Analog/Digital Converter (GE Healthcare). Fractions were collected and assayed for CPS by dot enzyme-linked immunosorbent assay (dot-ELISA) with an anti-*S. suis* serotype 9 rabbit polyclonal antibody (1/500) as the primary antibody and horseradish-peroxidase-conjugated (HRP-conjugated) goat anti-rabbit secondary antibody (1/3000). Fractions giving a positive RI signal and response with antibodies but no absorption at 280 and 254 nm were pooled and freeze dried. The purified material was dissolved in water, dialyzed against ddH_2O for 48 h at 4 °C, and finally freeze dried.

For **Method B**, bacterial pellet from the 6 L culture was resuspended in deionized water (ddH_2O) and washed 2 times. The bacteria were killed by heating at 60 °C for 45 min, which have been confirmed by the absence of growth on blood agar plates. Finally, the content of the tubes were lyophilized for 72 h. Then, bacterial cells were stirred in water (3 g dry cells in 100 mL of water) at room temperature overnight, cells removed by centrifugation at 12000*g* 20 min, solution dialyzed, dried, dissolved in 2 mL of water, insoluble material removed by centrifugation (12000*g*, 10 min), solution separated on Biogel P10 column (2.5 × 60 cm; Bio Rad, Hercules, CA) in 1% AcOH. Broad peak starting at void volume was collected in 2 fractions. The eluent was turbid and viscous after this step and was spun 120000 g for 1 h. After small amount of precipitate was discarded, solution became clear, colorless and not viscous. Both fractions from gel chromatography contained the same polysaccharide, second (lower molecular mass) fraction contained more impurities as indicated by nuclear magnetic resonance (NMR) (data not shown). Extraction of the remaining water-washed cells by 45% phenol at 70 °C produced no polysaccharides.

2.2. NMR spectroscopy

NMR experiments were carried out on a Bruker AVANCE III 600 MHz (^{1}H) spectrometer with 5 mm Z-gradient probe with acetone internal reference (2.225 ppm for ^{1}H and 31.45 ppm for ^{13}C) using standard pulse sequences cosygpprqf (gCOSY), mlevphpr (TOCSY) (mixing time 120 ms), roesyphpr (ROESY) (mixing time 500 ms), hsqcedetgp (HSQC), hsqcetgpml (HSQC-TOCSY) (80 ms TOCSY delay) and hmbcgplpndqf (HMBC) (100 ms long range transfer delay). Resolution was kept <3 Hz/pt in F2 in H–H correlations and <5 Hz/pt in F2 of H–C correlations. Number of points in F2 set so to have AQ about 0.8 s for H–H correlations, 0.24 s for C–H HSQC. Number of points in F1 was 1/4 of F2. The spectra were processed and analyzed using the Bruker Topspin 2.1 program.

2.3. Determination of neutral and amino sugars as alditol acetates

Monosaccharides were detected as reduced and acetylated derivatives (alditol acetates). Polysaccharide sample (0.2 mg) with inositol internal standard was dephosphorylated with 48% hydrofluoric acid (10 μL, 30 min, 30 °C), dried by air stream, and hydrolyzed with 3 M trifluoroacetic acid (120 °C, 3 h). The sample was then dried, reduced with $NaBD_4$, and reagent destroyed with 0.5 mL of AcOH. The obtained solution dried under the stream of air, dried twice with addition of MeOH (1 mL), acetylated with 0.4 mL Ac_2O - 0.4 mL pyridine for 30 min at 100 °C, dried, and finally analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) (Varian Saturn 2000 ion-trap instrument, capillary column DB-17, 160–260 °C by 4°/min).