Journal of Biotechnology 198 (2015) 46–52

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Simplified low-cost production of O-antigen from *Salmonella* Typhimurium Generalized Modules for Membrane Antigens (GMMA)

Eleonora Meloni[1], Anna Maria Colucci, Francesca Micoli, Luigi Sollai, Massimiliano Gavini[2], Allan Saul, Vito Di Cioccio, Calman A. MacLennan*

Novartis Vaccines Institute for Global Health, Via Fiorentina 1, 53100 Siena, Italy

ARTICLE INFO

Article history:
Received 27 October 2014
Received in revised form 16 January 2015
Accepted 26 January 2015
Available online 3 February 2015

Keywords:
GMMA
Salmonella
Lipopolysaccharide
O-antigen
Vaccine

ABSTRACT

The Novartis Vaccines Institute for Global Health is developing vaccines using outer membrane particles, known as Generalized Modules for Membrane Antigens (GMMA). These are blebs of outer membrane and periplasm, shed from the surface of living Gram-negative bacteria following the targeted deletion of proteins involved in maintaining the integrity of the inner and outer membranes. The current study investigates the use of GMMA as starting material for extraction of membrane components, focusing on the O-antigen polysaccharide portion of lipopolysaccharide from *Salmonella* Typhimurium. We show that the amount of O-antigen extracted from GMMA by acid hydrolysis is comparable to the quantity extracted from whole wild type bacteria, but with less protein and DNA contaminants. Compared to conventional purification, GMMA enabled a reduction in the number of purification steps required to obtain the O-antigen polysaccharide with the same purity. Purification processes from GMMA and bacteria were characterised by similar final yields. Use of GMMA as starting material provides the possibility to simplify the purification process of O-antigen, with a consequent decrease in manufacturing costs of O-antigen-based glyconjugate vaccines against *Salmonella* strains and potentially other Gram-negative bacteria.

1. Introduction

The cell envelopes of Gram-negative bacteria consist of three layers: a lipid–protein inner (cytoplasmic) membrane, an outer membrane, composed mainly of lipopolysaccharides (LPS) and proteins, and a thin rigid layer of peptidoglycan located in the periplasmic space. Peptidoglycan has the ability to interact with many proteins of the cell envelope, contributing to the stability of the inner and outer membranes (Godlewska et al., 2009). LPS consists of three main components: lipid A, core oligosaccharide and O-specific polysaccharide (or O-antigen), which is composed of repeating oligosaccharide units (Vines et al., 2005). The O-antigen is the most variable part of LPS. Variation between different O-antigens lies in their sugar composition, the arrangement of sugars, and the linkages between the sugars within the O-antigen repeating unit, as well between O-antigen units (Reeves and Wang, 2002).

In nature, Gram-negative bacteria normally shed parts of their outer membrane as blebs into the surrounding environment through bulging and 'pinching off' of the outer membrane. These blebs are known as native outer membrane vesicles and have been proposed as a means of delivering virulence factors to host cells. Their maximum production occurs during the end of log phase growth (Kuehn and Kesty, 2005). The blebs are almost free of cytoplasmic and inner membrane components and contain lipophilic proteins. This contrasts with detergent-extracted outer membrane vesicles derived from homogenized bacteria (Ferrari et al., 2006; Holst et al., 2009), which have been used as vaccines, *e.g.* MeNZB, an outer membrane vesicle vaccine which was used to control a *Neisseria meningitidis* serogroup B outbreak in New Zealand (Holst et al., 2009).

The spontaneous production of outer membrane particles can be enhanced by introducing targeted deletion of proteins involved

Abbreviations: GMMA, Generalized Modules for Membrane Antigens; LPS, lipopolysaccharide; TFF, tangential flow filtration; CDM, chemically defined medium; HMW, high molecular weight; MMW, medium molecular weight; LMW, low molecular weight.

* Corresponding author. Present address: Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, United Kingdom. Tel.: +44 (0)1223 495391; fax: +44 (0)1223 494919.
E-mail address: cm15@sanger.ac.uk (C.A. MacLennan).
[1] Phico Therapeutics Ltd, Babraham Research Campus, Babraham, Cambridge CB22 3AT, United Kingdom.
[2] Novartis Vaccines, Via Fiorentina 1, 53100 Siena, Italy.

http://dx.doi.org/10.1016/j.jbiotec.2015.01.020

Table 1
Primers used to generate *S.* Typhimurium SL1344 Δ*tolR* strain.

Primer	Sequence
tolR1	CTAGTCTAGAGCCATCATTATCCAGCGAAC
tolR2	AGCTTGATATCGGCTTACCCCTTGTTGCTTTC
tolR3	AGCTTGATATCAGTCTGCGTCCCGTTGGCTT
tolR4	CCGGGTACCGTTGCGCCAGTTTTGCCGCT
tolR5	AGCTTGATATCAAAGCCACGTTGTGTCTCAAAATCTC
tolR6	AGCTTGATATCTGAGGTCTGCCTCGTGAAGAAG

in maintaining the integrity of the inner and outer membranes. This leads to the production of Generalized Modules for Membrane Antigens (GMMA). For example, manipulation of the Tol-Pal system leads to the production of large amounts of GMMA from Gram-negative bacteria including *Escherichia coli, Shigella* and *Salmonella* (Berlanda et al., 2012). GMMA are spherical with a bilayer membrane and a variable diameter, depending on the bacterial parent strain. They are composed of outer membrane proteins, LPS, phospholipids and soluble periplasmic proteins (Mashburn-Warren et al., 2008).

Whole GMMA can either be used as vaccines themselves or as a source of components for subunit vaccines. Since GMMA are composed mainly of membrane components, the purification of these components is potentially more straightforward than purification from whole bacteria. We hypothesised that simplified processes can be used, thereby avoiding costly steps necessary to remove residual nucleic acids and proteins that can potentially interfere with downstream applications. A main problem with standard purification protocols starting from whole bacteria is the contamination of the end product with nucleic acids and proteins. Each of these impurities must be consistently removed to an acceptable residual level (less than 1% w/w compared to the amount of sugar) (Micoli et al., 2013) during downstream processing in order to ensure product safety (Shukla et al., 2008).

NVGH has optimized methods for GMMA production, using mutant strains with deletion of the *tolR* gene without loss of membrane integrity (Berlanda et al., 2012), and passage through a two-step Tangential Flow Filtration (TFF) process, in order to purify GMMA from bacterial suspensions and soluble proteins (Berlanda et al., 2012). GMMA constitute an affordable technology with the potential to have a marked impact on the manufacturing costs of vaccines against Gram-negative bacteria, making them particularly suitable for the development of low-cost vaccines against neglected infectious diseases of the developing world.

In the present study, we investigate the potential of using GMMA from *Salmonella* Typhimurium SL1344 Δ*tolR* as a source of O-antigen polysaccharide for glycoconjugate vaccine production (Micoli et al., 2013).

2. Materials and methods

2.1. Bacterial strains

Salmonella Typhimurium SL1344 was chosen as the parent strain. *S.* Typhimurium SL1344 Δ*tolR* was obtained by replacing *tolR* (Berlanda et al., 2008) with a kanamycin resistance gene (*kan*) as follows: The upstream and downstream regions of *tolR* were amplified using the primer pairs tolR1/tolR2 and tolR3/tolR4 (Table 1), respectively. The *kan* gene was amplified from pUC4K (Berlanda et al., 2012) using primer pairs tolR5/tolR6 (Table 1). The fragments were inserted into pBluescript (Stratagene) so that the *kan* gene interposed the flanking regions of *tolR*. The replacement construct (upstream region–*kan*–downstream region) was amplified using the primers tolR1/tolR4 and used to transform recombination prone cells of *S.* Typhimurium produced using the Lambda phage recombination system (red operon) encoded on pAJD433 (Berlanda et al., 2012). pAJD434 was subsequently removed from the mutant strain.

2.2. Growth and fermentation condition

S. Typhimurium SL1344 wild type and *S.* Typhimurium SL1344 Δ*tolR* were grown to 30 OD in 3 and 3.5 L, respectively, of chemically defined medium (CDM), using a 5 L fermenter (Sartorius B-plus). Temperature was maintained at 37 °C, pH fixed at 6.7 and controlled with 28% NH_4OH, and oxygen saturation set at 30% and maintained using an agitation controller. The composition of the CDM was: 13.3 g/L KH_2PO_4, 4 g/L $(NH_4)_2HPO_4$, 1.7 g/L citric acid monohydrate, 1 M $MgSO_4{\cdot}7H_2O$, 1 mL/L Trace Elements, 1 mL/L Trace Vitamins and 3% glycerol, pH 6.7. Trace Elements solution contained: 2.5 g/L $CoCl_2{\cdot}6H_2O$, 15 g/L $MnCl_2{\cdot}4H_2O$, 1.5 g/L $CuCl_2{\cdot}H_2O$, 3 g/L H_3BO_3, 2.5 g/L $Na_2MoO_4{\cdot}2H_2O$, 2.5 g/L $Zn(CH_3COO)_2{\cdot}H_2O$ and 100 mM ferric citrate. Trace Vitamins solution contained: 5 g/L thiamine, 10 g/L nicotinic acid, 10 g/L Ca-panthotenate and 10 g/L Pyridoxine-HCl, 10 mg/L B12. PPG 0.25 g/L was added to control foam. Histidine was added to the CDM with a starting concentration of 0.6 g/L and additional CDM medium feedings were carried out during the fermentation process, giving the final histidine addition of 1.2 g/L. Optical density was measured at 600 nm

2.3. Purification from total biomass

2.3.1. Biomass direct acid hydrolysis

Two litres of the 3 L *S.* Typhimurium SL1344 wild type fermentation broth were directly hydrolysed with 1% Acetic Acid (pH 4–4.5), 100 °C, 6 h (Fig. 1A). The resulting solution was neutralized by adding 28% NH_4OH (Sigma, 338818) to a pH of 6–7.

2.3.2. Standard O-antigen purification process from wild type *Salmonella*

Standard O-antigen purification from the hydrolysate was performed as previously described (Micoli et al., 2013) (Fig. 2A). At the end of hydrolysis, *S.* Typhimurium SL1344 wild type fermentation broth was passed through a 0.2 μm TFF membrane (Hydrosart Sartocon Slice 0.2 μm, Sartorius). 1.8 L of the resulting permeate were concentrated fivefold by TFF (Pressure in, Pin = 1.8–2.0 bar and transmembrane pressure, TMP = 0.9–1 bar), using a 30 kDa cut-off filter cassette (Hydrosart Sartocon Slice 200, Sartorius), then washed by diafiltration against 10 volumes of 1 M NaCl and then 10 volumes of water, maintaining the same volume.

Fifty millilitres of the 5× 30 kDa retentate was further processed. Citrate buffer (200 mM, pH 2.7) was added, to give a final concentration of 20 mM, pH 3. The solution was mixed at room temperature for 30 min, and further centrifuged for 30 min, at 4000 × *g* and 4 °C, and the resulting pellet was discarded. The O-antigen in the supernatant was purified by cationic exchange filtration, using a Sartobind S MA75 filter (Sartorius) equilibrated with 20 mM citrate pH 3 using the same equilibration conditions. In this step, protein impurities are reduced through the binding of proteins to the filter bed, while the O-antigen is eluted in the flow-through. The flow-through was collected, and 500 mM Na_2HPO_4, absolute EtOH and 5 M $CaCl_2$ were added to give a final concentration of 18 mM Na_2HPO_4, 24% EtOH (v/v) and 200 mM $CaCl_2$. The solution was mixed at room temperature for 30 min then centrifuged for 30 min at 4000 × *g* and 4 °C, and the resulting pellet (formed by nucleic acid) was discarded. Five millilitre-aliquots of the supernatant, containing O-antigen, were passed through a PD-10 desalting column (GE Healthcare Life Sciences) and analysed.

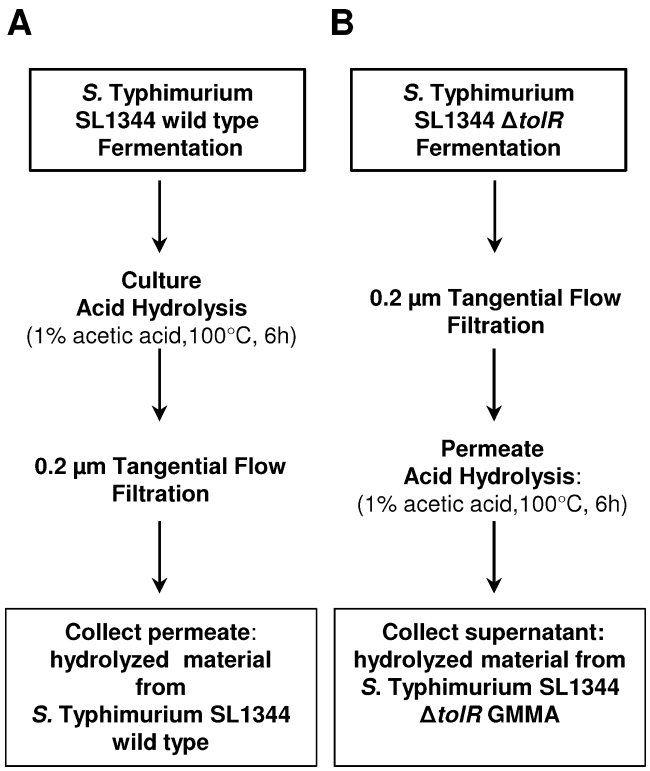

Fig. 1. Flow chart for O-antigen extraction from *S.* Typhimurium SL1344 wild type and *S.* Typhimurium SL1344 Δ*tolR* GMMA. *S.* Typhimurium SL1344 wild type was directly hydrolysed at the end of the fermentation process and then passed through a 0.2 μm TFF membrane, to separate the residual biomass from the permeate containing O-antigen. *Salmonella* Typhimurium SL1344 Δ*tolR* fermentation culture was processed by 0.2 μm TFF to collect the permeate containing GMMA, and then hydrolysed to release O-antigen.

2.4. Purification from GMMA

2.4.1. GMMA recovery and direct acid hydrolysis

3.5 L of *Salmonella* Typhimurium SL1344 Δ*tolR* fermentation broth were filtered through a 0.2 μm TFF cassette (Sartocon Slice Hydrosart 0.2 μm Sartorius) in order to separate the bacterial biomass from permeate containing GMMA and soluble proteins (Fig. 1B). 1.8 L of GMMA of the 0.2 μm TFF permeate were hydrolysed with 1% Acetic Acid (pH 4–4.5), 100 °C, 6 h (Fig. 1B). The resulting solution was neutralized by adding 28% NH_4OH (Sigma, 338818) to a pH of 6–7.

2.4.2. O-antigen purification process from GMMA

1.8 L of hydrolysed GMMA from the 0.2 μm TFF *Salmonella* Typhimurium SL1344 Δ*tolR* permeate were concentrated three-fold by TFF (Pin = 1.8–2.0 bar and transmembrane pressure, TMP = 0.9–1 bar), using a 30 kDa cut-off filter cassette and the sample washed by diafiltration against 10 volumes of 1 M NaCl and then 10 volumes of water, maintaining the same volume. The retentate was collected, and four aliquots of 50 mL were prepared and treated using various approaches, as described below (Fig. 2A–D).

2.4.2.1. Standard O-antigen purification process. The sample (50 mL, aliquot 1) was processed according to the standard purification protocol used for the *Salmonella* Typhimurium wild type biomass (Section 2.3.2 and Fig. 2, scheme A).

2.4.2.2. Citrate buffer precipitation and cationic exchange chromatography. Citrate buffer (200 mM, pH 2.7) was added to the 50 mL aliquot 2 giving a final concentration of 20 mM, pH 3. The solution was mixed at room temperature for 30 min and centrifuged for 30 min at 4000 × *g* and 4 °C. The supernatant was collected and passed through a Sartobind S MA75 filter, conditioned with the same buffer (Citrate buffer 20 mM, pH 3.0). The flow-through was collected, passed through a PD-10 desalting column and analysed (Fig. 2 scheme B, *i.e.* no EtOH and $CaCl_2$ precipitation step).

2.4.2.3. Citrate buffer and $CaCl_2$/EtOH precipitations. Citrate buffer (200 mM, pH 2.7) was added to the 50 mL aliquot 3, giving a final concentration of 20 mM, pH 3. The solution was mixed at room temperature for 30 min and further centrifuged for 30 min at 4000 × *g* and 4 °C. The supernatant was collected and 500 mM Na_2HPO_4, absolute EtOH and 5 M $CaCl_2$ were added to give final concentrations of 18 mM Na_2HPO_4, 24% EtOH (v/v) and 200 mM $CaCl_2$. The solution was mixed at room temperature for 30 min and further centrifuged at 4000 × *g* at 4 °C for 30 min, the supernatant was collected, passed through a PD-10 desalting column and analysed (Fig. 2 scheme C, *i.e.* no cationic exchange chromatography step).

2.4.2.4. Cationic exchange chromatography. Citrate buffer (200 mM, pH 2.7) was added to 50 mL aliquot 4, giving a final concentration of 20 mM, pH 3. The resulting solution was immediately passed through Sartobind S MA75 filter, conditioned with the same buffer (Citrate buffer 20 mM, pH 3.0). The flow-through was passed through a PD-10 desalting column and analysed (Fig. 2, scheme D, *i.e.* no citrate buffer precipitation and no EtOH/$CaCl_2$ precipitation steps).

2.5. Analytical size exclusion chromatography: TSK gel 3000 PWXL

Molecular size distribution of O-antigen populations was measured as previously described (Micoli et al., 2013). Samples were analysed by HPLC-Size Exclusion Chromatography, using a TSK gel 3000 PWXL column (30 cm × 7.8 mm; particle size 7 μm). Running buffer composition was 100 mM NaCl, 100 mM NaH_2PO_4 5%, CH_3CN pH 7 and the flow rate was of 0.5 mL/min. The O-antigen peaks were measured by a refractive index detector.

2.6. High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

Precise sugar quantification and composition analysis was performed by HPAEC–PAD, after acid hydrolysis of the O-antigen to release the monosaccharides constituting the sugar chain (Micoli et al., 2013). Commercial monomer sugars were used for preparation of calibration curves for Rha, Gal, Glc and Man quantification. O-antigen samples were diluted to have each sugar monomer in the range 0.5–10 μg/mL, and were hydrolysed at 100 °C for 4 h in 2 M trifluoroacetic acid.

2.7. Determination of total sugar by Phenol Sulphuric Acid Colorimetric Assay, protein and nucleic acid content of hydrolysed samples

In process control quantification of total sugar in each sample during the various steps of purification was determined by Phenol Sulphuric Acid Colorimetric Assay (Dubois et al., 1951). Protein content was measured by the Micro BCA Method (Thermo scientific Micro BCA Protein Assay Kit), following the manufacturer's instructions. Nucleic acid content was estimated measuring the absorbance at 260 nm, the maximum absorption wavelength of DNA. This method gives only an estimate of the upper limit of the nucleic acid content present in the sample, since at 260 nm other components (*e.g.* protein) can contribute some absorbance.

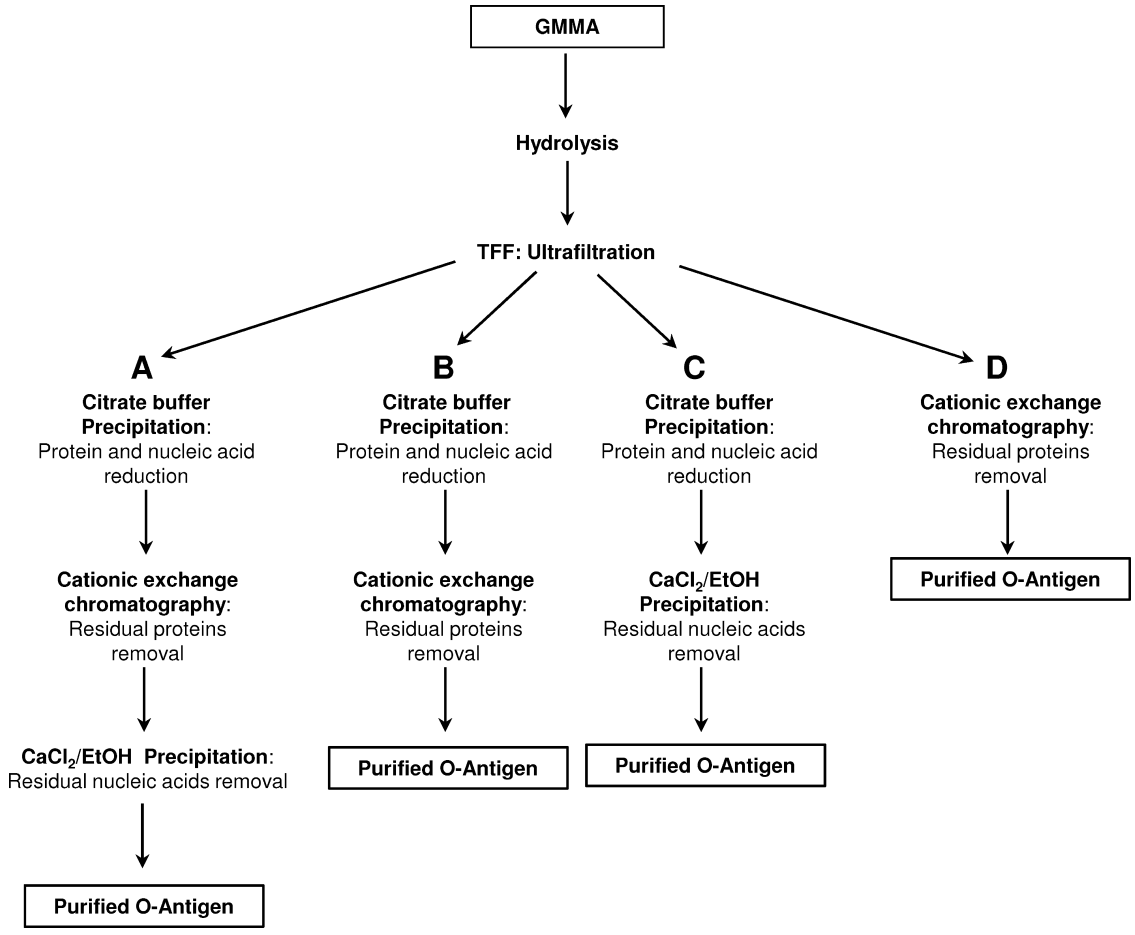

Fig. 2. O-antigen purification strategies starting from GMMA. To optimize the O-antigen purification protocol, we used different approaches starting with the hydrolysed GMMA: (A) GMMA sample was treated using the standard O-antigen purification protocol, already developed for the biomass; (B) only the first precipitation step and the cationic exchange chromatography were used; (C) citrate buffer and $CaCl_2$/EtOH precipitations were used avoiding the Sartobind S step; (D) only cationic exchange filter was used.

3. Results

3.1. Composition of initial hydrolyses

Whole *S.* Typhimurium SL1344 wild type and GMMA from *Salmonella* Typhimurium SL1344 Δ*tolR*, were hydrolysed and then analysed for total sugar, peptide and nucleic acid content. No vesicles equivalent to GMMA could be recovered from the *S.* Typhimurium SL1344 wild type fermentation broth. Following acid hydrolysis, the GMMA preparation had a comparable total sugar concentration to the wild type bacteria (0.86 g/L versus 1.0 g/L; Table 2). Hydrolysed material from GMMA contained much lower amounts of protein and nucleic acid contamination compared with material from the wild type fermentation culture, particularly with regards to nucleic acids (Table 2). These samples were analysed by SEC on a TSK gel 3000 PWXL HPLC-SEC column. The material from whole bacteria (solid line, Fig. 3A) gave three refractive index peaks corresponding to high molecular weight (HMW), medium molecular weight (MMW) and low molecular weight (LMW) O-antigen. The hydrolysed material from GMMA (dashed line, Fig. 3A) gave peaks with a similar elution time but with a relatively lower HMW O-antigen.

3.2. O-antigen purification from Salmonella Typhimurium SL1344 wild type bacteria and GMMA

Concentrations of sugar, protein and nucleic acids, following each step of the standard purification process following 0.2 μm TFF of hydrolysed wild type bacteria, are given in Table 2. We have previously verified good agreement between this assay and HPAEC-PAD (Micoli et al., 2013). To assess whether contaminating nucleic acids could impair performance of the phenol sulphuric acid assay in the current study, we treated DNA with 1% Acetic Acid at 100 °C for 6 h (the same treatment followed for O-antigen extraction) and added 62.5 μg/mL (final concentration), the equivalent of that present in the most impure intermediate of the process described in the study, to a standard sample of glucose at 100 μg/mL. Sugar quantification varied by a maximum of 10% from the concentrations recorded without the addition of nucleic acids, indicating minimal interference with the assay.

3.2.1. Standard purification of O-antigen from GMMA

Using the standard O-antigen purification (Section 2.4.2.1, Fig. 2, scheme A), no pellet was observed at the end of the precipitation steps, either the first step, using citrate buffer, which permits the co-precipitation of nucleic acids and proteins, or the $CaCl_2$/EtOH step indicating the absence of sufficient protein and nucleic acid for visible precipitation. Very low levels of nucleic acids were present in the GMMA sample, and following the first 30 kDa TFF and citrate buffer addition, the amount remained the same (Table 2). The content of residual proteins decreased by almost 50% at the end of the first precipitation step (Table 2), with a 15-fold reduction following cationic exchange chromatography.

Table 2
Purity and yield of *Salmonella* Typhimurium O-antigen through the standard and alternative purification processes starting with wild type bacterial biomass or GMMA. The end points of each of the four GMMA-derived O-antigen purification schemes (A to D) are given in bold.

Step	Sugar[a] (mg/L)	Protein[a] (mg/L)	Nucleic acid[a] (mg/L)	% Protein[b]	% Nucleic acids[b]	% Sugar yield[c]
Purification from biomass						
Starting hydrolysate	1000	680	567	68.0	56.7	
30 kDa TFF	916	164	570	18.0	62.2	
First precipitation, citrate buffer	938	18.0	160	1.9	17.0	102.4
Sartobind S MA75	744	2.8	2.8	0.4	0.4	81.2
Second precipitation $CaCl_2$/EtOH	650	1.6	2.4	0.2	0.3	71.0
Purification from GMMA						
Starting hydrolysate	860	144	11	16.7	1.2	
30 kDa TFF	824	70.7	2.7	8.6	0.4	
Standard purification						
First precipitation, citrate buffer	812	40.7	2.0	5.0	0.3	98.6
Sartobind S MA75 (**scheme B**)	618	2.7	2.0	0.4	0.3	75.0
Second precipitation, $CaCl_2$/EtOH (**scheme A**)	514	2.0	2.0	0.4	0.3	62.4
Alternative purifications (final purification)						
First precipitation citrate buffer and second precipitation (**scheme C**)	564	22.0	2.0	3.9	0.3	68.5
Only Sartobind S MA75 (**scheme D**)	590	2.0	1.3	0.3	0.2	71.7

[a] Per L of hydrolysate taking into account concentration in TFF steps and volume of sample.
[b] compared to sugar content.
[c] yield of sugar from 30 kDa hydrolysate.

3.2.2. Modified purification of O-antigen from GMMA

Although the first precipitation step, citrate buffer 20 mM, was insufficient to meet the target level of residual protein, optimal purification could still be achieved when the first citrate precipitation step was used in combination with cationic exchange chromatography, without the final $CaCl_2$/EtOH precipitation step (Section 2.4.2.2, Fig. 2, scheme B). At the end of these two steps, there was very low residual nucleic acid and proteins levels so the final precipitation step using the $CaCl_2$/EtOH could be removed (Table 2). A suboptimal result was obtained using just two precipitation steps (citrate buffer and $CaCl_2$/EtOH) without the Sartobind S MA75 filtration step (Section 2.4.2.3, Fig. 2, scheme C). The levels of residual proteins and nucleic acids remained higher than required (Table 2). However using only the Sartobind S MA75 filter, without either precipitation step gave a low level of impurities (Section 2.4.2.4, Fig. 2, scheme D). The amount of sugar in the cationic exchange chromatography flow though was no lower than the final sugar concentration in the standard purification protocol (Table 2). These data indicate that the cationic exchange chromatography step is responsible for the main reduction in impurities, while maintaining an optimum sugar yield, and that both precipitations steps involved in the O-antigen purification protocol can be removed when the starting material is GMMA.

The final yield of O-antigen obtained starting from GMMA and using only Sartobind S for protein removal was approximately 72% and almost identical to the final sugar yield obtained from *Salmonella* Typhimurium SL1344 wild type bacteria biomass following the standard purification process (Table 2).

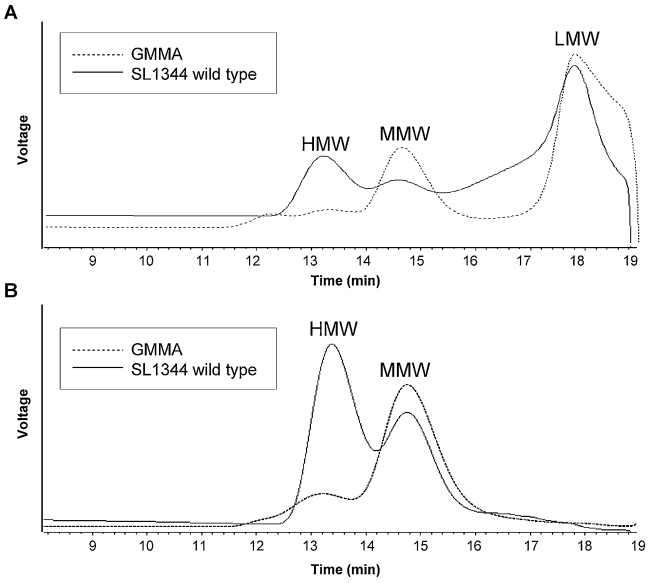

Fig. 3. *S.* Typhimurium SL1344 wild type and *S.* Typhimurium SL1344 Δ*tolR* GMMA HPLC-SEC profiles. (A) Following hydrolysis, but prior to purification, the refractive index channel for *S.* Typhimurium SL1344 wild type O-antigen (solid line) indicates the predominance of the high molecular weight (HMW) and low molecular weight (LMW) populations, whereas the main populations for *S.* Typhimurium SL1344 Δ*tolR* GMMA (dashed line) are medium molecular weight (MMW) and LMW O-antigen. (B) At the end of purification, LMW O-antigen is lost as indicated by profiles of pure O-antigen from both starting materials, with continued dominance of HMW O-antigen starting from wild type bacteria (solid line) and MMW O-antigen from GMMA (dashed line).

3.3. O-antigen characterization of O-antigen purified from GMMA

The GMMA O-antigen was characterised for sugar composition with HPAEC-PAD and the analysis confirmed the expected structure for *Salmonella* Typhimurium O-antigen in relation to composition of the sugar backbone of rhamnose, mannose and galactose (Table 3) (Micoli et al., 2014). However, glucosylation of O-antigen extracted from GMMA was higher than that from wild type *Salmonella*, with a molar ration of 0.49 relative to rhamnose, as compared with 0.08. Analysis by HPLC-SEC showed that the MMW population remained the most abundant one for O-antigen from GMMA, while the HMW population was dominant for O-antigen from wild type. The LMW fraction was completely lost following purification, regardless of whether the O-antigen was extracted from wild type bacteria or GMMA (Fig. 3B).

Table 3
Sugar composition of O-antigen from *Salmonella* Typhimurium SL1344 GMMA and wild type bacteria (molar ratios relative to Rha).

Sugar	*S.* Typhimurium SL1344 Δ*tolR* GMMA	*S.* Typhimurium SL1344 wild type
Rha	1.00	1.00
Man	1.00	1.00
Gal	1.08	1.10
Glc	0.49	0.08

4. Discussion

An ideal vaccine production system, especially for bacterial vaccines for developing countries, will encompass multiple antigens and enable vaccines to be rapidly tailored to local and changing antigenic phenotypes. Ideally, such vaccines will also need to be inexpensive to manufacture (Berlanda et al., 2012). We investigated the use of GMMA from *S.* Typhimurium SL1344 Δ*tolR*, to understand whether GMMA could be used as an alternative O-antigen source to wild type bacteria for the development of glycoconjugate vaccines. Although wild type Gram-negative bacteria can spontaneously shed vesicles equivalent to GMMA (termed native outer membrane vesicles or NOMV), no such vesicles could be recovered from the fermentation broth of wild type *S.* Typhimurium SL1344. Therefore, the introduction of a modification such as the deletion of *tolR*, described here, is an essential pre-requisite for the production of GMMA from *S.* Typhimurium.

GMMA from *Salmonella* Typhimurium SL1344 Δ*tolR* has two main characteristics at the end of the fermentation process, relating to the polysaccharide yield and impurities: The O-antigen concentration using GMMA as starting material is comparable with that using *Salmonella* Typhimurium SL1344 wild type biomass (respectively 1.56 g versus 1.80 g per 1.8 L of acid hydrolysate), but a lower amount of impurities is present in the GMMA preparation compared with the SL1344 wild type biomass (Table 2), indicating the potential of GMMA as starting material for O-antigen extraction. First, we applied the standard O-antigen purification protocol on GMMA. This method of O-antigen extraction from biomass involves several purification steps, which are required to remove large quantities of impurities consistently, present at the end of the initial acid hydrolysis step, as reported by Micoli et al. (2013). We have shown that, owing to the low level of impurities present in GMMA, this purification protocol can be simplified, removing the citrate buffer and $CaCl_2$/EtOH precipitation steps (Fig. 4) and leaving only the cation exchange filtration step for removal of impurities.

A maximum acceptable limit for nucleic acid and protein contamination in O-antigen-based *Salmonella* conjugate vaccines has yet to be stipulated (Kothari et al., 2014). We decided to set our upper limits for purified O-antigen from *S.* Typhimurium in the current study at 1% for both protein and nucleic acid content. These values are guided by the limits specified by the WHO of 1% (w/w with respect to polysaccharide content) protein and 2% (w/w with respect to polysaccharide content) nucleic acids for *Salmonella* Vi vaccines (WHO, 1994).

Starting from GMMA we obtained the same sugar yield as for the *Salmonella* Typhimurium SL1344 per litre of the fermentation (around 71%) using a TFF filtration step, to separate the GMMA-containing supernatant in the culture from the bacterial cells, and hydrolysis, followed by a TFF and a cation filtration step to remove impurities. From a bioprocess view, this compares very favourably with the best process previously developed for purification from whole bacterial cultures, comprising an acid hydrolysis step, TFF, precipitation and centrifugation, a cation filtration step and another precipitation and centrifugation step.

Associated with the generation of the GMMA producing strain, there was a change in the relative size of the O-antigen, with relatively more MMW and less HMW. Although this is not a direct consequence of the revised purification process (the ratios of HMW and MMW were similar following hydrolysis and after purification), the lower size results in a lower viscosity of the purified polysaccharide, and therefore leads to improvements in the subsequent vaccine production. In addition, the more homogeneous O-antigen population improves consistency and characterisation of the final conjugate. We have recently shown that glycoconjugate vaccine made with MMW O-antigen population of *S.* Typhimurium

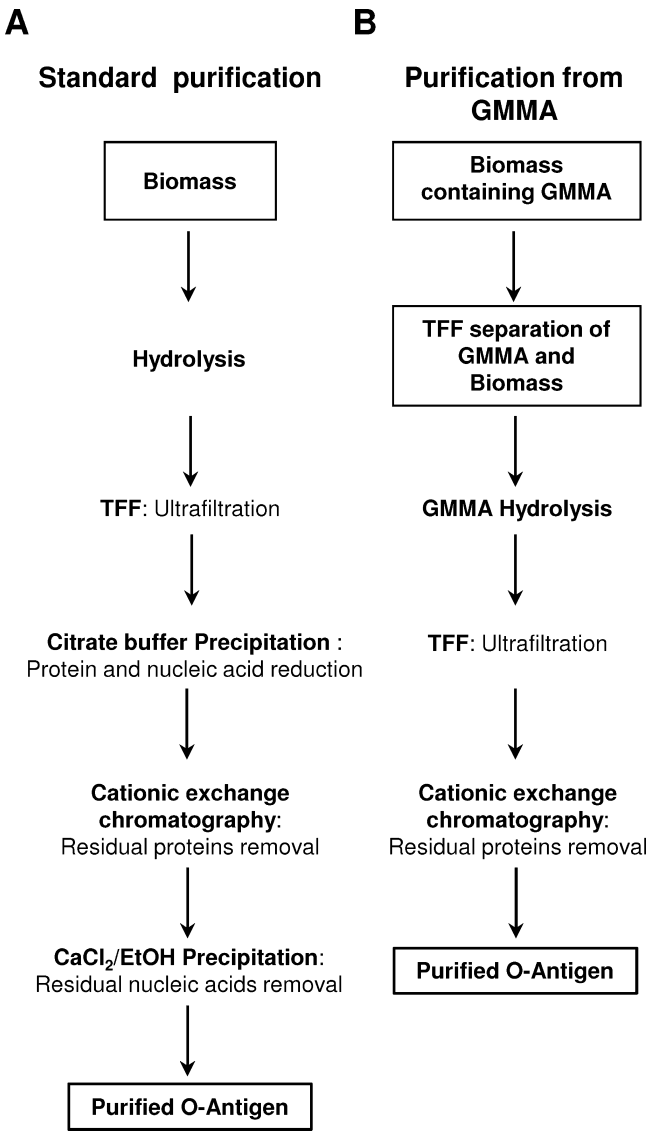

Fig. 4. Flow chart of O-antigen purification process starting from biomass and GMMA. With GMMA from *S.* Typhimurium SL1344 Δ*tolR* as starting material, the O-antigen purification process could be simplified compared with that used for purification from wild type *S.* Typhimurium SL1344, with the removal of the two precipitation steps, giving advantages in terms of time and cost, thus potentially reducing the unit vaccine cost.

is more immunogenic than an equivalent vaccine made with HMW O-antigen (Rondini et al., 2014).

Another difference between the O-antigen extracted from GMMA and that from wild type *Salmonella* is the glucosylation level of the purified O-antigen, with O-antigen from GMMA being over five times more glucosylated. In our recent work on *S.* Typhimurium O-antigen conjugate vaccines, we found that higher O-antigen glucosylation associated with greater immunogenicity (Rondini et al., 2014). These differences in relation to O-antigen size population and glucosylation level suggest additional potential benefits from using GMMA as the starting source of O-antigen for production of *S.* Typhimurium glycoconjugate vaccines. Further studies are required to understand what impact use of GMMA as starting material would have on O-antigen of other *Salmonella* strains and other Gram-negative bacteria.

A surprising result was the amount of GMMA in the supernatant of the bacterial culture. There was 1.0 g/L of sugar in the hydrolysate from the non-GMMA producing wild type bacteria and 0.87 g/L

from just the GMMA of the GMMA-producing bacteria. The yield of O-antigen as a percentage of the total sugar was similar from biomass and GMMA, suggesting that there is nearly as much LPS, and presumably outer membrane per litre in the GMMA alone from Δ*tolR* mutant bacteria, as there is in wild type bacteria.

In summary, GMMA have good potential for use as a starting source for O-antigen extraction, owing to both the large quantity of GMMA in cultures from mutant bacteria and the possibility of simplification of the standard purification process, with a reduction in production time and cost, ultimately leading to a decrease in the potential cost of the final vaccine. Suitability of the process at manufacturing scale could facilitate the robust generation of polysaccharide-based vaccines starting from GMMA, as the membrane component source, and reduce the manipulation of potentially pathogenic bacteria, with a consequent safety benefit during the O-antigen extraction process.

Acknowledgements

We thank Dr Renzo Alfini for experimental assistance and Drs Christiane Gerke and Omar Rossi for critically reviewing the manuscript.

References

Berlanda, S.F., Doro, F., Rodriguez-Ortega, M.J., Stella, M., Liberatori, S., Taddei, A.R., Serino, L., Gomes, M.D., Nesta, B., Fontana, M.R., Spagnuolo, A., Pizza, M., Norais, N., Grandi, G., 2008. Proteomics characterization of outer membrane vesicles from the extraintestinal pathogenic *Escherichia coli* DeltatolR IHE3034 mutant. Mol. Cell. Proteomics 7, 473–485, http://dx.doi.org/10.1074/mcp.M700295-MCP200.

Berlanda, S.F., Colucci, A.M., Maggiore, L., Sanzone, S., Rossi, O., Ferlenghi, I., Pesce, I., Caboni, M., Norais, N., Di, C.V., Saul, A., Gerke, C., 2012. High yield production process for Shigella outer membrane particles. PLoS ONE 7, e35616, http://dx.doi.org/10.1371/journal.pone.0035616.

Dubois, M., Gilles, K., Hamilton, J.K., Rebers, P.A., Smith, F., 1951. A colorimetric method for the determination of sugars. Nature 168, 167.

Ferrari, G., Garaguso, I., Adu-Bobie, J., Doro, F., Taddei, A.R., Biolchi, A., Brunelli, B., Giuliani, M.M., Pizza, M., Norais, N., Grandi, G., 2006. Outer membrane vesicles from group B *Neisseria meningitidis* delta gna33 mutant: proteomic and immunological comparison with detergent-derived outer membrane vesicles. Proteomics 6, 1856–1866, http://dx.doi.org/10.1002/pmic.200500164.

Godlewska, R., Wisniewska, K., Pietras, Z., Jagusztyn-Krynicka, E.K., 2009. Peptidoglycan-associated lipoprotein (Pal) of Gram-negative bacteria: function, structure, role in pathogenesis and potential application in immunoprophylaxis. FEMS Microbiol. Lett. 298, 1–11, http://dx.doi.org/10.1111/j.1574-6968.2009.01659.x.

Holst, J., Martin, D., Arnold, R., Huergo, C.C., Oster, P., O'Hallahan, J., Rosenqvist, E., 2009. Properties and clinical performance of vaccines containing outer membrane vesicles from *Neisseria meningitidis*. Vaccine 27 (Suppl. 2), B3–B12, http://dx.doi.org/10.1016/j.vaccine.2009.04.071.

Kothari, S., Kim, J.A., Kothari, N., Jones, C., Choe, W.S., Carbis, R., 2014. Purification of O-specific polysaccharide from lipopolysaccharide produced by Salmonella enterica serovar Paratyphi A. Vaccine 32, 2457–2462, http://dx.doi.org/10.1016/j.vaccine.2014.02.090.

Kuehn, M.J., Kesty, N.C., 2005. Bacterial outer membrane vesicles and the host-pathogen interaction. Genes Dev. 19, 2645–2655, http://dx.doi.org/10.1101/gad.1299905.

Mashburn-Warren, L., McLean, R.J., Whiteley, M., 2008. Gram-negative outer membrane vesicles: beyond the cell surface. Geobiology 6, 214–219, http://dx.doi.org/10.1111/j.1472-4669.2008.00157.x.

Micoli, F., Rondini, S., Gavini, M., Pisoni, I., Lanzilao, L., Colucci, A.M., Giannelli, C., Pippi, F., Sollai, L., Pinto, V., Berti, F., Maclennan, C.A., Martin, L.B., Saul, A., 2013. A scalable method for O-antigen purification applied to various Salmonella serovars. Anal. Biochem. 434, 136–145, http://dx.doi.org/10.1016/j.ab.2012.10.038.

Micoli, F., Ravenscroft, N., Cescutti, P., Stefanetti, G., Londero, S., Rondini, S., MacLennan, C.A., 2014. (2014) Structural analysis of O-polysaccharide chains extracted from different *Salmonella* Typhimurium strains. Carbohydr. Res. 385, http://dx.doi.org/10.1016/j.carres.2013.12.003.

Reeves, P.P., Wang, L., 2002. Genomic organization of LPS-specific loci. Curr. Top. Microbiol. Immunol. 264, 109–135.

Rondini, S., Micoli, F., Lanzilao, L., Gavini, M., Alfini, R., Brandt, C., Clare, S., Mastroeni, P., Saul, A., Maclennan, C.A., 2014. Design of glycoconjugate vaccines against invasive African *S.* Typhimurium. Infect. Immun., http://dx.doi.org/10.1128/IAI.03079-14.

Shukla, A.A., Jiang, C., Ma, J., Rubacha, M., Flansburg, L., Lee, S.S., 2008. Demonstration of robust host cell protein clearance in biopharmaceutical downstream processes. Biotechnol. Prog. 24, 615–622, http://dx.doi.org/10.1021/bp070396j.

Vines, E.D., Marolda, C.L., Balachandran, A., Valvano, M.A., 2005. Defective O-antigen polymerization in tolA and pal mutants of *Escherichia coli* in response to extracytoplasmic stress. J. Bacteriol. 187, 3359–3368, http://dx.doi.org/10.1128/JB.187.10.3359-3368.2005.

WHO (1994) Requirements for Vi polysaccharide typhoid vaccine. In: WHO Technical Reports Series, No. 840: 14-33. Geneva, Switzerland. Available at: http://www.who.int/biologicals/publications/trs/areas/vaccines/typhoid/WHO_TRS_840_A1.pdf Accessed 15th January 2015.