The Human Pathogen Streptococcus pyogenes Releases Lipoproteins as Lipoprotein-rich Membrane Vesicles* **

Massimiliano Biagini‡**, Manuela Garibaldi‡**, Susanna Aprea‡, Alfredo Pezzicoli‡, Francesco Doro‡††, Marco Becherelli‡, Anna Rita Taddei¶, Chiara Tani¶, Simona Tavarini¶, Marirosa Mora¶, Giuseppe Teti¶, Ugo D’Oro¶, Sandra Nuti¶, Marco Soriani¶, Immaculada Margariti¶, Rino Rappuoli¶, Guido Grandi¶‡‡‡, and Nathalie Norais‡

Bacterial lipoproteins are attractive vaccine candidates because they represent a major class of cell surface-exposed proteins in many bacteria and are considered as potential pathogen-associated molecular patterns sensed by Toll-like receptors with built-in adjuvanticity. Although Gram-negative lipoproteins have been extensively characterized, little is known about Gram-positive lipoproteins. We isolated from Streptococcus pyogenes a large amount of lipoproteins organized in vesicles. These vesicles were obtained by weakening the bacterial cell wall with a sublethal concentration of penicillin. Lipid and proteomic analysis of the vesicles revealed that they were enriched in phosphatidylglycerol and almost exclusively composed of lipoproteins. In association with lipoproteins, a few hypothetical proteins, percinillin-binding proteins, and several members of the ExPortal, a membrane microdomain responsible for the maturation of secreted proteins, were identified. The typical lipidic moiety was apparently not necessary for lipoprotein insertion in the vesicle bilayer because they were also recovered from the isogenic diacylglycerol transferase deletion mutant. The vesicles were not able to activate specific Toll-like receptor 2, indicating that lipoproteins organized in these vesicular structures do not act as pathogen-associated molecular patterns. In light of these findings, we propose to name these new structures Lipoprotein-rich Membrane Vesicles. Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.045880, 2138–2149, 2015.

Bacterial lipoproteins (Lpps)1 are a subset of membrane proteins that are covalently modified with a lipidic moiety at their N-terminal cysteine residue. It is commonly reported that Lpps of Gram-positive bacteria are processed by two key enzymes; the prolipoprotein diacylglycerol transferase (Lgt) and the lipoprotein signal peptidase (Lsp). The Lgt enzyme recognizes a so-called lipobox motif in the C-terminal region of the signal peptide of a premature lipoprotein and transfers a diacylglycerol moiety to the cysteine residue of the lipobox (1), (2). Subsequently, the Lsp enzyme cleaves the signal peptide resulting in a mature Lpp (3), (4). Nevertheless, recent reports have suggested that N-acylation occurs in bacteria that lack the Gram-negative homologous apolipoprotein N-acyltransferase (Lnt) gene responsible for this modification (5, 6), and that Lpp N-terminal could also be modified with an acetyl group in some Gram-positive (7).

Lpps have been described as virulence factors because they play critical roles in membrane stabilization, nutrient uptake, antibiotic resistance, bacterial adhesion to host cells, protein maturation and secretion and many of them still have unknown function (8). Several studies have suggested that bacterial Lpps are pathogen-associated molecular patterns (PAMPs) sensed by the mammalian host through Toll-like receptor 2 (TLR2) heterodimerized with TLR1 or TLR6 to induce innate immunity activation and to control adaptive immunity (9–12). TLR2 plays a critical role in the host response to the Gram-positive bacteria Staphylococcus aureus (13) and

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1 The abbreviations used are: CDM, chemically defined medium; Lpp, lipoprotein; EM, electron microscopy; LB, Luria Bertani; LMV, lipoprotein-rich membrane vesicles; Lgt, prolipoprotein diacylglycerol transferase; Lnt, apolipoprotein N-acyltransferase; Lsp, lipoprotein signal peptidase; OMV, outer membrane vesicles; PAMP, pathogen-associated molecular pattern; THB, Todd-Hewitt broth; TLR, Toll-Like receptor; TSB, Tryptic Soy broth.
Streptococcus agalactiae (14). Although TLR2 has been considered a receptor for various structurally unrelated PAMPs, recent studies have suggested that, via their lipid moiety, bacterial Lpps function as the major, if not the sole, ligand molecules responsible for TLR2 activation (15). Although Gram-negative Lpps have been widely studied, little information is available for Gram-positive Lpps (16) and the ways they are released into the bacterial extracellular compartment and reach the host immune system remain unclear.

We focused our attention on Lpps release by Streptococcus pyogenes. This Gram-positive bacterium is an important human pathogen that causes a wide range of diseases from superficial and self-limiting infection, e.g. pharyngitis and impetigo, to more systemic or invasive diseases like necrotizing fasciitis and septicemia (17). Understanding the role of bacterial Lpps in mediating innate and acquired immunity can be instrumental for the therapy and prophylaxis of human S. pyogenes infections. In this study, we showed that in S. pyogenes Lpps are released into the growth medium within vesicle-like structures in minute amounts. Conditions weakening the bacterial cell wall, such as the addition of sublethal concentrations of penicillin to the bacterial growth medium enhanced this phenomenon and allowed the recovery of sufficient material to enable an in-depth characterization. Proteomic analysis of the vesicles revealed that they were almost exclusively constituted of Lpps. A total of 28 Lpps were identified, representing more than 72% of the Lpps predicted from the genome of the strain under investigation. In addition, multiple transmembrane domain proteins were not found in abundance associated to the vesicles, indicating that vesicles were not representative of the bacterial membrane. We defined these vesicles as Lipoprotein-rich Membrane Vesicles (LMVs).

Common characteristics are shared between the LMVs and the ExPortal described for the first time by Rosch and Caparon (18). This asymmetric and distinct membrane microdomain has been reported to be enriched in anionic phospho lipids and acts in promoting the biogenesis of secreted proteins by coordinating interactions between nascent unfolded secretory proteins and the accessory factors required for their maturation (19–21). An association between ExPortal and peptidoglycan synthesis has also been reported (22). Similarly, LMVs are enriched in anionic phosphatidylglycerol, enzymes involved in protein maturation/secretion and cell wall biogenesis, suggesting that LMVs might derive from the ExPortal. Finally, we showed that LMVs do not induce TLR2 activation, indicating that the Lpps did not act as PAMPs when integrated into the LMVs.

**EXPERIMENTAL PROCEDURES**

**Bacterial Growth Conditions**—M1–3348 S. pyogenes strain was provided by the Istituto Superiore di Sanità, Rome, Italy, M1-SF370 by ATCC, M6-S43 by Laboratory of Bacterial Pathogenesis and Immunology, Rockefeller University, New York, M28-HRO-K-06 by University of Rostock. Other strains were available in house.

In-frame deletion mutant of M1–3348 S. pyogenes strain lacking the lgt gene (Δlgt) was constructed by splicing-by-overlapping-extension PCR. Briefly, in frame deleted gene product was amplified using the following specific primers: LGT-F1 tcgcgatcattatTTGAATTTCAAGAGATCTT, LGT-R2 AACATTGACAATACCTTGGAATTTTACAAGAAGTGTAGATAA, LGT-F3 TTATCGACTTCTTGTAAGTAATTCC-AACAGGTGTACATTT, LGT-R4 tggcgagctcTGAATATTATCAAGTG-GTG. The PCR product was cloned using BamHI and Xhol restriction sites in the temperature-sensitive vector pJR233 (23). Transformation and allelic exchange was performed under selective pressure and drug sensitive colonies were screened by PCR for the absence of the target allele. S. pyogenes wild type strain and the respective isogenic Δlgt mutant were grown in TSB or CDM medium at 37 °C, in a rotary shaker, to reach OD600 = 0.4, whereas S. agalactiae was grown in THB medium. From liquid cultures, bacteria were either collected by 10 min centrifugation at 4000 × g, or bacteria were treated with penicillin by addition of the same volume of medium containing penicillin at the concentration of 0.7 μg/ml for 80 min, unless specified.

**Vesicle Preparation by High-Speed Centrifugation**—Culture media of wild type and Δlgt strains were filtered through a 0.22-μm pore size filter (Millipore, Bedford, MA). The filtrates were subjected to high-speed centrifugation (200,000 × g for 90 min), and the pellets containing the vesicles were washed with PBS, centrifuged again at the same conditions and finally resuspended with PBS.

**Negative Staining Electron Microscopy**—Vesicles were fixed overnight in 2.5% (v/v) glutaraldehyde in PBS and then washed and resuspended in the same buffer. A drop of suspension was placed on Formvar/carbon-coated grids, and vesicles were adsorbed for 5 min. Grids were then washed with distilled water and blotted with a filter paper. For negative staining, grids were treated with 2% (w/v) uranyl acetate for 1 min, air-dried, and viewed with a JEM 1200 EX II transmission electron microscope (Jeol, Peabody, MA) operating at 80 kV.

**Denaturing Monodimensional Gel Electrophoresis (SDS-PAGE)**—Vesicles were denatured for 10 min at 95 °C in Laemmli buffer. 20 μg of proteins were loaded onto 4–12% (w/v) polyacrylamide gradient gels (Life Technologies, Carlsbad, CA). Gels were run in MOPS buffer (Life Technologies) and stained with Coomassie Blue R-250.

**In Gel Protein Digestion and MALDI-TOF Mass Spectrometry Analysis**—Protein bands were excised from the gels, washed with 50 mM ammonium bicarbonate (Fluka Chemie AG, Buchs, Switzerland), MS-grade acetonitrile (Sigma-Aldrich, St. Louis, MO) (1:1, v/v), washed once with pure acetonitrile, and air-dried. Dried spots were digested for 18 h at 37 °C in 20 μl of 5 mM ammonium bicarbonate and 12 ng/μl sequencing grade modified trypsin (Promega, Madison, WI). After digestion, 0.6 μl were spotted onto a matrix PAC target (Prespotted AnchorChip 96, set for proteomics, Bruker Daltonics, Bremen, Germany) and air-dried. Spots were washed with 0.6 μl of 70% ethanol, 0.1% trifluoroacetic acid. Mass spectra were acquired on an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) in reflectron, positive mode in the mass range of 700–3500 m/z. Spectra were externally calibrated by using a combination of standards prespotted on the target. MS spectra were analyzed with FlexAnalysis (version 2.4, Bruker Daltonics) using default parameters and manually revised.

**NanoLC-MS/MS and Protein Identification**—Peptides were separated by nano LC on a nanoAcquity UPLC system (Waters, Milford, MA) connected to an ESI Q-TOF Premier 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 a 2–40% of 98% acetonitrile, 0.1% formic acid solution at a flow rate of 250 nl/min. The eluted peptides were subjected to an auto-
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Molecular 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 four different ions 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 ProteinLynxGlobal Server, version 2.5 (Waters) to obtain the peak list file. Protein identification was carried out from the generated peak list using the Mascot software (version 2.2.03, Matrix Science Inc., Boston, MA). Mascot was run on a custom database comprising public S. pyogenes database and internal annotated S. pyogenes genomes (56,403 sequences; 16,305,282 residues). Search parameters were as follows: cleavage by trypsin; missed cleavage, 1; oxidation (M) and deamidation (N,O) as variable modification (mass tolerance, 100 ppm and 0.2 Da (for MALDI and ESI-Q-TOF analysis, respectively). Identifications were considered when the Mowse score (24) was significant according to Mascot output (equal or greater than 34 for the searches of this study). Peptide counts were defined as the number of unique peptides identified for a single protein with significant Mowse scores. Known contaminant signals (from keratins and trypsin) were manually excluded from MALDI spectra.

Bioinformatics—Re-annotation of M1-SF370 genome, using Glimmer version 3.0.2 compared with the original annotation (25) was performed to find proteins eventually missed or not classified as Lpps because of errors in the assignment of the initial start codon. In *silo* predictions with different algorithms (PSORT (26), LipoP (27) DOLOP (28), PredLipo (29) or bibliographic reference (30), as reported in Table I, were used to include all the possible Lpps. PSORTb version 3.0.2 was used for the prediction of protein cellular compartment ([http://www.psort.org/psortb)](http://www.psort.org/psortb/)) (31).

Western blot Analysis—Western blot was carried out on 0.22-μm filtered total culture supernatant, ultracentrifuged supernatant (secreted proteins), and pellet from ultracentrifuged supernatant (vesicles). 200 ml of culture supernatant were filtered to remove residual cells. The remaining material was ultracentrifuged at 200,000 *g* for 90 min. After ultracentrifugation, the pellet was resuspended in 200 μl of PBS. Fifteen microliters of the ultracentrifuged supernatant and the resuspended pellet were resolved by SDS-PAGE analysis. The remaining material was ultracentrifuged at 200,000 × *g* for 90 min. After ultracentrifugation, the pellet was resuspended in 200 μl of PBS. Fifteen microliters of the ultracentrifuged supernatant and the resuspended pellet were resolved by SDS-PAGE run in MOPS buffer and transferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA). After membrane saturation in PBS containing 3% (w/v) powdered milk, the membranes were incubated, with mice polyclonal antiserum (1:1000 dilution) in PBS containing 0.1% (w/v) BSA, were added to the bacterial suspension in 10 ml of goat anti-mouse IgG, F(ab’2) fragment, Chemiluminescent Substrate kit (Pierce, Rockford, IL) as described by the manufacturer.

Confocal Immunofluorescence Microscopy—S. pyogenes LMVs were visualized by standard immunofluorescence procedures. Briefly, paraformaldehyde was added to the LMV preparation to a final concentration of 2% (v/v) and fixed for 20 min at room temperature onto POLYSINE slides (Menzel-Glaser, Braunschweig, Germany). The slides were then washed and blocked with PBS containing 10% (v/v) normal goat serum and 3% (w/v) BSA (Sigma-Aldrich) for 30 min and incubated with a mouse polyclonal antiSPy1390 serum (1:500 dilution) and with 10-n-nonyl acridine orange (Sigma-Aldrich; 400 nM final concentration) diluted in PBS with 1% (w/v) BSA for 15 min at room temperature. The slides were then washed and stained with goat anti-mouse IgG Alexa Fluor 647-conjugated antibodies (Life Technologies, 1:1000 dilution) for 10 min at room temperature. ProLong Gold Antifade reagent (Life Technologies) was used to mount coverslips. The slides were analyzed with a Zeiss Observer LSM 710 confocal scanning microscope (Zeiss, Oberkochen, Germany).

Lipid Extraction and Separation by Thin Layer Chromatography (TLC)—Lipids were extracted from S. pyogenes total extract obtained after mechanical lysis of the bacteria or from the purified LMVs following the Bligh and Dyer protocol (33) using a solution of water/chloroform/methanol (0.9/1/1; v/v/v). Dried lipids were resuspended in 100 μl of chloroform and 2 μl were loaded onto a TLC silica gel plate (Merk-Millipore, Billerica, MA). Lipids were vertically resolved in a TLC chamber saturated by a solution of chloroform/ethanol/water/triethylamine (35/35/7/35; v/v/v/v). Lipids were stained with a nebulization of 0.05% (w/v) primulin (Sigma) in 1:1 acetone/water (v/v) and visualized by UV lamp. Lipid standards (Sigma) were: PA, Lα,α-phosphatidic acid; PE, 3-sn-phosphatidylethanolamine; S, sphingomyelin; PC, Lα,α-phosphatidylcholine; PG, Lα,α-phosphatidyl-DL-glycerol; LC, Lα,α-lisophosphatidylcholine; PS, 1,2-diacyl-sn-glycero-3-phospho-L-serine.

Membrane Protein Extraction—M1–3348 S. pyogenes strain grown in THB until midexponential phase was harvested and resuspended in 3 m guanidinium chloride, 25 mM Tris, pH 8.5 (lysis buffer), and mechanically disrupted in FastPrep® FP120 Bead Beater (Qbiogene, Inc., Carlsbad, CA) by seven cycles of 1 min. Unbroken cells and cellular debris were pelleted and discarded by 10 min of centrifugation at 4000 × *g* at 4 °C. The supernatant was then high-speed centrifuged at 200,000 × *g* for 3 h and the resulting pellet was analyzed by SDS-PAGE as reported above.

Recombinant Protein Purification of and Preparation of Immune Sera—The recombinant forms of the Lpps were cloned without the presequence and with a C-terminal His-tag as previously reported in Bensi et al. (32). The recombinant proteins were purified to homogeneity and used to immunize mice as previously described (34). Preparation of immune sera was performed as previously described (34).

FACS Analysis of the Vesicles—Vesicles were washed twice with PBS, suspended in newborn calf serum (NCS, Sigma), incubated for 20 min at room temperature and dispensed into a 96-well plate (20 μl/well). Eighty μl of pre-immune or immune mouse sera, diluted in PBS containing 0.1% (w/v) BSA, were added to the bacterial suspension to a final dilution of 1:200 and incubated on ice for 30 min. After washing twice with 0.1% (w/v) BSA in PBS, bacteria were incubated on ice for 30 min in 10 μl of goat anti-mouse IgG, F(ab’)2 fragment-specific, R-phycocyanin-conjugated (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) diluted 100-fold in PBS containing 0.1% (w/v) BSA, 20% (v/v) NCS. After incubation, bacteria were washed with PBS containing 0.1% (w/v) BSA, suspended in 200 μl PBS and analyzed using a FACScan Calibur cytometer (Becton Dickinson, Franklin Lakes, NJ) and Cell Quest Software (Becton Dickinson). In vitro Activity on Human TLR2 Stable Cells—HEK293-NF-kB-Luc cells (clone LP58), a cell line stably transfected with a reporter vector in which the luciferase gene is under the control of an NF-kB-dependent promoter, were previously produced by Cell and Molecular Technologies (CMT Inc., Phillipsburg, NJ) for Chiron Corporation under a service contract. These cells were transfected using Lipofectamine 2000 (Life Technologies) with pcDNA3.1-Hygro-FLAG-HTLR2 plasmid encoding for human TLR2 containing a FLAG epitope at the N terminus and a hygromycin resistance gene for selection. Transfected cells were cultured in the presence of hygromycin (250 μg/ml) and individual resistant clones were picked, expanded, and tested for expression of luciferase upon stimulation with the TLR2 agonist Pam3CSK4. The best responding clone was then selected for experiments. For
luciferase assay HEK293-FLAG-hTLR2-NF-kB-Luc cells (25 × 10^3 cells/well) were seeded into microclear 96-well flat bottom plates in 90 μl of complete medium in the absence of selection antibiotics. After overnight incubation, cells were stimulated in duplicates with different dilutions of stimuli (10 μl/well) for 6 h. Then, medium was discarded and cells were lysed with 20 μl of Passive Lysis Buffer (Promega) for 20 min at room temperature. Luciferase levels were measured by addition of 100 μl/well Luciferase Assay Substrate (Promega) using the LMax II 384 microplate reader (Molecular Devices, Sunnyvale, CA). Raw light units (RLU) from each sample (average of 2) were divided by the RLU of the control sample (PBS) and expressed as Fold Increase (FI).

Edman Degradation—Edman degradation was performed by Alphalyse A/S (Odense, Denmark) on proteins transferred from SDS-PAGE onto PVDF membrane stained by Coomassie Blue R-250.

RESULTS

S. pyogenes Lipoproteins are Released as LMVs

The presence of Lpps in growth media has been reported for several Gram-positive bacteria (14). We have recently reported that Lpps were not the major secreted proteins in the S. pyogenes secretome (32). Nevertheless, we investigated their presence in a growth medium from a midLog phase culture by Western blot using sera from mice immunized with recombinant forms of three Lpps: the putative protease maturation protein (SPy1390), the acid phosphatase (SPy1882) and the surface lipoprotein (SPy2000), available from our S. pyogenes vaccine discovery program (32, 34). The sera recognized the three Lpps with the expected molecular weight confirming their release into the growth medium (Fig. 1). Because of the hydrophobic nature of their lipid moiety, we hypothesized that Lpps were released as complex structures. To validate this hypothesis, the growth medium was centrifuged at high-speed and the pellet and supernatant were analyzed by Western blot using the same sera. Lpps were found mainly associated to the pellet indicating that they were released as high molecular weight structures (Fig. 1). We made the assumption that the bacterial cell wall could hamper the release of these structures and that weakening it by a β-lactamic antibiotic could potentially increase their release. Penicillin at a final concentration of 0.7 μg/ml was added to a midLog phase growth and the antibiotic treatment was allowed to pursue for 80 min (Fig 2A). These conditions allowed the recovery of about 1 mg of pelleted proteins from one liter of bacterial culture without observing...
on SDS-PAGE the EF-Tu, a cytoplasmic protein observed for higher concentration of penicillin or a longer period of incubation (data not shown). The electrophoretic profiles of the pelleted material derived from culture with or without penicillin were qualitatively comparable, although the amount of proteins recovered from the bacteria grown in presence of the antibiotic was about 20-fold higher. This observation indicated that penicillin amplified, but did not generate, a phenomenon occurring at a very low level in the standard growth conditions. A similar electrophoretic pattern was obtained with vancomycin, a glycopeptide inhibiting the cell wall biogenesis (data not shown). Moreover, the electrophoretic profile was different from the pattern of a *S. pyogenes* membrane preparation indicating that the material we isolated was not representative of the bacterial membrane (supplemental Fig. S1).

*S. pyogenes* Vesicles Are Almost Exclusively Constituted of Lpps—Proteins from the nine major SDS-PAGE bands were analyzed by Peptide Mass Fingerprint (PMF), leading to the identification of 10 proteins (Fig. 2A and Table II). Eight of them were Lpps including the three Lpps revealed by the Western blot analysis (Fig. 1). The two non-Lpp proteins were the putative penicillin-binding protein 1a (SPy1649) and the hypothetical protein (SPy0836). Although the N-terminal regions of Gram-negative Lpps are well known to be tri-acylated, the question of an N-acylation, N-acetylation or no N-modification of Gram-positive Lpps is still under investigations (7). Edman degradation was performed from the main Lpp bands transferred onto PVDF membrane. No sequence was obtained indicating a modification of the Lpp N-terminal residues. Unfortunately, no peptides corresponding to the modified N-terminal peptides generated a MS signal allowing the identification of the modification.

The pellet recovered from the high-speed centrifugation was also analyzed by electron microscopy and vesicle-like structures of 50 to 100 nm of diameter were observed (Fig. 2B). To show that the Lpps were associated to the vesicles, confocal microscopy was performed on the cell-free growth medium using two separated stainings. Lipids were stained with 10-μm–nonyl acridine orange and the peptidylprolyl isomerase (SPy1390) was detected with a polyclonal antibody revealed with a fluorescent secondary antibody. The co-localization of stainings confirmed the association of the Lpp SPy1390 to the vesicles (Fig. 2C). Moreover, the vesicles were also specifically recognized by the antiLpp peptidylprolyl isomerase (SPy1390) polyclonal antibody in FACS experiment indicating the surface exposure of this Lpp (Fig. 2D).

To confirm the high content of Lpps associated to the vesicles, the full SDS-PAGE lane was sliced, digested by trypsin and subjected to LC-MS/MS experiments (four biological replicates). One hundred and eleven proteins were identified (supplemental Table S1). The definition of Lpps in Gram-positive bacteria is still controversial. Using six references for Lpp prediction (software and bibliographic references reported in Table I), 39 Lpps were predicted from the M1-SF370

| Table I | List of software and bibliographic reference used for Lpp prediction |
|---------|---------------------------------------------------------------|
| PSORT  | First Version of Poort |
| 1999    | G-LPP Lipoprotein prediction tools |
| 2002    | LipoP 2003 |
|         | PredLipo 2006 |
|         | DOLIP 2008 |

Publication year
Number of predicted Lpps
Comments
Available link
PSORT http://psort.hgc.jp/form.html
LipoP http://www.cbs.dtu.dk/services/LipoP/
DOLIP http://www.mrc-lmb.cam.ac.uk/genomes/dolip/
List of the Lpps predicted from the M1-SF370 genome. The predicted Lpps are grouped as experimentally identified by mass spectrometry or not. (ii) This ORF is present in the M1-SF370 genome but not correctly annotated and SPy number refers to MGAS5005 strain. (*) Protein with newly assigned methionine for Lpp prediction was included in the table.

| SPy number | Annotation | PSORT | C+LPP | LipoP | Lei B. et al. | DOLOP | PredLipo |
|------------|------------|-------|-------|-------|---------------|-------|----------|
| M5005_Spy0249 | oligopeptide-binding protein (*) |  |  |  |  |  |  |
| SPy0453   | metal binding protein of ABC transporter (lipoprotein) |  |  |  |  |  |  |
| SPy0457   | putative cyclophilin-type protein |  |  |  |  |  |  |
| SPy1228   | putative lipoprotein |  |  |  |  |  |  |
| SPy1390   | putative protease maturation protein |  |  |  |  |  |  |
| SPy1882   | putative acid phosphatase |  |  |  |  |  |  |
| SPy2000   | surface lipoprotein |  |  |  |  |  |  |
| SPy2032   | putative ATP-binding cassette transporter-like protein |  |  |  |  |  |  |
| SPy0163   | putative ABC transporter (lipoprotein) |  |  |  |  |  |  |
| SPy0247   | conserved hypothetical protein SPy0247 |  |  |  |  |  |  |
| SPy0252   | putative sugar transporter sugar binding lipoprotein |  |  |  |  |  |  |
| SPy0317   | conserved hypothetical protein SPy0317 |  |  |  |  |  |  |
| SPy0319   | conserved hypothetical protein SPy0319 |  |  |  |  |  |  |
| SPy0351   | hypothetical protein SPy0351 |  |  |  |  |  |  |
| SPy0385   | ferrichrome ABC transporter (ferrichrome-binding protein) |  |  |  |  |  |  |
| SPy0604   | hypothetical protein SPy0604 |  |  |  |  |  |  |
| SPy0778   | putative ABC transporter (substrate-binding protein) |  |  |  |  |  |  |
| SPy0903   | putative ABC transporter (binding protein) |  |  |  |  |  |  |
| SPy1094   | conserved hypothetical protein SPy1094 |  |  |  |  |  |  |
| SPy1245   | putative phosphate ABC transporter |  |  |  |  |  |  |
| SPy1274   | putative amino acid ABC transporter |  |  |  |  |  |  |
| SPy1290   | hypothetical protein SPy1290 |  |  |  |  |  |  |
| SPy1294   | putative maltose/maltodextrin-binding protein |  |  |  |  |  |  |
| SPy1306   | maltose/maltodextrin-binding protein |  |  |  |  |  |  |
| SPy1686   | hypothetical protein SPy1686 |  |  |  |  |  |  |
| SPy1697   | hypothetical protein SPy1697 |  |  |  |  |  |  |
| SPy2033   | hypothetical protein SPy2033 |  |  |  |  |  |  |
| SPy2037   | conserved hypothetical protein SPy2037 |  |  |  |  |  |  |

| Number of Lpps experimentally identified | 22 | 22 | 24 | 26 | 23 | 25 |
|-----------------------------------------|----|----|----|----|----|----|

| Lpps not identified | PSORT | C+LPP | LipoP | Lei B. et al. | DOLOP | PredLipo |
|---------------------|-------|-------|-------|---------------|-------|----------|
| SPy0210             | hypothetical protein SPy0210 |  |  |  |  |  |
| SPy0251             | putative N-acetylmannosamine-6-P epimerase |  |  |  |  |  |
| SPy0857             | putative peptidoglycan hydrolase |  |  |  |  |  |
| SPy1361             | putative internalin A precursor |  |  |  |  |  |
| SPy1405             | hypothetical protein SPy1405 |  |  |  |  |  |
| SPy1558             | hypothetical protein SPy1558 |  |  |  |  |  |
| SPy1592             | conserved hypothetical protein SPy1592 |  |  |  |  |  |
| SPy1795             | putative ABC transporter (periplasmic binding protein) |  |  |  |  |  |
| SPy1972             | putative pullulanase |  |  |  |  |  |
| SPy2007             | putative laminin adhesion |  |  |  |  |  |
| SPy2066             | putative dipeptidase |  |  |  |  |  |

| Number of Lpps not identified | 7 | 6 | 9 | 7 | 8 | 8 |
|-------------------------------|---|---|---|---|---|---|

| Percentage of identified Lpps | 76 | 79 | 72 | 79 | 74 | 76 |
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### TABLE III

List of the most abundant non-Lpp proteins identified. Non-Lpp proteins identified from proteomic analysis were classified according to their biological function. Cellular localization was reported according to PSORTb prediction.

| Biological function                          | SPy N° | Annotation                                      | PSORTb               |
|---------------------------------------------|--------|------------------------------------------------|----------------------|
| Secretion and protein maturation            | SPy2216| Putative serine protease HtrA                   | Unknown              |
|                                             | SPy1154| Sortase A                                       | Cytoplasmic Membrane |
|                                             | SPy1842| Putative signal peptidase I                    | Cytoplasmic Membrane |
| Cell wall biogenesis                        | SPy1649| Putative penicillin-binding protein 1a         | Cytoplasmic Membrane |
|                                             | SPy0097| Putative penicillin-binding protein 1b         | Cytoplasmic Membrane |
|                                             | SPy2059| Penicillin-binding protein 2a                  | Cytoplasmic Membrane |
| Hypothetical protein                        | SPy0836| Hypothetical protein                            | Unknown              |
|                                             | SPy2065| Hypothetical protein                            | Unknown              |
| Secreted protein                            | SPy0167| Streptolysin O                                  | Extracellular        |
| Protein translation                         | SPy2092| 30S ribosomal protein S2                       | Cytoplasmic          |
|                                             | SPy0063| 50S ribosomal protein L5                       | Cytoplasmic          |
|                                             | SPy0461| 50S ribosomal protein L1                       | Cytoplasmic          |
|                                             | SPy2178| 30S ribosomal protein S4                       | Cytoplasmic          |
| Energy metabolism                           | SPy0731| Phosphopyruvate hydratase                      | Cytoplasmic          |
|                                             | SPy1031| Putative dihydrolipoamide dehydrogenase        | Cytoplasmic Membrane |

... (Table II)...

genome (Table II), 28 of which were identified in the vesicles representing more than 72% of the predicted Lpps (Table II and supplemental Table S1).

In order to better understand the nature of the LMVs, we examined the most abundant proteins applying a semi-quantitative measurement of protein abundance through peptide counts (35, 36). We arbitrarily defined a protein as “most abundant” if identified in at least three out of the four experiments with a total peptide counts superior to 17 (corresponding to 1% of the global peptide count within the four experiments). Twenty eight proteins were classified according to these criteria. They included the 10 proteins identified from the SDS-PAGE (Fig. 2A and Table II) validating the used of the peptide count approach in defining the most abundant proteins in the vesicles. Moreover 13 of these were classified as Lpps (supplemental Table S1) confirming the Lpp enrichment in the vesicles. The most abundant non-Lpp proteins identified are reported in Table III. With the exception of the six predicted cytoplasmic proteins, all the proteins could be related to the ExPortal (18, 22) because they were involved in the maturation of the secretory proteins (SPy1154 and SPy1842, SPy2216) (19, 21) and cell wall biogenesis (SPy1649, SPy0097 and SPy2059) (37–39) or were reported as ExPortal-substrate (SPy0167) (22). Considering the high number of proteins associated to the ExPortal, it is probable that the two hypothetical proteins (SPy0836 and SPy2065) might have ExPortal-associated functions. It was not possible to determine if the predicted cytoplasmic proteins of this group, as well as most of the less abundant proteins (supplemental Table S1), were LMV proteins or derived from light bacterial lysis (34, 40). Considering the high proportion of Lpps in the purified material, we named these structures as Lipoprotein-rich Membrane Vesicles (LMVs).

To define if other growth stress conditions could generate the release of LMVs, *S. pyogenes* was grown in the chemically defined medium established by Michelson (41). Fifty-five proteins were identified from the growth medium by LC-MS/MS (supplemental Table S2), of which 21 were either Lpps (Table II) or classified as LMV most abundant non-Lpp proteins (supplemental Table S2) indicating that other stress conditions allowed the release of Lpps. This set of identified protein was not found from the secretome analysis of *S. pyogenes* grown in rich medium (32).

The release of LMVs was not restricted to the analyzed strain because a similar pattern was obtained from 5 other *S. pyogenes* strains representative of the major M serotypes (M1, M4, M6, M28 and M75) (supplemental Fig. S2). Interestingly, LMVs were observed also for *S. agalactiae* strain 2603 V/R (supplemental Fig. S3) indicating that their release is not limited to *S. pyogenes*, but probably is a common phenomenon among Streptococcal species.

**LMV Lipid Composition is not Representative of the Bacterial Membrane**—The fact that vesicles were almost exclusively constituted of Lpps and were deprived of multiple transmembrane proteins indicated that they do not faithfully represent the standard bacterial membrane composition and suggested that they may derive from membrane microdomains. Such domains have been characterized by a local lipidic composition diverse from the bacterial membrane lipid composition (42, 43). Lipids from whole bacteria grown in presence or in absence of penicillin, and LMV lipids were extracted by chloroform/methanol and separated by thin layer chromatography (Fig. 3). No difference in the lipid pattern was observed from bacteria grown in the presence or absence of antibiotic, indicating that penicillin had no effect on the lipid composition (Fig. 3, lanes 2 and 3). Although an identical lipid pattern was observed in bacterial membrane and LMVs, the lipid proportion was different as highlighted by the two lipid spots labeled in Fig. 3. The lipid spot presenting a ratio frontis identical to the phosphatidyglycerol marker was more intense...
Fig. 3. S. pyogenes membrane and vesicle lipid composition is different. Lipids were extracted by chloroform/methanol from total extract of bacteria grown in presence (lane 2) or in absence (lane 3) of penicillin or from the purified vesicles (lane 4) and separated by thin layer chromatography. Lipids were stained with primulin and visualized by UV lamp. Circles highlight difference in lipid proportion. Lipid standard mixture (5 μg each) was loaded in lane 1: L-α-phosphatidyl-DL-glycerol (PG); 3-sn-phosphatidylethanolamine (PE); L-α-phosphatidic acid (PA); L-α-phosphatidylcholine (PC); 1,2-diacyl-sn-glycerol-3-phospho-L-serine (PS); sphingomyelin (S); L-α-lysophosphatidylcholine (LC).

in the LMV compared with whole bacteria (Fig. 3, lane 4 compared with lanes 2 and 3).

Lpp Lipid Moiety is not Necessary for the LMV Formation—In order to investigate if the lipidic moiety of the Lpps was necessary for the formation of the LMVs, we generated an isogenic knock-out strain in which the gene encoding for diacylglycerol transferase was deleted (Δglt). Because it is now well accepted that the S-diacylglyceryl moiety of the Lpps is responsible for TLR2 activation, we also tested TLR2-activation by LMVs isolated from Δglt. The addition of penicillin to the growth culture of the wild type strain induced a luciferase induction of only threefold (Fig. 5), whereas the increase of released LMVs was about 20-fold (Fig. 2). To assess if the measured induction was associated to the LMV, the growth medium was centrifuged at high-speed and the TLR2 activation was assessed from the supernatant. The supernatant of Δglt grown in presence or absence of penicillin did not induce any TLR2 activation (Fig. 5). This data reinforced the already reported role of the Lpp S-diacylglycerol moiety for TLR2 activation (14).

The addition of penicillin to the growth culture of the wild type strain induced a luciferase induction of only threefold (Fig. 5), whereas the increase of released LMVs was about 20-fold (Fig. 2). To assess if the measured induction was associated to the LMV, the growth medium was centrifuged at high-speed and the TLR2 activation was assessed from the supernatant. The TLR2 activation observed from the growth culture was not found associated to the LMV fraction. These data indicated that the Lpps contained in LMVs did not represent a form able to act as PAMPs, although we cannot exclude that the release of LMVs could be an intermediate state for the release of Lpps in a form able to activate the TLR2.

DISCUSSION

A number of Lpps have been implicated in the virulence mechanisms of bacterial pathogens and they are recognized by pattern recognition receptors of host immune systems (14). Despite this interest, Gram-positive Lpps remain uncharacterized and how they reach the host immune system is still
unknown. In this work, we showed by proteomics, electron microscopy and FACS analysis, that S. pyogenes Lpps are released in a vesicle-like structure that we named LMVs (Lipoprotein-rich Membrane Vesicles). Already in 1975, Kusaka described the release of diglyceride-rich membrane vesicles during protoplast formation in some Gram-positive bacteria (45), but no further investigations have been reported. Only very recently, a correlation between penicillin and release of Lpps has been reported in Streptococcus suis (46, 47). The observation that LMV release is increased in presence of penicillin allowed their deep characterization.

Proteomic analysis revealed Lpps are the major components of the LMVs (Fig. 2). More than 72% of the genome predicted Lpps were identified in the LMVs, making them different from the vesicles recently isolated from B. anthracis (48), S. aureus (49) and S. pneumoniae (50) in which Lpps are not the major components. In addition to the very high proportion of Lpps, the absence of multiple transmembrane proteins and the different lipid composition of the LMVs indicated that they might derive from membrane microdomains. Such domains have been proposed to be associated to the coupled process of transcription, translation and insertion of nascent membrane of exported proteins (42, 43, 51). The analysis of the LMVs revealed several characteristics of the ExPortal, an asymmetric and distinct membrane microdomain proposed by Rosch and Caparon for protein secretion and maturation (18) and cell wall biogenesis (22) in S. pyogenes. As for the ExPortal, LMVs are enriched in phosphatidylglycerol and among the fifteen most abundant non-Lpp proteins, seven could be associated to ExPortal functions. They are the three proteins involved in secreted protein maturation, HtrA serine protease (SPy2216) and Sortase A (SPy1154) both reported to be part of the ExPortal, as well as signal peptidase I (SPy1842); the three penicillin-binding proteins (SPy1649, SPy0097, and SPy2059) involved in the cell wall biogenesis; and the streptolysin O (SPy0167) that has been reported as an
ExPortal substrate (22). Two hypothetical proteins SPy0836 and SPy2065 were also identified. SPy0836 carries a common domain with the HlyD family secretion protein, specific for the secretion type I in Gram-negative bacteria (52), and no function could be assigned to SPy2065. A deeper study of these proteins might complete the characterization of the ExPortal. On the other hand, it should be of note that the SecA from the secretion system (20), or the MraY and MurN (22) involved in the first step of the cell wall biogenesis reported as part of the ExPortal and the signal peptidase II, involved in the Lpp maturation were not identified in our analysis. This apparent discrepancy probably reflects the limit of our analysis as well as the lack of deep characterization of the ExPortal. With the exception of the streptolysin O, no other cell wall or secreted proteins were found associated in high amount to the LMVs. This observation certainly reveals the transient association of these protein families with the ExPortal whereas the Lpps might remain more tightly associated.

Prelipoproteins are inserted in the microdomain prior to their maturation because nondiacylated Lpps were found associated to the LMVs released by the Δgt mutant, indicating that the lipid moieties were not required for an insertion into the LMVs. Nevertheless, the association of the unlipidated Lpps to the LMVs was not as tight as the mature forms.

At this stage of the investigation, we cannot state if the LVM formation is an active mechanism for the secretion/maturation of the Lpps or due to a locally different lipid composition. By cryo-transmission electron microscopy of frozen-hydrated section of plasmolysed B. subtilis and S. aureus, Matias and Beveridge (53, 54) observed vesicles within a space that they named “inner wall zone.” Weakening bacterial cell wall by penicillin might render the bacteria more sensitive to a hyper- tonic environment allowing the massive release of LMV by detachment from the membrane of microdomains of different lipid composition.

The activation of the TLR2 by Lpps released in the growth medium of Gram-positive bacteria has been reported (14). We confirmed that the diacylated moiety is responsible for the TLR2 activation because growth culture from Δgt mutant was not able to induce any activation. Nevertheless, we showed that the Lpps associated to the LMVs are not in a form accountable for the TLR2 activation, but it is not excluded that they represent an intermediate state for the final release of a TLR2-activating material. In this case, penicillin, the antibiotic choice for treatments of S. pyogenes infections (55, 56) would not only have a direct role on bacterial mortality, but would also contribute to the release of a material that activates the host immune system through TLR2 signaling. In addition, LMVs could also represent an excellent vector for antigen delivery via their potential intrinsic adjuvanticity.

Release of outer membrane vesicles (OMVs) is a ubiquitous process that occurs during normal bacterial growth of Gram-negative bacteria with several functions including toxin and virulence factor delivery to host cells, inter- and intraspecies cellular cross-talk, biofilm formation, rapid adaptation to variations in the external environment, genetic transformation and defense against host immune responses (57) (58). So far, we cannot attribute any biological function to the LMVs. Nevertheless the LMVs described in this study represent a suitable material to study the role of Lpps in the host-pathogen interactions and Lpp structures.

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This article contains supplemental Figs. S1 to S4, Tables S1 and S2 and 5 Data files.

** These authors contributed equally to this work.

†† Current address: Janssen, Newtonweg 1, 2333 CP Leiden, The Netherlands.

†§§ Current address: Center for Integrative Biology Via Sommarive, 9 38123 Povo, Italy.

REFERENCES

1. von Heijne, G. (1989) The structure of signal peptides from bacterial lipo- proteins. Protein Eng. 2, 531–534
2. Sutcliffe, I. C., and Harrington, D. J. (2002) Pattern searches for the identifica- tion of putative lipoprotein genes in Gram-positive bacterial genomes. Microbiology 148, 2065–2077
3. Hussain, M., Ichihara, S., and Mizushima, S. (1982) Mechanism of signal peptide cleavage in the biosynthesis of the major lipoprotein of the Escherichia coli outer membrane. J. Biol. Chem. 257, 5177–5182
4. Sankaran, K., and Wu, H. C. (1994) Lipid modification of bacterial prolipo- protein. Transfer of diacylglycerol moiety from phosphatidylglycerol. J. Biol. Chem. 269, 19701–19706
5. Kurokawa, K., Lee, H., Roh, K. B., Asanuma, M., Kim, Y. S., Nakayama, H., Shirasutsuchi, A., Choi, Y., Takeuchi, O., Kang, H. J., Dohmae, N., Nakana- shi, Y., Akira, S., Sekimizu, K., and Lee, B. L. (2009) The triacylated ATP binding cluster transporter substrate-binding lipoprotein of staphylococ- cus aureus functions as a native ligand for toll-like receptor 2. J. Biol. Chem. 284, 8406–8411
6. Vidal-Inigilardi, D., Lewenza, S., and Buddelmeijer, N. (2007) Identification of essential residues in apolipoprotein N-acetyltransferase, a member of the CN hydrolase family. J. Bacteriol. 189, 4456–4464
7. Kurokawa, K., Ryu, K. H., Ichikawa, R., Masuda, A., Kim, M. S., Lee, H., Chae, J. H., Shimizu, T., Saitoh, T., Kuwano, K., Akira, S., Dohmae, N., Nakaya- ma, H., and Lee, B. L. (2012) Novel bacterial lipoprotein struc- tures conserved in low-GC content gram-positive bacteria are recog- nized by Toll-like receptor 2. J. Biol. Chem. 287, 13170–13181
8. Kovacs-Simon, A., Titball, R. W., and Michell, S. L. (2011) Lipoproteins of bacterial pathogens. Infect Immun. 79, 548–561
9. Alexeopoulou, L., Thomas, V., Schnare, M., Lobet, Y., Anguita, J., Schoen, R. T., Medzhitov, R., Filavell, R. A. (2002) Hyporesponsiveness to vaccination with Borrelia burgdorferi OspA in humans and in TLR1- and TLR2-deficient mice. Nat. Med. 8, 878–884
10. Ozinsky, A., Underhill, D. M., Fontenot, J. D., Hajjar, A. M., Smith, K. D., Wilson, C. B., Schroeder, L., and Aderem, A. (2000) The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. Proc. Natl. Acad. Sci. U.S.A.

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11. Takeuchi, O., Kawai, T., Mihara, T. F., Moro, M., Radolf, J. D., Zychlinsky, A., Takeda, K., and Akira, S. (2001) Discrimination of bacterial lipoproteins by Toll-like receptor 6. Int. Immunol. 13, 933–940

12. Takeuchi, O., Sato, S., Horuchi, T., Hoshino, K., Takeda, K., Dong, Z., Modlin, R. L., and Akira, S. (2002) Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J. Immunol. 169, 10–14

13. Pietrocola, G., Arciola, C. R., Rindi, S., Di Poto, A., Missineo, A., Montanaro, L., and Speziale, P. (2011) Toll-like receptors (TLRs) in innate immune defense against Staphylococcus aureus. Int. J. Artif Organs 34, 799–810

14. Henneke, P., Dramsi, S., Mancuso, G., Chraibi, K., Pellegrini, E., Theilacker, C., Hubner, J., Santos-Suárez, T., Saito, G., Golenbock, D. T., Poyart, C., and Trieu-Cuot, P. (2006) Lipoproteins are critical TLR2 activating toxins in Streptococcus pyogenes. J. Immunol. 178, 6149–6158

15. Hashimoto, M., Tawaratsumida, K., Kariya, H., Kiyohara, A., Suda, Y., Kirkia, F., Kirkia, T., and Gotz, F. (2006) Not lipoteichoic acid but lipoproteins appear to be the dominant immunobiologically active compounds in Staphylococcus aureus. J. Immunol. 177, 3162–3169

16. Hutchings, M. I., Palmer, T., Harrington, D. J., and Sutcliffe, I. C. (2009) Molecular & Cellular Proteomics 14.8

17. Langlois, D. M., and Andreae, M. (2011) Group A streptococcal infections. Pediatr. Res. 32, 423–429

18. Rosch, J. W., and Caparon, M. G. (2005) The ExPortal: an organelle dedicated to the biogenesis of secreted proteins in Streptococcus pyogenes. Mol. Microbiol. 58, 959–968

19. Kline, K. A., Kau, A. L., Chen, S. L., Lim, A., Pinkner, J. S., Rosch, J. W., Hsu, F. F., and Caparon, M. G. (2007) Anionic lipids with a Hidden Markov Model. Biochem. Sci.

20. Henneke, P., Dramsi, S., Mancuso, G., Chraibi, K., Pellegrini, E., Theilacker, C., Hubner, J., Santos-Suárez, T., Saito, G., Golenbock, D. T., Poyart, C., and Trieu-Cuot, P. (2006) Lipoproteins are critical TLR2 activating toxins in Streptococcus pyogenes. J. Immunol. 178, 6149–6158

21. Enright, P., O’Connell, D., and Tigges, M. (2011) Trends in Biochem. Sci.

22. Rosch, J. W., Hsu, F. S., and Caparon, M. G. (2007) Anionic lipids enriched at the ExPortal of Streptococcus pyogenes. J. Bacteriol. 189, 801–806

23. Lyon, W. R., and Caparon, M. G. (2004) Role for serine protease HtrA (DegP) in the biogenesis of secreted proteins in Streptococcus pyogenes. Mol. Microbiol. 58, 959–968

24. Vega, L. A., Port, G. C., and Caparon, M. G. (2013) An association between peptidoglycan synthesis and organization of the Streptococcus pyogenes ExPortal. mBio 4, e00485–00413

25. Perez-Casal, J., Price, J. A., Maguen, E., and Scott, J. R. (1993) An M protein with a single C repeat prevents phagocytosis of Streptococcus pyogenes by human polymorphonuclear cells. J. Bacteriol. 172, 1625–1629

26. Perkins, D. N., Pappin, D. J., Creasy, D. M., and Cottrell, J. S. (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20, 3561–3567

27. Ferretti, J. J., McShan, W. M., Ajdic, D., Savic, D. J., Savic, G., Lyon, K., Primeaux, C., Sebat, J., Suytorov, A. N., Kenton, S., Lai, H. S., Lin, S. P., Qian, Y., Jia, H. G., Najar, F. Z., Ren, Q., Zhu, H., Song, L., White, J., Yuan, X., Clifton, S. W., Roe, B. A., and McLaughlin, R. (2001) Complete genome sequence of an M1 strain of Streptococcus pyogenes. Proc. Natl. Acad. Sci. U.S.A. 98, 4658–4663

28. Nakai, K., and Horton, P. (1999) PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem. Sci. 24, 34–36

29. Juncker, A. S., Willenbrock, H., Von Heijne, G., Brunak, S., Nielsen, H., and Krogh, A. (2003) Prediction of lipoprotein signal peptides in Gram-positive bacteria. Protein Sci. 12, 1652–1662

30. Madan Babu, M., and Sankaran, K. (2002) DOLOP – database of bacterial lipoproteins. Bioinformatics 18, 641–643

31. Bagos, P. G., Tsinigos, K. D., Liakopoulos, T. D., and Hamodrakas, S. J. (2008) Prediction of lipoprotein signal peptides in Gram-positive bacteria with a Hidden Markov Model. J. Proteome Res. 7, 5082–5093

32. Lei, B., Liu, M., Chesney, G. L., and Musser, J. M. (2004) Identification of a novel candidate vaccine antigens made by Streptococcus pyogenes: purification and characterization of 16 putative extracellular lipoproteins. J. Infect. Dis. 189, 79–89

33. Yu, N. Y., Wagner, J. R., Laird, M. R., Melli, G., Rey, S., Lo, R., Dao, P., Sahinlap, S. C., Ester, M., Foster, L. J., and Brinkman, F. S. (2010) PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. Bioinformatics 26, 1608–1615

34. Bligh, E. G., and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917

35. Lin, N. M., and Speziale, P. (2011) Toll-like receptors (TLRs) in innate immune defense against Staphylococcus aureus. Int. J. Artif Organs 34, 799–810

36. Michelson, M. N. (1964) Chemically defined medium for growth Streptococcus pyogenes. J. Bacteriol. 87, 158–164

37. Fishov, I., and Woldringh, C. L. (1999) Visualization of membrane domains enriched at the ExPortal of Streptococcus pyogenes. J. Bacteriol. 181, 801–806

38. Vanounou, S., Parola, A. H., and Fishov, I. (2003) Phosphatidylethanolamine and phosphatidylglycerol are segregated into different domains in bacterial membranes. J. Bacteriol. 185, 4658–4663

39. Vanounou, S., Parola, A. H., and Fishov, I. (2003) Phosphatidylethanolamine and phosphatidylglycerol are segregated into different domains in bacterial membrane. J. Bacteriol. 185, 4658–4663
50. Olaya-Abril, A., Prados-Rosales, R., McConnell, M. J., Martin-Pena, R., Gonzalez-Reyes, J. A., Jimenez-Munguia, I., Gomez-Gascon, L., Fernandez, J., Luque-Garcia, J. L., Garcia-Lidon, C., Estevez, H., Pachon, J., Obando, I., Casadevall, A., Pirofski, L. A., and Rodriguez-Ortega, M. J. (2014) Characterization of protective extracellular membrane-derived vesicles produced by Streptococcus pneumoniae. *J. Proteomics* **106C**, 46–60

51. Binenbaum, Z., Parola, A. H., Zaritsky, A., and Fishov, I. (1999) Transcription- and translation-dependent changes in membrane dynamics in bacteria: testing the transertion model for domain formation. *Mol. Microbiol.* **32**, 1173–1182

52. Pimenta, A. L., Racher, K., Jamieson, L., Blight, M. A., and Holland, I. B. (2005) Mutations in HlyD, part of the type 1 translocator for hemolysin secretion, affect the folding of the secreted toxin. *J. Bacteriol.* **187**, 7471–7480

53. Matias, V. R., and Beveridge, T. J. (2005) Cryo-electron microscopy reveals native polymeric cell wall structure in Bacillus subtilis 168 and the existence of a periplasmic space. *Mol. Microbiol.* **56**, 240–251

54. Matias, V. R., and Beveridge, T. J. (2007) Cryo-electron microscopy of cell division in Staphylococcus aureus reveals a midzone between nascent cross walls. *Mol. Microbiol.* **64**, 195–206

55. Bisno, A. L., Gerber, M. A., Gwaltney, J. M., Jr., Kaplan, E. L., and Schwartz, R. H. (2002) Practice guidelines for the diagnosis and management of group A streptococcal pharyngitis. Infectious Diseases Society of America. *Clin. Infect. Dis.* **35**, 113–125

56. Dajani, A., Taubert, K., Ferrieri, P., Peter, G., and Shulman, S. (1995) Treatment of acute streptococcal pharyngitis and prevention of rheumatic fever: a statement for health professionals. Committee on rheumatic fever, endocarditis, and kawasaki disease of the council on cardiovascular disease in the young, the american heart association. *Pediatrics* **96**, 758–764

57. Kulp, A., and Kuehn, M. J. (2010) Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annu. Rev. Microbiol.* **64**, 163–184

58. Ellis, T. N., and Kuehn, M. J. (2010) Virulence and immunomodulatory roles of bacterial outer membrane vesicles. *Microbiol. Mol. Biol. Rev.* **74**, 81–94