



HR-MAS NMR reveals a pH-dependent LPS alteration by de-O-acetylation at abequose in the O-antigen of *Salmonella enterica* serovar Typhimurium

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ABSTRACT

NMR spectroscopy can detect biomolecules like lipopolysaccharide directly on the surface of the cell, thus avoiding isolation and purification, and providing a more realistic description than the one derived from *in vitro* studies. Here we present a high-resolution magic-angle spinning NMR study of the O-antigen of *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) performed directly on the cells showing the alteration of its acetylation state over time. The O-antigen region of *S. Typhimurium* consists of the repeating unit [$\rightarrow 2$]- α -D-Manp-($1 \rightarrow 4$)- α -L-Rhap-($1 \rightarrow 3$)- α -D-Galp-($1 \rightarrow$] where Man stands for mannose, Rha for rhamnose, and Gal for galactose. Man is substituted with abequose (Abe) O-acetylated at carbon 2. Our studies revealed that the appearance of de-O-acetylated O-antigen in the stationary growth phase is due to the de-O-acetylation of already synthesized O-acetylated O-antigen and that this reaction is caused by the metabolism-induced basic pH of the growth medium. The labile O-acetylation of the O-antigen we observed in *S. Typhimurium* generates non-stoichiometric O-acetylation states and therefore changes the nature of an immunogenic epitope.

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1. Introduction

Salmonella enterica subspecies 1 serovar Typhimurium (*S. Typhimurium*) is one of the most prevalent causes for human gastroenteritis. It can cause diarrhea, abdominal pain, and nausea.¹ In addition to its two type three secretion systems (TTSS) encoded by the genes in the *Salmonella* pathogenicity islands I and II, lipopolysaccharide (LPS) is one of its main virulence factors.^{2,3} LPS can structurally be divided into three parts: lipid A, inner and outer core, and the repetitive O-antigen subunit.⁴ While inner and outer core are conserved among different *Salmonella enterica* strains, the O-antigen region is highly variable.^{5–7} The O-antigen of *S. Typhimurium* consists of 70–100 repeating [$\rightarrow 2$]- α -D-Manp-($1 \rightarrow 4$)- α -L-Rhap-($1 \rightarrow 3$)- α -D-Galp-($1 \rightarrow$] units, where Man stands for mannose, Rha for rhamnose, and Gal for galactose (Fig. 1). The Man residue is substituted at carbon 3 (C3) with α -linked abequose (Abe; 3,6-dideoxy-D-xylo-hexose), which is O-acetylated at

C2. The Gal residue is non-stoichiometrically substituted with a glucose (Glc) residue, either in α -($1 \rightarrow 6$) or α -($1 \rightarrow 4$) linkage.^{5,8,9} The O-antigen region of *Salmonella* also provides the basis for the serological classification scheme by Kauffman and White, for example, O-antigenic factor 5 is defined as the O-linked acetyl group at Abe C2.⁵

S. Typhimurium LPS is synthesized via the wzy-dependent pathway in which one O-antigen subunit is assembled on the cytoplasmic side of the inner membrane on the lipid carrier undecaprenylphosphate by subsequent action of glycosyltransferases. This lipid-linked oligosaccharide is then translocated to the periplasmic side by Wzx³ where the single O-antigen subunits are polymerized by Wzy. The complete polymerized O-antigen is subsequently transferred to the preassembled lipid A core by the O-antigen ligase WaaL.¹⁰ The acetylation at the Abe C2 takes place in the cytoplasm and is catalyzed by the OafA acetyltransferase that uses acetyl-CoA as acetyl group donor.¹¹ Addition of the Glc residue only occurs after polymerization of the LPS chain and is non-stoichiometrical.¹²

The structure, length, and the integrity of the O-antigen are important virulence factors in *Salmonella* infections as the O-antigen is involved in resistance to phagocytosis, antimicrobial peptides and serum complement.^{13–15} The lack of abequose O-acetylation does not diminish the virulence of *S. Typhimurium* in a murine infection model.¹⁶ However, its importance in immune

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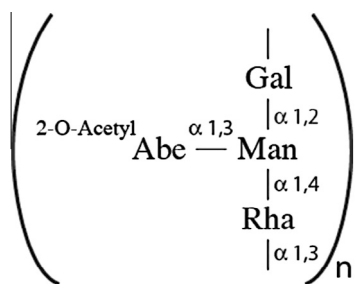


Figure 1. Repeat unit of O-antigen in *S. Typhimurium*.

response can be deduced from the fact that a monoclonal secretory IgA that recognizes O-acetylated abequeose as the determining part of the antigen can protect mice against oral challenge with *S. Typhimurium*.^{16,17} Also in other bacterial species, the presence of O-acetyl groups in the O-antigen has been shown to influence the O-antigen antigenicity and thus, the immunogenicity of vaccines developed using the polysaccharide part of the LPS as immunogen.^{18–20} The knowledge of the exact repeating unit of the O-antigen, including the occurrence and position of O-acetyl groups, is therefore a major prerequisite for the development of vaccine candidates based on LPS.

Salmonella O-antigen has been studied on extracted LPS using solution NMR spectroscopy.^{8,9,21,22} Using high-resolution magic angle spinning (HR-MAS) NMR spectroscopy of intact bacterial cells²³ we were able to observe that the O-acetylation state of *S. Typhimurium* O-antigen is not constant but evolves during the cell

growth phase and that the O-acetylation at the abequeose C2 defining O-antigenic factor 5 decreases during the stationary phase. This demonstrates that HR-MAS NMR can be employed to analyze small alterations in LPS. In fact, the ¹H on-cell NMR spectrum of intact cells of *S. Typhimurium* grown to late exponential phase shows that peak integrals of the anomeric protons (H1) of Abe, Man, Gal, and Rha are comparable, in accordance with the repeat unit^{5,8,9} and that Abe is completely O-acetylated.²³ Over time we observed the progressive appearance of Abe de-O-acetylated at C2. We found that the evolution of the O-acetylation state of the O-antigen of *S. Typhimurium* was correlated to the change of the pH in the unbuffered Luria–Bertani (LB) growth medium during bacterial growth, as the presence of de-O-acetylated Abe was associated with an increase of the pH of the medium during growth. This effect was more pronounced in alive cells than in inactivated ones.²³

Here, we investigated in more detail the evolution of the O-acetylation state of Abe C2 in the O-antigen of *S. Typhimurium*.

2. Results

2.1. De-O-acetylated O-antigen is not newly synthesized

To determine if the O-antigen de-O-acetylated at Abe C2 which characterizes the *S. Typhimurium* cells at late growth phases is newly synthesized or if it is due to the de-O-acetylation of the previously synthesized O-acetylated O-antigen we measured the ¹H MAS NMR spectra of aliquots of *S. Typhimurium* intact cells taken from the same culture at given time points (Fig. 2). The experiment is similar to the one reported in Figure 2D–F in our previous study.²³ The main difference is that here the aliquots were

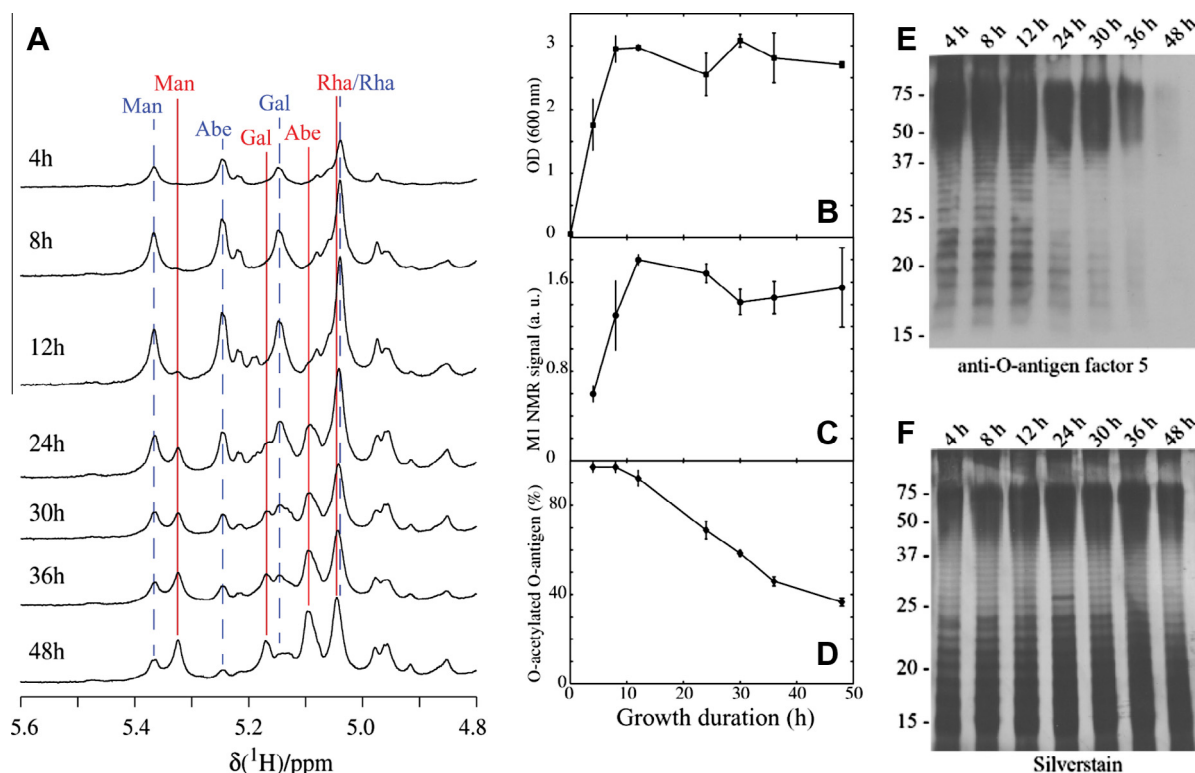


Figure 2. The O-antigen of *S. Typhimurium* alters during the stationary phase. (A) ¹H MAS NMR spectra (anomeric region) of intact cells of *S. Typhimurium* SL1344 grown in LB. The corresponding growth times are indicated. The assignment of the anomeric protons of the O-antigen is reported, the blue broken line and the red line correspond to the O-antigen which is O-acetylated and de-O-acetylated at Abe C2, respectively. The MAS spinning frequency was 5 kHz. (B) Optical density of the cell cultures (OD₆₀₀/mL) used in the NMR experiments. (C) Integrals of the NMR signal of Man H1 normalized to the integral of the signal of a fixed amount of TSP. (D) Fraction of the O-antigen O-acetylated at Abe C2 determined from the deconvolution of the Man H1 NMR signals. The average of three series and the corresponding maximum absolute deviation are reported. (E) Immunoblot of SDS–PAGE of proteinase K-treated whole cell extracts of *S. Typhimurium* SL1344 of the given time points developed with anti-factor 5 antiserum. (F) Silverstain of SDS–PAGE of proteinase K-treated whole cell extracts of *S. Typhimurium* SL1344 of the given time points.

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