High Yield Production Process for *Shigella* Outer Membrane Particles

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**Abstract**
Gram-negative bacteria naturally shed particles that consist of outer membrane lipids, outer membrane proteins, and soluble periplasmic components. These particles have been proposed for use as vaccines but the yield has been problematic. We developed a high yielding production process of genetically derived outer membrane particles from the human pathogen *Shigella sonnei*. Yields of approximately 100 milligrams of membrane-associated proteins per liter of fermentation were obtained from cultures of *S. sonnei* ΔtolR ΔgalU at optical densities of 30–45 in a 5 L fermenter. Proteomic analysis of the purified particles showed the preparation to primarily contain predicted outer membrane and periplasmic proteins. These were highly immunogenic in mice. The production of these outer membrane particles from high density cultivation of bacteria supports the feasibility of scaling up this approach as an affordable manufacturing process. Furthermore, we demonstrate the feasibility of using this process with other genetic manipulations e.g. abolition of O antigen synthesis and modification of the lipopolysaccharide structure in order to modify the immunogenicity or reactogenicity of the particles. This work provides the basis for a large scale manufacturing process of Generalized Modules of Membrane Antigens (GMMA) for production of vaccines from Gram-negative bacteria.

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**Introduction**

*Shigella* spp. are Gram-negative bacteria that infect the intestinal epithelium and cause dysentery. In 1999 the World Health Organization estimated an annual burden of 164.7 million shigellosis cases throughout the year of which 163.2 million occur in developing countries, including 1.1 million deaths, mostly in children younger than 5 years of age [1]. Four serogroups have been identified: *S. dysenteriae* (15 serotypes), *S. boydii* (20 serotypes), *S. flexneri* (14 serotypes) and *S. sonnei* (1 serotype) [2]. No vaccine is currently available. So far, vaccine candidates based on O antigen synthesis and modification of the lipopolysaccharide structure in order to modify the immunogenicity or reactogenicity of the particles. This work provides the basis for a large scale manufacturing process of Generalized Modules of Membrane Antigens (GMMA) for production of vaccines from Gram-negative bacteria.

Thus, the orientation of components in the membrane of the outer membrane particles is the same as in the bacterial outer membrane and the components in the outer face of the bacterial outer membrane are also in the outer face of the outer membrane particles [7]. Outer membrane particles are naturally shed at low concentration. Mutations such as the deletion of gene *gna33* in *Neisseria meningitidis* [10] or modifications of the tol-pal pathway of *Escherichia coli* could potentially alter the membrane composition of the outer membrane vesicles derived from homogenized bacteria they are almost free of cytoplasmic and inner membrane components and maintain lipoproteins. The outer membrane particles used for these proteomic studies have been derived in small quantities from cells grown to low cell density.
It has been previously proposed that outer membrane particles could be exploited for use as vaccines [10,12]. The immunogenicity of outer membrane particles from a variety of Gram-negative bacteria has been studied. Consistent with their high content of stimulators of the innate immune system, e.g. lipopolysaccharide (LPS) [7] and Toll-like receptor 2 (TLR2) agonists [14], they are strongly immunogenic in the absence of adjuvant. They have been shown to induce protection in mice against multiple pathogens, including Salmonella enterica serovar Typhimurium [15], Helicobacter pylori [16], Vibrio cholerae [17,18], or to elicit antibodies in mice with in vitro bacterial activity, e.g. for Neisseria meningitidis [19]. Recently, outer membrane particles from Shigella flexneri 2a have been shown to confer protection in mice after mucosal immunization [20]. Although these studies suggest that outer membrane particles may form the basis of vaccines [15,17,18], there remain several problems: their reactogenicity and the difficulty of purifying them in the quantity and at costs that would make them attractive as vaccines for the public sector most impacted by diseases such as shigellosis.

The problem of reactogenicity is amenable to genetic manipulation. A variety of strategies has been examined to attenuate the pyrogenicity of LPS by modifying genes involved in lipid A biosynthesis, e.g. msbB and htrB in Shigella and E. coli or fyuA in Neisseria that are required for complete acylation and thereby pyrogenicity of lipid A [21–24]. However, a major remaining difficulty is developing a scalable method for the high volume and low unit cost production of vaccines based on this method.

In this paper we show that high purity outer membrane particles from Shigella sonnei mutant strains can be produced from fermentation in chemically defined medium with high yield using a simple purification process thus making production of inexpensive vaccines feasible. We believe that this process will be widely applicable for production of Gram-negative membrane antigens and thus call it the ‘Generalized Modules for Membrane Antigens (GMMA)’ process. In the literature, outer membrane particles that are either naturally released or produced by genetically modified strains are usually referred to as outer membrane vesicles (OMV). The same term has also been used for the vesicles derived by detergent-extraction of homogenized bacteria currently used as vaccines, e.g. MenZB, an outer membrane vesicle vaccine used to control Neisseria meningitidis type B infections in New Zealand. In order to differentiate the two substantially different types of OMV [10] we chose the term GMMA to specify the particles released from the surface of intact cells used in this study.

**Methods**

**Construction of Shigella Sonnei 53G Mutants**

Shigella sonnei 53G [25] was chosen as parent strain. The null mutants tolR [13], galU [26], and msbB1 [21] were obtained by replacing the gene coding sequence with a resistance cassette [27]. Kanamycin was used for tolR, chloramphenicol for galU and erythromycin for msbB1. To achieve this, we used a three step PCR protocol to fuse the gene upstream and downstream regions to the resistance gene. Briefly, the upstream and downstream regions of the gene were amplified from Shigella sonnei 53G genomic DNA with the primer pairs gene.AB.500-5/gene.ABL.3 and gene.ABL.5/gene.AB.500-3, respectively (details of target ‘gene’, antibiotic cassette ‘AB’ and sequence are reported in Table 1). The kanamycin cassette was amplified from pUC4K [28] and the cat gene from pKOBEG [29] using the primers ampli.AB.5/ampli.AB.3 (Table 1). Finally the three amplified fragments were fused together by mixing 100 ng of each in a PCR reaction containing the gene.AB.500-5/gene.AB.500-3 primers. The linear fragment to delete tolR was used to transform recombination-prone Shigella sonnei 53G carrying pMD134 to obtain the respective deletion mutant S. sonnei ΔtolR. Recombination-prone S. sonnei ΔtolR was then transformed with the linear fragment for the deletion of galU, resulting in mutant strain S. sonnei ΔgalU. A clone of S. sonnei ΔtolR lacking the virulence plasmid, S. sonnei –pSS ΔtolR, was selected by white appearance on congo red agar. The curing of the virulence plasmid (pSS) was confirmed by the absence of the origin of replication and the plasmid encoded gene wzy when using primers pSS.503G.wzyF/pSS.503G.wzyR and pSS.503G.wzyF/pSS.503G.wzyR respectively (Table 1). Two functional msbB genes are present in Shigella [21]. In the ΔtolR background, the copy located on the virulence plasmid (msbB2) was removed by curing the plasmid and the plasmid pΔmsbBkoccery was constructed to delete the gene msbB1 on the chromosome. Upstream and downstream flanking regions of the msbB1 gene were amplified by PCR with the XhoI.msbB5’/EcoRV.msbB5’ .R and EcoRV.msbB3’/XhoI.msbB3’ .R primers, respectively. Both products were cloned into the pBluescript (Stratagegen) vector in Max Efficiency® E. coli DH15™-T1R (Invitrogen). The erm erythromycin resistance gene [30] was amplified with primers EcoRV.Ery.F/EcoRV.Ery.R and was inserted into the EcoRV site between the flanking regions generating pΔmsbBkoccery. Primers XhoI.msbB5’/XhoI.msbB3’ .R were used to amplify by PCR a linear fragment from pΔmsbBkoccery plasmid, containing the resistance cassette flanked by msbB1 flanking regions that was used to transform the recombination-prone plasmid-cured Shigella sonnei 53G ΔtolR strain to generate the msbB knockout mutant. Recombination-prone

| Table 1. Primers used in this study. |
|-------------------------------------|
| tolR.Kan.500-5 TCTGGAATCGAATTCTTCG  |
| tolR.Kan.L3 ATTTTGGAGAAGAGGTTCCCTTCTGTTGTTG  |
| tolR.Kan.L5 TTCAAGAGAGGAGCTCCCTTAAACATCGTGTTTCTCGG  |
| tolR.Kan.300-5 TTGCCTCTGTTAATCTCCG  |
| ampli.Kan.5- ATGGACCCTTATCAACGGGGAAAC  |
| ampli.Kan-3 TTAGAAAACACTCATGACCATAAA  |
| galU.Cm.500-5 AAAATCAACGGTGTCGAGAG  |
| galU.Cm.5-3 CGAAATGATCCTTCTGACATACATATAATTCTTCGAGCTTCC  |
| galU.Cm.L-3 CGCTGTGATCGTATCTTGATATTG  |
| galU.Cm.ext-5 GCTGGTGCTGTATATCGCGGCG  |
| galU.Cm.Cm-5 GCGCAGACGAGAAGAGATAA  |
| galU.Cm.Cm-5 CGGGACCAATATAACGCTTGA  |
| XbaI.msbB5’ .F CTAGTCTAGAAGTGCTTTCAGTGGGTGACG  |
| EcoRV.msbB5’ .R AGCTTGATATCAGAGTGTGTTGATAGTGCAGTATC  |
| EcoRV.msbB3’ .F AGCTTGATATCGGCAATTCAACCGTATAAG  |
| XbaI.msbB3’ .R CCCGCACAGGGGGAGAAGGTGGTAAACAGACAG  |
| EcoRV.Ery.F AGCTTGATATCACTGAGGTGTGATAGTGCAGTATC  |
| EcoRV.Ery.R AGCTTGATATCACTTCTGACCTTCTGGAAAGCT  |
| pSso.503G.oriF CGTACCTAACATTAACGCGG  |
| pSso.503G.oriR GATTCCACATCTCCACCC  |
| pSso.503G.wzyF CGTTAGGTTTCCAGGTTCCT  |
| pSso.503G.wzyR TTACATTATATCCCTCGGCA  |

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Shigella sonnei 53G cells were produced by using the highly proficient homologous recombination system as previously described [31] encoded on pAJD434 [32]. pAJD434 was subsequently removed from the mutant strains.

Bacterial Strain Growth Conditions and Media

Shigella sonnei and E. coli strains were routinely cultured in Luria-Bertani (LB) medium. When required, kanamycin (30 μg/mL), chloramphenicol (20 μg/mL), trimethoprim (100 μg/mL), or ampicillin (100 μg/mL) were added. Tryptic soy agar (30 g/L tryptic soy broth, 15 g/L agar) supplemented with 150 mg/L tryptone and 2 g/L yeast extract was used to prepare the medium for the growth of S. sonnei tolR mutant strains. E. coli strains were routinely cultured in tryptic soy broth, 15 g/L agar) supplemented with 150 mg/L tryptone and 2 g/L yeast extract.

GMMA was collected and sterile filtered using a 0.22 μm pore size cassette (Sartocon HYDROSART, Sartorius) in order to separate GMMA that remain in the retentate from soluble proteins (filtrate). After five diafiltration steps using PBS, the retentate containing the GMMA was collected and sterile filtered using a 0.22 μm Express™ PLUS stericup (Millipore).

Protein Quantification

Proteins were quantified by Bradford method, using bovine serum albumin as standard. GMMA were boiled for 10 minutes in 3.0 M guanidine hydrochloride prior quantification.

Negative Staining Electron Microscopy

A drop of 5 μL of GMMA suspension was placed on copper formvar/carbon-coated grids and adsorbed for 5 min. Grids were then washed with few drops of distilled water and blotted with a Whatman filter paper. For negative staining, grids were treated with 2% uranyl acetate in ddH2O for 1 min, air-dried and viewed with a CM100 transmission electron microscope (Philips, Eindhoven, the Netherlands) operating at 80 kV. Electron micrographs were recorded at a nominal magnification of 60000×.

Denaturing Mono-Dimensional Electrophoresis

GMMA were denatured for 3 min at 95°C in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 2% (wt/vol) SDS. 20 μg of proteins were loaded onto 12% (wt/vol) or 4–12% (wt/vol) polyacrylamide gels (BioRad, Hercules, U.S.A.). Gels were run in 3-(N-morpholino)-propanesulfonic acid (MOPS) buffer (BioRad) and were stained with Coomassie Blue R-250.

Two-Dimensional Electrophoresis

Two hundred micrograms of GMMA were separated by 2-dimensional electrophoresis (2-DE) as previously described [10]. Briefly, proteins were separated in the first dimension on a non linear pH 3–11 gradient and in the second dimension on a linear 4–12% polyacrylamide gradient unless specified in text. Gels were stained with colloidal Coomassie G-250 [33].

Densitometry Analysis

SDS-PAGE and 2-DE gels were scanned with an Image Quant 400 (GE Healthcare). Images were analyzed with the software Image master 2D Platinum 6.0 (Amersham Biosciences).

In-Gel Protein Digestion and MALDI-TOF Analysis

Protein spots were excised from the gels and processed as previously described [13]. Mass spectra were acquired on a Ultraflex MALDI-TOF-TOF mass spectrometer (Bruker Dal-
tonics) in reflectron, positive mode, in the mass range of 900 to 3,500 Da. Spectra were externally calibrated by using a combination of standards pre-spotted on the target (Bruker Daltonics). MS spectra were analyzed by Protein Mass Fingerprint (PMF) with flexAnalysis (flexAnalysis version 2.4, Bruker Daltonics). Monoisotopic peaks were annotated with flexAnalysis default parameters and manually revised. Protein identification was carried from the generated peak list using the Mascot program (Mascot server version 2.2.01, Matrix Science). Mascot was run on a database containing protein sequences deduced from seven sequenced Shigella genomes, downloaded from NCBI or from the Wellcome Trust Sanger Institute database. Genomes used were from strains Shigella sonnei 53G, Shigella flexneri 2a str. 301, Shigella flexneri 2a str. 245T7, Shigella sonnei Ss046, Shigella boydii Sb227, Shigella flexneri 5 str. 8401, Shigella boydii CDC 3083-94. Search parameters, mass tolerance, known contaminant ions, validation and handling of multiple matches were performed as described previously [13].

Protein Precipitation and In-solution Protein Digestion

Proteins from supernatants or purified GMMA were precipitated by adding TCA and deoxycholate to a final concentration of 10% and 0.04%, respectively. The precipitation was allowed to proceed for 30 min at 4°C. The precipitate was recovered by 10 min centrifugation at 20,000 x g at 4°C. The pellet was washed once with 10% TCA (wt/vol) and twice with absolute ethanol, dried with Speedvac (Labconco, Kansas City, U.S.A). For analysis by SDS-PAGE, the precipitates were resuspended with 200 mM Tris-HCl, pH 8.8, and quantified. For LC-MS/MS analysis 20 μg of GMMA were precipitated and resuspended in 50 μL, 6 M guanidinium chloride, 5 mM DTT, 200 mM Tris-HCl, pH 8.0. Denaturation proceeded for 60 min at 60°C. Prior to digestion, the solution was diluted 1:3 with a solution of 100 mM Tris-HCl, pH 8.0, 5 mM DTT and 5 μg of trypsin (Promega) were added into the diluted solution. Digestion was carried out overnight at 37°C. The reaction was stopped by adding formic acid to 0.1%. Peptides were extracted using Oasis extraction cartridges (HLB 1cc (30 mg) extraction cartridges, Waters, Milford, MA, USA) and analyzed by LC-MS/MS.

Protein Identification by Nano-LC-MS/MS

Peptides were separated by nano-LC on a NanoAcquity UPLC system (Waters) connected to a Q-ToF Premier ESI mass spectrometer equipped with a nanospray source (Waters). Samples were loaded onto a NanoAcquity 1.7 μm BEH130 C18 column (75 μm x 25 mm; Waters) through a NanoAcquity 5 μm Symmetry C18 trap column (180 μm x 20 mm; Waters). Peptides were eluted with a 120 min gradient of 2-40% acetonitrile (98%), 0.1% formic acid solution at a flow rate of 250 nL/min. The eluted peptides were subjected to an automated data-dependent acquisition using the MassLynx software, version 4.1 (Waters) where an MS survey scan was used to automatically select multicharged peptides over the m/z ratio range of 300–2000 for further MS/MS fragmentation. Up to eight different peptides were individually subjected to MS/MS fragmentation following each MS survey scan. After data acquisition, individual MS/MS spectra were combined, smoothed, and centroided using ProteinLynx, version 3.5 (Waters) to obtain the peak list file. The Mascot Daemon application (Matrix Science Ltd., London, UK) was used for the automatic submission of data files to in-house licensed Mascot, version 2.2.1, running on a local server. The Mascot search parameters were set to (i) 2 as the number of allowed missed cleavages (only for trypsin digestion), (ii) methionine oxidation as variable modifications, (iii) 0.05 Da as the peptide tolerance, and (iv) 0.05 Da as the MS/MS tolerance. Only significant hits were considered as defined by the Mascot scoring and probability system.

Bioinformatics

Prediction of protein localization was carried out using PSORTb v3.0 [34] and Lipo program [35].

Mouse Immunizations

Outbred CD1 mice (female, 4 to 6 weeks of age) received three injections of GMMA via the subcutaneous route on days 0, 21, and 35. Each injection contained GMMA normalized to 0.2 μg or 2 μg of protein and formulated in PBS only, with Freund's adjuvant (FA), or adsorbed onto aluminum hydroxide (alum), 2 mg/mL, in a final volume of 100 μL. If Freund's adjuvant was used, Freund's complete adjuvant (FCA) was used for the first immunization, Freund's incomplete adjuvant (ICFA) was used for the second and third immunization. Control mice received either adjuvant or PBS alone. Blood samples were collected before immunization and 14 days after the second and third injection. The animal experiments complied with the relevant guidelines of Italy and the institutional policies of Novartis. The animal protocol was approved by the Animal Welfare Body of Novartis Vaccines and Diagnostics, Siena, Italy, approval number AEC 2009-05.

Western Blot

GMMA were boiled in loading buffer and loaded on 12% (wt/vol) polyacrylamide-SDS gels (BioRad) or on 2D gels as described. Gels were run in MOPS buffer (BioRad) and protein were subsequently transferred onto nitrocellulose membrane using Trans-blot transfer medium (BioRad). The membranes were blocked in PBS containing 3% (wt/vol) powdered milk, then incubated with mouse polyclonal antisera diluted (1:1000) in PBS containing 3% (wt/vol) milk for 90 min at 37°C. Membranes were washed three times with PBS containing Tween 20, 0.1% (vol/vol) milk and then incubated with sheep anti-mouse horseradish peroxidase-conjugated IgG (GE Healthcare, UK Limited), diluted (1:7500) in PBS containing 3% (wt/vol) milk. Colorimetric staining was performed, after washing the membranes, with SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce, Rockford, U.S.A.) as described by the manufacturer. Positive signals were related to the corresponding proteins by comparing the Western blot membrane to the gel using Ponceau staining of the membrane as a reference and aligning the images with Image master 2D Platinum 6.0.

Enzyme-linked Immunosorbent Assay (ELISA)

To measure Shigella sonnei GMMA-specific immunoglobulin G (IgG) in mice serum, Nunc Maxisorp 96-well plates were coated overnight at 2 to 8°C with 100 μL/well of a 0.5 μg/mL suspension of Shigella sonnei 53G –pSS ΔωR GMMA, purified from defined medium with 2 μM ferric citrate in the same way as the GMMA in the vaccine, diluted in phosphate-buffered saline (PBS). Plates were then washed three times with 300 μL/well of phosphate-buffered saline containing 0.05% (vol/vol) Tween 20 (PBST) and blocked with PBS containing 1% (wt/vol) BSA for 60 min at 37°C. Serial dilutions of reference and sample sera were prepared in PBST, 1% (wt/vol) BSA in a separate dilution plate, and 100 μL/well of each serial dilution was transferred to the coated plate, incubated for 2 hours at 37°C, and then washed as described above. Bound antibody was detected using a goat anti-mouse IgG conjugated to alkaline phosphatase, diluted in PBST,


| A | B | C | D | E |
|---|---|---|---|---|
| **Outer membrane** | | | | |
| 1 | 3 | outer membrane channel protein [S. flexneri 2a str. 301] | tolC | gi|56480244 |
| 2 | 3 | outer membrane porin protein C [S. sonnei Ss046] | ompC | gi|74312736 |
| 3 | 3 | outer membrane protein A [S. sonnei Ss046] | ompA | gi|74311514 |
| 4 | 3 | outer membrane protein induced after carbon starvation [S. flexneri 5 str. 8401] | sip | gi|110616891 |
| 5 | 3 | outer membrane protein X [S. flexneri 2a str. 301] | ompX | gi|56479734 |
| 6 | 2 | outer membrane protein assembly factor YaeT [S. Flexneri 2a str. 301] | yaeT | gi|24111612 |
| 7 | 2 | outer membrane protein C [S. boydii CDC 3083-94] | ompC | gi|187733369 |
| 8 | 2 | outer membrane receptor FepA [Shigella sonnei Ss046] | fepA | gi|74311118 |
| 9 | 2 | ferrichrome outer membrane transporter [Shigella sonnei Ss046] | fhuA | gi|74310771 |
| 10 | 2 | colicin I receptor [Shigella sonnei Ss046] | cirA | gi|74312677 |
| 11 | 2 | maltoporin [Shigella flexneri 2a str. 301] | lamB | gi|56480532 |
| 12 | 4 | putative ferric siderophore receptor [S. sonnei Ss046] | iutA | gi|74313972 |
| 13 | 2 | outer membrane protein W [Shigella sonnei Ss046] | yciD | gi|74312394 |
| 14 | 2 | serine protease [S. flexneri 2a str. 301] | sigA | gi|24114232 |
| **Outer membrane Lipoproteins** | | | | |
| 15 | 3 | murein lipoprotein [S. flexneri 2a str. 301] | lpp | gi|24113066 |
| 16 | 2 | outer membrane lipoprotein LolB [S. flexneri 2a str. 301] | lolB | gi|24112608 |
| 17 | 3 | peptidoglycan-associated outer membrane lipoprotein [S. flexneri 2a str. 301] | pal | gi|56479690 |
| 18 | 1 | entericidin B membrane lipoprotein [S. flexneri 2a str. 301] | ecnB | gi|24115506 |
| 19 | 1 | hypothetical protein S2067 [S. flexneri 2a str. 2457T] | yedD | gi|30063370 |
| 20 | 1 | hypothetical protein S4565 [S. flexneri 2a str. 2457T] | yjeI | gi|30065519 |
| 21 | 1 | hypothetical protein SF0398 [S. flexneri 2a str. 301] | ybaY | gi|24111837 |
| 22 | 1 | RpoE-regulated lipoprotein [S. flexneri 2a str. 301] | SF2485 | gi|24113773 |
| 23 | 1 | hypothetical protein SSON_2966 [S. Sonnei Ss046] | SSON_2966 | gi|74313380 |
| 24 | 1 | lipoprotein [S. flexneri 2a str. 2457T] | nlpB | gi|30063856 |
| 25 | 2 | entry exclusion protein 2 [S. sonnei Ss046] | exc | gi|145294038 |
| 26 | 2 | LPS-assembly lipoprotein RpiB [S. dysenteriae Sd197] | rpiB | gi|82775909 |
| 27 | 2 | putative pectinesterase [S. sonnei Ss046] | ybhC | gi|74313130 |
| 28 | 4 | outer membrane protein assembly complex subunit YIO [Shigella sonnei Ss046] | SSON_2721 | gi|74313154 |
| 29 | 3 | outer membrane lipoprotein [S. flexneri 2a str. 301] | yraP | gi|24114441 |
| 30 | 3 | DNA-binding transcriptional activator OsmE [S. flexneri 2a str. 301] | osmE | gi|24112862 |
| 31 | 1 | outer membrane protein [S. flexneri 2a str. 301] | slyB | gi|24113033 |
| **Periplasm** | | | | |
| 32 | 3 | FKBP-type peptidyl-prolyl cis-trans isomerase [S. Flexneri 2a str. 301] | fkpA | gi|24114611 |
| 33 | 3 | histidine-binding periplasmic protein of high-affinity histidine transport system [S. sonnei Ss046] | hisJ | gi|74312826 |
| 34 | 3 | serine endoprotease [S. flexneri 2a str. 301] | htrA | gi|24111599 |
| 35 | 3 | translocation protein TolB [S. flexneri 2a str. 2457T] | tolB | gi|30062097 |
| 36 | 1 | molybdate transporter periplasmic protein [S. flexneri 2a str. 301] | modA | gi|24111968 |
| 37 | 1 | peptidyl-prolyl cis-trans isomerase A (rotamase A) [S. flexneri 2a str. 301] | ppA | gi|24114628 |
| 38 | 1 | peptidyl-prolyl cis-trans isomerase SurA [S. flexneri 2a str. 301] | surA | gi|24111499 |
| 39 | 1 | periplasmic oligopeptide binding protein [S. flexneri 2a str. 2457T] | oppA | gi|30062764 |
| 40 | 1 | periplasmic protein [S. flexneri 2a str. 2457T] | osmY | gi|30065614 |
| 41 | 2 | arginine 3rd transport system periplasmic binding protein [S. sonnei Ss046] | artJ | gi|74311404 |
| 42 | 2 | bifunctional UDP-sugar hydrolase/S^'-nucleotidase [S. sonnei Ss046] | ushA | gi|74311061 |
| 43 | 2 | cysteine transporter subunit [S. sonnei Ss046] | fliY | gi|74311733 |
| 44 | 2 | glucan biosynthesis protein G [S. flexneri 5 str. 8401] | mdoG | gi|110805056 |
| 45 | 2 | thiosulfate transporter subunit [S. sonnei Ss046] | cyiP | gi|74312961 |
| 46 | 2 | hypothetical protein SBO_2040 [Shigella boydii Sb227] | ycdO | gi|825445054 |
1% (wt/vol) BSA to 1:5000 and incubated for 2 hours at 37°C. After a wash with PBS, 100 μL/well of 9-nitrophenyl phosphate substrate dissolved in diethanolamine buffer (1 M, pH 9.8) was added, and after 20 minutes optical densities were measured with an ELx800 (BioTek) plate reader at 405 and 490 nm wavelength.

Results

Statistical Analysis

Antibody levels (ELISA units) in different groups after the third immunization were compared by non-parametric Kruskal-Wallis and Mann-Whitney tests. A p value of 0.05 was considered to be significant. For multiple comparisons the p value considered to be significant in each of the comparisons was adjusted according to the number of analyses.

Results

Generation of a Shigella Sonnei 53G Strain Capable of Overproducing Modified GMMA

The first aim of the study was to investigate if Shigella sonnei 53G could be developed as a strain suitable to overproduce GMMA through modification of the Tol-Pal system. A null mutation of the tolR gene was introduced as this has previously been demonstrated to result in overproduction of GMMA in E. coli [11,13]. The mutation in the tolR gene led to the release of large amounts of GMMA from the surface of S. sonnei 53G as assessed by SDS page (Fig. 1A). The deletion of tolR had no detectable influence on bacterial growth (data not shown). In addition, to test if GMMA overproduction is also feasible in strains with additional genetic modifications we removed the O antigen of the LPS, either by deletion of galU as the biosynthesis genes for the O antigen in Shigella are encoded on the plasmid [36]. GMMA obtained from S. sonnei ΔtolR ΔgalU showed a similar protein profile to GMMA from S. sonnei 53G ΔtolR ΔgalU as the biosynthesis genes for the O antigen in Shigella are encoded on the plasmid [36]. GMMA obtained from S. sonnei ΔtolR ΔgalU showed a similar protein profile to GMMA from S. sonnei ΔtolR ΔgalU with minor differences in the 37 kDa to 50 kDa range and proteins smaller than 30 kDa appeared to be less abundant in S. sonnei ΔtolR ΔgalU (Fig. 1A).

Also GMMA obtained from the plasmid-cured S. sonnei ΔtolR mutant (S. sonnei –pSS ΔtolR) showed a nearly identical protein pattern to GMMA from S. sonnei ΔtolR (Fig. 1A).

Furthermore, the genes mshB1 and mshB2 involved in lipopolysaccharide biosynthesis were deleted since these deletions have previously been reported to decrease LPS toxicity in Shigella [21]. As the gene mshB2 is encoded on the virulence plasmid and thus absent in S. sonnei –pSS ΔtolR we deleted the chromosomal gene mshB1 in this strain to generate a mutant strain lacking mshB1 and mshB2. For simplicity the ΔmshB1ΔmshB2 mutant is referred to as the ΔmshB mutant. The ΔmshB mutant was selected at 37°C on LB plates and grew in LB and yeast extract at 37°C with a duplication time of about 55 min compared to a duplication time of about 28 min for the single ΔtolR mutant. In the defined medium developed for fermentation, the plasmid-cured ΔtolR ΔmshB mutant strain (S. sonnei –pSS ΔtolR ΔmshB) was able to grow to high optical density (OD) at 30°C, but grew poorly at 37°C. Thus,
for generation of GMMA from *S. sonnei* ΔtolR ΔmshB cultivation in chemically defined medium at a growth temperature of 30°C was chosen. GMMA from *S. sonnei* ΔpSS ΔtolR ΔmshB produced under these conditions showed a similar protein pattern to GMMA generated by *S. sonnei* ΔpSS ΔtolR and *S. sonnei* ΔtolR with only minor variation in relative amounts of proteins visible by SDS-PAGE in the 45–75 kDa range (Fig. 1A). In order to test if the lower temperature would change the GMMA composition we compared GMMA derived from *S. sonnei* ΔpSS ΔtolR at 30°C or 37°C. Only few differences were detected as highlighted in Fig. 1B, indicating that GMMA can be generated at 30°C without major effects on the composition. In conclusion, deletion of *tolR* greatly enhanced GMMA release while additional genetic modification of the strain or a change in growth temperature only had minor effects on the protein composition visible by SDS-PAGE.

**High Density Cultivation of Shigella Sonnei**

To investigate the feasibility to produce GMMA at large scale, *S. sonnei* 53G ΔtolR ΔgalU, *S. sonnei* 53G ΔpSS ΔtolR, and *S. sonnei* 53G ΔpSS ΔtolR ΔmshB were tested for their capacity to grow to high densities in a 5 liter reactor. Starter cultures were grown in flasks to OD 0.8 and were then transferred to the 5 L fermenter to reach a starting OD of 0.02. Dissolved oxygen was maintained at 30% saturation. The pH was maintained at 7.2 in HTMC or at 6.7 in SSDM and the temperature was kept constant either at 57°C or at 30°C when *S. sonnei* 53G ΔpSS ΔtolR ΔmshB was used. Under these conditions, cultures with optical densities of 45 to 80 were obtained.

Iron-regulated proteins have previously been shown to be important in vaccine formulations against *Pasteurella* and *Salmonella* [37,38]. Thus, we evaluated if the GMMA process would allow the upregulation of iron-regulated proteins. Growth of *S. sonnei* 53G – pSS ΔtolR with 0.2 μM iron concentration in chemically defined medium led to the induction of iron-regulated proteins but hindered high density cultivation of bacteria. The addition of 2 μM iron to the medium was sufficient to allow optimal growth and the induction of three iron-regulated proteins visible by SDS-PAGE (Fig. 1C), identified by protein mass fingerprint analysis of GMMA generated from *S. sonnei* ΔtolR ΔgalU grown in HTMC, [*P. multocida* (gi|74311118), *IntA* (gi|74313972) and Colicin I receptor (gi|74312677)]. The expression of these proteins was reduced when bacteria were grown in 200 μM iron (Fig. 1C). In bacteria grown in HTMC the iron-regulated proteins are expressed to a similar level as in chemically defined medium with 200 μM iron (data not shown). Their presence was confirmed by protein mass fingerprint analysis of GMMA generated from *S. sonnei* ΔtolR ΔgalU grown in HTMC (Table 2, proteins 9, 10, 12). Growth of *S. sonnei* 53G – pSS ΔtolR ΔmshB at 30°C in defined medium with 2 μM iron also enhanced expression of FepA and IntA, Colicin I receptor (marked in Fig. 1C) was less expressed than in GMMA from *S. sonnei* 53G ΔpSS ΔtolR prepared from cultures grown at 37°C (data not shown).

**Purification of GMMA from High Density Culture Supernatant**

So far, GMMA have always been purified from flask cultures by ultracentrifugation [13]. Cultures were centrifuged at low speed (4000 g) to separate biomass from supernatant which was subsequently filtered through a 0.22 μm filter. GMMA present in the supernatant were collected by ultracentrifugation, washed, and then resuspended and stored in PBS [10,13]. Since this technique is not suitable for large volumes we developed a scalable purification method to purify GMMA from high density cultures using tangential flow filtration (TFF). In TFF, also known as crossflow filtration, the feed stream is pumped tangentially across the surface of the membrane rather than into the filter as in conventional ‘dead-end’ filtration. A proportion of the soluble components and particles smaller than the membrane’s pores penetrates the filter (permeate). The remainder (retentate) is circulated back to the reservoir and over the filter again. In this way, the larger particles do not build up at the surface of the filter but are swept away by the tangential flow allowing smaller molecules to continuously reach and pass through the membrane. This feature makes TFF an efficient process for size separation, concentration and diafiltration.
GMMA were purified from fermentation cultures in a 2-step TFF process. In the first step, the culture supernatant that contains the GMMA was separated from the bacteria using a 0.2 μm filter. In this step, the bacteria remained in the retentate and GMMA transferred into the filtrate. In the second filtration step using a 0.1 μm filter, GMMA were separated from soluble components present in the culture supernatant, including proteins secreted by the bacteria or released by lysis. In this step, GMMA were retained by the filter and collected and concentrated in the retentate whereas soluble proteins passed through the filter. We tested this purification process under two slightly different conditions. Firstly, when the fermentation culture of *S. sonnei ΔtolR ΔgaiU* reached OD 45, the culture was transferred directly from the fermentor to the first TFF and the culture supernatant containing the GMMA was collected. The retained biomass was washed with 5 volumes of PBS buffer (dialfiltration) to recover remaining GMMA and the filtrate containing these GMMA was combined with the culture supernatant. In the slightly modified purification process tested, this dialfiltration step was omitted. Proteins were quantified by Bradford method. In the purification performed with dialfiltration of the biomass the total protein content of the TFF 0.2 μm filtrate was approximately 1.5 g/L of fermentation culture of which 15% was GMMA-associated as determined by separation of the soluble components from the high molecular weight portion (GMMA) via an ultracentrifugation step (Fig. 2 and Table 3). In the second TFF step, GMMA were concentrated in the retentate and washed with five volumes of PBS to remove remaining soluble proteins. As TFF is usually performed under non-sterile conditions, the final retentate was sterilized by filtration through a 0.22 μm filter. An aliquot of the sterilized retentate was subjected to ultracentrifugation to determine the content of GMMA as above. As shown in Fig. 2 most of the proteins in the retentate are GMMA-associated.

Table 3. Yield, purity, and recovery rate of GMMA by the high yield production process.

| Protein content [mg/L fermentation] | Fermentation A OD 45 | Fermentation B1 OD 30 | Fermentation B2 OD 39 |
|------------------------------------|----------------------|-----------------------|----------------------|
| **0.2 μm TFF permeate**            |                      |                       |                      |
| Total protein*                     | 1465                 | 1237                  | 797                  |
| GMMA-associated protein            | 214                  | 143                   | 138                  |
| Soluble protein                    | 1251                 | 1094                  | 659                  |
| **0.1 μm TFF retentate**           |                      |                       |                      |
| Total protein*                     | 108                  | 144                   | 118                  |
| GMMA-associated protein            | 120                  | 127                   | 114                  |
| Soluble protein                    | 14                   | 5                     | 3                    |
| GMMA-associated protein per OD     | 2.7 mg/L/OD          | 4.2 mg/L/OD           | 2.9 mg/L/OD          |
| **Purity of GMMA after 0.1 μm TFF [%]** |                      |                       |                      |
| GMMA (GMMA-protein/total protein) | 90                   | 88                    | 97                   |
| Soluble protein (sol. protein/total protein) | 10 | 3 | 3 |
| **Recovery of GMMA by 0.1 μm TFF [%]** |                      |                       |                      |
| GMMA-protein after 0.1 μm TFF/0.2 μm | 56 | 89 | 83 |

*Total protein amount calculated as sum of GMMA-associated protein and soluble protein.

**Total protein amount measured directly by Bradford assay.

*Shigella sonnei ΔtolR ΔgaiU was grown in HTMC in a 5 L fermenter to high densities of OD 45 (A), OD 30 (B1) and OD 39 (B2) and GMMA were purified using 2-step TFF. Purification from fermentation A was performed including 5 diafiltration steps of the biomass, for GMMA purification from fermentations B1 and B2 the biomass was not subjected to dialfiltration. The GMMA content in the permeate of the 0.2 TFF step (culture supernatant) and the retentate of the 0.1 μm TFF (purified GMMA) were determined by separation of GMMA from soluble protein by ultracentrifugation. Protein was quantified using Bradford assay. All samples were normalized to amount per liter fermentation broth. To compare the yields from different ODs, yields are also expressed as amount per liter fermentation per OD.

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40±20 nm of outer membrane particles produced by *E. coli tol-pal* mutants [11].

**Characterization of GMMA Protein Content**

GMMA purified by TFF from *S. sonnei* 53G ΔtolR ΔgalU grown in high density culture were characterized to confirm their integrity and to analyze their protein content. One- and two-dimensional SDS-PAGE of GMMA and densitometry analysis (Fig. 1D and Fig. 4) were used to determine the protein profile and to study relative protein quantities of the most abundant proteins. Most of the Coomassie blue-stained bands and spots were identified using peptide mass fingerprint (Table 2). OmpA and OmpC are known to be among the most abundant proteins present in the outer membrane. In fact, densitometry analysis of GMMA from *S. sonnei* ΔtolR ΔgalU grown in HTMC and analyzed by 1D SDS-PAGE indicated that OmpA and OmpC together contribute for 45% of the total protein; OmpX, 9%; Slp, 6%; YifO, 5.6%; TolB, 2.3%; TolC 1.4%; and YaeT, 1.8% (Fig. 1D). With the exception of the predicted periplasmic protein TolB, all of these proteins are predicted to be associated with the outer membrane. YifO is predicted to be an outer membrane lipoprotein. OmpA, OmpC, OmpX, Slp, TolC, and YaeT are predicted to be outer membrane proteins. Thus, the seven most abundant outer membrane-associated proteins account for approximately 69% of the protein amount in GMMA. Further densitometry analysis after 2D SDS-PAGE determined that there are approximately equal quantities of OmpA and OmpC (OmpA:OmpC is 1:0.83 by densitometry of a Coomassie blue-stained gel). In order to identify the diverse and less expressed proteins, GMMA were studied by proteolytic digestion and reverse
Visible bands were identified by protein mass fingerprint. OmpA and OmpC were quantified with Image master 2D Platinum 6.0.  

B) Sera from mice GMMA were separated in the first dimension on a non-linear pH 3–11 gradient, and in the second dimension on a 4–12% polyacrylamide gradient. Gel containing 20 μg of GMMA protein from S. sonnei ΔtolR ΔgalU was blotted and the membrane was incubated with sera from immunized mice with GMMA from S. sonnei ΔtolR ΔgalU in combination with Freund’s adjuvant. Several reactive proteins were identified. The numbers behind the names refer to the position of the proteins in Table 2. C) To verify that the signal observed in the 2D Western blot was due exclusively to antibody raised upon immunization with GMMA, 10 μg of GMMA were separated by 1D SDS-PAGE (12% PA) and stained with Coomassie (1) or transferred to a membrane. Western blots were developed using (2) sera raised against GMMA from S. sonnei ΔtolR ΔgalU as used for the 2D Western blot in B, (3) preimmune serum, (4) sera raised in mice immunized with Freund’s adjuvant or (5) PBS, or (6) secondary antibody only. A signal could only be observed when sera raised against GMMA were used (2).

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Figure 4. 2D gel electrophoresis of Shigella sonnei ΔtolR ΔgalU GMMA and immunoblot. A) 200 μg of proteins from S. sonnei ΔtolR ΔgalU GMMA were separated in the first dimension on a non-linear pH 3–11 gradient, and in the second dimension on a 4–12% polyacrylamide gradient. Visible bands were identified by protein mass fingerprint. OmpA and OmpC were quantified with Image master 2D Platinum 6.0. B) Sera from mice immunized with GMMA from S. sonnei ΔtolR ΔgalU were used to study the subset of proteins present in GMMA that are able to raise antibodies. A 2D gel containing 20 μg of GMMA protein from S. sonnei ΔtolR ΔgalU was blotted and the membrane was incubated with sera from immunized mice with GMMA from S. sonnei ΔtolR ΔgalU in combination with Freund’s adjuvant. Several reactive proteins were identified. The numbers behind the names refer to the position of the proteins in Table 2. C) To verify that the signal observed in the 2D Western blot was due exclusively to antibody raised upon immunization with GMMA, 10 μg of GMMA were separated by 1D SDS-PAGE (12% PA) and stained with Coomassie (1) or transferred to a membrane. Western blots were developed using (2) sera raised against GMMA from S. sonnei ΔtolR ΔgalU as used for the 2D Western blot in B, (3) preimmune serum, (4) sera raised in mice immunized with Freund’s adjuvant or (5) PBS, or (6) secondary antibody only. A signal could only be observed when sera raised against GMMA were used (2).
antiagonens, and to reduce the endotoxic activity [10,13,19,21,26,40,41]. GMMA could potentially be a safe, effective and low cost vaccine but need a practical way of manufacture at scale.

*Shigella sonnei* 53G was chosen for a first approach to develop a scalable process and a null mutation of the *tolR* gene was introduced to overproduce GMMA as previously described for *E. coli* [13]. To verify that the process is applicable to produce GMMA harboring modified lipid A, which would be more suitable for use as vaccine, and/or lacking the O antigen of the LPS we grew high density cultures of *S. sonnei* ΔtolR ΔgalU, *S. sonnei*–pSS ΔtolR (cured of the virulence plasmid pSS), and *S. sonnei*–pSS ΔtolR ΔmsbB in a 5 L fermenter in complex (HTMC) or chemically defined medium. Chemically defined medium was used to avoid contamination from proteins present in complex media and to have the possibility to regulate iron concentration.

Bacteria were removed from the culture supernatant by a tangential flow filtration step using a 0.2 μm membrane. A second tangential flow filtration step with a 0.1 μm membrane was used to concentrate GMMA and to remove soluble proteins. This choice of appropriate molecular weight membranes allowed the purification of GMMA in an easy, efficient, and scalable process. After purification, approximately 90% of all protein was consistently GMMA-associated with reproducible yields of more than 100 mg of GMMA-associated protein per liter fermentation volume from OD 30–45 cultures of *S. sonnei* ΔtolR ΔgalU. The integrity of GMMA obtained by this process was confirmed using electron microscopy. The purity and yield can be likely increased as indicated by fermentations with *S. sonnei*–pSS ΔtolR ΔmsbB to densities of 65 and 80. Furthermore, first results obtained by quantitative amino acid analysis of different types of GMMA indicated an at least two-fold higher protein amount in the GMMA preparations than determined by the Bradford assay used in this study (data not shown). Still, assuming an average yield of 100 mg/L fermentation and a dosage of 25 μg as used for the MenZB outer membrane vesicle meningococcal vaccine, at least 400,000 doses could be obtained from a 100 L fermenter.

A proteomic approach confirmed that *Shigella sonnei* 53G ΔtolR ΔgalU–derived GMMA are composed mostly of outer membrane and periplasmic components. They conserve lipophilic polypeptides. Only a small number of cytoplasmic components and one inner membrane protein were predicted. Thus, the proteomic analysis of GMMA obtained from an OD 45 culture revealed a similar composition as previously seen in proteomic analyses of outer membrane particles that were obtained from cultures at early logarithmic phase to avoid impurities by cytoplasmic proteins [10,13].

In accordance with previous reports [15,17] GMMA were highly immunogenic in mice with titers around 1:100,000 after administration of 2 μg of GMMA with and without adjuvant. A 10-fold lower dosage of GMMA (without adjuvant) resulted in only a 3-fold reduction and still very high antibody titers suggesting that low amounts of GMMA might be sufficient for vaccination. GMMA from the *msbB* mutant *S. sonnei* strain did not show a difference in immunogenicity which was expected due to a recent report that the resulting lipid A modification does not affect LPS recognition in mice [42]. Immunoblots confirmed that antibodies to proteins, including outer membrane proteins OmpA, OmpX, and YaeT, strongly contributed to the reactivity of the sera. Interestingly, the outer membrane protein OmpC which represents about 20% of protein in GMMA was not detected by sera raised against GMMA. Previously, an immunoproteomic analysis of isolated outer membrane proteins of *Shigella flexneri* 2a [43] also failed to detect OmpC as immunogenic protein. This could suggest that either OmpC is not immunogenic or that epitopes potentially recognized by antibodies are not maintained after SDS-PAGE. This might also apply to other membrane proteins that were not found by the Western blot analysis even though not all reactive proteins could be identified.

The *msbB* mutant strain of *Shigella* lacking the genes *msbB1* and *msbB2* [21] was generated to investigate if the production process was applicable to GMMA with modified lipid A. A previous report [21] had shown that these deletions result in the synthesis of a penta-acylated lipid A instead of a hexa-acylated lipid A in *Shigella* [21]. While the *S. sonnei*–pSS ΔtolR ΔmsbB mutant grows in rich media at 37°C temperature, its growth is impaired in the chemically defined medium developed for fermentation at 37°C but shows a normal growth in this medium at 30°C. Previously,
Figure 6. ELISA analysis of sera reactivity against GMMA. Groups 1–6 received 2 μg of GMMA with or without Freund’s adjuvant (FA), group 1) GMMA from S. sonnei ΔtolR ΔmsbB (grown in HTMC, 37°C), 2) GMMA of group 1 plus FA, 3) GMMA S. sonnei –pSS ΔtolR (defined medium, 37°C), 4) GMMA of group 3 plus FA, 5) GMMA from S. sonnei –pSS ΔtolR ΔmsbB (defined medium, 30°C), 6) GMMA of group 5 plus FA. Group 7 received 0.2 μg of GMMA from S. sonnei –pSS ΔtolR ΔmsbB. Control groups were immunized with PBS alone (group 8) or FA alone (group 9). Sera from individual mice obtained 14 days after the third immunization and pooled preimmune sera from each group respectively were assayed in dilutions of 1:1000, 1:10,000, and 1:100,000 on GMMA from S. sonnei 53G –pSS ΔtolR as coating and arbitrary units were calculated. Data are presented as scatter plots of ELISA units determined in individual mice (groups 1–9) or of the pooled preimmune sera (pre). The horizontal lines represent the geometric mean. All groups receiving GMMA showed higher GMMA to each other and with and without FA. No statistically significant differences were found (n.s.). Reduction of the immunization dosage of S. sonnei –pSS ΔtolR ΔmsbB GMMA to 0.2 μg (group 7) resulted in statistically significant reduction of ELISA units in the sera of the immunized animals compared to sera of mice immunized with 2 μg of the same GMMA (group 5) as determined by Mann-Whitney test (p = 0.0047). All groups receiving GMMA showed higher S. sonnei –pSS ΔtolR-specific antibody responses than groups immunized with PBS or FA alone (Mann-Whitney, p<0.003). For all comparisons a p value smaller than 0.05 was considered to be significant.

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Supporting Information

Figure S1 GMMA enrichment and purity after TFF without diafiltration of the biomass. GMMA were purified from a 5 L fermentation culture of S. sonnei ΔtolR ΔmsbB grown in HTMC at 37°C to OD 39 (fermentation B2 in Table 3) using 2-step TFF. In the first step, the culture supernatant which contains the GMMA was separated from the bacteria using a 0.2 μm filter without further diafiltration of the biomass. To determine the amount of GMMA in the permeate GMMA were separated from soluble proteins by ultracentrifugation. After ultracentrifugation, the pellet (GMMA) was resuspended in the initial volume of the centrifuged material to normalize all samples to fermentation volume. Equivalent volumes of the 0.2 μm filtrate before ultracentrifugation (1), the resuspended GMMA pellet (2), and the supernatant of the ultracentrifugation (3) were separated by SDS-PAGE indicating that the change in temperature does not have major effects on GMMA composition.

In summary, we have identified an easy process to produce large quantities of GMMA from high density culture. GMMA purified from fermentation are extremely pure particles composed almost exclusively of outer membrane and periplasmic components. The simplicity and high yield of the process support its applicability for large scale manufacturing. We have also shown that this process can be used with strains genetically modified to reduce reactogenicity or to remove immunodominant antigens, e.g. the O antigen. While this work focused on Shigella sonnei, we believe that this technology is an innovative platform for efficient vaccine manufacturing for Gram-negative bacteria.

Supporting Information

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(TIF)
normalized all samples to fermentation volume. Equivalent volumes of the retentate before ultracentrifugation (1), the resuspended in the visible amount of proteins are highlighted by arrows. suggesting good reproducibility of the process. Minor differences in the visible amount of proteins are highlighted by arrows. (TIF)

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Author Contributions

Conceived and designed the experiments: FBS AMC SS NN VDC AS CG. Performed the experiments: FBS AMC LM SS OR IF IP MC VDC. Analyzed the data: FBS LM SS OR IF IP CG. Contributed reagents/materials/analysis tools: IF NN. Wrote the paper: FBS CG.

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Table 7

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