Characterization of Generalized Modules for Membrane Antigens (GMMA) vaccine candidates against nontyphoidal *Salmonella*

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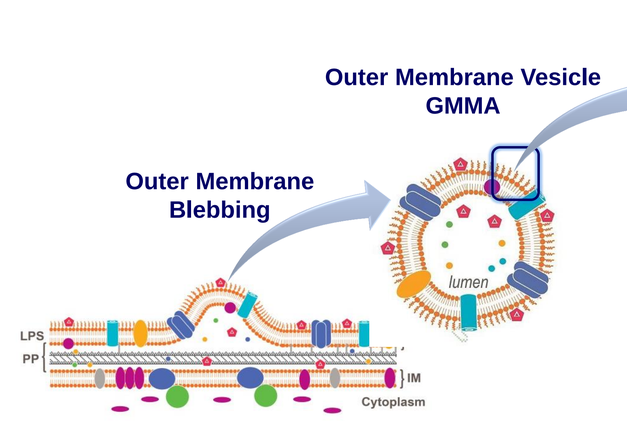
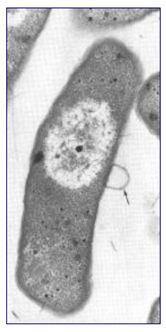
INTRODUCTION

- **Invasive nontyphoidal *Salmonella* disease (iNTS)** is a leading cause of bloodstream infections in Africa and no licensed vaccines are currently available. The most common pathogens are ***Salmonella* Typhimurium (STm)** and ***Salmonella* Enteritidis (SEn)**[1].
- **The O-antigen (OAg)** portion of **lipopolysaccharide (LPS**) is a **key antigen for protective immunity** and represents a target for the development of effective vaccines against iNTS[2].
- With the aim to develop a **bivalent OAg-based vaccine** against STm and SEn, **Generalized Modules for Membrane Antigens (GMMA)** were proposed as an attractive **OAg delivery system**[3].
- **Gram-negative bacteria** naturally shed **outer membrane vesicles (OMV)** which **imitate the bacterial surface**, maintaining structure, composition, and orientation of outer membrane components, including LPS and OAg[4].
- Wild type GMMA-producing strains were firstly genetically manipulated **to increase GMMA production (Δ*tolR*)** and further mutated **to reduce LPS reactogenicity (Δ*msbB*, Δ*htrB*, Δ*pagP*)**[5].
- Advantages of GMMA vaccines include **simplicity and low cost of manufacture**, **co-delivery of multiple antigens** in the context of a membrane and **bacterial "danger signals"** triggering innate immunity, obtaining a **self-adjuvanting activity**.
- **GMMA platform** has been **applied to different pathogens** (*Salmonella*, *Shigella*, Meningococcus) for the production of vaccines for low and middle income countries[6-7].



- Methods to determine **quality**, **consistency of production** and **stability** of OMV vaccines are of fundamental importance. It is important to characterize the **key antigens** displayed **on OMV surface** and presented to the immune system, and also **OMV as particles.**



STm and SEn GMMA-producing strains

Genetic manipulation

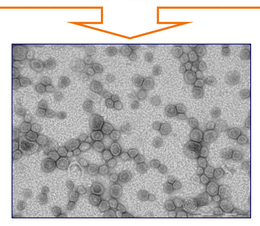
1. Increase GMMA production (Δ*tolR*)
2. Reduce lipid A toxicity and innate system over-stimulation (Δ*msbB*, Δ*htrB*, Δ*pagP*)

Fermentation

Purification

Micro-filtration 0.22 µm permeate collection

Ultra-filtration 300 kDa retentate collection



Purified GMMA

- In the context of **identification of the most suitable GMMA candidate** vaccines against iNTS, we have developed a **panel of analytical methods for LPS/OAg and GMMA characterization**[3].
- Our aim was to evaluate the **impact of genetic mutations** introduced into GMMA-producing strains **on OAg expression, structure and composition**, and the resulting impact on **GMMA immunogenicity**.
- **Particle size distribution** of GMMA from different mutants was evaluated by comparing three different methods: **Dynamic Light Scattering (DLS)**, **Multi-Angle Light Scattering (MALS) coupled with High Performance Liquid Chromatography - Size Exclusion Chromatography (HPLC-SEC)**, and **Nanoparticle Tracking Analysis (NTA)**[8].

References

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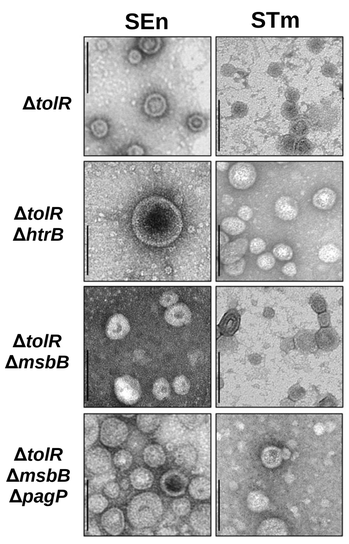
RESULTS

GMMA analytical characterization

- Analyses were performed **directly on STm and SEn GMMA** from different mutated strains and **on their OAg**.
- OAg extracted[9] from GMMA were compared to OAg produced by corresponding **wild type strains**.



Analyses on extracted OAg

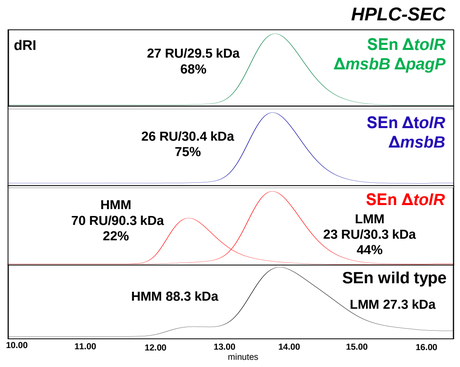
Test category	Analytical method
OAg size distribution	HPLC-SEC
Relative % of OAg with different chain length	HPLC-SEC/Semicarbazide Phenol sulfuric acid assay
OAg sugar composition, glycosidic linkages and absolute configuration	GLC/GLC-MS HPAEC-PAD
OAg identity *O*-acetylation pattern and level	NMR GLC

Analyses on GMMA

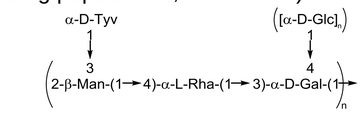
Test category	Analytical method
Purity	HPLC-SEC
Protein content	micro BCA SDS-PAGE
OAg content/ identification	HPAEC-PAD
Lipid A content/ identification	HPLC-SEC/Semicarbazide MALDI-TOF MS
Particle size distribution/ aggregation	DLS HPLC-SEC/MALS NTA

OAg size distribution

HPLC-SEC



- **Δ*tolR* mutation impact on OAg size distribution** (High/low molecular mass OAg populations, HMM/LMM).



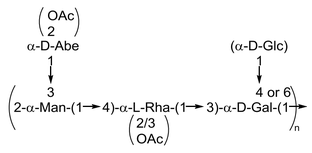
***S.* Enteritidis OAg repeating unit**

OAg sugar composition

HPAEC-PAD

OAg	Tyv	Rha	Man	Gal	Glc
SEn wild type	1.00	1.00	1.00	1.08	0.19
SEn Δ*tolR* (HMM OAg)	0.99	1.00	1.04	1.05	0.12
SEn Δ*tolR* (LMM OAg)	0.93	1.00	0.99	1.07	0.11
SEn Δ*tolR* Δ*msbB*	0.97	1.00	1.00	1.05	0.09
SEn Δ*tolR* Δ*msbB* Δ*pagP*	0.97	1.00	0.99	1.06	0.08

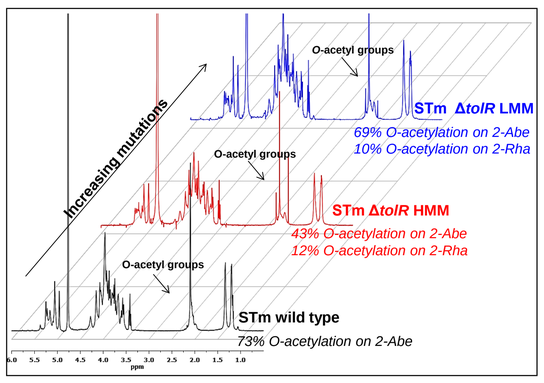
- **Similar sugar composition** of OAg populations from all GMMA, in agreement with OAg from wild type strain.



***S.* Typhimurium OAg repeating unit**

OAg identity and O-acetylation degree

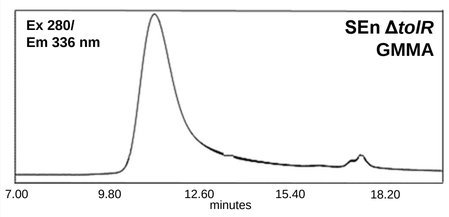
^{1}H NMR



- **No major impact** of mutations introduced **on *O*-acetylation levels**. **Additional *O*-acetyl groups** in OAg populations extracted from STm Δ*tolR* GMMA (not only on 2-Abe but also on 2-Rha).

GMMA purity

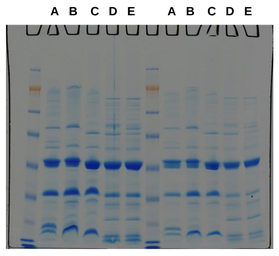
HPLC-SEC



- Low % of free soluble proteins and nucleic acid contaminants (<5%), indicating **GMMA good purity**.

GMMA protein content

SDS-PAGE



Protein profile of GMMA from SEn Δ*tolR* (A), SEn Δ*tolR* Δ*msbB* Δ*pagP* (B), STm Δ*tolR* (C), STm Δ*tolR* Δ*htrB* (D) and STm Δ*tolR* Δ*msbB* (E) strains.

- **No major changes in the protein pattern** of GMMA produced by lipid A mutated strains, compared to GMMA with no lipid A modification (Δ*tolR*).

GMMA OAg and lipid A content

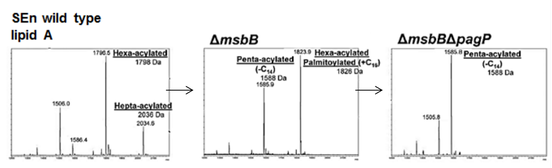
Micro BCA/HPAEC-PAD/HPLC-SEC

GMMA	w/w OAg/ GMMA protein ratio	Molar % OAg chains/ total LPS	nmol lipid A/ mg GMMA protein
SEn Δ*tolR*	0.6	14	156.6
SEn Δ*tolR* Δ*msbB*	1.7	22	240.1
SEn Δ*tolR* Δ*msbB* Δ*pagP*	1.5	12	528.0
STm Δ*tolR*	0.7	10	172.8
STm Δ*tolR* Δ*msbB*	0.03	<1	108.2
STm Δ*tolR* Δ*htrB*	0.02	0.45	154.8

- **Impact on OAg production** after lipid A detoxification mutations introduced in some strains.
- Large portion of **LPS molecules** containing **just core**.

GMMA Lipid A structure

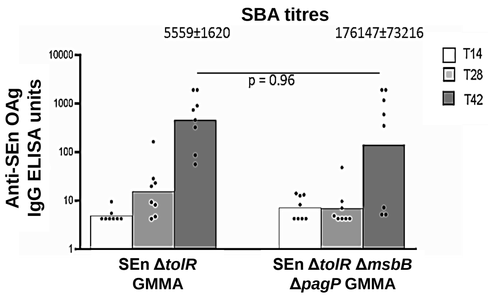
MALDI-TOF MS



- Combination of **Δ*msbB*-Δ*pagP* mutations** as **optimal approach to minimize STm and SEn GMMA reactogenicity**, resulting in **pure penta-acylated lipid A**.

GMMA immunogenicity

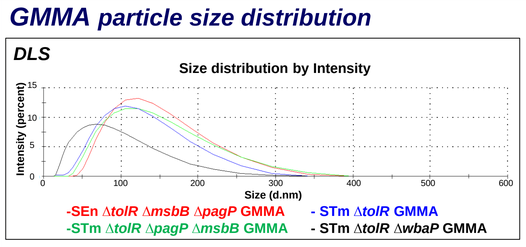
ELISA/SBA

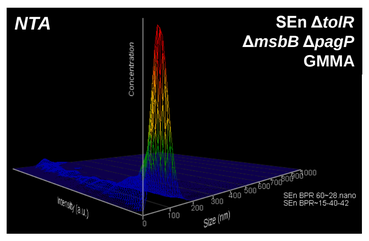


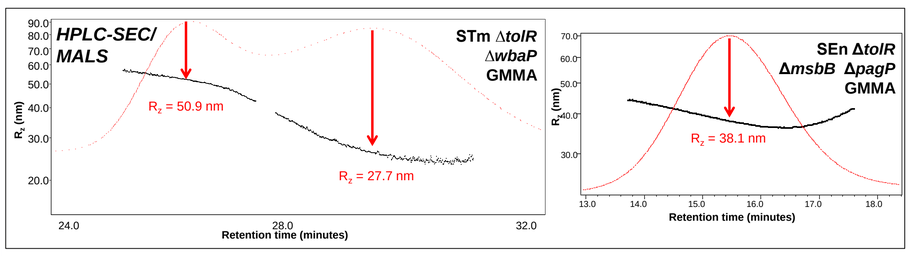
- CD1 mice immunised at days 0 and 28.
- 1 µg OAg dose + Alhydrogel (0.7 mg/mL Al^{3+}).
- Anti-OAg IgG geometric means and individual Abs levels induced.
- SBA results of pooled sera at day 42.

- **High levels of serotype-specific anti-OAg-specific IgG functional antibodies** induced by all GMMA, with no significant differences **despite variations in OAg density and chain length**, when tested in mice **at the same OAg dose**.
- Anti-OAg IgG response **booster effect** shown by all GMMA.

GMMA particle size distribution







Correlation between GMMA size and their main features

GMMA	OAg presence	Lipid A structure	DLS Z-average (d) nm	HPLC-SEC/MALS 2 x R_z nm	NTA Mode/ Mean (d) nm	OAg chains/ GMMA	OAg size (kDa)	ζ-potential (mV)
SEn Δ*tolR* Δ*msbB* Δ*pagP*	OAg$^+$	Penta	111.1	76.2	106.0/91.9	2812	30.0	-3.2
STm Δ*tolR* Δ*pagP* Δ*msbB*	OAg$^+$	Penta	103.5	72.2	102.5/78.6	2204	34.6	-3.3
STm Δ*tolR*	OAg$^+$	Hepta/hexa	91.5	73.8	95.8/85.6	768	32.9	-2.7
STm Δ*tolR* Δ*wbaP*	OAg$^-$	Hepta/hexa	57.6	101.8 (Peak 1) 55.4 (Peak 2)	90.1/62.6	/	/	-9.8

- **Strengths and weaknesses** shown by each methodology, providing **complementary information** and allowing a **more complete evaluation of GMMA size**.
- SEC-MALS and NTA able to better **distinguish populations of different size** and preferable to DLS for analysis of **bimodal samples**.
- **Real time visualization with simultaneously tracking and counting of individual particles** by NTA, but results deeply dependent on the choice of **data analysis parameters**.
- **Higher DLS Z-average diameters compared to MALS size for OAg-positive GMMA. Hydrodynamic diameter increases with the number of OAg chains per particle.**

CONCLUSIONS

- **A comprehensive panel of analytical methods has been assembled for GMMA characterization with particular attention to their surface OAg, which is key target of protective antibody response.**
- **Such methods are of fundamental importance in the process of vaccine development, to ensure consistency of production and to monitor stability of GMMA over time.**
- **Mutations introduced in GMMA producing strains to enhance blebbing and reduce LPS toxicity can impact OAg expression levels and structural characteristics. Careful characterization is needed to identify optimal GMMA candidates for inclusion in a vaccine against iNTS.**
- **Simplicity of manufacturing process, coupled with encouraging immunogenicity data, make the GMMA approach particularly attractive for the development of a vaccine against iNTS.**

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