Proteomic and Metabolomic Analyses of *Xylella fastidiosa* OMV-Enriched Fractions Reveal Association with Virulence Factors and Signaling Molecules of the DSF Family

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ABSTRACT

Xylella fastidiosa releases outer membrane vesicles (OMVs) known to play a role in the systemic dissemination of this pathogen. OMVs inhibit bacterial attachment to xylem wall and traffic lipases/esterases that act on the degradation of plant cell wall. Here, we extended the characterization of *X. fastidiosa* OMVs by identifying proteins and metabolites potentially associated with OMVs produced by Temecula1, a Pierce's disease strain, and by 9a5c and Fb7, two citrus variegated chlorosis strains. These results strengthen that one of the OMVs multiple functions is to carry determinants of virulence, such as lipases/esterases, adhesins, proteases, porins, and a pectin lyase-like protein.

For the first time, we show that the two citrus variegated chlorosis strains produce *X. fastidiosa* diffusible signaling factor 2 (DSF2) and citrus variegated chlorosis-DSF (likewise, Temecula1) and most importantly, that these compounds of the DSF (*X. fastidiosa* DSF) family are associated with OMV-enriched fractions. Altogether, our findings widen the potential functions of *X. fastidiosa* OMVs in intercellular signaling and host-pathogen interactions.

Keywords: citrus variegated chlorosis, diffusible signaling factor, outer membrane vesicle, Pierce's disease

Outer membrane vesicles (OMVs) are spherical particles of ~30 to 300 nm released into extracellular milieu by all Gram-negative bacteria studied (Jan 2017; Schwechheimer and Kuehn 2015). OMVs are currently considered an additional type of the bacterial secretion system (type 0 secretion system) that circumvents the arduous task of secreting compounds through the Gram-negative bacterial envelope (Guerrero-Mandujano et al. 2017). As a rule, the surface of OMVs reflects the bacterial outer membrane composition, including its phospholipids, membrane proteins, and lipopolysaccharides. The OMV lumen primarily contains periplasmic components trapped during the vesiculation process plus various metabolites, nucleic acids, and cytoplasmic and inner-membrane proteins (Jan 2017; Schwechheimer and Kuehn 2015; Toyofuku et al. 2019). OMVs have been proposed to traffic hydrophobic signaling molecules embedded in their lipid bilayer (Brameyer et al. 2018; Lynch and Alegado 2017).

OMVs can spread far from producer cells to perform functions in the environment, and their cargo, such as signaling molecules and virulence factors, would be duly protected from degradation until reaching the target site (Guerrero-Mandujano et al. 2017; Kulp and Kuehn 2010; Lynch and Alegado 2017). For instance, these bacterial secreted nanoparticles play key roles in cell signaling and quorum sensing (Brameyer et al. 2018; Mashburn and Whiteley 2005; Toyofuku et al. 2017), interspecies communication (Amano et al. 2010; Lynch and Alegado 2017), horizontal gene transfer (Fulsundar et al. 2014; Grull et al. 2018), nutrient acquisition (Li et al. 2016), defense against environmental stressors (Volgers et al. 2018), antimicrobial activity (Kadurugamuwa and Beveridge 1996), and onset of metazoan immune response (Kaparakis-Liaskos and Ferrero 2015; Manning and Kuehn 2011; Pathirana and Kaparakis-Liaskos 2016).

Although the production of OMVs and their various related functions have been mostly investigated in animal pathogens, bacterial phytopathogens have been shown to release OMVs that participate in biofilm formation, virulence, and modulation of plant immunity (Katsir and Bahar 2017). For example, OMVs produced by *Xanthomonas campestris* pv. *campestris* (Sidhu et al. 2008; Solé et al. 2015) and *Pseudomonas syringae* pv. *tomato* (Chowdhury and Jagannadham 2013) contain virulence-associated proteins, such as protease, xylanase, and chitinase. Moreover, *X. campestris* pv. *campestris* and *Xanthomonas oryzae* pv. *oryzae* OMVs can induce typical immune responses in *Arabidopsis thaliana* (Bahar et al. 2014, 2016). In *Xylella fastidiosa*, OMVs function as antiadhesive nanoparticles, inhibiting bacterial attachment to plant cells and thus, facilitating the systemic dissemination of the phytopathogen (Ionescu et al. 2014).

X. fastidiosa OMVs have been shown to transport lipases/esterases that degrade plant cell wall (Nascimento et al. 2016). Thus, as with other pathogenic bacteria (Toyofuku et al. 2019), *X. fastidiosa* may use OMVs to transport virulence factors. This seems to be quite relevant, because *X. fastidiosa* does not possess a type 3 secretion system (T3SS) (Van Sluys et al. 2002), which is

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commonly used by most phytopathogenic bacteria to secrete protein effectors that enhance virulence and/or suppress plant defenses (Büttner 2016). However, *X. fastidiosa* genome does encode the proteins required for assembly and function of type 1 secretion system (T1SS), type 2 secretion system (T2SS), and type 5 secretion system (T5SS) (Simpson et al. 2000; Van Sluys et al. 2003). Although T1SS seems to be important for detoxification of *X. fastidiosa* cells within the plant host (Reddy et al. 2007), its T2SS has been related to secretion of proteases and plant cell wall degrading enzymes (Rapicavoli et al. 2018). *X. fastidiosa* has six genes predicted to encode autotransporters from the T5SS family, one of which is the adhesin XatA (Matsumoto et al. 2012). In addition, three other genes encoding trimeric autotransporters from the T5SS family are found in *X. fastidiosa* genome, two of each are known as the afimbrial adhesins XadA1 and XadA2 (Caserta et al. 2010). A complete type 4 secretion system (T4SS) involved in conjugation and DNA transfer among bacteria is encoded in some *X. fastidiosa* plasmids (Burbank and Van Horn 2017). Although, homologous genes of the plasmidial T4SS are also found in the chromosome of *X. fastidiosa* (Marques et al. 2001; Simpson et al. 2000), currently nothing is known regarding their function.

Expressions of several of the virulence and/or pathogenicity determinants in *X. fastidiosa* are regulated by a signaling pathway mediated by *X. fastidiosa* diffusible signaling factor (DSF) (Chatterjee et al. 2008; Wang et al. 2012). *X. fastidiosa* DSF comprises a family of related unsaturated fatty acids (Ionescu et al. 2016) that regulates bacterial behavior in a cell density-dependent manner, modulating the transition from less adhesive and motile phenotype to more adhesive sessile cells. Although the motile phenotype is critical for systemic plant colonization, the adherent cells form a biofilm both in the insect vectors and in the xylem (Chatterjee et al. 2008). Regulation of pathogenicity factors (*rpf*) genes is responsible for production and sensing of *X. fastidiosa* DSF (Chatterjee et al. 2008). Accordingly, a mutant blocked in production of *X. fastidiosa* DSF ($\Delta rpfF$) has a less adhesive phenotype, is hypervirulent to plant host, and is impaired in insect colonization and transmission (Ionescu et al. 2013; Newman et al. 2004).

X. fastidiosa is receiving an increasing attention since it reemerged as a plant pathogen of global importance. Once confined to the Americas, this pathogen is spreading throughout Europe (Sicard et al. 2018). This bacterium is transmitted by ubiquitous generalists xylem sap-feeding insects (Almeida and Nunney 2015; Redak et al. 2004), being the causative agent of several important crop diseases (Janse and Obradovic 2010; Rapicavoli et al. 2018), such as the Pierce's disease (PD) of grapevine (Hopkins and Purcell 2002), the citrus variegated chlorosis (CVC) of orange tree (Li et al. 1999), and the quick decline syndrome of olive tree (Cariddi et al. 2014; Della Coletta et al. 2016).

In this study, we extended the characterization of *X. fastidiosa* OMVs by analyzing the protein and lipid composition of OMVs produced by three different strains, one of them associated with PD (strain Temecula1) and two of them associated with CVC (strains 9a5c and Fb7). Our results show, for the first time, that, other than several virulence-related proteins, *X. fastidiosa* OMVs also seem to traffic *X. fastidiosa* DSF, thus expanding our understanding of the functions played by this secretion mechanism in host colonization, virulence, and onset of plant diseases.

MATERIALS AND METHODS

***X. fastidiosa* strains and growth conditions.** *X. fastidiosa* PD strain Temecula1 (Newman et al. 2003; Van Sluys et al. 2003) and the CVC strains 9a5c (Li et al. 1999; Simpson et al. 2000) and Fb7 (da Silva et al. 2007) were routinely cultured for 5 to 7 days in periwinkle wilt glucose (PWG) agar medium, which is periwinkle wilt (PW) medium (Davis et al. 1981) plus 0.5% glucose. To obtain liquid bacterial cultures, cells were harvested from the agar plates and inoculated in PWG broth (filtered through 0.22- μ m membrane) with optical densities at 600 nm (OD_{600}) of 0.05 to 0.1. Cultures

were maintained for 7 days at 28°C in a rotary shaker at 100 rotations per minute until they reached an OD_{600} of 0.8 to 1.2. Total protein content of bacterial cultures was determined using Bradford reagent (Bio-Rad Laboratories, Inc.).

Nanoparticle tracking analysis of cell-free culture supernatants. Concentration and size distribution of nanoparticles in *X. fastidiosa* culture supernatants were determined by nanoparticle tracking analysis (NTA) using a NanoSight LM10 system (NanoSight Ltd.) following a previously described procedure (Ionescu et al. 2014). Briefly, cells were removed by two cycles of centrifugation at $16,000 \times g$ for 20 min each at 4°C, and the cell-free culture supernatant was carefully removed and kept on ice. Each sample, in triplicate, was diluted in 0.22- μ m filtered phosphate buffered saline (PBS) (pH 7.4) to a concentration of 10^8 to 10^9 particles per milliliter and loaded in the NanoSight equipment. A monochromatic laser beam at 405 nm was applied, and three videos of 30 s were recorded, with sample mixing between recordings. Camera sensitivity and detection threshold were set to 14. Ambient temperature ranged from 20 to 22°C. The videos were analyzed using the NTA software (version 2.3), with the parameters minimal expected particle size, minimum track length, and blur setting all set to automatic. NanoSight particle analysis from PWG medium (blank sample) was subtracted from each sample measurement (Turner et al. 2018). NTA was performed in three biological replicates (three independent cultures) of each *X. fastidiosa* strain, and the average was calculated and plotted as particle size versus number of particles per milliliter. Statistically significant differences on nanoparticles counting between the different strains were determined with the Fisher's least significant difference (LSD) test.

Preparation of OMV-enriched fraction. OMVs enrichment from *X. fastidiosa* culture supernatants was performed using sequential centrifugation as previously described (Ionescu et al. 2014; Matsumoto et al. 2012) from three independent cultures for each strain. Briefly, *X. fastidiosa* cells were pelleted from 400 ml cultures by centrifugation at $8,000 \times g$ for 15 min at 4°C. The cell-free culture supernatant was carefully collected and centrifuged at $38,000 \times g$ for 1 h at 4°C in a P28S rotor (Hitachi) to remove cell debris. The $38,000 \times g$ supernatant was collected and centrifuged at $144,000 \times g$ for 5 h at 4°C (P28S rotor; Hitachi). The high-speed centrifugation pellets enriched in OMVs were immediately processed for scanning electron microscopy, shotgun proteomics, and metabolomics.

Scanning electron microscopy. The OMV-enriched fraction suspended in PBS (pH 7.4) was spotted on glass coverslips and fixed with 10% glutaraldehyde solution in PBS (pH 7.4) in 24-well plates. Next, the samples were gently immersed in 1% osmium tetroxide buffer for 1 h, washed three times with cacodylate buffer, treated with 1% tannic acid for 30 min, and washed twice with deionized water. Then, the samples were impregnated with osmium tetroxide for 30 min, rinsed for three times with deionized water, and gradually dehydrated with ethanol concentrations starting at 50% for 10 min, with 10% increments every time. After critical point drying, samples were coated with a layer of 25- to 50-nm gold and kept dehydrated until analyzed in a scanning electron microscope model FEI Quanta 250 FEG operating at 20 to 30 kV.

Label-free shotgun proteomics. Three independent OMV-enriched preparations (pellet after high-speed ultracentrifugation) for each *X. fastidiosa* strain were suspended in 8 M urea buffer containing protease inhibitors (cOmplete; Roche). Next, they were pooled, concentrated by freeze drying, and kept dried at room temperature until the shotgun proteomic analysis. The dried samples were reconstituted in PBS, and 300 μ g of each sample was precipitated with four times the volume of ProteoExtract Protein Precipitation Kit (Calbiochem) according to the manufacturer's instructions. The samples were reconstituted in 100 μ l of 6 M urea in 50 mM triethylammonium bicarbonate (TEAB) plus 5 mM dithiothreitol (DTT) and incubated at 37°C for 30 min with 1,000-rpm shaking. Next, 15 mM iodoacetamide (IAA) was added

followed by incubation at room temperature for 30 min. The IAA was then neutralized with 30 mM DTT in incubation for 10 min. Lys-C/trypsin was added (1:25 enzyme/total protein) followed by incubation at 37°C for 4 h. TEAB (550 µl of 50 mM) was added to dilute the urea and activate trypsin digestion overnight. The digested peptides were desalted with Aspire RP30 Desalting Tips (Thermo Scientific), vacuum dried, and suspended in 45 µl of 50 mM TEAB. Peptides were quantified by Pierce quantitative fluorometric assay (Thermo Scientific), and 1 µg was analyzed on a QExactive mass spectrometer (Thermo Scientific) coupled with an Easy-LC source (Thermo Scientific) and a nanospray ionization source. The peptides were loaded onto a Trap (100 µm; C18 100-Å 5U) and desalted online before separation using a reversed-phase (75 µm; C18 200-Å 3U) column. The duration of the peptide separation gradient was 60 min using 0.1% formic acid and 100% acetonitrile (ACN) for solvents A and B, respectively. The data were acquired using a data-dependent MS/MS (tandem mass spectrometry) method, which had a full scan range of 300 to 1,600 Da and a resolution of 70,000. The resolution of the MS/MS method was 17,500, and the insulation width was 2 *m/z*, with normalized collision energy of 27. The nanospray source was operated using a spray voltage of 2.2 kV and a transfer capillary temperature heated to 250°C.

The raw data were analyzed using X! Tandem and viewed using the Scaffold Software v.4. (Proteome Software, Inc.). Samples were searched against UniProt databases appended with the cRAP database, which recognizes common laboratory contaminants. Reverse decoy databases were also applied to the database before the X! Tandem searches. The coding sequences (CDSs) annotated in the genomes of the Temecula1 and 9a5c strains were used as reference for identification of the proteins at their respective proteomes. The 9a5c strain genome was used as a reference for Fb7 strain. The proteins identified were filtered in the Scaffold software based on the following criteria: 1.0% false discovery rate (FDR) at protein level (following the prophet algorithm: <http://proteinprophet.sourceforge.net/>), minimum number of two peptides, and 0.1% FDR at peptide level. Proteins sequences identified were examined for the presence of a signal peptide (Petersen et al. 2011) and the potential (SecP score) of being secreted by a nonclassical protein secretion system (Bendtsen et al. 2005).

Metabolomics. For metabolome analysis, three independent OMV-enriched preparations for each *X. fastidiosa* strain were pooled and dried in an Eppendorf Concentrator 5301. Methanol was

then added to the precipitate, and the suspension was centrifuged for 5 min at 12,000 × *g* followed by filtration in a 0.45-µm PVDF (polyvinylidene difluoride) membrane. The samples were then injected into the Shimadzu UFLC-20AD chromatograph coupled to a Bruker TOF mass spectrometer. The suspension was separated with a Phenomenex Kinetex C18 100 × 2.1 mm with 2.6-µm particle size and 100-Å pore size. Mobile phase A consisted of 10 mM NH₄Ac in water (pH 8), and mobile phase B consisted of 95% ACN + 5% mobile phase solution A. Binary gradient program was 0 to 1 min, 5%; 1 to 12 min, 40%; 12 to 18 min, 80%; 18 to 20 min, 80%; 20 to 21 min, 5%; and 21 to 24 min, 5%. Flow was set at 400 µl, and oven temperature was maintained at 40°C. Mass spectrometer parameters were used as follows: negative mode, -3,800 V; offset, 500 V; gas temperature, 250°C; 10 liters/min; scan range, 50 to 1,000 *m/z*; and scan rate, 1 Hz/s. The compounds that had their molecular masses resolved were identified, and their identities were predicted with ProbMetab software (Silva et al. 2014) and the KEGG database (<https://www.kegg.jp/>) for the *X. fastidiosa* metabolic model inferred from its complete genome. Methanol solutions of synthetic 2-cis-tetradecenoic acid (*X. fastidiosa* DSF1), 12-methyltetradecanoic (CVC-DSF), 2-cis-hexadecenoic acid (*X. fastidiosa* DSF2), and octadecanoic acid (C18:0) (Beaulieu et al. 2013) were run as standards. Both the OMV extracts and synthetic standards were compared with a blank sample (PWG medium), which was extracted as described above.

RESULTS

***X. fastidiosa* CVC strains release OMVs.** *X. fastidiosa* PD strain Temecula1 has been shown to release OMVs in culture supernatants (Ionescu et al. 2014; Matsumoto et al. 2012; Nascimento et al. 2016) and in the xylem (Ionescu et al. 2014). The number and size distribution of nanoparticles, which include OMVs, in culture supernatants of *X. fastidiosa* strains were estimated by NTA. After 7 days of culture in PWG, the CVC strains 9a5c and Fb7 released nanoparticles (1 to 3 × 10⁸/ml) with average size of 100 nm, similar to Temecula1 (Fig. 1A). The three strains presented comparable cell densities and total protein content (Fig. 1B), but strain Fb7 exhibited 60% higher nanoparticles count per 1 ml (*P* < 0.05 in Fisher's LSD test) than strains 9a5c and Temecula1 (Fig. 1A). Given the remarkably adhesive phenotype of 9a5c strain and the mucoid-like phenotype of Fb7, CFU assessments

Fig. 1. Size determination and quantification of nanoparticles released by *Xylella fastidiosa* Pierce's disease and citrus variegated chlorosis strains. **A**, Nanoparticles, which include outer membrane vesicles secreted by strains Temecula1, 9a5c, and Fb7 present in cell-free culture supernatants, were assessed by nanoparticle tracking analysis (NTA) to determine their size distribution and concentration per milliliter. **B**, Bacterial cell density (optical density at 600 nm [OD₆₀₀] per 1 ml) and total protein (absorbance at 595 nm [Abs₅₉₅] per 1 ml) of the cell cultures used for NTA were determined. Error bars represent standard deviation of the mean of three biological replicates, with three technical replicates each.

were not reliable for these two strains, and as such, it was not possible to determine their nanoparticle production per cell number. However, Temecula1 produced 170 ± 79 nanoparticles per CFU after 7 days growth in PWG medium, which is approximately three times more than previously observed (51 ± 20 nanoparticles per CFU) after 4 days of growth in Pierce's disease 3 (PD3) medium (Ionescu et al. 2014). Although NTA does not identify the type of the nanoparticle material in the cell-free culture supernatants, the detected nanoparticles are known to include OMVs (Chan et al. 2017; Ionescu et al. 2014).

To further investigate the production of OMVs by Temecula1, 9a5c, and Fb7 strains, OMV-enriched preparations were evaluated by scanning electron microscopy (Fig. 2A to F). No detectable variations in form and size range of these nanoparticles among the different strains were seen in culture suspensions (Fig. 2G to I).

***X. fastidiosa* OMVs carry porins, adhesins, and hydrolases.** Using label-free shotgun proteomics, we identified a large set of proteins potentially associated with the OMVs secreted by Temecula1 and two CVC strains (9a5c and Fb7) (Supplementary Tables S1, S2, and S3). This approach identified, with a high degree of confidence (1.0% FDR at the protein level), 122, 152, and 122 OMV-associated proteins for Temecula1, 9a5c, and Fb7, respectively. These were then ranked based on their relative abundances according to Scaffold software quantitation (Scaffold score). Among the potentially OMV-associated proteins, we have identified that 65, 72, and 79% are predicted as secreted proteins in strains Temecula1, 9a5c, and Fb7, respectively, based on signal peptide analysis and the SecP score. Moreover, about one-third of the identified proteins are predicted to be located on the outer membrane. Indeed, several of the identified proteins are

Fig. 2. Electron scanning micrographs of outer membrane vesicles (OMVs) from strains Temecula1, 9a5c, and Fb7. **A to F**, OMV preparations from culture supernatants. **A to C**, Isolated OMVs or **D to F**, clusters of OMVs are shown. **G to I**, OMVs associated with cells. **A, D, and G**, Strain Temecula1. **B, E, and H**, Strain 9a5c. **C, F, and I**, Strain Fb7. Scale bars = 1 μ m.

well-known outer membrane proteins, such as OmpW, OmpA, MopB, PorinO, TonB-dependent receptor, and XadA1 (Nascimento et al. 2016).

Of the 199 unique proteins detected in the OMV-enriched preparations, 67 were shared by the three strains (Fig. 3A), whereas 26, 31, and 12 proteins seemed to be exclusive to strains Temecula1, 9a5c, and Fb7, respectively. The 35 most abundant proteins identified in the OMV-enriched fraction for each one of three *X. fastidiosa* strains comprise 58 unique proteins (Table 1). The three strains share 45 of these 58 proteins (Fig. 3B), although there is a great variation in their abundances indicated by the ranking positions in each strain's OMV proteome (Table 1). Ten of the 45 shared top abundant proteins (Table 1) were already found to be OMV associated as well as soluble proteins in Temecula1 culture supernatant (Nascimento et al. 2016).

The OMVs of the three strains have in common the afimbrial adhesins XadA1, Hsf/XadA2, and XadA3 but with great variation in their relative abundances. Although XadA1 seems relatively more abundant than XadA3 in Temecula1 and Fb7, the opposite situation is verified for 9a5c (Table 1). Another protein also common to all three strains is Omp1X (PD1063), heretofore known as Ax21, which was previously reported to be OMV associated (Pierce et al. 2014). The OMV-enriched fraction of the three strains also contains the autotransporter XatA1 (PD0528), previously described to be OMV associated in Temecula1 (Matsumoto et al. 2012). Up to six distinct porins were detected on OMVs-enriched fractions of the three strains.

Several hydrolases (lipases/esterases, proteases, and peptidases) were also detected, with lipases/esterases LesA (PD1702/XF0357) and LesB (PD1703/XF0358) among the most abundant in the OMV proteome (Table 1). Although in lower abundance, we highlight the identification of the XF0119/PD0090 protein in the OMV-enriched preparations of the three strains. This CDS encodes a domain characteristic of pectin lyases and has 31% sequence similarity to a *X. oryzae* pectate lyase (data not shown).

Among the selected proteins listed in Table 1, just a few seem to be exclusive to one or two strains (Fig. 3B). We highlight the BamA protein (XF1046) and the XF1496 TonB-dependent receptor that were detected only in the OMVs of the citrus strains (Fb7 and 9a5c). However, the CDS annotated as L-ascorbate oxidase (XF2677/PD2039) was detected in the OMV-enriched proteome of Fb7 and Temecula1 strains but not in 9a5c (Table 1).

Although hemagglutinin PspA (XF2775/PD2118; HxfA) was detected in Temecula1 OMVs, a paralogous hemagglutinin (XF0889/PD1792; HxfB) was found in the OMVs of strain 9a5c. However, none of the two hemagglutinins were found in the OMVs of Fb7, a strain that presents a mucoid-like phenotype (unpublished observation).

***X. fastidiosa* OMVs carry *X. fastidiosa* DSF2 and CVC-DSF.** Aiming to complement the characterization of OMVs composition, we performed an untargeted metabolomics approach using mass spectrometry. Approximately 275 compounds from an OMV-enriched fraction of strains Temecula1, 9a5c, and Fb7 had their molecular masses resolved, and their identities were predicted using ProbMetab and the KEGG database. The list of likely metabolites predicted in *X. fastidiosa* OMV-enriched fraction includes fatty acids, free amino acids, carbohydrates, and phenolic compounds (Supplementary Tables S4, S5, and S6). For the purpose of this work, however, we focused our attention on the fatty acid content of the OMVs, particularly those of the *X. fastidiosa* DSF family (Beaulieu et al. 2013; Colnaghi Simionato et al. 2007; Ionescu et al. 2016).

Our metabolome analysis of Temecula1, 9a5c, and Fb7 OMV-enriched fractions indicated that all three strains produce CVC-DSF, *X. fastidiosa* DSF2 (C16:1), and C18:0, the identities of which were further confirmed by comparative analysis with synthetic standards (Fig. 4). These results show, for the first time, that the two CVC strains produce *X. fastidiosa* DSF2 and CVC-DSF (likewise Temecula1) and most importantly, that these compounds were found in association with OMVs. However, *X. fastidiosa* DSF1 (C14:1) was not detected in either of the OMVs-enriched fractions from the three *X. fastidiosa* strains.

DISCUSSION

Initially suggested as artifacts of bacterial growth, OMVs have a plethora of functions demonstrated in intraspecies and interspecies cell-cell communication, mediating pathogenesis as well as beneficial ecological interactions (Kulp and Kuehn 2010; Lynch and Alegado 2017; Pathirana and Kaparakis-Liaskos 2016; Schwechheimer and Kuehn 2015; Toyofuku et al. 2019). Although somewhat underappreciated, the implications of OMVs released by bacterial plant pathogens for host colonization have begun to be unraveled (Chowdhury and Jagannadham 2013; Katsir and Bahar

Fig. 3. Shared and exclusive outer membrane vesicle (OMV)-associated proteins in three *Xylella fastidiosa* strains. The protein compositions of OMV-enriched preparations of strains Temecula1, 9a5c, and Fb7 were determined using label-free shotgun proteomics of three biological replicates. The Venn diagrams indicate shared and exclusive proteins identified **A**, in the total proteome of OMVs and **B**, among the 35 most abundant proteins (Table 1) for each one of three strains.

2017; Sidhu et al. 2008). For instance, *X. fastidiosa* OMVs seem to facilitate systemic dispersal of bacterial cells in the plant host, increasing virulence (Ionescu et al. 2014) and traffic lipases/esterases that have the potential to act on the degradation of plant cell wall in sites distant from OMV-producing cells (Nascimento et al. 2016).

The work reported here further explores the importance and complexity of OMVs in *X. fastidiosa* by expanding the set of proteins potentially associated with native OMVs produced by one

PD and two CVC wild-type strains and determining the metabolites predicted in OMVs-enriched fractions. It is worth mentioning that we did not apply any intervention to increase OMV production. Enhancement of vesiculation by a variety of external stimuli can improve OMV yield but may change the composition of native OMVs (Klimentová and Stulik 2015). Thus, for the purpose of this work, we followed previously described protocols to obtain native OMV-enriched preparations by using sequential centrifugation (Gasperini et al. 2018; Ionescu et al. 2014; Kulp et al. 2015; Mantri

TABLE 1. Top abundant proteins identified in the outer membrane vesicle (OMV)-enriched preparations from *Xylella fastidiosa* strains Temecula1, 9a5c, and Fb7

CDS number _{CVC}	CDS number _{PD} ^a	CDS symbol	Gene product	Ranking (scaffold score)		
				Temecula1	9a5c	Fb7
XF0357	PD1702	LesA	Lipase/Esterase LesA	1	14	7
XF0358	PD1703	LesB	Lipase/Esterase LesB	2	24	11
XF0872	PD1807	OmpW	Outer membrane protein W	3	52	1
XF1026	PD0313	PspB	Serine Protease PspB	4	1	45
XF1516	PD0731	XadA1	Adhesin XadA1	5	61	2
XF0343	PD1709	OmpA/mopB	Outer membrane protein A	6	2	9
XF1036	PD0318		Porin	7	12	3
XF2237	PD1283		TonB-dependent receptor	8	4	4
XF1851	PD0950		Serine protease	9	11	19
XF0363	PD1699		Peptidoglycan-associated lipoprotein	10	7	13
XF1547	PD0757	Lpp	Membrane lipoprotein Lpp	11	18	23
XF1981	PD0824	XadA3	Adhesin XadA3	12	3	20
XF2713	PD2065	CirA	Porin CirA	13	5	8
XF0975	PD0270	OprP	Porin O OprP	14	9	10
XF2586	PD1964	TolC	Outer membrane TolC	15	8	6
XF2775	PD2118	PspA	Hemagglutinin PspA	16	–	–
XF0550	PD1589	BtuB	Porin BtuB	17	10	36
XF1529	PD0744	XadA2/Hsf	Adhesin XadA2/Hsf	18	152	64
XF1226	PD0503		Protein of unknown function	19	31	55
XF0083	PD0062	FimA	Fimbrial protein FimA	20	36	–
XF2407	PD1427	RTX	Hemolysin toxin protein RTX	21	35	94
XF2628	PD1996	EF-Tu	Elongation factor-Tu	22	39	–
XF0339	PD1711		TonB-dependent receptor	23	32	14
XF1287	PD0540		Protein of unknown function	24	13	5
XF2137	PD1200	BfeA	Ferric enterobactin receptor BfeA	25	118	40
XF0081	PD0060	FimD	Outer membrane protein FimD	26	20	–
XF0531	PD0956		Cysteine/serine peptidase	27	74	30
XF0668	PD1506	RTX	Hemolysin toxin protein RTX	28	17	109
XF1827	PD1040	OsmC/OhrA	Hydroperoxide reductase OsmC/OhrA	29	79	108
XF0898	PD1786		Membrane lipoprotein	30	29	104
XF2349	PD0528		Autotransporter beta-domain (XatA1)	31	16	68
XF1803	PD1063		Omp1X	32	6	26
XF0820	PD1850		Zn-dependent amino- or carboxypeptidase	33	90	58
XF2677	PD2039	Aoo	L-ascorbate oxidase Aoo	34	–	12
XF1811	PD1055	Slp	Outer membrane protein Slp	35	40	21
XF0395	PD1672	Bfr	Bacterioferritin Bfr	37	65	33
XF2151	PD1211	LesC	Lipase/Esterase LesC	40	147	15
XF0615	PD1538	GroL	Chaperonin GroL	41	30	116
XF1896	PD0895	OmpA	Outer membrane protein A	44	33	17
XF1911	PD0880		Membrane lipoprotein	46	34	24
XF0321	PD0264	OprO	Porin O OprO	47	22	77
XF1887	PD0904		Cysteine/serine peptidase PS-46	50	19	32
XF1897	PD0894	TolB	TolB protein	51	23	16
XF0781	PD1879	EstA	Lipase/Esterase	52	43	22
XF0816	PD1853		Zn-dependent peptidase	53	44	29
XF1434	PD0955		Cysteine/serine peptidase	54	109	27
XF0855	PD1820	NlpD	Lipoprotein NlpD	60	27	47
XF0464	PD1620	BamB	Outer membrane protein BamB	62	38	35
XF2349	PD1379		Autotransporter beta-domain	63	16	68
XF1628	PD1151	YdiY	Outer membrane protein YdiY	123	28	53
XF1577	PD0978		DUF2184	–	15	–
XF1011	PD0305	RTX	Hemolysin toxin protein RTX	–	21	–
XF1046	PD0326	BamA	Outer membrane protein assembly factor BamA	–	25	28
XF1496	PD0711		Porin	–	26	46
XF1384	–	PqaA	Alpha/beta-Hydrolases PqaA	–	57	18
XF2551	PD1934		Dipeptidyl aminopeptidase protein 6	–	64	34
XF1024	PD0312		Membrane lipoprotein	–	–	25
XF1745	–		Alpha/beta-Hydrolases	–	–	31

^a Coding sequences (CDSs) in bold have been previously reported as OMV associated in Temecula1 (Nascimento et al. 2016). CVC, citrus variegated chlorosis; PD, Pierce's disease.

et al. 2015; Matsumoto et al. 2012; Nascimento et al. 2016; Wai et al. 2003; Yun et al. 2017). The sequential centrifugations, in which both pellet and supernatants were carefully removed, certainly have greatly diminished the presence of molecules not associated with OMVs in the final OMV-enriched preparations. However, because no additional purification steps were performed to get highly purified OMVs (Chutkan et al. 2013; Klimentová and Stulik 2015), the proteins and metabolites that we have identified are referred to as potentially associated with OMVs.

Among the set of proteins identified in the OMVs-enriched fractions of Temecula1, 9a5c, and Fb7 strains, we highlight XadA1, a known Temecula1 OMV marker (Ionescu et al. 2014), and the two other afimbrial adhesins (Hsf/XadA2 and XadA3). The presence of adhesins and hemagglutinins among the OMV-associated proteins might be important to modulate attachment of these nanoparticles to surfaces. As previously shown, *X. fastidiosa* OMVs reduce cell attachment to surfaces, such as the xylem vessels, perhaps by competing with cells for attachment sites (Ionescu et al. 2014).

OMVs of the three strains may hold a capacity to secrete and/or internalize specific molecules given that six porins were identified in the OMV proteome. For instance, it has been suggested that antimicrobial-resistant *Escherichia coli* OMVs might take up β -lactam antibiotics into their lumens through increased quantity of OmpC, OmpF, and OmpW (Kim et al. 2018). Moreover, porins and enzymes related to benzoate degradation were uniquely found in OMVs prepared from *Pseudomonas putida* cultured in media containing benzoate as the energy source (Choi et al. 2014). A related point to consider is that the CDS XF2237/PD1283 (TonB-dependent receptor) is one of the most abundant proteins in the OMVs of all three strains. TonB-dependent receptors are bacterial

outer membrane proteins that bind and transport siderophores as well as vitamin B12, nickel complexes, and carbohydrates (Noinaj et al. 2010).

Unlike closely related pathogens, *X. fastidiosa* lacks the T3SS (Simpson et al. 2000; Van Sluys et al. 2002). Thus, the release of OMVs would be an advantageous strategy for *X. fastidiosa* cells to deliver virulence effectors without the requirement of direct proximity to target cells. Our findings corroborate previous studies that investigated smaller sets of OMV proteins in *X. fastidiosa* (Ionescu et al. 2014; Matsumoto et al. 2012; Nascimento et al. 2016; Pierce et al. 2014), hence strengthening one of the OMVs multiple functions, which is to carry determinants of virulence. Accordingly, we have found the lipases/esterases LesA (PD1702/XF0357) and LesB (PD1703/XF0358) as OMV-associated proteins in the three strains that we have investigated (Table 1). These enzymes have been previously described as soluble secreted proteins associated with OMVs and have a relevant role in the virulence of the Temecula1 strain (Nascimento et al. 2016). In addition, we also identified the PtrA protease (XF0531/PD0956) in the OMVs-enriched fraction of the three strains. This enzyme plays an important role in the control of cell proliferation, biofilm formation, and virulence of strain Temecula1, and deletion of this gene results in a hypervirulence phenotype (Gouran et al. 2016).

Amid the proteins potentially associated with *X. fastidiosa* OMVs, some caught our attention as potential virulence factors that deserve future functional investigation. One is a putative pectin lyase (XF0119/PD0090) that may act in plant cell wall degradation. Enzymes of the pectin lyase family are recognized as virulence factors in phytopathogens (Hugouvieux-Cotte-Pattat et al. 2014), but their function and/or activity remain unknown in *X. fastidiosa*.

Fig. 4. Chromatograms of compounds of the *Xylella fastidiosa* diffusible signaling factor (DSF) family identified in outer membrane vesicle-enriched preparations of *X. fastidiosa* strains. **A to D**, Extracted ions chromatograms (XICs) of strain Temecula1. **E to H**, XICs of strain 9a5c. **I to L**, XICs of strain Fb7. **M to P**, XICs of the standards *X. fastidiosa* DSF1, citrus variegated chlorosis (CVC)-DSF, *X. fastidiosa* DSF2, and C18:0, respectively.

Another is XF2677/PD2039, which is found in the OMV-enriched fraction of Temecula1 and Fb7 strains. This CDS is annotated as L-ascorbate oxidase, which catalyzes the oxidation of ascorbic acid to dehydroascorbic acid. Action of this enzyme could potentially result in depletion of ascorbate produced by the plant host, affecting its primary defense line against external oxidants (Akram et al. 2017).

It is well known that *X. fastidiosa* Temecula1 is capable of producing a range of carboxylic acids with >12 carbons, among them the signaling molecules 2-cis-tetradecenoic acid (*X. fastidiosa* DSF1) and 2-cis-hexadecenoic acid (*X. fastidiosa* DSF2) (Beaulieu et al. 2013; Ionescu et al. 2016). Moreover, an additional compound, 12-methyltetradecanoic acid (CVC-DSF), has been shown to be produced by strain 9a5c (Colnaghi Simionato et al. 2007). We have shown that *X. fastidiosa* DSF2 and CVC-DSF, but not *X. fastidiosa* DSF1, were detected among OMVs-associated metabolites in all three strains. Our failure in detecting *X. fastidiosa* DSF1 in *X. fastidiosa* OMVs might be because of its low level of production in the growth condition that we have used. Accordingly, a previous

report also failed in detecting *X. fastidiosa* DSF1 in Temecula1 culture supernatants, despite the fact that this molecule was detected on expression of *X. fastidiosa* RpfF (*X. fastidiosa* DSF synthase) in *Pantoea agglomerans* as surrogate host (Ionescu et al. 2016). Another explanation of why *X. fastidiosa* DSF1 (C14:1) was not detected in the OMV-enriched fractions is its relative low hydrophobicity. As such, *X. fastidiosa* DSF1 does not require OMVs to be carried and instead, diffuses freely to the extracellular environment. Short chain- and medium chain-length DSF species as *X. fastidiosa* DSF1 can be predicted to be sufficiently soluble in water based on their partition coefficient. However, *X. fastidiosa* DSF2 (C16:1) is estimated to have a much lower solubility in water (Ionescu et al. 2016). *X. fastidiosa* DSF2 can be acquired by cells and subsequently interact with the DSF sensor RpfC (Ionescu et al. 2016). Based on these observations, these authors hypothesized that *X. fastidiosa* DSF2 (and other more hydrophobic DSFs) would interact with hydrophobic matrices, such as bacterial membranes, and perhaps, would be delivered through OMVs. Taking these remarks together, we suggest that *X. fastidiosa* may use OMVs to

Fig. 5. A simplified model for intercellular signaling mediated by outer membrane vesicles (OMVs) and diffusible signaling factors (DSFs) in *Xylella fastidiosa*. OMVs are formed at the bacterial cell surface, carrying proteins anchored to the outer membrane (OM) and present in the periplasm (PP), such as proteases, porins, and adhesins, among other functions (represented by geometric forms). Metabolites are also transported, including members of the DSF family (*X. fastidiosa* DSFs), which are represented by stars of different colors. Although more hydrophobic *X. fastidiosa* DSFs, such as *X. fastidiosa* DSF2 (red stars) and citrus variegated chlorosis (CVC)-DSF (green stars), would be enriched to the OMVs, shorter-chain *X. fastidiosa* DSFs, such as *X. fastidiosa* DSF1 (blue stars), would be found predominantly free in the extracellular milieu. The secreted OMVs would deliver *X. fastidiosa* DSF and other compounds to neighboring or distant target cells. Target cells can sense *X. fastidiosa* DSF through the histidine kinase receptor RpfC anchored in the inner membrane (IM), triggering quorum-sensing responses.

traffic its highly hydrophobic signaling molecules, similar to other bacteria (Brameyer et al. 2018; Li et al. 2016; Mashburn and Whiteley 2005; Morinaga et al. 2018; Toyofuku et al. 2017).

Other than their role in microbial quorum sensing (Mashburn and Whiteley 2005), the hydrophobic signaling molecules transported by OMVs have been suggested to participate in the biogenesis of these vesicles (Mashburn-Warren et al. 2008; Schertzer and Whiteley 2012). For instance, the *Pseudomonas aeruginosa* signaling molecule 2-heptyl-3-hydroxy-4-quinolone mediates its own insertion into the outer membrane, increasing membrane curvature and leading to the formation of OMVs (Florez et al. 2017; Schwechheimer and Kuehn 2015). Moreover, phospholipid accumulation in the outer leaflet of the outer membrane has been shown to trigger OMV formation and suggested as a general mechanism of OMV biogenesis conserved among Gram-negative bacteria (Roier et al. 2016).

It remains to be investigated if *X. fastidiosa* DSFs would play a role in the biogenesis of *X. fastidiosa* OMVs. It is worth noting that the hypervirulent mutant $\Delta rpfF$ of Temecula1 does not produce *X. fastidiosa* DSFs (Ionescu et al. 2016) and overproduces OMVs (Ionescu et al. 2014). Moreover, $\Delta rpfF$ OMVs do not seem to differ from the wild-type strain OMVs, at least regarding their role as antiadhesive nanoparticles and by the presence of the OMV marker protein XadA1 (Ionescu et al. 2014). Thus, future characterization of OMVs produced by the available mutants in the DSF signaling pathway will be a valuable approach to investigate the regulation of OMV biogenesis as well as their role in cell–cell signaling in *X. fastidiosa*.

Based on the current knowledge along with the results described in this work, we suggest that more hydrophobic *X. fastidiosa* DSFs (Ionescu et al. 2016), such as *X. fastidiosa* DSF2 and CVC-DSF, may be enriched in OMVs, possibly embedded on its lipid bilayer (Fig. 5). Furthermore, *X. fastidiosa* DSF1, a shorter-chain signaling fatty acid (Beaulieu et al. 2013), would be found predominantly free in the extracellular milieu (Fig. 5). Therefore, other than their important functions in host colonization and virulence (Ionescu et al. 2014; Nascimento et al. 2016), *X. fastidiosa* OMVs would also serve to deliver *X. fastidiosa* DSF2 to neighboring or distant cells. This would activate its receptor RpfC, a histidine kinase of the quorum-sensing signaling pathway (Chatterjee et al. 2008). The strategy of *X. fastidiosa* DSF delivery through OMVs would overcome the limitation of dispersing hydrophobic molecules in an aqueous milieu, such as xylem sap.

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LITERATURE CITED

- Akram, N. A., et al. 2017. Ascorbic acid—A potential oxidant scavenger and its role in plant development and abiotic stress tolerance. *Front. Plant Sci.* 8: 613.
- Almeida, R. P. P., and Nunney, L. 2015. How do plant diseases caused by *Xylella fastidiosa* Emerge? *Plant Dis.* 99:1457-1467.
- Amano, A., et al. 2010. Outer membrane vesicles function as offensive weapons in host-parasite interactions. *Microbes Infect.* 12:791-798.
- Bahar, O., et al. 2014. The *Xanthomonas* Ax21 protein is processed by the general secretory system and is secreted in association with outer membrane vesicles. *PeerJ* 2:ARTN e242.
- Bahar, O., et al. 2016. Bacterial outer membrane vesicles induce plant immune responses. *Mol. Plant-Microbe Interact.* 29:374-384.
- Beaulieu, E. D., et al. 2013. Characterization of a diffusible signaling factor from *Xylella fastidiosa*. *MBio* 4:e00539-12.
- Bendtsen, J. D., et al. 2005. Non-classical protein secretion in bacteria. *BMC Microbiol.* 5:ARTN 58.
- Brameyer, S., et al. 2018. Outer membrane vesicles facilitate trafficking of the hydrophobic signaling molecule CAI-1 between *Vibrio harveyi* cells. *J. Bacteriol.* 200:e00740-17.
- Burbank, L. P., and Van Horn, C. R. 2017. Conjugative plasmid transfer in *Xylella fastidiosa* is dependent on tra and trb operon functions. *J. Bacteriol.* 199:e00388-17.
- Büttner, D. 2016. Behind the lines-actions of bacterial type III effector proteins in plant cells. *FEMS Microbiol. Rev.* 40:894-937.
- Cariddi, C., et al. 2014. Isolation of a *Xylella fastidiosa* strain infecting olive and oleander in Apulia, Italy. *J. Plant Pathol.* 96:425-429.
- Caserta, R., et al. 2010. Expression of *Xylella fastidiosa* fimbrial and afimbrial proteins during biofilm formation. *Appl. Environ. Microbiol.* 76:4250-4259.
- Chan, K. W., et al. 2017. Antibiotics and iron-limiting conditions and their effect on the production and composition of outer membrane vesicles secreted from clinical isolates of extraintestinal pathogenic *E. coli*. *Proteomics Clin. Appl.* 11:1600091.
- Chatterjee, S., et al. 2008. Living in two worlds: The plant and insect lifestyles of *Xylella fastidiosa*. *Annu. Rev. Phytopathol.* 46:243-271.
- Choi, C. W., et al. 2014. Proteomic characterization of the outer membrane vesicle of *Pseudomonas putida* KT2440. *J. Proteome Res.* 13:4298-4309.
- Chowdhury, C., and Jagannadham, M. V. 2013. Virulence factors are released in association with outer membrane vesicles of *Pseudomonas syringae* pv. tomato T1 during normal growth. *Biochim. Biophys. Acta Proteins Proteomics* 1834:231-239.
- Chutkan, H., et al. 2013. Quantitative and qualitative preparations of bacterial outer membrane vesicles. *Methods Mol. Biol.* 966:259-272.
- Colnaghi Simionato, A. V., et al. 2007. Characterization of a putative *Xylella fastidiosa* diffusible signal factor by HRGC-ESI-MS. *J. Mass Spectrom.* 42: 490-496.
- da Silva, V. S., et al. 2007. Comparative genomic characterization of citrus-associated *Xylella fastidiosa* strains. *BMC Genomics* 8:474.
- Davis, M. J., et al. 1981. Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. *Curr. Microbiol.* 6:309-314.
- Della Coletta, H., et al. 2016. First report of olive leaf scorch in Brazil, associated with *Xylella fastidiosa* subsp. *pauca*. *Phytopathol. Mediterr.* 55: 130-135.
- Florez, C., et al. 2017. Membrane distribution of the pseudomonas quinolone signal modulates outer membrane vesicle production in *Pseudomonas aeruginosa*. *MBio* 8:e01034-17.
- Fulsundar, S., et al. 2014. Gene transfer potential of outer membrane vesicles of *Acinetobacter baylyi* and effects of stress on vesiculation. *Appl. Environ. Microbiol.* 80:3469-3483.
- Gasperini, G., et al. 2018. Outer membrane vesicles (OMV)-based and proteomics-driven antigen selection identifies novel factors contributing to bordetella pertussis adhesion to epithelial cells. *Mol. Cell. Proteomics* 17: 205-215.
- Gouran, H., et al. 2016. The secreted protease PrtA controls cell growth, biofilm formation and pathogenicity in *Xylella fastidiosa*. *Sci. Rep.* 6: 31098.
- Grull, M. P., et al. 2018. Small extracellular particles with big potential for horizontal gene transfer: Membrane vesicles and gene transfer agents. *FEMS Microbiol. Lett.* 365:fny192.
- Guerrero-Mandujano, A., et al. 2017. The outer membrane vesicles: Secretion system type zero. *Traffic* 18:425-432.
- Hopkins, D. L., and Purcell, A. H. 2002. *Xylella fastidiosa*: Cause of Pierce's disease of grapevine and other emergent diseases. *Plant Dis.* 86:1056-1066.
- Hugouvieux-Cotte-Pattat, N., et al. 2014. Bacterial pectate lyases, structural and functional diversity. *Environ. Microbiol. Rep.* 6:427-440.
- Ionescu, M., et al. 2013. Diffusible signal factor (DSF) synthase RpfF of *Xylella fastidiosa* is a multifunctional protein also required for response to DSF. *J. Bacteriol.* 195:5273-5284.
- Ionescu, M., et al. 2014. *Xylella fastidiosa* outer membrane vesicles modulate plant colonization by blocking attachment to surfaces. *Proc. Natl. Acad. Sci. USA* 111:E3910-E3918.
- Ionescu, M., et al. 2016. Promiscuous diffusible signal factor production and responsiveness of the *Xylella fastidiosa* Rpf system. *MBio* 7:e01054-16.
- Jan, A. T. 2017. Outer membrane vesicles (OMVs) of gram-negative bacteria: A perspective update. *Front. Microbiol.* 8:1053.
- Janse, J. D., and Obradovic, A. 2010. *Xylella fastidiosa*: Its biology, diagnosis, control and risks. *J. Plant Pathol.* 92:S35-S48.
- Kadurugamuwa, J. L., and Beveridge, T. J. 1996. Bacteriolytic effect of membrane vesicles from *Pseudomonas aeruginosa* on other bacteria including pathogens: Conceptually new antibiotics. *J. Bacteriol.* 178: 2767-2774.
- Kaparakis-Liaskos, M., and Ferrero, R. L. 2015. Immune modulation by bacterial outer membrane vesicles. *Nat. Rev. Immunol.* 15:375-387.
- Katsir, L., and Bahar, O. 2017. Bacterial outer membrane vesicles at the plant-pathogen interface. *PLoS Pathog* 13:ARTN e1006306.
- Kim, S. W., et al. 2018. Outer membrane vesicles from beta-lactam-resistant *Escherichia coli* enable the survival of beta-lactam susceptible *E. coli* in the presence of beta-lactam antibiotics. *Sci. Rep.* 8:ARTN 5402.

- Klimentová, J., and Stulik, J. 2015. Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria. *Microbiol. Res.* 170: 1-9.
- Kulp, A., and Kuehn, M. J. 2010. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annu. Rev. Microbiol.* 64: 163-184.
- Kulp, A. J., et al. 2015. Genome-wide assessment of outer membrane vesicle production in *Escherichia coli*. *PLoS One* 10:ARTN e0139200.
- Li, J., et al. 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, W. B., et al. 1999. A triply cloned strain of *Xylella fastidiosa* multiplies and induces symptoms of citrus variegated chlorosis in sweet orange. *Curr. Microbiol.* 39:106-108.
- Lynch, J. B., and Alegado, R. A. 2017. Spheres of hope, packets of doom: The good and bad of outer membrane vesicles in interspecies and ecological dynamics. *J. Bacteriol.* 199:e00012-17.
- Manning, A. J., and Kuehn, M. J. 2011. Contribution of bacterial outer membrane vesicles to innate bacterial defense. *BMC Microbiol.* 11:258.
- Mantri, C. K., et al. 2015. Fimbriae-mediated outer membrane vesicle production and invasion of *Porphyromonas gingivalis*. *MicrobiologyOpen* 4: 53-65.
- Marques, M. V., et al. 2001. Genetic organization of plasmid pXF51 from the plant pathogen *Xylella fastidiosa*. *Plasmid* 45:184-199.
- Mashburn, L. M., and Whiteley, M. 2005. Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature* 437:422-425.
- Mashburn-Warren, L., et al. 2008. Interaction of quorum signals with outer membrane lipids: Insights into prokaryotic membrane vesicle formation. *Mol. Microbiol.* 69:491-502.
- Matsumoto, A., et al. 2012. XatA, an AT-1 autotransporter important for the virulence of *Xylella fastidiosa* Temecula1. *MicrobiologyOpen* 1:33-45.
- Morinaga, K., et al. 2018. *Paracoccus denitrificans* can utilize various long-chain N-acyl homoserine lactones and sequester them in membrane vesicles. *Environ. Microbiol. Rep.* 10:651-654.
- Nascimento, R., et al. 2016. The type II secreted lipase/esterase LesA is a key virulence factor required for *Xylella fastidiosa* pathogenesis in grapevines. *Sci. Rep.* 6:18598.
- Newman, K. L., et al. 2003. Use of a green fluorescent strain for analysis of *Xylella fastidiosa* colonization of *Vitis vinifera*. *Appl. Environ. Microbiol.* 69:7319-7327.
- Newman, K. L., et al. 2004. Cell-cell signaling controls *Xylella fastidiosa* interactions with both insects and plants. *Proc. Natl. Acad. Sci. USA* 101: 1737-1742.
- Noinaj, N., et al. 2010. TonB-dependent transporters: Regulation, structure, and function. *Annu. Rev. Microbiol.* 64:43-60.
- Pathirana, R. D., and Kaparakis-Liaskos, M. 2016. Bacterial membrane vesicles: Biogenesis, immune regulation and pathogenesis. *Cell. Microbiol.* 18: 1518-1524.
- Petersen, T. N., et al. 2011. SignalP 4.0: Discriminating signal peptides from transmembrane regions. *Nat. Methods* 8:785-786.
- Pierce, B. K., et al. 2014. The *Xylella fastidiosa* PD1063 protein is secreted in association with outer membrane vesicles. *PLoS One* 9:ARTN e113504.
- Rapicavoli, J., et al. 2018. *Xylella fastidiosa*: An examination of a re-emerging plant pathogen. *Mol. Plant Pathol.* 19:786-800.
- Redak, R. A., et al. 2004. The biology of xylem fluid-feeding insect vectors of *Xylella fastidiosa* and their relation to disease epidemiology. *Annu. Rev. Entomol.* 49:243-270.
- Reddy, J. D., et al. 2007. ToIC is required for pathogenicity of *Xylella fastidiosa* in *Vitis vinifera* grapevines. *Mol. Plant-Microbe Interact.* 20:403-410.
- Roier, S., et al. 2016. A novel mechanism for the biogenesis of outer membrane vesicles in Gram-negative bacteria. *Nat. Commun.* 7:10515.
- Schertzer, J. W., and Whiteley, M. 2012. A bilayer-couple model of bacterial outer membrane vesicle biogenesis. *MBio* 3:e00297-11.
- Schwechheimer, C., and Kuehn, M. J. 2015. Outer-membrane vesicles from Gram-negative bacteria: Biogenesis and functions. *Nat. Rev. Microbiol.* 13: 605-619.
- Sicard, A., et al. 2018. *Xylella fastidiosa*: Insights into an emerging plant pathogen. *Annu. Rev. Phytopathol.* 56:181-202.
- Sidhu, V. K., et al. 2008. Analysis of outer membrane vesicle associated proteins isolated from the plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris*. *BMC Microbiol.* 8:87.
- Silva, R. R., et al. 2014. ProbMetab: An R package for Bayesian probabilistic annotation of LC-MS-based metabolomics. *Bioinformatics* 30:1336-1337.
- Simpson, A. J., et al. 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. *Nature* 406:151-157.
- Solé, M., et al. 2015. *Xanthomonas campestris* pv. *vesicatoria* secretes proteases and xylanases via the Xps type II secretion system and outer membrane vesicles. *J. Bacteriol.* 197:2879-2893.
- Toyofuku, M., et al. 2017. Membrane vesicle-mediated bacterial communication. *ISME J.* 11:1504-1509.
- Toyofuku, M., et al. 2019. Types and origins of bacterial membrane vesicles. *Nat. Rev. Microbiol.* 17:13-24.
- Turner, L., et al. 2018. *Helicobacter pylori* outer membrane vesicle size determines their mechanisms of host cell entry and protein content. *Front. Immunol.* 9:1466.
- Van Sluys, M. A., et al. 2002. Comparative genomic analysis of plant-associated bacteria. *Annu. Rev. Phytopathol.* 40:169-189.
- Van Sluys, M. A., et al. 2003. Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. *J. Bacteriol.* 185:1018-1026.
- Volgers, C., et al. 2018. Gram-negative bacterial membrane vesicle release in response to the host-environment: Different threats, same trick? *Crit. Rev. Microbiol.* 44:258-273.
- Wai, S. N., et al. 2003. Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin. *Cell* 115:25-35.
- Wang, N., et al. 2012. RpfF-dependent regulon of *Xylella fastidiosa*. *Phytopathology* 102:1045-1053.
- Yun, S. H., et al. 2017. Proteomic characterization of the outer membrane vesicle of the halophilic marine bacterium *Novosphingobium pentaromaticivorans* US6-1. *J. Microbiol.* 55:56-62.