Analysis of Outer Membrane Vesicles Indicates That Glycerophospholipid Metabolism Contributes to Early Symbiosis Between *Sinorhizobium fredii* HH103 and Soybean

Dongzhi Li, Ziqi Li, Jing Wu, Zhide Tang, Fuli Xie, Dasong Chen, Hui Lin, and Youguo Li[†]

State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan 430070, People's Republic of China

Accepted 2 January 2022.

Gram-negative bacteria can produce outer membrane vesicles (OMVs), and most functional studies of OMVs have been focused on mammalian-bacterial interactions. However, research on the OMVs of rhizobia is still limited. In this work, we isolated and purified OMVs from *Sinorhizobium fredii* HH103 under free-living conditions that were set as control (C-OMVs) and symbiosis-mimicking conditions that were induced by genistein (G-OMVs). The soybean roots treated with G-OMVs displayed significant deformation of root hairs. G-OMVs significantly induced the expression of nodulation genes related to early symbiosis, while they inhibited that of the defense genes of soybean. Proteomics analysis identified a total of 93 differential proteins between C-OMVs and G-OMVs, which are mainly associated with ribosome synthesis, flagellar assembly, two-component system, ABC transporters, oxidative phosphorylation, nitrogen metabolism, quorum sensing, glycerophospholipid metabolism, and peptidoglycan biosynthesis. A total of 45 differential lipids were identified through lipidomics analysis. Correlation analysis of OMV proteome and lipidome data revealed that glycerophospholipid metabolism is the enriched Kyoto Encyclopedia of Genes and Genomes metabolic pathway, and the expression of phosphatidylserine decarboxylase was significantly up-regulated in G-OMVs. The changes in three lipids related to symbiosis in the glycerophospholipid metabolism pathway were verified by enzyme-linked immunosorbent assay. Our results indicate that glycerophospholipid metabolism contributes to rhizobia-soybean symbiosis via OMVs.

Keywords: correlation analysis, early symbiosis, lipidomics, outer membrane vesicles, proteomics, rhizobia

[†]Corresponding author: Y. Li; youguoli@mail.hzau.edu.cn

Funding: This work was supported by the funds from the National Key Research and Development Program of China (grant no. 2019YFA0904704), the National Natural Science Foundation of China (grant no. 31970267), the National Natural Science Foundation of China for the Youth (grant no. 32000193) and Hubei Natural Science Foundation (grant no. 2020CFA008).

*The e-Xtra logo stands for “electronic extra” and indicates there are supplementary figures and tables published online.

The author(s) declare no conflict of interest.

Rhizobia can form symbiotic structures called nodules with legumes to convert nitrogen in the air into ammonia to provide nitrogen sources for plants; and plants, in turn, provide carbon sources and nutrients for rhizobia (Udvardi and Poole 2013). Stable colonization of rhizobia on plants is a prerequisite for effective nodulation and nitrogen fixation. Symbiosis response between rhizobia and host plants is a key step for the successful infection of rhizobia (Via et al. 2016). However, similar to the infection of pathogens on plants, the infection process of rhizobia on leguminous plants to form nodules will trigger the immune response of the host. During the process of infection, it remains elusive how rhizobia evade the innate immunity and defense response of the host for colonization and intracellular invasion. In recent years, numerous studies have reported the function of Nop proteins of the type III secretion system in the early infection process of rhizobia on legumes (Jiménez-Guerrero et al. 2017, 2020; Sugawara et al. 2018; Xin et al. 2012). Some genes related to lipid synthesis and metabolism have also been reported to play crucial roles in the symbiosis between rhizobia and legumes. For example, the *pmtA* and *pmtX1* genes of *Bradyrhizobium japonicum* encode a phospholipid *N*-methyltransferase, which is essential for the effective symbiosis between *Bradyrhizobium japonicum* and soybean (Hacker et al. 2008). In *Sinorhizobium meliloti*, the *pmtA* and *pcs* genes are key genes in the phosphatidylcholine (PC) methylation pathway and the PC synthase pathway, respectively (de Rudder et al. 1999). In the $\Delta pmtA\Delta pcs$ double mutant of *S. meliloti*, PC was completely absent and the mutant exhibited severe growth defects without the formation of effective nodules (Aktas et al. 2010). In addition, the Δpsd mutant could not synthesize phosphatidylserine (PS) decarboxylase and decarboxylate PS to form phosphatidylethanolamine (PE), resulting in the accumulation of PS during growth, which accounted for about 20% of the total lipids. Compared with the wild type, the Δpsd mutant had fewer nodules and exhibited a delay in nodulation (Vences-Guzmán et al. 2008). Orn lipids (OLs) have been found to play an important role in the symbiosis between *Rhizobium tropici* and *Phaseolus vulgaris* (Vences-Guzmán et al. 2011). $\Delta olsE$ or $\Delta olsC$ could not hydroxylate OLs, leading to serious nodulation defects (Vences-Guzmán et al. 2011). However, to the best of our knowledge, there has been no report on the pathway through which these lipid metabolism-related molecules interact with host plants.

Recently, there has been increasing research on outer membrane vesicles (OMVs) or outer-inner membrane vesicles (O-IMVs) of bacteria. OMVs and O-IMVs are vesicle-like physiological structures containing biologically active substances and are ubiquitous in gram-negative bacteria. They range from

Copyright © 2022 The Author(s). This is an open access article distributed under the CC BY-NC-ND 4.0 International license.

10 to 300 nm in diameter and contain lipopolysaccharides (LPS), outer membrane proteins, peptidoglycans, phospholipids, RNA, toxins, signal molecules, periplasmic components wrapped by the outer membrane during the formation process and some other substances (Chatterjee and Chaudhuri 2011; Ellis and Kuehn 2010; Hagemann et al. 2014; Katsir and Bahar 2017; Kulp and Kuehn 2010; Li et al. 2016; Pérez-Cruz et al. 2015; Vanhove et al. 2015). Currently, some researchers believe that OMVs should be regarded as a secretion system, because some macromolecules can be secreted in the form of OMVs from the inside of the bacteria to the outside and enter the host cell to interfere with the physiological functions of the host. The secretion system is defined as the type 0 secretion system (Guerrero-Mandujano et al. 2017). Studies in the past few decades have demonstrated that almost all gram-negative bacteria can produce OMVs, though most functional studies of OMVs have been carried out on animal pathogens, especially in mammalian-bacterial interactions (Deo et al. 2018, 2020; Koeppen et al. 2016; Yang et al. 2020). Relatively, the interaction between OMVs and plants has been less studied. However, in recent years, many studies have reported that OMVs play various roles in plant-pathogen interactions, such as promoting bacterial virulence (Altindis et al. 2014; Vanhove et al. 2015), mediating cell-to-cell communication (Mashburn and Whiteley 2005), improving tolerance to various stresses (MacDonald and Kuehn 2013; McBroom and Kuehn 2007), facilitating nutrient acquisition (Elhenawy et al. 2014; Lin et al. 2017), and regulating the formation of biofilms (Schooling and Beveridge 2006; Yonezawa et al. 2009). There have been several studies concerning the OMVs of pathogens and their role in mediating plant immune responses in recent years (Bahar et al. 2016; McMillan et al. 2021). For instance, the OMVs of four kinds of plant-pathogenic bacteria, including *Xanthomonas oryzae*, were isolated and were then used to treat *Arabidopsis thaliana*. The leaves, cell cultures, and seedlings of *Arabidopsis* were tested, and the results suggested that OMVs can quickly induce plant active oxygen burst, medium alkalization, and defense gene expression. It was demonstrated that EF-Tu is present in OMVs (Bahar et al. 2016). Recently, it was found that an EF-Tu receptor (EFR) from *Arabidopsis* in sweet orange can sense the EF-Tu present in the OMVs secreted by *Xylella fastidiosa* (Mitre et al. 2021). Besides, a previous study has shown that treatment of *A. thaliana* with OMVs from *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* could activate plant immune response and provide protection against the infection of bacterial and oomycete pathogens (McMillan et al. 2021).

The above research on OMVs has mostly been focused on the interaction between pathogens and plants. However, there has been very limited research on OMVs of symbiotic bacteria such as rhizobia. So far, only two studies have reported OMVs in rhizobia; one study used proteomics to compare the expression profiles of periplasmic space proteins and OMVs of *Rhizobium etli* (Taboada et al. 2019b), and the other study reported the protein profiles of OMVs from *R. etli* before and after naringenin induction (Taboada et al. 2019a). However, the function of rhizobial OMVs remains largely unknown and there has been little research on other components besides proteins in OMVs. From the above review, it can be inferred that the lipids of rhizobia may mediate rhizobia-plant symbiosis through OMVs.

Soybean is an important oil crop worldwide. It can form nodules with soybean rhizobia to fix nitrogen, which can greatly reduce the use of nitrogen fertilizer in agricultural production. Therefore, elucidation of the molecular mechanism of rhizobium-soybean nodulation and its effect on nitrogen fixation is of great significance for improving the efficiency of symbiotic nitrogen fixation, enhancing competitive nodulation, and expanding the host range of nodulation. *Sinorhizobium fredii* HH103 is a model strain of soybean rhizobia, owing to its fast growth rate and clear

genetic background, making it a good material for studying soybean-rhizobium interaction.

Omics methods may help to determine the exact changes in the composition of OMVs from *S. fredii* HH103 under free-living (C-OMVs) and symbiosis-mimicking conditions (G-OMVs). Lipidomics and proteomics can be used to identify lipids, proteins, and associated pathways that are crucial for understanding the components in OMVs from different sources and the mechanisms underlying the effect of OMVs on early symbiotic infection. Most of the current studies of OMVs have been focused on using proteomics to identify the virulence factor genes of gram-negative pathogens (Aguilera et al. 2014; Kunsmann et al. 2015; Park et al. 2011). However, few studies have been focused on the proteins and lipids in C-OMVs and G-OMVs from rhizobia that affect the early rhizobium-soybean symbiotic infection. Recent advances in lipidomics and proteomics technologies greatly facilitate the identification and characterization of natural products and lipids (Ngo et al. 2013; Zeng et al. 2017). In this regard, integrated lipid-proteomics (Haraszi et al. 2016), which couples nontargeted lipidomics with label-free proteomics, is a good method for identifying the lipids or proteins responsible for the different effects of OMVs from different sources on early infection events in soybeans.

Here, we first used the classic methods to isolate, purify, and identify the OMVs of *S. fredii* HH103 and obtained evidence for the existence of OMVs in rhizobia. Then, we conducted an integrated analysis of untargeted lipidomics and label free-based proteomics to comprehensively understand how early rhizobia-legume symbiosis is affected by lipid changes. The findings are expected to reveal how rhizobial lipids and related molecules in their metabolic pathways mediate rhizobium-legume interactions via OMVs and provide new insights into the participation of other biomolecules in the symbiosis process through OMVs.

RESULTS

Isolation, purification, and identification of OMVs from *S. fredii* HH103.

To investigate the role of OMVs secreted by *S. fredii* HH103 in early symbiosis, we first isolated and purified the OMVs secreted by *S. fredii* HH103 under free-living conditions (C-OMVs) and symbiosis-mimicking conditions (G-OMVs). In order to determine the reliability of the symbiosis-mimicking conditions, we added genistein, a flavonoid in soybean, to the medium at a final concentration of 10 μ M and measured the expression of the symbiotic gene *nodABC* at different induction times. The quantitative reverse transcription PCR (qRT-PCR) results revealed that the relative expression of *nodABC* reached the maximum (Supplementary Fig. S1) after 6 h of genistein induction. Transmission electron microscopy (TEM) of C-OMVs and G-OMVs revealed a clear membrane-like structure, indicating that the obtained pellets were free of bacteria and were OMVs of high purity (Fig. 1A and B).

In order to determine the size distribution of OMVs, C-OMVs and G-OMVs were analyzed by dynamic light scattering (DLS). The results showed that the average diameter of C-OMVs was approximately 82 nm, whereas that of G-OMVs was approximately 56 nm (Fig. 1C), which is consistent with the size range of OMVs in gram-negative bacteria (Beveridge 1999), indicating that the average diameter of G-OMVs was smaller than that of C-OMVs. In short, all of the observations above indicated the integrity and purity of OMVs preparation in this work.

OMVs were further used for qualitative and quantitative analysis of their proteins by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and bovine serum albumin (BSA) methods. The Coomassie brilliant blue staining results showed that the protein content and abundance in OMVs was significantly different between the two conditions (Fig. 1D). Then,

the protein concentration of OMVs was measured by the BSA method. The protein concentration of C-OMVs was 81.7 μg per liter per optical density at 600 nm (OD_{600}) unit, while that of G-OMVs was 210.1 μg per liter per OD_{600} unit (Fig. 1E). Thus, the protein concentration of G-OMVs was more than twice that of C-OMVs.

NanoSight NS3000 Nanoparticle Tracking Analyzer (NTA) was used to determine the abundance of OMVs. The results showed that the concentration of G-OMVs was around seven times more than that of C-OMVs. The concentration of G-OMVs was about 11×10^8 particles per liter per OD unit, while that of C-OMVs was about 1.5×10^8 particles per liter per OD unit (Fig. 1F).

Taken together, these results provided direct evidence that soybean rhizobia can produce OMVs under both free-living and

symbiosis-mimicking conditions and the diameter of G-OMVs is less than that of C-OMVs but the number and concentration of proteins were significantly greater than those of C-OMVs.

G-OMV treatment caused significant deformation of soybean root hairs and decreased the expression of defense genes.

In order to investigate the effect of OMVs on soybean early infection events, we soaked the roots of soybean seedlings with the solution prepared with OMVs for 12 h. The results showed that the bacterial solution of *S. fredii* HH103 caused significant curling of root hairs (Fig. 2A) and the morphology of root hairs was not significantly altered after phosphate buffered saline (PBS) and C-OMV treatments (Fig. 2B and C) but was significantly

Fig. 1. Isolation and identification of outer membrane vesicles (OMVs) from *Sinorhizobium fredii* HH103 under different conditions. **A**, Transmission electron microscopy images of negatively stained OMVs (C-OMVs) and **B**, those induced by genistein (G-OMVs). Arrows indicate OMVs. Scale bar = 200 nm. **C**, Size distribution measured by dynamic light scattering of C-OMVs and G-OMVs. **D**, Protein profiles analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of OMVs under different conditions. Molecular weight markers (kDa) are indicated on the left. M = prestained marker, CK = C-OMVs, and Gen = G-OMVs. Three replicates were performed for each set of samples. **E**, The concentration of proteins in OMVs. Two asterisks (**) indicate extremely significant difference at the $P < 0.01$ level (Student's t test). The error bars represent the standard deviations of three independent experiments. **F**, The abundance of OMVs.

deformed after G-OMV treatment (Fig. 2D and E). Moreover, treatment with G-OMVs at the 1× and 5× concentrations significantly induced the deformation of soybean root hairs, but different concentrations C-OMV treatment had no or very weak effect on that of the soybean root hairs (Fig. 2F and G). In addition, we determined the expression levels of symbiosis genes and defense genes in the host plants during early symbiosis after treatment with OMVs. The qRT-PCR results showed that the expression of plant symbiotic gene *NIN* was significantly up-regulated after G-OMV treatment, while that of defense genes *PR1* and *PR5* was significantly down-regulated compared with that under C-OMV treatment (Fig. 2H). The above results indicated that some components in G-OMVs are involved in the early recognition and infection of rhizobia on soybean. Therefore, we next analyzed the main components in OMVs in order to identify the key components that affect the early symbiosis.

Protein profile of OMVs from *S. fredii* HH103.

The C-OMVs and G-OMVs from *S. fredii* HH103 were evaluated by profiling the proteome. Through liquid chromatography-tandem mass spectrometry (LC-MS/MS) identification and a search against the database, we identified 889 unique peptides corresponding to 192 proteins. Based on a cut-off value of 1.5-fold change, the abundance of some proteins was found to be significantly different between C-OMVs and G-OMVs, and a total of 93 differentially expressed proteins (DEPs) were identified. Among these proteins, 82 proteins were up-regulated and 11 proteins were down-regulated in G-OMVs relative to in C-OMVs (Supplementary Table S1). There were 30 DEPs that can be quantified (Table 1).

Based on gene ontology (GO) category enrichment, we then used all 93 DEPs to carry out protein functional analysis using the UniProt Gene Ontology Annotation (GOA) database integrated with InterProScan software. According to their functional characteristics, the 93 DEPs were classified into the biological process (BP), cellular component (CC), and molecular function (MF) categories (Supplementary Fig. S2). The main functional BP categories included organic substance metabolic process, cellular metabolic process, nitrogen compound metabolic process, primary metabolic process, and biosynthetic process, which were partly involved in cellular component biogenesis, regulation of biological process, response to stress and cellular response to stimulus. For the MF proteins, binding, assembly, catalytic activity, and transporter activity were the most abundant categories. The intracellular, cell periphery, plasma membrane, and external encapsulating structure were the most abundant CC categories.

In addition, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed to understand the molecular pathways that contain the DEPs. The 93 proteins were assigned to 36 pathways (Supplementary Table S2). These proteins were mainly distributed in ribosome synthesis, flagellar assembly, two-component system, ABC transporters, oxidative phosphorylation, nitrogen metabolism, quorum sensing, glycerophospholipid metabolism, peptidoglycan biosynthesis and other categories (Fig. 3).

Lipid profile of OMVs from *S. fredii* HH103.

Changes in the lipid profiles of C-OMVs and G-OMVs were analyzed using the lipidomics method based on LC-MS/MS technology. The significantly differential lipids were screened based on the criteria of principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) model VIP ≥ 1 , fold change ≥ 1.2 or ≤ 0.83 and a *P* value < 0.05 . In order to assess the rationality of the candidate lipids and more comprehensively and visually present the link between the lipid profile and the samples, we performed a hierarchical cluster analysis based on the significantly differential lipids in each group of samples. This approach helped to accurately select marker lipids

and investigate changes in related metabolic processes (Fig. 4). Finally, 45 significantly differential lipids were detected (Supplementary Table S3), among which 35 lipids were up-regulated, while 10 were down-regulated in G-OMVs relative to in C-OMVs. These lipids mainly included sphingolipids, saccharolipids, glycerophospholipids, and glycerolipids (Fig. 5A). In lipid subclasses, the levels of monoglyceride and dimethyl PE were significantly down-regulated, whereas those of lyso-phosphatidylinositol, sphingomyelin phytosphingosine, triglyceride (TG), and monoglycosylceramide were significantly up-regulated (Table 2; Supplementary Fig. S3).

We then submitted the differential lipids to the KEGG website for the analysis of relevant pathways and extracted information from pathways that these differential lipids were mainly involved in, the metabolic pathways of glycerophospholipid metabolism, biosynthesis of secondary metabolites, sphingolipid metabolism, glycerolipid metabolism, and alpha-linolenic acid metabolism (Fig. 5B).

Correlation analysis of proteome and lipidome data of OMVs from *S. fredii* HH103.

To analyze the correlation between the significantly differential proteins and significantly differential lipids obtained from the proteomics and lipidomics analysis of OMVs, we took the average of the expression of each omics as the expression value of the group of samples. In order to visually reflect the differences in the expression patterns of significantly differential proteins and lipids, we calculated the Pearson correlation coefficients of expression for the differential proteins and differential lipids and carried out Pearson correlation hierarchical cluster analysis. Then, the R heatmap package was used to visualize the heatmap. There were only two samples of data after taking the average, so the correlation result was +1 or -1 (Supplementary Fig. S4). In order to screen the lipids and proteins with significant differences at key node positions in the network, we used Cytoscape software to perform a Pearson correlation network analysis on the proteins and lipids with significant differences. This coexpression network involved a total of 30 differential proteins and 45 differential lipids, forming a close interaction network. These results indicated that the differential proteins and differential lipids in C-OMVs and G-OMVs have high correlations (Supplementary Fig. S5).

Furthermore, in order to investigate the common metabolic pathways involved in differential proteins and differential lipids, we simultaneously mapped the differential proteins and differential lipids to the KEGG pathway database and obtained information about their common pathways. Then, we conducted a mapping analysis based on the differences in proteins and lipids. Finally, glycerophospholipid metabolism was identified as the KEGG metabolic pathway associated with both differential proteins and differential lipids. When we visualized the glycerophospholipid metabolism, the proteins and lipids with differential abundance were clearly mapped in KEGG. The results showed that one protein and five lipids were involved in glycerophospholipid metabolism. Among them, PS decarboxylase, phosphatidylinositol (PI), and lyso-PC (LPC) were up-regulated in G-OMVs, PS and PC were down-regulated in G-OMVs, and the expression of PE showed no significant change (Supplementary Fig. S6).

Verification of differential lipids in the glycerophospholipid metabolism pathway by enzyme-linked immunosorbent assay (ELISA).

To confirm the changes of representative lipids in the glycerophospholipid metabolism pathway identified above, we used the ELISA method to quantify the abundance of PI, PC, and PS in C-OMVs and G-OMVs. The results showed that, in G-OMVs, PS and PC were dramatically decreased, while PI was significantly increased compared with those in C-OMVs (Fig. 5C, D, and E). These results were consistent with those of the lipidome analysis.

Fig. 2. Effect of outer membrane vesicle (OMV) treatment on the early infection events of soybean seedlings. **A to D**, Root hair morphology of soybean seedlings treated with control and genistein-induced OMVs (C-OMVs and G-OMVs, respectively). Phosphate buffered saline (PBS) and bacterial solution served as negative and positive controls, respectively. Scale bars = 50 μ m. **E**, Statistics on the number of deformed root hairs of soybean seedlings treated with C-OMVs and G-OMVs for 12 h. PBS and bacterial solution served as the negative and positive controls, respectively. **F**, Various concentrations of G-OMVs. **G**, Various concentrations of C-OMVs. PBS served as the control. **H**, Relative transcription levels of symbiosis and defense genes in soybean seedlings after C-OMV and G-OMV treatments. One asterisk (*) indicates significant difference at the $P < 0.05$ level and two asterisks (**) indicate extremely significant difference at the $P < 0.01$ level (Student's t test). Different lower case letters (a, b, c) indicate that the mean values are significantly different from each other by S-N-K test at $P < 0.05$. The error bars represent the standard deviations of three independent experiments.

DISCUSSION

OMVs are a type of vesicle-like physiological structure formed by budding from the cell surface. They are called exosomes in animals (Wu and Liu 2018) and extracellular vesicles in plants (Cai et al. 2018), while, in gram-negative bacteria, they are called OMVs and O-IMVs. OMVs can be found in almost all gram-negative pathogens, such as *P. aeruginosa* (Lin et al. 2017), *Xanthomonas oryzae* (Bahar et al. 2016), and *Neisseria gonorrhoeae* (Deo et al. 2018). OMVs mostly play the role of delivering virulence factors. However, there have been very few reports on the OMVs secreted by symbiotic nitrogen-fixing bacteria. Two studies of OMVs in *R. etli* were published in 2019 in which OMVs of *R. etli* CE3 were isolated by lyophilizing the fermentation supernatant (Taboada et al. 2019a and b).

In this study, we, for the first time, isolated and purified the OMVs produced by soybean rhizobia *S. fredii* HH103 under symbiosis-mimicking conditions. Ultracentrifugation combined with OptiPrep density gradient centrifugation was used to obtain the C-OMVs and G-OMVs of *S. fredii*. The purified OMVs had obvious membrane boundaries, with a diameter range of 20 to 200 nm, which is consistent with the characteristics of gram-negative bacteria previously reported (Roier et al. 2016). Compared with the lyophilization concentration method (Taboada et al. 2019a and b), the modified method resulted in obviously higher membrane integrity and purity of OMVs.

A previous study has reported that the OMVs of pathogenic bacteria can induce protective immunity in *Arabidopsis* (McMillan et al. 2021). In this study, treatment of Zhonghuang 13 soybean seedlings with G-OMVs for 12 h resulted in significant deformation of root hairs. The expression of nodulation genes related to early symbiosis, including *GmNIN*, was induced and expression of the defense genes of soybean was inhibited in response to G-OMV

treatment. All these results suggest that OMVs participate in the early infection process of rhizobia on soybean. It can be speculated that certain components in G-OMVs are involved in the early rhizobium-soybean symbiosis.

Recently, some studies have shown that lipid A remodeling affects the survival of *Burkholderia pseudomallei* in host cells and activates caspase-11-mediated cytotoxicity to promote recognition and infection of *B. pseudomallei* on the host (Norris et al. 2018). It

Fig. 3. Kyoto Encyclopedia of Genes and Genomes pathways of the differentially expressed proteins between control outer membrane vesicles (C-OMVs) and those induced by genistein (G-OMVs).

Table 1. All quantifiable significantly different proteins of control outer membrane vesicles (C-OMVs) and those induced by genistein (G-OMVs)^a

Protein accession	FC	Regulated type	P value	Gene name	Subcellular localization	KO no.
G9AB39	8.494	Up	<0.01	<i>flgG</i>	Extracellular	K02392
G9A4U4	7.184	Up	<0.01	<i>rpsH</i>	Cytoplasmic	K02994
G9AG23	6.371	Up	<0.01	<i>exsH</i>	Extracellular	
G9AFF6	5.579	Up	<0.01	<i>SFHH103_05322</i>	Extracellular	K01406
G9A220	5.243	Up	<0.01	<i>lktA</i>	Outer membrane	
G9A8V7	5.154	Up	0.02	<i>SFHH103_02192</i>	Extracellular	
G9A4V4	5.051	Up	<0.01	<i>rpoA</i>	Cytoplasmic	K03040
G9A285	3.740	Up	<0.01	<i>sucB</i>	Cytoplasmic	K00658
G9A7C3	3.234	Up	<0.01	<i>SFHH103_01656</i>	Extracellular	
G9A4Q3	3.057	Up	0.04	<i>rne</i>	Cytoplasmic	K08300
G9A342	3.053	Up	<0.01	<i>rpsF</i>	Cytoplasmic	K02990
G9A604	2.472	Up	<0.01	<i>rpsA</i>	Cytoplasmic	K02945
G9A4R9	2.372	Up	<0.01	<i>rplA</i>	Cytoplasmic	K02863
G9A4V2	2.093	Up	<0.01	<i>rpsM</i>	Cytoplasmic	K02952
G9A4S2	1.773	Up	<0.01	<i>rpoB</i>	Cytoplasmic	K03043
G9A106	1.738	Up	<0.01	<i>SFHH103_00407</i>	Extracellular	
G9AB33	1.700	Up	<0.01	<i>flgF</i>	Extracellular	K02391
G9A5N8	1.581	Up	<0.01	<i>rpsB</i>	Cytoplasmic	K02967
G9A174	1.502	Up	0.03	<i>groEl</i>	Cytoplasmic	K04077
G9AB47	0.298	Down	<0.01	<i>flaB</i>	Extracellular	K02406
G9A1V6	0.326	Down	<0.01	<i>SFHH103_00546</i>	Outer membrane	
G9AB46	0.350	Down	0.03	<i>flaC</i>	Extracellular	K02406
G9A823	0.402	Down	0.02	<i>ilvC</i>	Cytoplasmic	K00053
G9A1R0	0.432	Down	<0.01	<i>talC3</i>	Cytoplasmic	K00616
G9A4U2	0.466	Down	0.02	<i>rplE</i>	Cytoplasmic	K02931
G9AB57	0.487	Down	<0.01	<i>flgK</i>	Extracellular	K02396
G9ABJ1	0.544	Down	<0.01	<i>cpoB</i>	Periplasmic	
G9A6X8	0.569	Down	<0.01	<i>purC</i>	Cytoplasmic	K01923
G9A4T3	0.592	Down	<0.01	<i>rplB</i>	Periplasmic	K02886
G9A7Y0	0.663	Down	<0.01	<i>SFHH103_01865</i>	Extracellular	

^a The data are the average of three replicates. Fold change (FC) is calculated as the average of the treated group relative to the control group. *P* value (Student's *t* test) < 0.05 is considered statistically significant. The significantly differential lipids were screened based on the criteria of FC ≥ 1.5 or ≤ 0.67 and *P* value < 0.05. KO = Kyoto Encyclopedia of Genes and Genomes Orthology database.

has also been found that LPS or lipid A remodeling in *Francisella* spp. would lead to changes in membrane integrity and fluidity, affecting the survival and virulence of bacteria (Li et al. 2012). In this study, we analyzed the lipid profile of OMVs from *S. fredii* HH103 and found that the lipid composition of G-OMVs and C-OMVs was significantly different. Specifically, G-OMVs had higher levels of TG and LPC and lower levels of PC and PS. Therefore, lipid remodeling may affect the recognition of the host plant on OMVs, thereby reducing the immune response of the host plant and promoting the early symbiosis between rhizobia and soybean. Moreover, it has been shown that plants have pattern recognition receptors to recognize LPS. For instance, the plant *Arabidopsis thaliana* specifically sensed LPS of *Pseudomonas* and *Xanthomonas* species, and the LPS receptor is a lectin S-domain-1 receptor-like kinase LORE (SD1-29) (Ranf et al. 2015).

Glycerophospholipids play an important role in the survival of bacteria and their interaction with the host. It has been found that lack of PE is lethal to *Escherichia coli*, and supplementation of divalent cations in the medium can inhibit this lethality (Shi et al. 1993). Moreover, PE is a precursor for many important virulence factors, including LPS (Ham et al. 2011; Raetz et al. 2007). Phosphatidylglycerol (PG) plays an essential role in bacterial assembly. Previous studies have shown that treatment of

Pseudomonas spp. with toxic organic solvents would change the content of PG in the cell membrane (Fang et al. 2000; Murzyn et al. 2005). Phosphatidylcholine (PC) can be synthesized in at least 15% of bacteria, such as *Sinorhizobium meliloti*, *Bradyrhizobium japonicum*, and *Pseudomonas* spp. (de Rudder et al. 2000; Kondakova et al. 2015; Minder et al. 2004). PC plays an important role in the cell membrane formation of *P. aeruginosa* and participates in the resistance of *P. putida* to heavy metals (Boeris and Lucchesi 2012). Notably, the integrated analysis of proteomics and lipidomics revealed that glycerophospholipid metabolism is the KEGG metabolic pathway shared by differential proteins and lipids. Therefore, glycerophospholipid metabolism in rhizobia may be involved in the rhizobium-soybean interaction.

Importantly, the expression of PS decarboxylase proenzyme (PsdA) was significantly up-regulated in G-OMVs while that of PS was down-regulated, which is consistent with findings in *S. meliloti* (Vences-Guzmán et al. 2008). In *S. meliloti*, the Δpsd mutant could not decarboxylate PS with a delayed nodulation phenotype, there were almost no bacteroids in the root nodules, and the mutant could not fix nitrogen (Vences-Guzmán et al. 2008). Our research and previous studies together demonstrate that PS decarboxylase plays an important role in early rhizobia-legume symbiosis via OMVs.

Fig. 4. Hierarchical clustering heat map of differential lipids between control outer membrane vesicles (C-OMVs) and those induced by genistein (G-OMVs).

Interestingly, the Tol-Pal system proteins TolB and Pal were also found to be significantly upregulated in G-OMVs. TolB was identified in the OMVs of *R. etli* CE3 treated with and without exogenous naringenin (Taboada et al. 2019a). Recent studies have reported that, in *S. meliloti*, the Tol-Pal system proteins can interact with the polar growth factor Rgs proteins, which is indispensable for maintaining the normal growth and rod-shaped cell morphology of *S. meliloti* (Krol et al. 2020). In addition, it has been found that *tolB* or *pal* mutants of *Escherichia coli* decreased the motility and the ability to adhere and form attaching and effacing lesions in host cells (Hirakawa et al. 2020). Moreover, the *tolB* mutant of *Citrobacter rodentium* exhibits low virulence in mice (Hirakawa et al. 2020). It can be speculated that the interaction between the Tol-Pal proteins in rhizobia and the host can promote the infection of rhizobia on the host plant in early symbiosis.

In summary, the combined proteomics and lipidomics analysis of C-OMVs and G-OMVs revealed the enrichment of glycerophospholipid metabolism pathway and that PS decarboxylase probably plays an important role in early rhizobia-soybean symbiosis. This study, for the first time, reports the lipid profile of rhizobial OMVs and its relationship with the protein profile and elucidates that the PS decarboxylase in rhizobia mediates the rhizobia-legume interaction via the OMVs. In addition, our findings provide a perspective for studying other biomolecules such as LPS and peptidoglycan that participate in rhizobia-legume symbiosis through OMVs.

MATERIALS AND METHODS

Bacterial strain and growth conditions.

Sinorhizobium fredii HH103 was cultured at 28°C in tryptone yeast (TY) medium. TY medium was supplemented by 100 µg of streptomycin per milliliter and 10 µM genistein.

Isolation and purification of OMVs secreted by *S. fredii* HH103.

S. fredii HH103 was cultured in 1 liter of TY liquid medium at 28°C, with shaking, at 220 rpm. When reaching the midlogarithmic phase, 10 µM genistein (symbiosis-mimicking conditions) or an equal volume of absolute ethanol (free-living conditions) was added to the culture, and the culture was then continued for 6 h under the same conditions. Isolation and purification of OMVs were carried out at 4°C, as previously described with minor modifications (Deo et al. 2018). After centrifugation ($3,841 \times g$ for 20 min at 4°C), the supernatant was passed through microporous membrane (0.45 and 0.22 µm) to remove any remaining rhizobium cells and cellular debris. The filtered supernatant was then transferred to an ultrafiltration centrifuge tube with a molecular weight cutoff of 100 kDa ($4,000 \times g$, 10 min), repeated several times until the final liquid volume reached about 50 ml. The concentrated liquid was then transferred to a sterile ultracentrifugation tube and was ultracentrifuged, to pellet OMVs using a SW32 Ti fixed angle rotor (L-90K centrifuge; Beckman Coulter) at $265,000 \times g$ for 1 h at 4°C. The pelleted OMVs were

Fig. 5. Lipid classification and Kyoto Encyclopedia of Genes and Genomes pathway analysis. **A**, Classification of the significantly differential lipids between control outer membrane vesicles (C-OMVs) and those induced by genistein (G-OMVs). The 45 differential lipids are involved in sphingolipids (4), saccharolipids (2), glycerophospholipids (10), and glycerolipids (29). **B**, Signaling pathways of the differential lipids involved in C-OMVs and G-OMVs. MP = metabolic pathways, GPM = glycerophospholipid metabolism, BSM = biosynthesis of secondary metabolites, SPM = sphingolipid metabolism, GLM = glycerolipid metabolism, ALAM = alpha-linolenic acid metabolism. **C to E**, Contents of phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylcholine (PC) detected in an enzyme-linked immunosorbent assay. Two asterisks (**) indicate extremely significant difference at the $P < 0.01$ level (Student's *t* test). The error bars represent the standard deviations of three independent experiments.

washed once with PBS to remove liquid medium and soluble proteins. In order to isolate OMVs of higher purity, crude OMVs were then subjected to OptiPrep density gradient ultracentrifugation, by mixing with 60% (wt/vol) Optiprep to acquire a final suspension concentration of 55% OMVs. The OptiPrep OMVs suspension (8 ml) was then laid at the bottom of a SW32 Ti centrifuge tube (Beckman Coulter) and 5 ml of discontinuous OptiPrep gradient (50, 45, 40, 35, 30, and 25%), decreasing from the bottom to the top, were centrifuged at $265,000 \times g$ for 16 h, at 4°C , using a SW32 Ti rotor. The purified OMVs accumulated at the 35 to 40% interface were collected, were resuspended in PBS, were pelleted by ultracentrifugation at $265,000 \times g$ for 1 h, at 4°C , and were used immediately or kept at -80°C . The protein concentration of the prepared OMVs was measured using the BSA method (Thermo Scientific). OMVs were subjected to β -mercaptoethanol denaturation and were then analyzed by SDS-PAGE.

TEM and DLS.

Formvar-coated (300 mesh) copper grids were placed on a drop of OMV suspension (20 μl) for 10 min, followed by staining with 2% phosphotungstic acid for 15 min. The grids were then air-dried and viewed with a Hitachi (Tokyo) transmission electron microscope (H-7650) at 100 kV, and images were acquired using TIA software (FEI Melbourne).

The particle size of the OMVs was measured by DLS. Samples were diluted at 1:1,000 in PBS to a total volume of 1.5 ml and the scatter was recorded, using a Zetasizer Nano ZS (Malvern Instruments) to obtain the average hydrodynamic radius.

Treatment of the roots of soybean seedlings by OMVs.

Large and plump Zhonghuang 13 soybean seeds were picked, were sterilized by 75% ethanol and 1% sodium hypochlorite solution, and were washed about ten times with distilled water. The sterilized seeds were spread on a 1% water agar plate for germination at 28°C . Upon observation twice a day, the germinated seeds were picked out in time and were stored at 4°C . Then, the roots of the germinated soybean seedlings were soaked for 12 h in the solution prepared with OMVs, and the changes in root hairs were observed and were photographed with an upright microscope. The statistical analysis of the

number of deformed root hairs was performed by randomly choosing five seedlings to count the total number, with three independent replicates for each treatment group.

qRT-PCR.

Total RNA was extracted from soybean roots treated with OMVs. The roots were harvested and total RNA was extracted by the TRIzol Reagent (Invitrogen), according to the protocol, and then, genomic DNA was removed with DNaseI (Fermentas) and immediately frozen in liquid nitrogen and was stored at -80°C . The complementary DNA was synthesized using random primers and qRT-PCR was performed using the SYBR Green Master kits (Roche). The data were analyzed using the $2^{-\Delta\Delta\text{CT}}$ method with *Tef* as the internal reference gene. Three biological replicates were carried out for each experiment. $P < 0.05$ or $P < 0.01$ (Student's *t* test) was considered statistically significant.

Measurement of glycerophospholipid content.

C-OMVs and G-OMVs samples were prepared according to the method described above and the content of glycerophospholipids was determined by ELISA. The experimental procedure followed the protocol of the ELISA kit, which was obtained from Shanghai Xinyu Biotechnology Co. Ltd.

Protein extraction and digestion.

Purified C-OMVs and G-OMVs were sonicated three times on the ice by a high intensity ultrasonic processor (Scientz) with a volume of lysis buffer of four folds (8 M urea, 1% protease inhibitor cocktail). Centrifugation at $12,000 \times g$ and 4°C for 10 min was performed to remove the remaining debris. Finally, the supernatant was collected and the protein concentration was measured with a BCA kit according to manufacturer instructions. For digestion, the protein solution was reduced with 5 mM dithiothreitol for 30 min at 56°C and was alkylated with 11 mM iodoacetamide for 15 min at room temperature in darkness. The protein sample was then diluted by adding 100 mM TEAB to urea concentration lower than 2 M. Finally, trypsin was added at a 1:50 trypsin-to-protein mass ratio for the first digestion overnight and a 1:100 trypsin-to-protein mass ratio for 4 h of second digestion.

LC-MS/MS analysis.

The tryptic peptides were dissolved in 0.1% formic acid (solvent A), directly loaded onto a home-made reversed-phase analytical column (15 cm length, 75 μm i.d.). The gradient comprised an increase from 5 to 25% solvent B (0.1% formic acid in 90% acetonitrile [ACN]) over 90 min, 25 to 35% in 22 min, and climbing to 80% in 4 min, then holding at 80% for the last 4 min, all at a constant flow rate of 500 nL/min on an EASY-nLC 1000 ultra-performance liquid chromatography (UPLC) system.

The peptides were subjected to nano-spray ionization source, followed by MS/MS in Q Exactive Plus (Thermo Scientific) coupled online to the UPLC. The applied electrospray voltage was 2.1 kV. The *m/z* scan range was 350 to 1,800 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were then selected for MS/MS, using a normalized collision energy (NCE) setting of 28, and the fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure alternated between one MS scan followed by ten MS/MS scans with 30-s dynamic exclusion. Automatic gain control (AGC) was set at 5E4, and the fixed first mass was set as 100 *m/z*.

Database search.

The obtained MS/MS data were processed using the Maxquant search engine (v.1.5.2.8). Tandem mass spectra were searched against the *Rhizobium fredii* strain HH103_1117943_PR_20191127 (6,073 sequences) database concatenated with the

Table 2. Different lipid subclasses of control outer membrane vesicles (C-OMVs) and those induced by genistein (G-OMVs)^a

Lipid subclasses	FC	P value
Lysyl-phosphatidylglycerol (LPG)	0.90	>0.05
Triglyceride (TG)	1.44	<0.001
Sulfoquinovosyl diacylglycerol (SQDG)	0.89	<0.001
Phosphatidylethanolamine (PE)	0.87	<0.001
Monosialodihexosylganglioside (GM3)	0.96	>0.05
Lyso-phosphatidylcholine (LPC)	1.18	<0.001
Monoglyceride (MG)	0.73	<0.001
Phosphatidylinositol (PI)	0.99	>0.05
Phosphatidylserine (PS)	0.86	<0.001
Monoglycosylceramide (CerG1)	1.28	<0.001
Lyso-PI (LPI)	2.56	<0.001
Phosphatidylcholine (PC)	0.90	<0.001
Dihexosylceramides (CerG2)	0.89	<0.001
Dimethyl PE	0.80	<0.001
Ceramides (Cer)	1.18	<0.001
Sphingomyelin phytosphingosine (phSM)	1.50	<0.001
Sphingomyelin (SM)	0.99	>0.05
Phosphatidylglycerol (PG)	0.91	<0.001
Digalactosyldiacylglycerol (DGDG)	0.96	>0.05

^a Data are the average of seven replicates. Fold change (FC) is calculated as the average of the treated group relative to the control group. *P* value (Student's *t* test) < 0.05 is considered statistically significant. The significantly differential lipids were screened based on the criteria of $\text{FC} \geq 1.2$ or ≤ 0.83 and *P* value < 0.05.

reverse decoy database. Trypsin/P was specified as cleavage enzyme, allowing up to two missing cleavages. The mass tolerance for precursor ions was set as 20 ppm in the First search and 5 ppm in the Main search, and the mass tolerance for fragment ions was set as 0.02 Da. Carbamidomethyl on Cys was specified as fixed modification, and acetylation modification and oxidation on Met were specified as variable modifications. The false discovery rate (FDR) was adjusted to <1% and the minimum score for modified peptides was set >40.

Lipid extraction.

Purified C-OMVs and G-OMVs were thawed on ice until no ice was observed in the tubes. The lipid extraction was performed following a previous method with minor modifications (Sarafian et al. 2014). Briefly, 100 µl of OMVs was extracted with 300 µl of precooled isopropanol (IPA) and 10 µl of SPLASH internal standard stock and was then vortexed for 1 min and incubated for 10 min at room temperature. The mixture was then stored overnight at -20°C to promote protein precipitation. Samples were centrifuged for 20 min at 1,707 × g, and the supernatant was resuspended with IPA/ACN/H₂O (2:1:1 vol/vol/vol) and was stored at -80°C until LC-MS analysis. Equal amounts of all samples were combined as a QC sample for LC-MS system adjustment and quality control (Dunn et al. 2011).

UPLC-MS/MS analysis and data processing.

Chromatographic conditions were as follows. The chromatographic column used was a CSH C18 chromatographic column (1.7 µm, 2.1 × 100 mm; Waters). The mobile phase in the positive ion mode was an aqueous solution containing 10 mM ammonium formate, 0.1% formic acid, and 60% ACN (liquid A), and a solution containing 10 mM ammonium formate, 0.1% formic acid, 90% isopropanol, and 10% ACN (liquid B). The mobile phase in negative ion mode was an aqueous solution containing 10 mM ammonium formate and 60% ACN (liquid A) and a solution containing 10 mM ammonium formate, 90% isopropanol, and 10% ACN (liquid B). The following gradient was used for elution: 0 to 2 min, 40 to 43% solution B; 2 to 2.1 min, 43 to 50% solution B; 2.1 to 7 min, 50 to 54% solution B; 7 to 7.1 min, 54 to 70% solution B; 7.1 to 13 min, 70 to 99% solution B; 13 to 13.1 min, 99 to 40% solution B, 13.1 to 15 min, 40% solution B. The flow rate was 0.35 ml/min, the column temperature was 55°C, and the injection volume was 5 µl. Mass spectrometry conditions were as follows. A Q Exactive mass spectrometer (Thermo Fisher Scientific) was used to collect primary and secondary MS data. The mass-to-nucleus ratio of the MS scan range was 200 to 2,000, the first-order resolution was 70,000, the AGC was 3e6, and the maximum injection time was 100 ms. According to the precursor ion intensity, Top3 was selected for fragmentation and the secondary information was collected. The secondary resolution was 17,500; AGC was 1e5; maximum injection time was 50 ms; and the fragmentation energy (stepped NCE) was set to 15, 30, and 45 eV. Ion source (electrospray ionization) parameter settings were: Sheath gas flow rate, 40; Aux gas flow rate, 10; spray voltage (kV) positive ion mode, 3.80; negative ion mode, 3.20; ion transfer tube temperature (Capillary temp), 320°C; and auxiliary gas heater temperature (Aux gas heater temp), 350°C. During instrument testing, in order to provide more reliable experimental results, the samples were randomly sorted to reduce systematic errors. Every 10 samples were interspersed with a QC sample for testing.

The raw data exported by LipidSearch were imported into metaX (Wen et al. 2017) for data preprocessing and subsequent analysis. The data preprocessing included the following steps. First, lipid molecules that were missing in >50% of QC samples and >80% of the experimental samples were deleted; second, the k-nearest neighbor algorithm was used to fill in the missing

values; third, probabilistic quotient normalization was used to normalize the data to obtain the relative peak area; finally, in all QC samples, the relative peak area coefficient of variation >30% of lipid molecules was deleted.

Statistical analysis.

Multivariate statistical analysis and univariate analysis were combined to screen differential lipids between groups. The multivariate statistical analysis methods included PCA and PLS-DA, and the univariate analysis included fold change and Student's *t* test. PCA between the comparative analysis groups (two sets of samples) was calculated, and the distribution and separation trends of the two sets of samples were observed. The PCA was calculated after log₂ logarithmic transformation of the data, and the scaling method was Par. After log₂ logarithmic transformation of the data, the PLS-DA model between the comparison groups (two sets of samples) was established, and the scaling method was Par. Sevenfold cross-validation was performed when building the model. In order to test the quality of the model, 200 times of response permutation testing were performed on the PLS-DA model. Analysis of fold changes and *t* test were performed on the data, and the *P* value pairs of the *t* test were corrected by FDR to obtain *t*.test_p.value_BHcorrect (*q*-value).

Bioinformatics analysis.

GO on proteomic annotations was derived from the UniProt-GOA database. First, the system converts the protein ID to UniProt ID, then uses UniProt ID to match GO ID, and retrieves the corresponding information from the UniProt-GOA database according to GO ID. If there is no protein information in the UniProt-GOA database, an algorithm software based on protein sequence, InterProScan, will be used to predict the GO function of the protein. These proteins are then classified according to CC, MF, or BP. KEGG can combine currently known protein interaction network information, such as pathways and related complexes (the "Pathway" database), genes and gene products (the "Gene" database), biological complexes and relevant reactions ("compound and reaction" database), and other information. The KEGG pathway database is used to annotate protein and lipid pathways. First, the KEGG online service tool KAAS is used to annotate the submitted proteins and lipids, and KEGG mapper is then used to match the annotated proteins or lipids into the corresponding pathways in the database.

ACKNOWLEDGMENTS

We thank Y. Ai from the College of Plant Science and Technology of Huazhong Agricultural University and Y. Liu and W. Song from PTM Bio for their kind help in data analysis and preparation of figures.

AUTHOR-RECOMMENDED INTERNET RESOURCES

InterProScan software: <http://www.ebi.ac.uk/interpro>

UniProt Gene Ontology Annotation database: <https://www.ebi.ac.uk/GOA>

LITERATURE CITED

- Aguilera, L., Toloza, L., Giménez, R., Odena, A., Oliveira, E., Aguilar, J., Badia, J., and Baldomà, L. 2014. Proteomic analysis of outer membrane vesicles from the probiotic strain *Escherichia coli* Nissle 1917. *Proteomics* 14:222-229.
- Aktas, M., Wessel, M., Hacker, S., Klüsener, S., Gleichenhagen, J., and Narberhaus, F. 2010. Phosphatidylcholine biosynthesis and its significance in bacteria interacting with eukaryotic cells. *Eur. J. Cell Biol.* 89:888-894.
- Altindis, E., Fu, Y., and Mekalanos, J. J. 2014. Proteomic analysis of *Vibrio cholerae* outer membrane vesicles. *Proc. Natl. Acad. Sci. U.S.A.* 111:E1548-E1556.

- Bahar, O., Mordukhovich, G., Luu, D. D., Schwessinger, B., Daudi, A., Jehle, A. K., Felix, G., and Ronald, P. C. 2016. Bacterial outer membrane vesicles induce plant immune responses. *Mol. Plant-Microbe Interact.* 29:374-384.
- Beveridge, T. J. 1999. Structures of gram-negative cell walls and their derived membrane vesicles. *J. Bacteriol.* 181:4725-4733.
- Boeris, P. S., and Lucchesi, G. I. 2012. The phosphatidylcholine synthase of *Pseudomonas putida* A ATCC 12633 is responsible for the synthesis of phosphatidylcholine, which acts as a temporary reservoir for Al3+. *Microbiology (Reading)* 158:1249-1257.
- Cai, Q., Qiao, L., Wang, M., He, B., Lin, F. M., Palmquist, J., Huang, S. D., and Jin, H. 2018. Plants send small RNAs in extracellular vesicles to fungal pathogen to silence virulence genes. *Science* 360:1126-1129.
- Chatterjee, D., and Chaudhuri, K. 2011. Association of cholera toxin with *Vibrio cholerae* outer membrane vesicles which are internalized by human intestinal epithelial cells. *FEBS Lett.* 585:1357-1362.
- de Rudder, K. E., López-Lara, I. M., and Geiger, O. 2000. Inactivation of the gene for phospholipid N-methyltransferase in *Sinorhizobium meliloti*: Phosphatidylcholine is required for normal growth. *Mol. Microbiol.* 37:763-772.
- de Rudder, K. E., Sohlenkamp, C., and Geiger, O. 1999. Plant-exuded choline is used for rhizobial membrane lipid biosynthesis by phosphatidylcholine synthase. *J. Biol. Chem.* 274:20011-20016.
- Deo, P., Chow, S. H., Han, M. L., Speir, M., Huang, C., Schittenhelm, R. B., Dhital, S., Emery, J., Li, J., Kile, B. T., Vince, J. E., Lawlor, K. E., and Naderer, T. 2020. Mitochondrial dysfunction caused by outer membrane vesicles from gram-negative bacteria activates intrinsic apoptosis and inflammation. *Nat. Microbiol.* 5:1418-1427.
- Deo, P., Chow, S. H., Hay, I. D., Kleinfeld, O., Costin, A., Elgass, K. D., Jiang, J. H., Ramm, G., Gabriel, K., Dougan, G., Lithgow, T., Heinz, E., and Naderer, T. 2018. Outer membrane vesicles from *Neisseria gonorrhoeae* target PorB to mitochondria and induce apoptosis. *PLoS Pathog.* 14:e1006945.
- Dunn, W. B., Broadhurst, D., Begley, P., Zelena, E., Francis-McIntyre, S., Anderson, N., Brown, M., Knowles, J. D., Halsall, A., Haselden, J. N., Nicholls, A. W., Wilson, I. D., Kell, D. B., and Goodacre, R.; Human Serum Metabolome (HUSERMET) Consortium. 2011. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. *Nat. Protoc.* 6:1060-1083.
- Elhenawy, W., Debelyy, M. O., and Feldman, M. F. 2014. Preferential packing of acidic glycosidases and proteases into *Bacteroides* outer membrane vesicles. *MBio* 5:e00909-e00914.
- Ellis, T. N., and Kuehn, M. J. 2010. Virulence and immunomodulatory roles of bacterial outer membrane vesicles. *Microbiol. Mol. Biol. Rev.* 74:81-94.
- Fang, J., Barcelona, M. J., and Alvarez, P. J. 2000. Phospholipid compositional changes of five pseudomonad archetypes grown with and without toluene. *Appl. Microbiol. Biotechnol.* 54:382-389.
- Guerrero-Mandujano, A., Hernández-Cortez, C., Ibarra, J. A., and Castro-Escarpullí, G. 2017. The outer membrane vesicles: Secretion system type zero. *Traffic* 18:425-432.
- Hacker, S., Gödeke, J., Lindemann, A., Mesa, S., Pessi, G., and Narberhaus, F. 2008. Global consequences of phosphatidylcholine reduction in *Bradyrhizobium japonicum*. *Mol. Genet. Genomics* 280:59-72.
- Hagemann, S., Stöger, L., Kappellmann, M., Hassl, I., Ellinger, A., and Velimirov, B. 2014. DNA-bearing membrane vesicles produced by *Ahrensia kielensis* and *Pseudoalteromonas marina*. *J. Basic Microbiol.* 54:1062-1072.
- Ham, H., Sreelatha, A., and Orth, K. 2011. Manipulation of host membranes by bacterial effectors. *Nat. Rev. Microbiol.* 9:635-646.
- Haraszti, R. A., Didiot, M. C., Sapp, E., Leszyk, J., Shaffer, S. A., Rockwell, H. E., Gao, F., Narain, N. R., DiFiglia, M., Kiebish, M. A., Aronin, N., and Khvorova, A. 2016. High-resolution proteomic and lipidomic analysis of exosomes and microvesicles from different cell sources. *J. Extracell. Vesicles* 5:32570.
- Hirakawa, H., Suzue, K., Takita, A., Awazu, C., Kurushima, J., and Tomita, H. 2020. Roles of the Tol-Pal system in the type III secretion system and flagella-mediated virulence in enterohemorrhagic *Escherichia coli*. *Sci. Rep.* 10:15173.
- Jiménez-Guerrero, I., Acosta-Jurado, S., Medina, C., Ollero, F. J., Alias-Villegas, C., Vinardell, J. M., Pérez-Montaña, F., and López-Baena, F. J. 2020. The *Sinorhizobium fredii* HH103 type III secretion system effector NopC blocks nodulation with *Lotus japonicus* Gifu. *J. Exp. Bot.* 71:6043-6056.
- Jiménez-Guerrero, I., Pérez-Montaña, F., Medina, C., Ollero, F. J., and López-Baena, F. J. 2017. The *Sinorhizobium (Ensifer) fredii* HH103 nodulation outer protein NopI is a determinant for efficient nodulation of soybean and cowpea plants. *Appl. Environ. Microbiol.* 83:e02770-16.
- Katsir, L., and Bahar, O. 2017. Bacterial outer membrane vesicles at the plant-pathogen interface. *PLoS Pathog.* 13:e1006306.
- Koeppen, K., Hampton, T. H., Jarek, M., Scharfe, M., Gerber, S. A., Mielcarz, D. W., Demers, E. G., Dolben, E. L., Hammond, J. H., Hogan, D. A., and Stanton, B. A. 2016. A novel mechanism of host-pathogen interaction through sRNA in bacterial outer membrane vesicles. *PLoS Pathog.* 12:e1005672.
- Kondakova, T., D'Heygère, F., Feuilloley, M. J., Orange, N., Heipieper, H. J., and Duclairoir Poc, C. 2015. Glycerophospholipid synthesis and functions in *Pseudomonas*. *Chem. Phys. Lipids* 190:27-42.
- Krol, E., Yau, H. C. L., Lechner, M., Schäper, S., Bange, G., Vollmer, W., and Becker, A. 2020. Tol-Pal system and Rgs proteins interact to promote unipolar growth and cell division in *Sinorhizobium meliloti*. *MBio* 11:e00306-20.
- Kulp, A., and Kuehn, M. J. 2010. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annu. Rev. Microbiol.* 64:163-184.
- Kunsmann, L., Rüter, C., Bauwens, A., Greune, L., Glüder, M., Kemper, B., Fruth, A., Wai, S. N., He, X., Llobes, R., Schmidt, M. A., Dobrindt, U., Mellmann, A., Karch, H., and Bielaszewska, M. 2015. Virulence from vesicles: Novel mechanisms of host cell injury by *Escherichia coli* O104:H4 outbreak strain. *Sci. Rep.* 5:13252.
- Li, J., Azam, F., and Zhang, S. 2016. Outer membrane vesicles containing signalling molecules and active hydrolytic enzymes released by a coral pathogen *Vibrio shilonii* AK1. *Environ. Microbiol.* 18:3850-3866.
- Li, Y., Powell, D. A., Shaffer, S. A., Rasko, D. A., Pelletier, M. R., Leszyk, J. D., Scott, A. J., Masoudi, A., Goodlett, D. R., Wang, X., Raetz, C. R., and Ernst, R. K. 2012. LPS remodeling is an evolved survival strategy for bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 109:8716-8721.
- Lin, J., Zhang, W., Cheng, J., Yang, X., Zhu, K., Wang, Y., Wei, G., Qian, P. Y., Luo, Z. Q., and Shen, X. 2017. A *Pseudomonas* T6SS effector recruits PQS-containing outer membrane vesicles for iron acquisition. *Nat. Commun.* 8:14888.
- MacDonald, I. A., and Kuehn, M. J. 2013. Stress-induced outer membrane vesicle production by *Pseudomonas aeruginosa*. *J. Bacteriol.* 195:2971-2981.
- Mashburn, L. M., and Whiteley, M. 2005. Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature* 437:422-425.
- McBroom, A. J., and Kuehn, M. J. 2007. Release of outer membrane vesicles by gram-negative bacteria is a novel envelope stress response. *Mol. Microbiol.* 63:545-558.
- McMillan, H. M., Zebell, S. G., Ristaino, J. B., Dong, X., and Kuehn, M. J. 2021. Protective plant immune responses are elicited by bacterial outer membrane vesicles. *Cell Rep.* 34:108645.
- Minder, A. C., de Rudder, K. E. E., Narberhaus, F., Fischer, H.-M., Hennecke, H., and Geiger, O. 2004. Phosphatidylcholine levels in *Bradyrhizobium japonicum* membranes are critical for an efficient symbiosis with the soybean host plant. *Mol. Microbiol.* 39:1186-1198.
- Mitre, L. K., Teixeira-Silva, N. S., Rybak, K., Magalhães, D. M., de Souza-Neto, R. R., Robatzek, S., Zipfel, C., and de Souza, A. A. 2021. The *Arabidopsis* immune receptor EFR increases resistance to the bacterial pathogens *Xanthomonas* and *Xylella* in transgenic sweet orange. *Plant Biotechnol. J.* 19:1294-1296.
- Murzyn, K., Róg, T., and Pasenkiewicz-Gierula, M. 2005. Phosphatidylethanolamine-phosphatidylglycerol bilayer as a model of the inner bacterial membrane. *Biophys. J.* 88:1091-1103.
- Ngo, L. T., Okogun, J. I., and Folk, W. R. 2013. 21st century natural product research and drug development and traditional medicines. *Nat. Prod. Rep.* 30:584-592.
- Norris, M. H., Somprasong, N., Schweizer, H. P., and Tuanyok, A. 2018. Lipid A remodeling is a pathoadaptive mechanism that impacts lipopolysaccharide recognition and intracellular survival of *Burkholderia pseudomallei*. *Infect. Immun.* 86:e00360-18.
- Park, S. B., Jang, H. B., Nho, S. W., Cha, I. S., Hikima, J., Ohtani, M., Aoki, T., and Jung, T. S. 2011. Outer membrane vesicles as a candidate vaccine against edwardsielliosis. *PLoS One* 6:e17629.
- Pérez-Cruz, C., Delgado, L., López-Iglesias, C., and Mercade, E. 2015. Outer-inner membrane vesicles naturally secreted by gram-negative pathogenic bacteria. *PLoS One* 10:e0116896.
- Raetz, C. R., Reynolds, C. M., Trent, M. S., and Bishop, R. E. 2007. Lipid A modification systems in gram-negative bacteria. *Annu. Rev. Biochem.* 76:295-329.

- Ranf, S., Gisch, N., Schäffer, M., Illig, T., Westphal, L., Knirel, Y. A., Sánchez-Carballo, P. M., Zähringer, U., Hückelhoven, R., Lee, J., and Scheel, D. 2015. A lectin S-domain receptor kinase mediates lipopolysaccharide sensing in *Arabidopsis thaliana*. *Nat. Immunol.* 16:426-433.
- Roier, S., Zingl, F. G., Cakar, F., Durakovic, S., Kohl, P., Eichmann, T. O., Klug, L., Gadermaier, B., Weinzerl, K., Prassl, R., Lass, A., Daum, G., Reidl, J., Feldman, M. F., and Schild, S. 2016. A novel mechanism for the biogenesis of outer membrane vesicles in gram-negative bacteria. *Nat. Commun.* 7:10515.
- Sarafian, M. H., Gaudin, M., Lewis, M. R., Martin, F. P., Holmes, E., Nicholson, J. K., and Dumas, M. E. 2014. Objective set of criteria for optimization of sample preparation procedures for ultra-high throughput untargeted blood plasma lipid profiling by ultra performance liquid chromatography-mass spectrometry. *Anal. Chem.* 86:5766-5774.
- Schooling, S. R., and Beveridge, T. J. 2006. Membrane vesicles: An overlooked component of the matrices of biofilms. *J. Bacteriol.* 188:5945-5957.
- Shi, W., Bogdanov, M., Dowhan, W., and Zusman, D. R. 1993. The pss and psd genes are required for motility and chemotaxis in *Escherichia coli*. *J. Bacteriol.* 175:7711-7714.
- Sugawara, M., Takahashi, S., Umehara, Y., Iwano, H., Tsurumaru, H., Otake, H., Suzuki, Y., Kondo, H., Konno, Y., Yamakawa, T., Sato, S., Mitsui, H., and Minamisawa, K. 2018. Variation in bradyrhizobial NopP effector determines symbiotic incompatibility with Rj2-soybeans via effector-triggered immunity. *Nat. Commun.* 9:3139.
- Taboada, H., Dunn, M. F., Meneses, N., Vargas-Lagunas, C., Buchs, N., Andrade-Domínguez, A., and Encarnación, S. 2019a. Qualitative changes in proteins contained in outer membrane vesicles produced by *Rhizobium etli* grown in the presence of the *nod* gene inducer naringenin. *Arch. Microbiol.* 201:1173-1194.
- Taboada, H., Meneses, N., Dunn, M. F., Vargas-Lagunas, C., Buchs, N., Castro-Mondragón, J. A., Heller, M., and Encarnación, S. 2019b. Proteins in the periplasmic space and outer membrane vesicles of *Rhizobium etli* CE3 grown in minimal medium are largely distinct and change with growth phase. *Microbiology (Reading)* 165:638-650.
- Udvardi, M., and Poole, P. S. 2013. Transport and metabolism in legume-rhizobia symbioses. *Annu. Rev. Plant Biol.* 64:781-805.
- Vanhove, A. S., Duperthuy, M., Charrière, G. M., Le Roux, F., Goudenège, D., Gourbal, B., Kieffer-Jaquinod, S., Couté, Y., Wai, S. N., and Destoumieux-Garzon, D. 2015. Outer membrane vesicles are vehicles for the delivery of *Vibrio tasmaniensis* virulence factors to oyster immune cells. *Environ. Microbiol.* 17:1152-1165.
- Vences-Guzmán, M. A., Geiger, O., and Sohlenkamp, C. 2008. *Sinorhizobium meliloti* mutants deficient in phosphatidylserine decarboxylase accumulate phosphatidylserine and are strongly affected during symbiosis with alfalfa. *J. Bacteriol.* 190:6846-6856.
- Vences-Guzmán, M. A., Guan, Z., Ormeño-Orrillo, E., González-Silva, N., López-Lara, I. M., Martínez-Romero, E., Geiger, O., and Sohlenkamp, C. 2011. Hydroxylated ornithine lipids increase stress tolerance in *Rhizobium tropici* CIAT899. *Mol. Microbiol.* 79:1496-1514.
- Via, V. D., Zanetti, M. E., and Blanco, F. 2016. How legumes recognize rhizobia. *Plant Signal. Behav.* 11:e1120396.
- Wen, B., Mei, Z., Zeng, C., and Liu, S. 2017. metaX: A flexible and comprehensive software for processing metabolomics data. *BMC Bioinformatics* 18:183.
- Wu, C. X., and Liu, Z. F. 2018. Proteomic profiling of sweat exosome suggests its involvement in skin immunity. *J. Invest. Dermatol.* 138:89-97.
- Xin, D. W., Liao, S., Xie, Z. P., Hann, D. R., Steinle, L., Boller, T., and Staehelin, C. 2012. Functional analysis of NopM, a novel E3 ubiquitin ligase (NEL) domain effector of *Rhizobium* sp. strain NGR234. *PLoS Pathog.* 8:e1002707.
- Yang, J., Hwang, I., Lee, E., Shin, S. J., Lee, E. J., Rhee, J. H., and Yu, J. W. 2020. Bacterial outer membrane vesicle-mediated cytosolic delivery of flagellin triggers host NLRC4 canonical inflammasome signaling. *Front. Immunol.* 11:581165.
- Yonezawa, H., Osaki, T., Kurata, S., Fukuda, M., Kawakami, H., Ochiai, K., Hanawa, T., and Kamiya, S. 2009. Outer membrane vesicles of *Helicobacter pylori* TK1402 are involved in biofilm formation. *BMC Microbiol.* 9:197.
- Zeng, C., Wen, B., Hou, G., Lei, L., Mei, Z., Jia, X., Chen, X., Zhu, W., Li, J., Kuang, Y., Zeng, W., Su, J., Liu, S., Peng, C., and Chen, X. 2017. Lipidomics profiling reveals the role of glycerophospholipid metabolism in psoriasis. *Gigascience* 6:1-11.