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

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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, penicillin-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 diacylglyceryl 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 diacylglyceryl 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 diacylglyceryl 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 diacylgyceryl 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 phospholipids 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 (Δ*lgt*) was constructed by splicing-by-overlapping-extension PCR. Briefly, in frame deleted gene product was amplified using the following specific primers: LGT-F1 tcgcggatccAATTATTGAAATTCAGAGATCTT, LGT-R2 AAATGTAACACCTGTTGGATTACTTACAAGAACTGCTGATAA, LGT-F3 TTATCGCAGTTCTTGTAAGTAATCCAACAGGTGTTACATTT, LGT-R4 tggcgagctcTGAATATTATCAAGTGCTGGT. The PCR product was cloned using BamHI and XhoI restriction sites in the temperature-sensitive vector pJRS233 (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 Δ*lgt* mutant were grown in TSB or CDM medium at 37 °C, in a rotary shaker, to reach OD_{600} = 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 Δ*lgt* 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 Laemli 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-

mated 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. pyogen*es genomes (56,403 sequences; 16,305,282 residues). Search parameters were as follows: cleavage by trypsin; missed cleavage, 1; oxidation (M) and deamidation (N,Q) 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 silico* 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/) (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 bacterial cells. Fifteen microliters were collected for subsequent SDS-PAGE analysis. The remaining material was ultracentrifuged at 200,000 $\times$ *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 antisera (1:1000 dilution) in PBS containing 3% (w/v) powdered milk for 90 min at 37 °C. Mouse polyclonal antisera were raised from mouse 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). Membranes were washed three times with PBS containing 0.1% (v/v) Tween-20 and then incubated with sheep anti-mouse horseradish peroxidase-conjugated IgG (GE Healthcare, Little Chalfont, Buckinghamshire, UK) (1:7500 diluted) in PBS containing 3% (w/v) powdered milk. Signals from probed blot were developed with SuperSignal West Pico 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 POLYSINETM 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 plates (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, L-α-phosphatidic acid; PE, 3-*sn*-phosphatidylethanolamine; S, sphingomyelin; PC, L-α-phosphatidylcholine; PG, L-α-phosphatidyl-DL-glycerol; LC, L-α-lysophosphatidylcholine; 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 $\times$ *g* at 4 °C. The supernatant was then high-speed centrifuged at 200,000 $\times$ *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 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 ml of goat anti-mouse IgG, F(ab')2 fragment-specific-R, phycoerythrin-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 FACS 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 PAM_3CSK_4. 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.

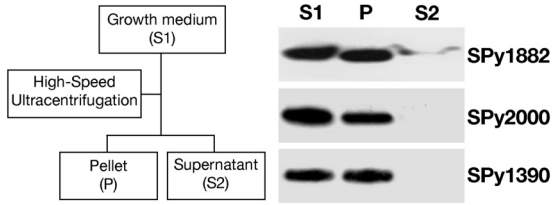

FIG. 1. **Lpps are released into the growth medium as high molecular weight structures.** *S. pyogenes* strain M1–3348 was grown until midexponential phase in THB medium. Growth medium (S1) was high-speed centrifuged producing supernatant (S2) and pellet (P). Aliquots from S1, S2, and P were normalized to the same volume and separated by SDS-PAGE. Proteins were then transferred onto a nitrocellulose membrane and analyzed by Western blot using mouse polyclonal antibodies antiSPy1882, SPy2000, and SPy1390.

RESULTS

S. pyogenes Lpps are Released in the Growth Medium and Penicillin Increases Their Release—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 2*A*). These conditions allowed the recovery of about 1 mg of pelleted proteins from one liter of bacterial culture without observing

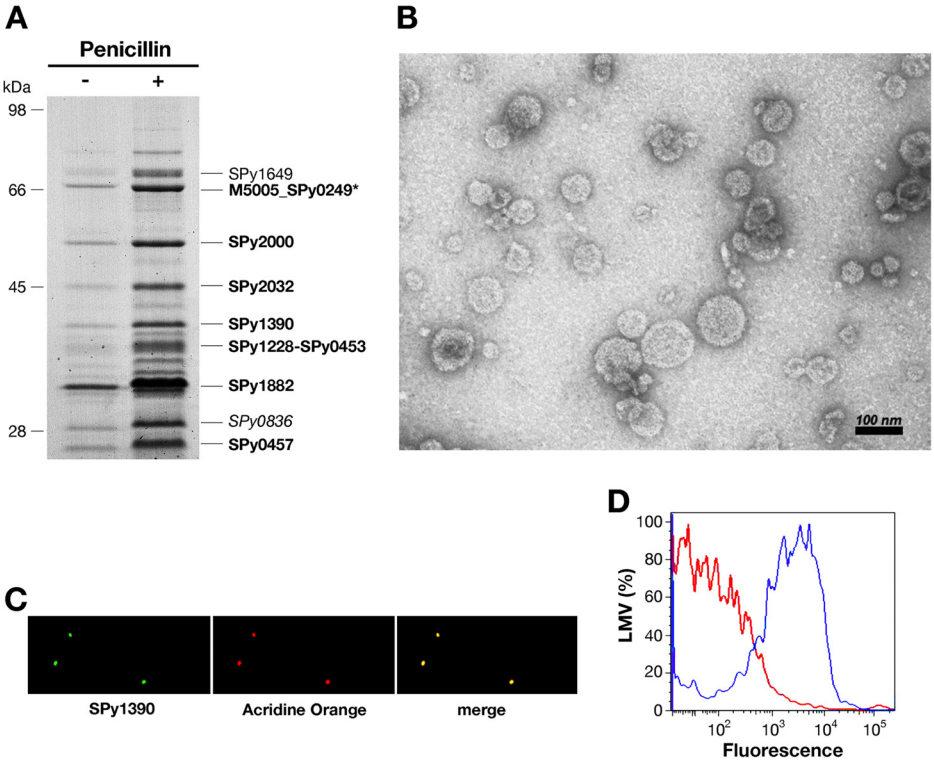

FIG. 2. **Lpps are released as LMVs.** *A*, *S. pyogenes* strain M1–3348 was grown until midexponential phase in THB medium. The culture volume was twofold diluted with prewarmed THB medium without or with penicillin at 0.7 μg/ml final concentration. After 80 min, culture medium was high-speed centrifuged, and pellets was solubilized in PBS and analyzed by SDS-PAGE. Proteins from the major Blue Coomassie stained bands were identified by Peptide Mass Fingerprint. Lpps are reported in bold. *B*, Pellet derived from penicillin treatment (LMVs) was also prepared for negative staining and viewed by electron microscopy. *C*, LMVs were stained with the 10-*n*-nonylacridine orange to evidence anionic lipids (central panel) and with antiLpp peptidylprolyl isomerase (SPy1390) polyclonal antibody (left panel); the merge of the two staining is reported in the right panel, corroborating the association between Lpps and LMVs. *D*, Lpp SPy1390 accessibility on the LMV surface was confirmed by FACS analysis. * Protein identified from the MGAS5005 genome.

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. 2*A* 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. 2*B*). 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-*n*-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. 2*C*). 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. 2*D*).

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

	Lipoprotein prediction tools					
	PSORT	*G+LPP*	*LipoP*	*Lei B et al.*	*DOLOP*	*PredLipo*
Publication year	1999	2002	2003	2004	2006	2008
Number of predicted Lpps	**29**	**28**	**33**	**33**	**31**	**33**
Comments	First Version of Psort	Application of a revised version of Prosite PS0013	Good performaces for Gram positive, although trained against Gram negative	Manual revision of localization prediction from existing software		
Available link						
PSORT	*http://psort.hgc.jp/form.html*					
LipoP	*http://www.cbs.dtu.dk/services/LipoP/*					
DOLOP	*http://www.mrc-lmb.cam.ac.uk/genomes/dolop/*					
PredLipo	*http://biophysics.biol.uoa.gr/PRED-LIPO/*					

TABLE II

List of the Lpps predicted from the M1-SF370 genome. The predicted Lpps are grouped as experimentally identified by mass spectrometry or not. (#) 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	G+LPP	LipoP	Lei B. *et al*	DOLOP	PredLipo
		Lpps identified						
PMF & LC-MS/MS	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				●		
LC-MS/MS	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						
	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**

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	Unkown
	SPy2065	Hypothetical protein	Unkown
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 component E3	Cytoplasmic

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 these criteria. They included the 10 proteins identified from the SDS-PAGE (Fig. 2*A* 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 phosphatidylglycerol marker was more intense

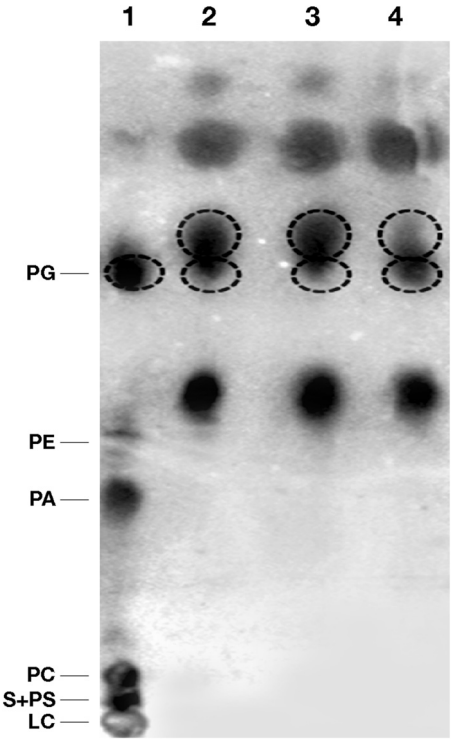

FIG. 3. ***S. pyogenes* membrane and vesicle lipid composition is different.** Lipids were extracted by chloroform/methanol from total extract of bacteria grown in presence (lane 2) or in absence (lane 3) of penicillin or from the purified vesicles (lane 4) and separated by thin layer chromatography. 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-glycero-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 diacylglyceryl transferase was deleted (Δ*lgt*), (supplemental Fig. S4). Because it is now well accepted that the S-diacylglyceryl moiety of the Lpps is responsible for the TLR2 activation (44), the absence of diacylated Lpps in the mutant strain was confirmed by the absence of TLR2 activation by the mutant growth supernatant as previously reported (14) (see below). Wild type and mutant strains were grown in presence of penicillin, and the ultracentrifuged pellets obtained from the same volume of culture were characterized. The electrophoresis patterns of the wild type and mutant pellets were comparable although the amount of proteins isolated from the mutant vesicles was lower (Fig. 4*A*). In order to discriminate if the observed difference was because of a reduction of LMVs release by the mutant or a reduction of LMV protein content, we analyzed both vesicles by FACS (Fig. 4*B*). The same number of events occurred by unit of time indicating that the LMV concentration was comparable. Moreover, wild type and mutant LMV diameters were similar although the heterogeneity of mutant LMV was higher (Fig. 4*B*). The data indicated that the lipidic moiety was not necessary for the formation of the vesicles and the Lpps are presumably shuttled to the LMVs via their signal sequence. The reduced amount of proteins associated to the mutant LMVs could be explained by Lpp release into the growth medium because of their lack of Lpp lipid membrane anchor. To assess this point, the growth medium of Δ*ltg* was ultracentrifuged and the Lpp repartition between pellet (containing the LMVs) and supernatant (soluble free molecule) was assessed by Western blot with the previously used polyclonal sera (antiSPy1390, SPy1882, SPy2000, Fig. 4*C*). Although the Lpps were mainly present in the LMVs derived from the wild type strain (Fig. 1), in the isogenic mutant the three Lpps were either completely (acid phosphatase, SPy1882) or partially (surface lipoprotein, SPy2000 and putative protease maturation protein, SPy1390) found in the high-speed centrifugation supernatant.

LMV-associated Lpps do not Activate the TLR2 Pathway—We assessed if the LMVs could be the structure responsible for TLR2-activation. In order to discriminate the role of the vesicle lipids and/or the lipo- or proteic- moieties of the Lpps, we also tested TLR2-activation by LMVs isolated from Δ*lgt*. HEK-TLR2 cells transfected with a plasmid encoding luciferase gene under the control of NF-kB dependent promoter were incubated with serial dilution of cell-free *S. pyogenes* supernatant. The supernatant of Δ*lgt* 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-diacylglyceryl 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 pellet and 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

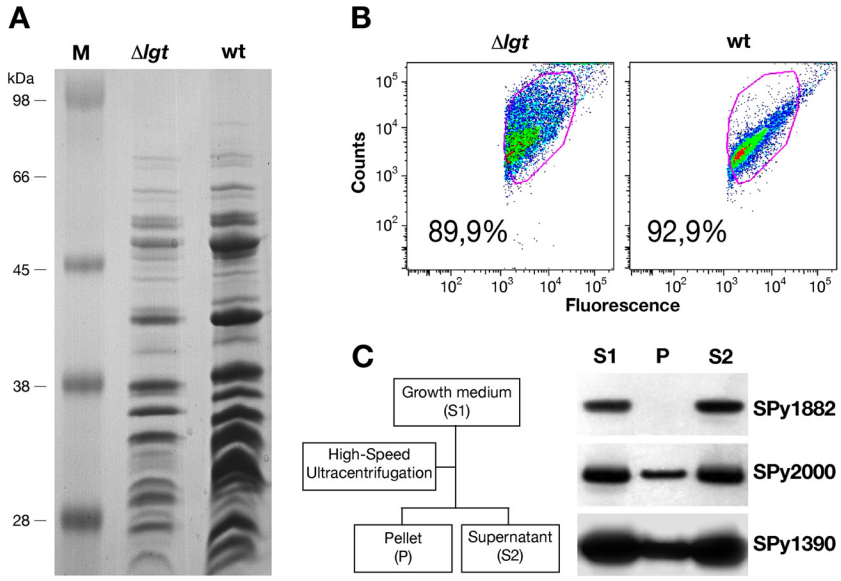

FIG. 4. **The Δ*lgt* mutant strain releases vesicles in the growth medium.** *A*, Vesicles purified from the same culture volume of M1–3348 wild type and Δ*lgt* mutant strains grown in presence of penicillin were analyzed by SDS-PAGE. *B*, The same volumes of vesicles were analyzed by FACS. *C*, Δ*lgt* mutant strain was grown in presence of penicillin and culture medium (S1) was then high speed-centrifuged producing supernatant (S2) and vesicle containing pellet (P). Aliquots from S1, S2, and P were normalized to the same volume and separated by SDS-PAGE. Proteins were then transferred onto a nitrocellulose membrane and analyzed by Western blot using mouse polyclonal antibodies antiSPy1892, SPy2000, and SPy1390.

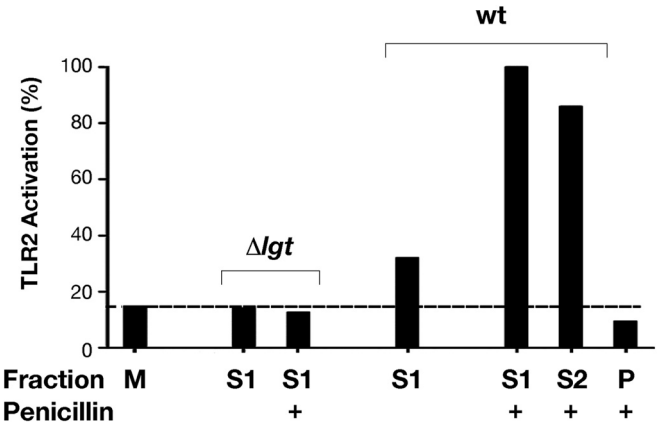

FIG. 5. ***S. pyogenes* LMVs do not activate TLR2.** HEK293-cells stable transfected with FLAG-human TLR2 were stimulated with different dilutions of cell-free culture medium and LMVs purified from M1–3348 wild type and Δ*lgt* mutant strains grown in presence or absence of penicillin. Luciferase expression was measured after 6 h of stimulation. All samples were normalized to the equivalent volume of starting material. TLR2 activation is expressed as percentage of the luciferase expression measured for the LMVs purified from M1–3348 wild type grown in presence of penicillin. S1, S2, and P refer to the starting material and ultracentrifuge fractions as reported in Figs. 1 and 4.

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 Δ*lgt* 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 LMV 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 hypertonic 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 Δ*lgt* 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 of 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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S This article contains supplemental Figs. S1 to S4, Tables S1 and S2 and 5 Data files.

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