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Structural analysis of the O-acetylated O-polysaccharide isolated from *Salmonella paratyphi* A and used for vaccine preparation

N. Ravenscroft [a], P. Cescutti [b], M. Gavini [c], G. Stefanetti [c], C. A. MacLennan [c], L. B. Martin [c], F. Micoli [c,*]

[a] Department of Chemistry, University of Cape Town, Rondebosch 7701, South Africa
[b] Department of Life Sciences, Blg. C11, Università di Trieste, via L. Giorgieri 1, 34127 Trieste, Italy
[c] Novartis Vaccines Institute for Global Health, Via Fiorentina 1, I-53100 Siena, Italy

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ABSTRACT

Salmonella paratyphi A is increasingly recognized as a common cause of enteric fever cases and there are no licensed vaccines against this infection. Antibodies directed against the O-polysaccharide of the lipopolysaccharide of *Salmonella* are protective and conjugation of the O-polysaccharide to a carrier protein represents a promising strategy for vaccine development. O-Acetylation of *S. paratyphi* A O-polysaccharide is considered important for the immunogenicity of S. *paratyphi* A conjugate vaccines.

Here, as part of a programme to produce a bivalent conjugate vaccine against both *S. typhi* and *S. paratyphi* A diseases, we have fully elucidated the O-polysaccharide structure of *S. paratyphi* A by use of HPLC–SEC, HPAEC–PAD/CD, GLC, GLC–MS, 1D and 2D-NMR spectroscopy. In particular, chemical and NMR studies identified the presence of *O*-acetyl groups on C-2 and C-3 of rhamnose in the lipopolysaccharide repeating unit, at variance with previous reports of O-acetylation at a single position. Moreover HR-MAS NMR analysis performed directly on bacterial pellets from several strains of *S. paratyphi* A also showed O-acetylation on C-2 and C-3 of rhamnose, thus this pattern is common and not an artefact from O-polysaccharide purification. Conjugation of the O-polysaccharide to the carrier protein had little impact on O-acetylation and therefore should not adversely affect the immunogenicity of the vaccine.

1. Introduction

Salmonella enterica serovars are responsible for invasive disease in the developing world. Most cases of enteric fever are caused by *Salmonella enterica* serovar *typhi* (*S. typhi*). The annual global burden of disease due to typhoid fever was estimated at approximately 22 million illnesses in 2000 with a case-fatality rate of 1% resulting in more than 200,000 deaths.[1] The highest incidence was reported in South Asia,[1] and in pre-school and school-aged children.[2–4] An additional 5.4 million cases of enteric fever in 2000 were caused by *Salmonella enterica* serovar *paratyphi* A (*S. paratyphi* A), with children mainly affected.[1] A more recent revised estimate of the global burden of typhoid and paratyphoid fever has reported 26.9 million and 4.9 million adjusted cases of *S. typhi* and *S. paratyphi* A, respectively, in 2010.[5] *S. paratyphi* A is an increasingly common cause of enteric fever in the Indian subcontinent and Southeast Asia, with data suggesting it may account for up to 50% of enteric fever cases.[6]

There are licensed vaccines against *S. typhi*,[7] but they do not offer protection against enteric fever caused by *S. paratyphi* A. The increasing frequency of *S. paratyphi* A as a cause of enteric fever and the emergence of antibiotic resistance[8] are of great concern, particularly because there are no licensed vaccines against *S. paratyphi* A infection. Furthermore, enteric fever caused by *S. paratyphi* A and *S. typhi* is clinically indistinguishable. As a result, in areas with high incidence of paratyphoid fever there is the risk that even a highly effective *S. typhi* vaccine will be poorly efficacious against enteric fever as a whole. To address enteric fever more broadly, a combination vaccine that protects against both *S. typhi* and *S. paratyphi* A would be a valuable public health tool.

The O-polysaccharide of the lipopolysaccharide (LPS) of *S. paratyphi* A is a potentially protective antigen and therefore the target for vaccine development. Phase 1 and 2 clinical studies with an O-polysaccharide-TT vaccine[9] were conducted 14 years ago in Vietnamese adults and children and found to be safe and immunogenic.[10] The Chengdu Institutes of Biological Products in China together with the Lanzhou Institute are currently conducting

* Corresponding author. Tel.: +39 0577 539087; fax: +39 0577 243540.
E-mail address: francesca.micoli@novartis.com (F. Micoli).

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a Phase 2 trial of this vaccine.[6,11] Other O-polysaccharide glycoconjugates using DT and CRM_{197} have been developed and tested in preclinical studies by the International Vaccine Institute (IVI)[6] and Novartis Vaccines Institute for Global Health (NVGH), respectively.[12] Both have been developed alongside Vi conjugate vaccines in order to be used in bivalent combinations and protect against both aetiologic agents of enteric fever.

Salmonella LPS consists of lipid A linked to the 3-deoxy-D-manno-octulosonic acid (Kdo) terminus of a conserved core region, which is linked to the variable O-polysaccharide chain. In *S. paratyphi* A, the chain repeating unit consists of a trisaccharide backbone composed of rhamnose (Rha), mannose (Man) and galactose (Gal), with a terminal paratose (Par) at C-3 of Man (which confers serogroup specificity: factor 2) and terminal glucose (Glc) at C-6 of Gal (Fig. 1).

A common feature shared by various O-polysaccharides is the presence of decorations such as *O*-acetyl groups. It has been reported that not only the presence but also the position of *O*-acetyl groups in the O-polysaccharide may influence the immune response.[13] The *S. paratyphi* A O-polysaccharide was reported to require *O*-acetyl groups in order to elicit serum LPS antibody with bactericidal activity in mice.[9] In the case of *S. paratyphi* A, a single site of O-acetylation has been reported, on C-3 of the Rha residue as part of the repeating O-polysaccharide chain[9,10,14,15] or on C-2 of the same monomer.[16] However, from analysis of the ^{13}C NMR spectrum, Konadu et al. suggested the presence of two different *O*-acetyl groups, the second one on the Par residue, akin to that found on C-2 of abequose for *S. typhimurium*.[17] The authors highlighted the need for further investigation given the important role that *O*-acetyl groups seem to play in immunogenicity.[9,10]

In the O-polysaccharide purification strategy adopted by NVGH, the labile linkage between the Kdo of the core and the lipid A is cleaved directly in the bacterial growth medium;[18] the O-polysaccharide, still linked to the core, is then easily purified from the cells and can be used for the production of conjugate vaccines. For conjugation to CRM_{197}, the *S. paratyphi* O-polysaccharide is linked to the carrier protein through the terminal Kdo, with the aim to preserve the O-polysaccharide antigen structure.[12] Here the O-polysaccharide structure has been elucidated by use of HPLC–SEC, HPAEC–PAD/CD, GLC, GLC–MS and 1D and 2D NMR studies. Heterogeneity due to non-stoichiometric glucosylation and O-acetylation resulted in complex NMR spectra and simpler structures were chemically derived to facilitate full assignment of the native O-polysaccharide structure. In particular, the presence of *O*-acetyl groups on both C-2 and C-3 of Rha was identified. The same pattern of O-acetylation was found in the O-polysaccharide-CRM_{197} conjugate and on the surface carbohydrate of *S. paratyphi* A bacteria. This confirmed that the purification and the conjugation processes do not alter the structure of the O-polysaccharide antigen, including retention of *O*-acetyl groups considered important for the immunogenicity of the *S. paratyphi* A vaccine.

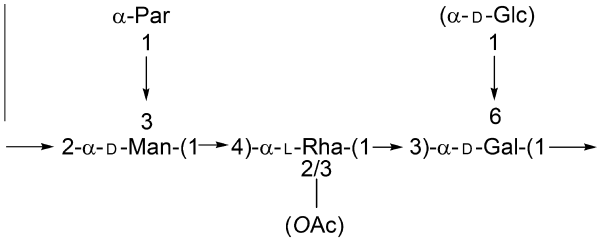

Figure 1. Structure of the repeating unit of *Salmonella paratyphi* A O-polysaccharide showing the positions of O-acetylation. The structural features that vary with the strain of origin are shown in curved brackets.

2. Results and discussion

2.1. Structural analysis of OPS

Purified O-polysaccharide (**OPS**) showed one main population with an average molecular mass of 40–45 kDa.[12,18] Sugar composition analysis of the **OPS** by GLC–MS analysis of the alditol acetate derivatives showed the presence of Rha, Man, Gal, Glc and a component eluting before Rha in the molar ratio 1.00:1.00:1.00:0.76:0.17, respectively. The early eluting component was identified by GLC–MS as a 3,6-dideoxy-hexose, ascribed to the expected Par, which was partially destroyed during acid hydrolysis. In the absence of a commercially available monomer standard, Par was quantified by ^1H NMR analysis which yielded the expected molar ratio of 1:1 with respect to Rha from the ratio of the corresponding H-6 resonances. Incomplete glucosylation is in agreement with HPAEC–PAD data (74%) previously reported.[18] GLC analysis of the chiral glycosides of the **OPS** showed that the hexoses were in the D absolute configuration and Rha in the L absolute configuration. As before, the absence of a commercial standard meant that analysis for Par could not be performed, and its D configuration was established by NMR glycosylation shifts. According to GLC and GLC–MS of the partially-methylated alditol acetate (PMAA) derivatives, **OPS** contains terminal non-reducing 3,6 dideoxyhexose, identified as Par by NMR (t-Par), 4-linked Rha (4-Rha), terminal non-reducing glucose (t-Glc), 3-linked Gal (3-Gal), 2,3-linked Man (2,3-Man) and 3,6-linked-Gal (3,6-Gal) in the molar ratio 0.16:∼0.94:∼0.71:0.30:1.00:0.67. These data are consistent with the **OPS** containing a mixture of ∼70% of the pentasaccharide repeating unit (containing Glc-(1→6)-Gal) and ∼30% of the tetrasaccharide repeating unit (without terminal glucose).[18] In addition to glucosylation, another source of the structural heterogeneity of the **OPS** is due to variable O-acetylation. Our earlier studies on several lots indicated a total *O*-acetyl content of 65–80% per repeating unit determined by ^1H NMR following in-tube de-O-acetylation using sodium deuteroxide. In this study, the total O-acetylation levels determined by HPAEC–CD were in agreement with values found by ^1H NMR and ranged from 65% to 75% per repeating unit for **OPS** lots evaluated.

The ^1H NMR spectrum of the **OPS** confirmed the presence of the two deoxy sugars with diagnostic signals for the H-3s of Par and H-6 of Rha (split) and Par, but the anomeric region could not be readily assigned due to overlap and the presence of multiple peaks. The complexity of the **OPS** spectrum was attributed to the twinning of some signals from incomplete glucosylation and the presence of both the pentasaccharide (major) and tetrasaccharide (minor) repeating units. This was further exacerbated by additional twinning of signals due to partial O-acetylation (shown by signals at 2.20 and 2.18 ppm) and the presence of small peaks from the core region and process residuals. Elucidation of these complex NMR spectra was achieved after full assignment of the ^1H and ^{13}C NMR spectra of simpler saccharides derived from **OPS**.

2.2. Characterization of OPS Smith degradation products

Complete periodate oxidation of **OPS**, followed by reduction with $NaBH_4$ and mild acid hydrolysis (Smith degradation) yielded an oligosaccharide (**OPS-S1**) as the major product. Full assignment of **OPS-S1** aided in the NMR interpretation of the polymer obtained after partial Smith degradation to reduce the level of glucosylation (**OPS-S2**), which in turn facilitated elucidation of the de-O-acetylated **OPS** (**OPS-D**) spectra and finally the spectra obtained for **OPS**.

The permethylated **OPS-S1** gave the mass spectrum (Fig. S1A) containing three ions at *m*/*z* 718.4, 723.4 and 739.4 corresponding to the parent adducts $[M+NH_4]^+$, $[M+Na]^+$, and $[M+K]^+$, respectively.