

1 **Biophysical and proteomic analyses suggest functions of *Pseudomonas syringae* pv *tomato***  
2 **DC3000 extracellular vesicles in bacterial growth during plant infection**

3

4 Martin Janda<sup>1,2,+</sup>, Christina Ludwig<sup>3</sup>, Katarzyna Rybak<sup>1</sup>, Chen Meng<sup>3</sup>, Egidio Stigliano<sup>4,5</sup>, Leon  
5 Botzenhardt<sup>1</sup>, Beata Szulc<sup>1</sup>, Jan Sklenar<sup>4</sup>, Frank L.H. Menke<sup>4</sup>, Jacob G. Malone<sup>5,6</sup>, Andreas  
6 Brachmann<sup>1</sup>, Andreas Klingl<sup>1</sup> and Silke Robatzek<sup>1#</sup>

7

8 <sup>1</sup>LMU Biocenter, Ludwig-Maximilian-University of Munich, Großhaderner Strasse 4, 82152  
9 Martinsried, DE

10 <sup>2</sup>University of Chemistry and Technology Prague, Department of Biochemistry and  
11 Microbiology, Technická 5, Prague 6 – Dejvice 166 28, CZ

12 <sup>3</sup>Bavarian Center for Biomolecular Mass Spectrometry (BayBioMS), Technical University of  
13 Munich, Gregor-Mendel-Strasse 4, 85354 Freising, DE

14 <sup>4</sup>The Sainsbury Laboratory, Norwich Research Park, NR4 7UH, UK

15 <sup>5</sup>John Innes Centre, Norwich Research Park, NR4 7UH, UK

16 <sup>6</sup>University of East Anglia, Norwich Research Park, NR4 7TJ, UK

17 <sup>+</sup>Present address: Faculty of Science, University of South Bohemia in České Budějovice,  
18 Branišovská 1645/31a, České Budějovice, CZ

19

20 #Correspondence: Silke Robatzek  
21 LMU Biocenter  
22 Tel.: +49-2180-74124  
23 Email: [robatzek@bio.lmu.de](mailto:robatzek@bio.lmu.de)

24

25

26 Short title: Analysis of *Pto* DC3000 extracellular vesicles

27

28 Key words: Extracellular vesicles, EVs, *Pto* DC3000, proteomics, pattern-triggered immunity,

29 PTI, nanoparticle tracking analysis, NTA, *Arabidopsis thaliana*

30

31 Manuscript information: Summary (180 words), text excluding references (10.450 words). The

32 number of figures: 8 main figures; 6 supplemental figures; 2 main tables; 6 supplemental tables.

## 33 **Summary**

34 Vesiculation is a process employed by Gram-negative bacteria to release extracellular vesicles  
35 (EVs) into the environment. Bacterial EVs contain molecular cargo from the donor bacterium  
36 and play important roles in bacterial survival and growth. Here, we describe EV production in  
37 plant-pathogenic *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto* DC3000), the causal agent  
38 of bacterial speck disease. Cultured *Pto* DC3000 exhibited EV structures both on the cell  
39 surface and in the vicinity of bacterial cells, observed as outer membrane vesicle (OMV)  
40 release. We used in-solution trypsin digestion coupled to mass spectrometry to identify 369  
41 proteins enriched in EVs recovered from cultured *Pto* DC3000. The predicted localization  
42 profile of EV proteins supports the production of EVs also in the form of outer-inner-membrane  
43 vesicles (OIMVs). EV production varied slightly between bacterial lifestyles and also occurred  
44 *in planta*. The potential contribution of EVs to *Pto* DC3000 plant infection was assessed using  
45 plant treatments and bioinformatic analysis of the EV-enriched proteins. While these results  
46 identify immunogenic activities of the EVs, they also point at roles for EVs in bacterial defences  
47 and nutrient acquisition by *Pto* DC3000.

48

## 49 **Introduction**

50 Successful colonization of hosts depends on the ability of microbes to defend themselves  
51 against host immune responses and acquire nutrients. Bacterial pathogens use macromolecular  
52 translocation systems and deliver virulence proteins, so-called effectors, to circumvent host  
53 immunity (Buttner & Bonas, 2010). *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000 is the  
54 causal agent of bacterial speck, a common disease that occurs in tomato production worldwide  
55 (Mansfield *et al.*, 2012, Wilson *et al.*, 2002). *Pto* DC3000 is a Gram-negative bacterium that  
56 invades through openings in the plant surface and propagates in the apoplast, where it takes up  
57 nutrients and proliferates (Melotto *et al.*, 2006, Xin & He, 2013, Xin *et al.*, 2018). Plants

58 respond rapidly to colonization by microbes, activating innate defence strategies, which can  
59 broadly be categorized into pattern-triggered immunity (PTI) activated by microbe-associated  
60 molecular patterns (MAMPs) and effector-triggered immunity (ETI) induced upon recognition  
61 of virulence factors or their actions (Couto D. & Zipfel, 2016, Dodds & Rathjen, 2010).  
62 Virulence of *Pto* DC3000 largely depends on the Type-III secretion system and its secreted  
63 effectors (Kvitko *et al.*, 2009, Nobori *et al.*, 2018, Nobori *et al.*, 2019, Nomura *et al.*, 2006). A  
64 number of Type-III secreted effectors from *Pto* DC3000 and their *in planta* targets have been  
65 identified, many involved in immune suppression and some with roles in gaining access to  
66 nutrients (Wei *et al.*, 2018, Xin *et al.*, 2018). For example, genes encoding proteins for  
67 siderophore biosynthesis are upregulated *in planta* (Nobori *et al.*, 2018). In addition, pathogenic  
68 bacteria need to adapt to the host environment, resisting the defences induced by the host  
69 immune system.

70

71 The survival of infectious Gram-negative bacteria is greatly enhanced by releasing extracellular  
72 vesicles (EVs), a process widely studied in the context of bacteria pathogenic to humans  
73 (Schwechheimer & Kuehn, 2015). During infection, bacterial EVs can counteract the effect of  
74 antimicrobial peptides (Roszkowiak *et al.*, 2019). They also perform immunomodulatory  
75 functions by delivering virulence factors to recipient cells resulting in immune-suppression  
76 (Kaparakis-Liaskos & Ferrero, 2015), despite having the capacity to activate defences due to  
77 their immunogenic cargoes (Kaparakis-Liaskos & Ferrero, 2015). More recently, a number of  
78 studies provide evidence that plant pathogenic bacteria, including cultured *Pto* bacteria release  
79 EVs (Bahar *et al.*, 2016, Chowdhury & Jagannadham, 2013, McMillan *et al.*, 2020). Although  
80 insights into both immunogenic and virulent roles have been achieved, little is currently known  
81 about the importance of EV production in bacterial infection success.

82

83 EVs are cytosol-containing membrane “nano” spheres that provide selection, storage and  
84 protection against degradation of enclosed cargoes in a highly dynamic and environmental cue-  
85 responsive manner (Bielska *et al.*, 2019, Rybak & Robotzek, 2019, Schwechheimer & Kuehn,  
86 2015). Gram-negative bacteria actively form EVs by budding and shedding of the outer  
87 membrane, producing so-called outer membrane vesicles (OMVs) (Raposo & Stoorvogel,  
88 2013, Roier *et al.*, 2016). Outer-inner-membrane vesicles (OIMVs) have also been described,  
89 involving a different mode of release such as endolysin-triggered cell lysis (Perez-Cruz *et al.*,  
90 2015, Toyofuku *et al.*, 2019). EVs can also be produced in the form of elongated, tube-shaped  
91 vesicles as observed in Gram-negative *Francisella* spp. (McCaig *et al.*, 2013, Sampath *et al.*,  
92 2018). As insufficient biomarkers are available to convincingly probe their origin, in particular  
93 for *P. syringae*, we will collectively refer to these vesicles as EVs. Notably, EV formation seems  
94 to be an essential process since no bacterial mutant lacking vesicle release has been reported so  
95 far and genetic reduction of vesiculation results in mutants with growth defects (McBroom *et*  
96 *al.*, 2006).

97  
98 Previous studies revealed a number of molecular cargoes present in EVs from phytopathogenic  
99 bacteria of the *Agrobacterium tumefaciens*, *P. syringae*, *Xanthomonas campestris* and *Xylella*  
100 *fastidiosa* species. These EV-associated proteins include degradative enzymes, Type-II-  
101 secreted virulence-associated proteins, components of the Type-III secretion system and its  
102 secreted proteins (Feitosa-Junior *et al.*, 2019, Chowdhury & Jagannadham, 2013, Knoke *et al.*,  
103 2020, Nascimento *et al.*, 2016, Sidhu *et al.*, 2008, Sole *et al.*, 2015). While respective genetic  
104 deletions of EV-associated degradative enzymes reduced bacterial virulence, the role of EVs in  
105 their delivery remained unanswered (Nascimento *et al.*, 2016, Sidhu *et al.*, 2008, Sole *et al.*,  
106 2015). A previous seminal study described the production of EVs as a mechanism, by which *X.*  
107 *fastidiosa* regulates its attachment to host cells and thus the promotes systemic infection

108 (Ionescu *et al.*, 2014). However, elongation factor Tu (EF-Tu) and lipopolysaccharides (LPS)  
109 are abundant components of EVs from *P. syringae*, *X. campestris*, *X. oryzae* and *X. fastidiosa*  
110 (Bahar *et al.*, 2016, Feitosa-Junior *et al.*, 2019, Chowdhury & Jagannadham, 2013, Sidhu *et al.*,  
111 2008). Both represent MAMPs, with EV-associated EF-Tu shown to activate a prototypic PTI  
112 response in a receptor-dependent manner (Bahar *et al.*, 2016). Further results indicate that EV-  
113 induced immunity, triggered with EVs recovered from pathogenic *Pto* DC3000 and the  
114 commensal *P. fluorescens*, protects plants against *Pto* DC3000 infection (McMillan *et al.*,  
115 2020). These studies hint at some contrasting roles that EVs from bacterial phytopathogens  
116 could play during plant infection.

117

118 Here, we used nanoparticle tracking analysis (NTA) to describe the production and the  
119 biophysical properties of EVs from *Pto* DC3000 in different growth conditions including their  
120 accumulation *in planta*. Analysis of *Pto* DC3000 cellular, outer membrane (OM) and EV  
121 proteomes by mass spectrometry identified 369 EV-enriched proteins. The potential  
122 contribution to bacterial growth *in planta* of these proteins was assessed using bioinformatic  
123 analysis as well as exploring plant responses to EVs. These findings expand our understanding  
124 of the functions of EVs in bacterial infection of plants.

125

## 126 **Results**

### 127 ***Pto* DC3000 bacteria vesiculate and produce EVs in culture**

128 We first examined the morphology of *Pto* DC3000 cultures by scanning electron microscopy  
129 (SEM). The bacteria displayed multiple spherical structures protruding from their cell surfaces,  
130 with diameters in the range of 20-120 nm (Fig. 1A; S1A). These vesicle-like structures appeared  
131 to be released from the surface, as similarly sized vesicular structures could also be observed in  
132 the vicinity of the bacteria (Fig. S1A). To determine whether these structures were released

133 from the bacterial cell surface, supernatants of planktonic *Pto* DC3000 cultures were filtered  
134 through 0.22 $\mu$ m-pore membranes to remove intact bacteria and measured by Nanoparticle  
135 Tracking Analysis (NTA) before (fluid sample) and after sucrose density gradient  
136 centrifugation followed by ultracentrifugation (gradient-enriched sample) (Fig. S2A). Density  
137 gradient centrifugation is used to separate EVs from other extracellular materials (Klimentova  
138 & Stulik, 2015). NTA measures particle number (concentration), particle size (median diameter  
139 and distribution), and particle surface charge (mean  $\zeta$ -potential). Both sample types exhibited a  
140 polydisperse sized population of spherical structures with a diameter ranging from  $\sim$  50 to 200  
141 nm and median sizes of 100 nm and 115 nm for fluid samples and gradient enriched samples,  
142 respectively (Fig. 1C, 1G, S1B). This could suggest that *Pto* DC3000 releases vesicles from  
143 different biogenesis routes.

144

145 Pellets obtained from gradient enriched samples were further examined by SEM and revealed  
146 numerous spherical structures (Fig. 1B), yet in this analysis the vesicles diameter ranged  
147 between 25 and 170 nm with a median around 50 nm. It is possible that conditions used for  
148 SEM and NTA differ in their capacity to hydrate the vesicles and/or that NTA underestimates  
149 smaller particles (Bachurski *et al.*, 2019). In addition, co-purifying filamentous structures could  
150 be detected (Fig. 1B). To determine whether EV production is an active process, EVs were  
151 quantified from culture supernatants of *Pto* DC3000 over cultivation time, with increasing  
152 particle numbers observed with bacterial density (Fig. 1D, 1E, S2B). While the total amount of  
153 EVs recovered from bacteria at late exponential growth was higher compared with early growth  
154 stages (Fig. 1D, 1E), calculation of the amount of EVs produced per bacteria showed that  
155 numbers were similar between growth stages (Fig. 1E). The median diameter and  $\zeta$ -potential of  
156 EVs was comparable across growth stages (Fig. 1F, 1G, 1H).

157

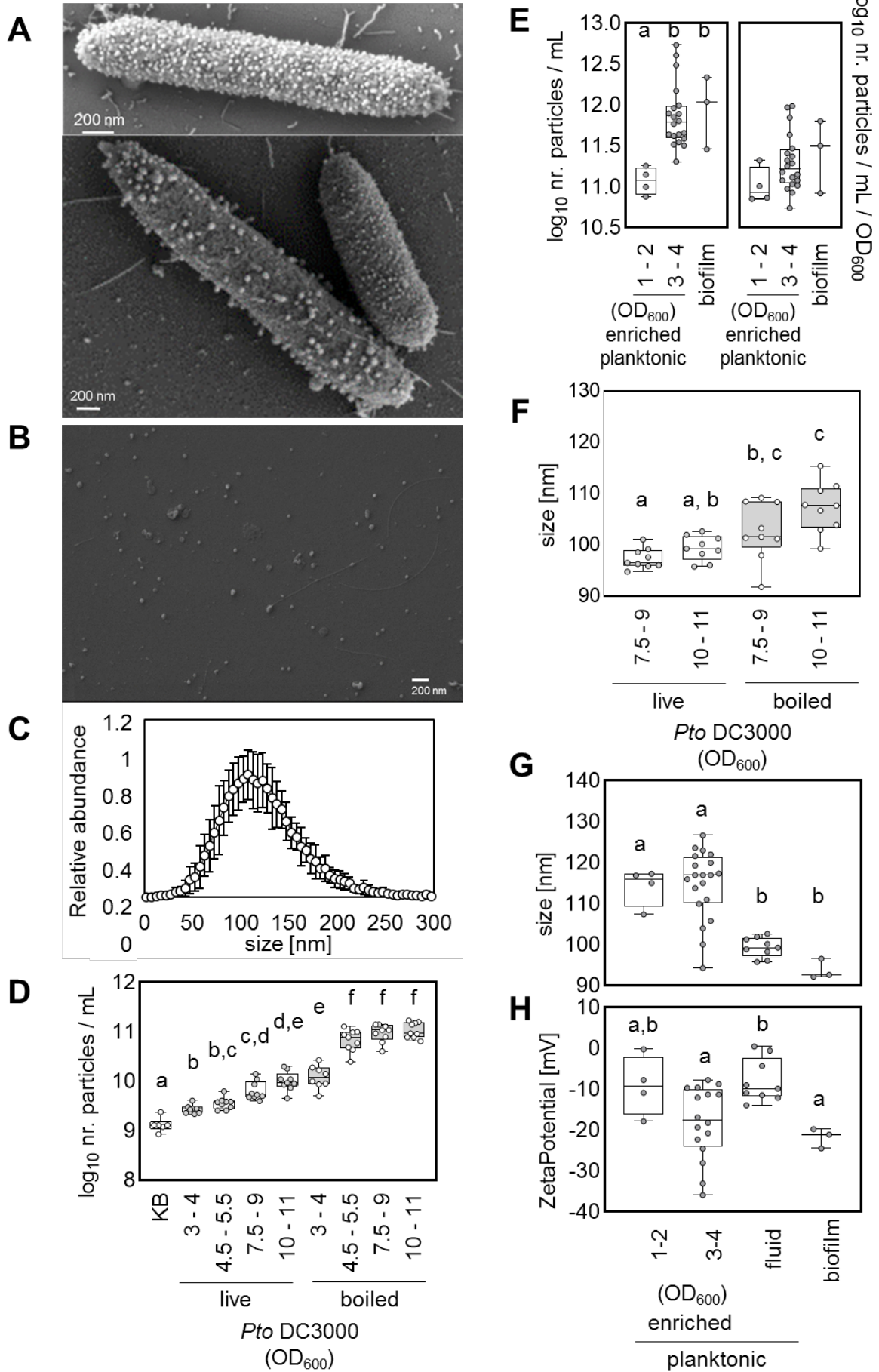
158 Quantification of EVs from *Pto* DC3000 cultures that were incubated in fresh media followed  
159 by heat inactivation showed increased vesicle numbers (Fig. 1D). This suggests that heat  
160 inactivation could additionally trigger the production of vesicles, i.e. from cellular debris and/or  
161 through a process described as explosive cell lysis (Toyofuku *et al.*, 2019). Given this increase  
162 and the distinct size of the vesicles from heat inactivated bacterial cultures (Fig. 1F), it suggests  
163 that the vesicles recovered from culture samples without heat inactivation are predominantly  
164 produced from bacteria as an active process.

165

166 EVs were also isolated from biofilm grown *Pto* DC3000 cultures (Fig. S2C, S2D). The median  
167 diameter of these EVs was smaller compared with EVs from planktonic gradient enriched *Pto*  
168 DC3000 EVs but had a similar size to EVs from fluid samples (Fig. 1G). The mean  $\zeta$ -potential  
169 of EVs from biofilm samples was similar to EVs from planktonic gradient enriched samples  
170 but more negative than EVs from fluid samples of planktonic *Pto* DC3000 cultures (Fig. 1H).  
171 Thus, EVs show diverse biophysical properties depending on bacterial lifestyle, further refuting  
172 the possibility that the purified particles are solely formed through nonspecific assembly of shed  
173 membrane fragments.

174



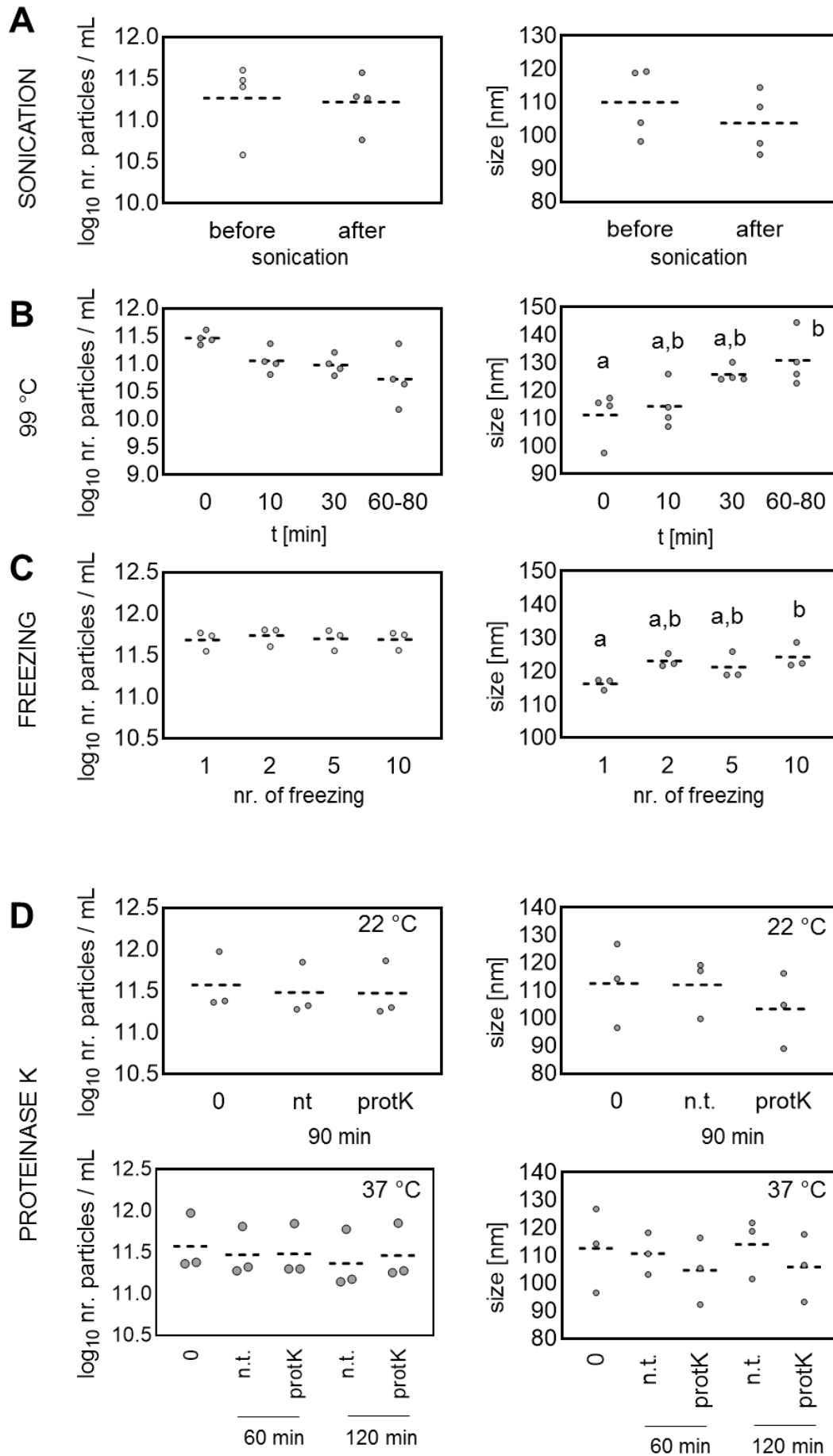


**Figure 1. *Pto* DC3000 release extracellular vesicles with differing biophysical parameters depending on lifestyle.** **A)** Representative SEM micrographs of *Pto* DC3000 growth in planktonic culture ( $OD_{600} = 3-4$ ). **B)** Representative SEM micrograph of gradient enriched *Pto* DC3000 EVs purified from planktonic culture ( $OD_{600} = 3-4$ ); scale bars represent 200 nm. For A and B, the micrographs were selected as representatives from three independent samples. For D-H each dot in the boxplot represents an independent sample. **C)** Size profile of gradient enriched EVs from *Pto* DC3000 planktonic cultures ( $OD_{600} = 3-4$ ), the values represent mean and standard deviations from  $n = 20$ . Concentration **(D)** and size **(F)** of EVs from fluid samples before (live) and after heat inactivation of bacteria (boiled); King's B (KB) medium. Concentration **(E)**, size **(G)** and  $\zeta$ -potential **(H)** of EVs from planktonic cultures (enriched = gradient enriched  $OD_{600} = 1-2$  and  $3-4$  and fluid samples  $OD_{600} = 7.5 - 11$ ) and of EVs from biofilm cultures. For D and F  $n = 8-9$  independent samples, for samples in E and G  $n = 4$  for  $OD_{600} = 1-2$  and  $n = 20$  for  $OD_{600} = 3-4$  for gradient enriched samples,  $n = 9$  for fluid samples and  $n=3$  for biofilm. The box in boxplots extends from 25<sup>th</sup> to 75<sup>th</sup> percentiles, whiskers go down to the minimal value and up to the maximal value, the line in the middle of the box is plotted at the median. Different letters indicate significant (Welsch's ANOVA with Dunnett's T3 multiple comparisons post hoc test;  $p < 0.05$ ).

175

176 To investigate whether the biophysical parameters of *Pto* DC3000 EVs could change upon  
177 mechanical treatments, we subjected the samples to sonication, heating and freezing. In  
178 addition, we tested the effect of incubation with Proteinase K, a treatment used to deplete the  
179 EV samples of extravesicular proteins (Metruccio *et al.*, 2016). None of the treatments  
180 significantly affected the particle concentration (Fig. 2). Also, particle size was not significantly  
181 changed upon sonication and Proteinase K treatments (Fig. 2A, 2D). A significant increase in  
182 particle size was observed after ten freeze-thaw cycles and longer heat exposure (Fig. 2B, 2C).  
183 These observations suggest that EVs are affected by more extreme temperature treatments,  
184 maybe forming higher aggregates, while sonication, shorter heat incubation, fewer freeze-thaw  
185 cycles and Proteinase K treatments showed no significant effects.

186



**Figure 2. Disruptive treatments cause minor changes to biophysical parameters of gradient enriched EVs.** Concentration and size analysis of *Pto* DC3000 EVs from planktonic cultures ( $OD_{600} = 3-4$ ). Effects of **A**) sonication (10 times 30 s); **B**) 99 °C for 80 min; **C**) freezing and thawing (up to ten times); **D**) Proteinase K treatment (10  $\mu\text{g}/\text{mL}$ ) at 22 °C and 37 °C for up to 120 min. Individual circles represent particles characteristics from independent samples;  $n = 3$  to 4. Different letters indicate significant difference (One-way ANOVA with Tukey post hoc test;  $p < 0.05$ ).

187

## 188 *Pto* DC3000 bacteria produce EVs *in planta*

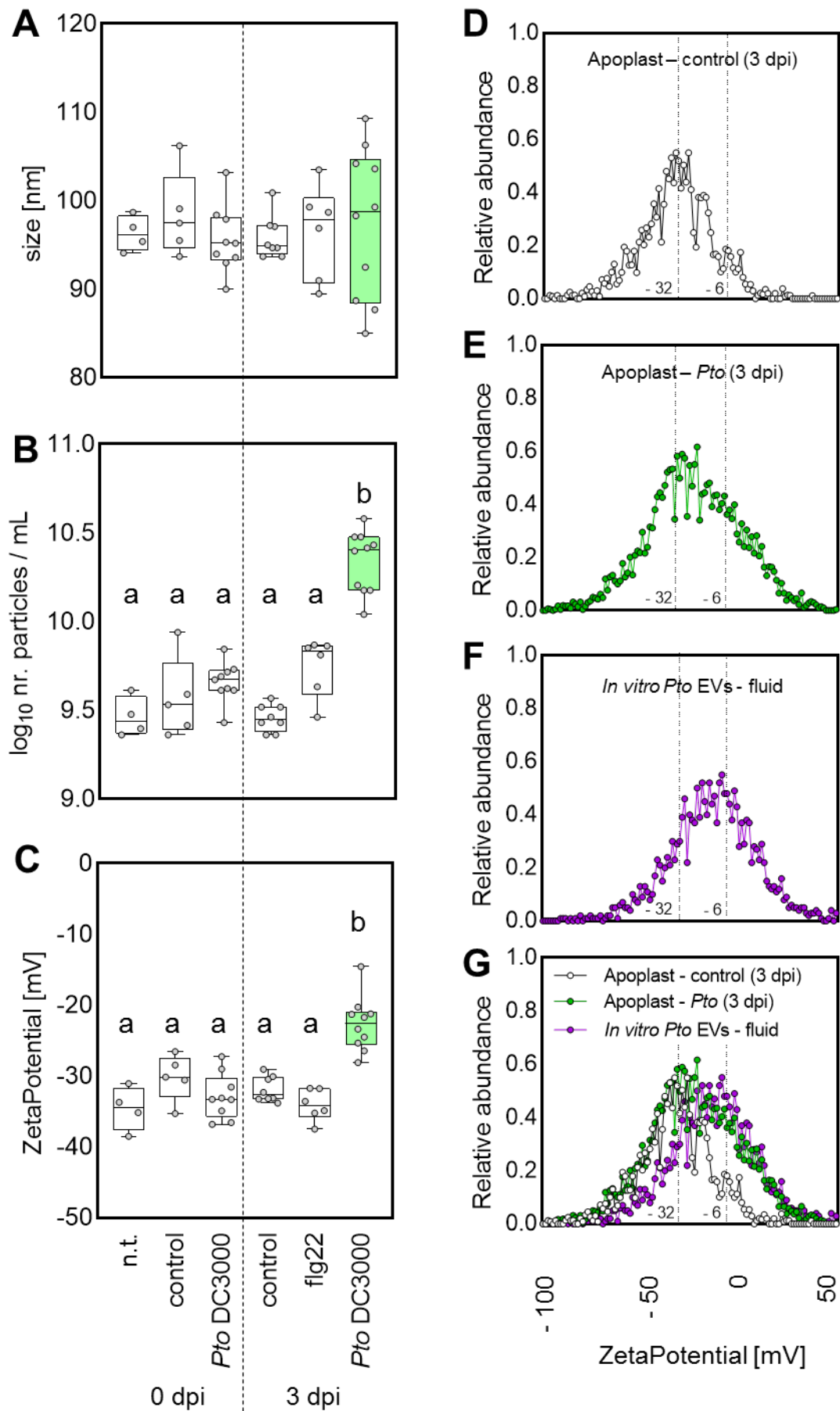
189 To determine whether *Pto* DC3000 releases vesicles *in planta*, apoplastic fluids were recovered  
190 from *Pto* DC3000-infected and control-treated *A. thaliana* leaf tissues at different time points.  
191 The apoplastic fluids were collected, filtered to remove intact bacteria and then directly  
192 characterized by NTA without density gradient centrifugation and ultracentrifugation. In these  
193 apoplastic fluid samples, we identified particles with a median diameter of  $\sim 96$  nm (Fig. 3A),  
194 which increased in abundance upon infection with *Pto* DC3000 (Fig. 3B), consistent with  
195 previous findings (Rutter & Innes, 2017). Increased particle abundance correlated with the  
196 bacterial infection time and titers (Fig. 3B, Fig. S3A, S3B). We also analysed EVs from  
197 apoplastic fluids of plants, which were co-treated with 100 nM flg22 and *Pto* DC3000. Particle  
198 numbers were lower than those recovered from *Pto* DC3000 infection only, consistent with  
199 induced plant defences (Fig. S3C). Taken together, the higher particle numbers and  
200 polydisperse particle size isolated from *Pto* DC3000-infected plants compared to flg22  
201 immune-stimulated plants hints at bacterial-derived EVs present in *A. thaliana* apoplastic fluids  
202 (Fig. 3B).

203

204 Since *Pto* DC3000 (fluid sample) and *A. thaliana* (apoplastic fluid samples) EVs did not  
205 significantly differ in diameter (Fig. 1G, 3A), we focused on the charge of EVs, reflecting the  
206 different surface composition of bacterial (prokaryotic) and plant-derived (eukaryotic) EVs.  
207 Evaluation of the mean  $\zeta$ -potential identified significantly less negatively charged EVs  
208 recovered from apoplastic fluids of *Pto* DC3000-infected plants at three days post infection

209 compared with control treatments and earlier time points (Fig. 3C, 3D, 3E, 3G). This time point  
210 correlated with *in planta* bacterial proliferation and depended on bacterial inoculum (Fig. S3A,  
211 S3B). Plotting the relative particle abundance over particle charge, the  $\zeta$ -potential profiles of  
212 EVs recovered from apoplastic fluids of untreated, control-treated and flg22-treated *A. thaliana*  
213 identified major peaks around -32 mV (Fig. 3C, 3D, S3J). By contrast, the  $\zeta$ -potential profile of  
214 EVs recovered from apoplastic fluids of *Pto* DC3000-infected *A. thaliana* had a broader  
215 distribution with a similar major peak around -32 mV and an additional shoulder around -10  
216 mV (Fig. 3E, 3G). Comparison of the different  $\zeta$ -potential profiles revealed similarities of the  
217 major -32 mV peak across all plant samples, likely representing a plant-derived EV pool (Fig.  
218 3C, 3D, S3J, S3K). Notably, the shoulder around -10 mV detected from apoplastic fluids of *Pto*  
219 DC3000-infected plant samples showed large overlap with the  $\zeta$ -potential profile of EV  
220 recovered from *Pto* DC3000 cultures (fluid samples), with a peak from -20 to 0 mV (Fig. 3E,  
221 3F, 3G). This could, therefore, represent a bacterial-derived EV pool. Since the  $\zeta$ -potential  
222 profiles of EVs recovered from apoplastic fluids of flg22-treated *A. thaliana* did not differ  
223 between untreated or control-treated leaves (Fig. 3C, S3J, S3K), we found no evidence that  
224 plant EVs modulate their surface charge during infection. Thus, our data strongly suggest that  
225 *Pto* DC3000 release EVs during plant infection.

226



**Figure 3. *Pto* DC3000 release EVs *in planta*.** Size, concentration and charge measurements of apoplastic fluids from *A. thaliana* plants infected with *Pto* DC3000 and treated as indicated. **A)** Size of the particles. **B)** Particle concentration in apoplastic fluids. **C)**  $\zeta$ potential of the particles. For A-C the variants represent: n.t. – non-treatment; control – 10 mM MgCl<sub>2</sub>; *Pto* DC3000 (OD<sub>600</sub> = 0.0006); 100 nM flg22. **D-G)** The profile of  $\zeta$ -potential for particles detected in Arabidopsis apoplast treated with MgCl<sub>2</sub> (control; D) for 3 days, with *Pto* DC3000 (*Pto*; OD<sub>600</sub> = 0.0006; E) for 3 days and for EVs from *Pto* DC3000 grown in culture (fluid; F). The dots represent the mean from 8 (D, G\_white), 10 (E, G\_green) and 13 (F, G\_purple). For A-C each dot in the boxplot represents an independent sample. The box in boxplots extends from 25<sup>th</sup> to 75<sup>th</sup> percentiles, whiskers go down to the minimal value and up to the maximal value, the line in the middle of the box is plotted at the median. Different letters indicate significant difference (One-way ANOVA with Tukey post hoc test;  $p < 0.05$ ). The green colour is highlighting the particles from *Pto* DC3000 infected plants (3 dpi).

227

### 228 **EV samples purified from *Pto* DC3000 cultures trigger plant immune responses**

229 Bacterial EVs contain immunogenic molecules such as EF-Tu and LPS, and can contain  
230 digestive enzymes and effectors that undermine host defences (Bahar *et al.*, 2016, Feitosa-  
231 Junior *et al.*, 2019, Knoke *et al.*, 2020, Rybak & Robatzek, 2019). To determine the effect of  
232 EVs from *Pto* DC3000 on plant cells, we first examined the ability of the *Pto* DC3000 EVs to  
233 modulate the outcome of bacterial infection. We pre-treated *A. thaliana* leaves with *Pto*  
234 DC3000 EVs, which limited the growth of subsequently infected *Pto* DC3000 bacteria *in planta*  
235 (Fig. 4A, S4A). Thus, the immunogenic potential of *Pto* DC3000 EVs is sufficient to restrict  
236 bacterial colonization, consistent with recent observations (McMillan *et al.*, 2020). Since  
237 MAMP treatment mediates anti-bacterial protection through the induction of plant immune  
238 reactions (Zipfel *et al.*, 2004), we next evaluated defence gene expression to EV treatment using  
239 *pFRK1::GUS* reporter lines (Kunze *et al.*, 2004). Seedlings were treated with purified EVs  
240 isolated from *Pto* DC3000 cultures, and GUS staining was measured after 18 h. We observed a  
241 significant induction of *pFRK1::GUS* expression triggered by the EVs albeit lower when  
242 compared to flg22 treatments (Fig. 4B, 4C). EV-induced *FRK1* upregulation is in agreement  
243 with previous observations (Bahar *et al.*, 2016). We also tested whether treatment with *Pto*  
244 DC3000 EVs could arrest seedling growth, a prototypic PTI response of plants to continual  
245 MAMP stimulation (Bredow *et al.*, 2019). Unexpectedly, we observed no significant growth

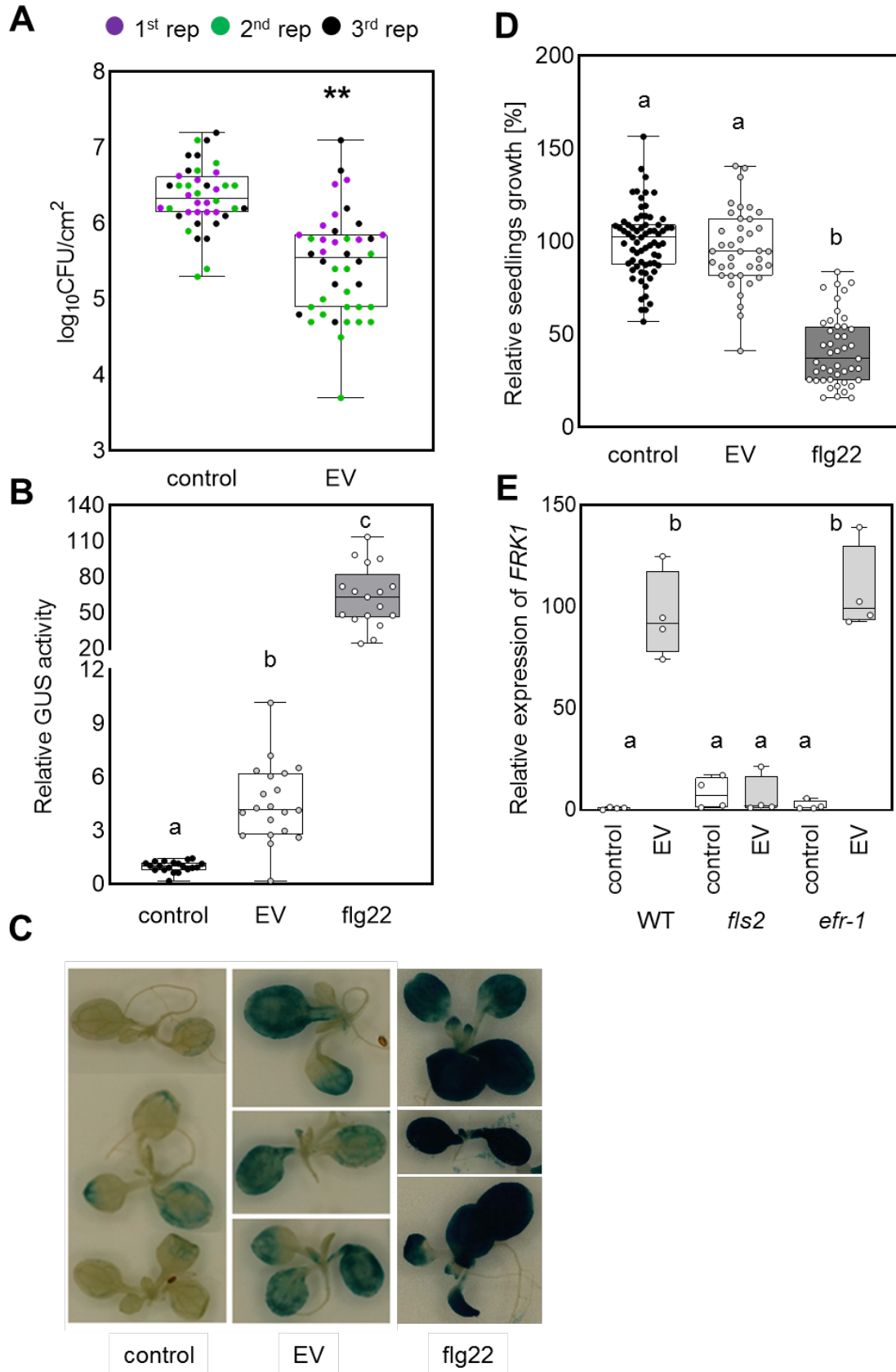
246 reduction (Fig. 4D). This suggests that immune induction by *Pto* DC3000 EVs does not affect  
247 plant growth, unlike treatment with flg22 (Fig. S4B) (Bredow *et al.*, 2019).

248

249 A previous study demonstrated that bacterial (*X. campestris*) EV activation of *FRK1* expression  
250 depends on the EF-Tu Receptor (EFR), which is responsible for detection of the immunogenic  
251 peptide elf18 derived from bacterial EF-Tu (Bahar *et al.*, 2016, Zipfel *et al.*, 2006). To  
252 determine the pathway, by which the *Pto* DC3000 EVs trigger immune responses, we treated  
253 *efr-1* and *flagellin sensing 2 (fls2)* mutants, the latter responsible for recognition of the  
254 immunogenic peptide flg22 of bacterial flagellin in *A. thaliana* (Zipfel *et al.*, 2004), with *Pto*  
255 DC3000 EVs and monitored *FRK1* gene expression. The *Pto* DC3000 EVs triggered *FRK1*  
256 gene expression in wild type and *efr-1* mutants to similar levels (Fig. 4E). No *FRK1* induction  
257 was observed in *fls2* mutants. Thus, the EVs isolated from *Pto* DC3000 cultures must contain  
258 bacterial flagellin. Notably, SEM analysis of gradient enriched EV samples showed the co-  
259 purification of filament-like structures (Fig. 1B), which could represent detached bacterial  
260 flagellar or pili. This suggests that co-purifying flagellin molecules may trigger plant immune  
261 responses.

262





**Figure 4. Immunogenic effects of *Pto* DC3000 EVs.** A) *Pto* DC3000 growth (CFU) after infection into leaves of *A. thaliana* without and with EV pre-treatment at 3 dpi (control = 0.02 mM EDTA). Three biological repeats consisting each 12 independent samples were

performed. The dots with the same colour represent independent samples from one biological repeat. **B)** Quantification of *pFRKI::GUS* activity in seedlings incubated without and with EVs (concentration  $\approx 1.10^{10}$ ) or with 100 nM flg22 for 18 h. **C)** Representative pictures of *pFRKI::GUS* seedlings incubated without and with EVs (concentration  $\approx 1.10^{10}$ ) or with 100 nM flg22 for 18 h. **D)** Fresh weight of seedlings grown without and with EVs (concentration  $\approx 1.10^{10}$ ) for 8 days. For control  $n = 69$ ; for 100 nM flg22 treatment  $n = 44$  and for EV treatment  $n = 39$  of independent samples. **E)** Relative *FRKI* gene expression in seedlings of the indicated genotypes incubated without and with EVs (concentration  $\approx 1.10^{10}$ ) for 5 h (control = 0.02 mM EDTA), four independent samples were used for each variant. For A, B, D, E each dot in the boxplot represents an independent sample. The box in boxplots extends from 25th to 75th percentiles, whiskers go down to the minimal value and up to the maximal value, the line in the middle of the box is plotted at the median. Asterisks indicate statistical significances (two tailed Welsch's t-test;  $p < 0.01$ ) in A; different letters indicate significant differences (Welsch's ANOVA with Dunnett's T3 multiple comparisons post hoc test;  $p < 0.05$ ) in B, D and E.

263

264 **EVs from cultured *Pto* DC3000 are enriched in proteins with predicted roles in transport**  
265 **and antimicrobial peptide resistance**

266 To gain insights into the function of *Pto* DC3000 EVs during the infection process, we  
267 characterized the proteome of EVs using liquid chromatography-based tandem mass  
268 spectrometry (LC-MS/MS). The *Pto* DC3000 EV-associated proteins were isolated from  
269 planktonic *Pto* DC3000 cultures by gradient enrichment. The proteomes of whole cells (WC)  
270 (Park *et al.*, 2014) and the outer membrane (OM) (Choi *et al.*, 2011) from bacteria grown to  
271 late exponential phase ( $OD_{600} = 3-4$ ; Fig. S2B) were also analysed and compared with the EV  
272 proteome. As expected, we detected the most proteins from the WC sample ( $n = 1587$ ), followed  
273 by the EV sample ( $n = 890$ ) and 212 proteins in OM samples (Fig. 5A, Table S1). In total, 2898  
274 proteins were identified over all samples, of which 1899 proteins were identified at least in three  
275 samples per sample type (WC, EV or OM). These proteins were taken forward for further  
276 analysis (Table S1). Similar protein intensity distributions were obtained for all samples (LFQ  
277 values were generated by MaxQuant, Fig. S5) and the four replicate measurements per sample  
278 type fell into sample clusters on the first and second principal components, suggesting a  
279 systematic difference in the proteomes of these three sample types (Fig. 5B). By comparing the  
280 proteomes of EV and WC, we identified 369 EV-enriched proteins, consisting of 162 proteins

281 exclusively identified in at least three replicates of EV sample (EV unique; Fig. 5C) and 207  
282 proteins significantly higher in the EV compared with WC (Fig. 5C; Table S1).

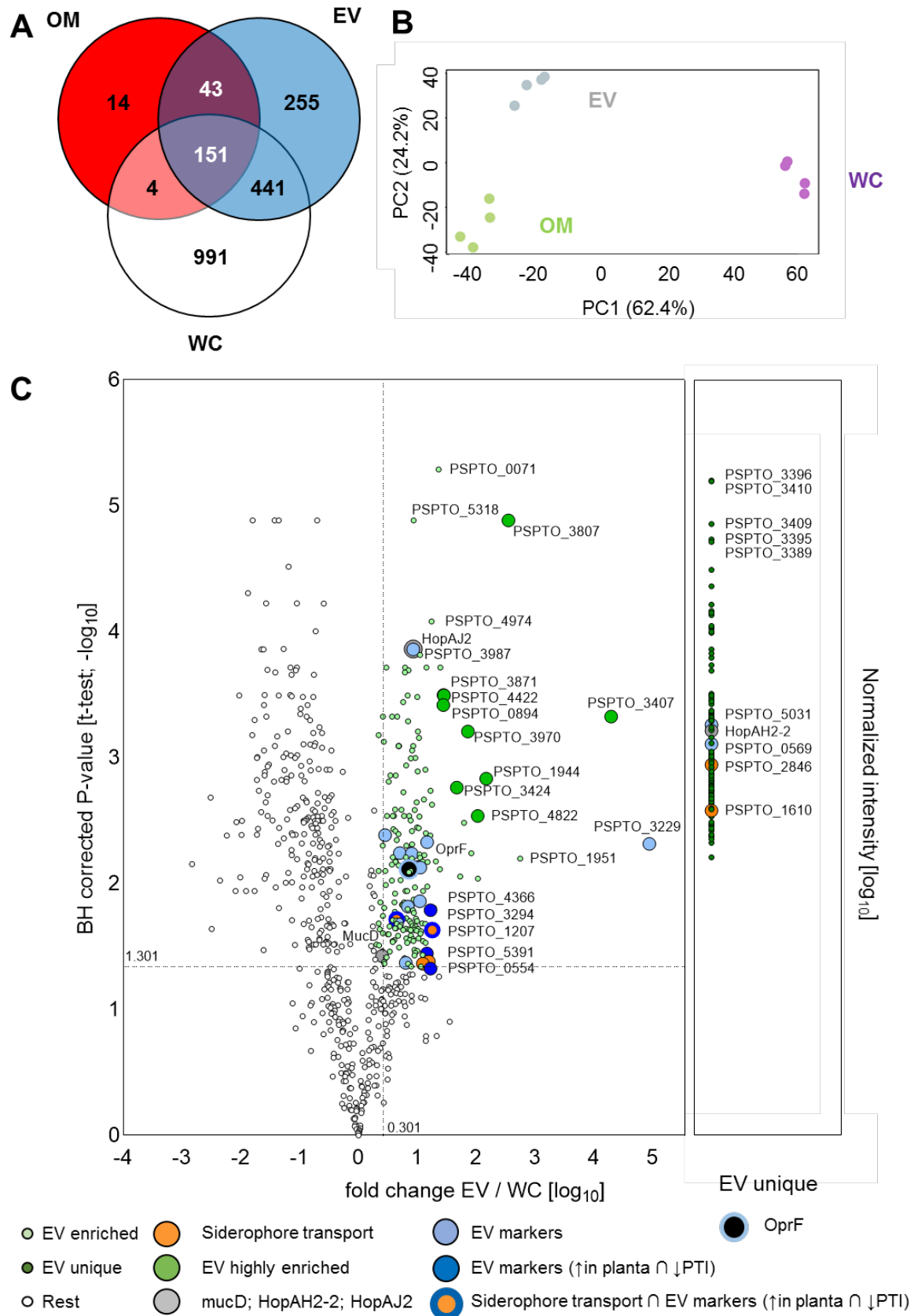
283

284 Of the nine labelled highly-enriched EV and the five EV-unique proteins (Fig. 5C), eight have  
285 unknown subcellular localization (PSPTO\_3807; PSPTO\_4822; PSPTO\_0894; PSPTO\_3407;  
286 PSPTO\_3424; PSPTO\_3970, PSPTO\_3396; and PSPTO\_3409). This could indicate that these  
287 proteins are predominantly present in EVs, a localization not included in the predictions. Four  
288 proteins are annotated as lipoproteins, reported in other Gram-negative bacteria to mediate the  
289 cross-linking of the peptidoglycan (PG) layer with the OM and thus playing roles in the  
290 production of OMVs (Schwechheimer and Kuehn, 2015). PSPTO\_3409 is the locus tag for the  
291 ATP-dependent ClpP-1 protease. Clp proteases were previously shown to regulate quorum  
292 sensing, in turn affecting OMV production and biofilm formation (Figaj *et al.*, 2019, Hall *et al.*,  
293 2017). The other were annotated as hypothetical proteins.

294

295 The EV-enriched proteome included proteins related to virulence (Fig. 5C, Table S1), such as  
296 MucD (PSPTO\_4221) (Wang *et al.*, 2019), HopAJ2 (PSPTO\_4817) (Vinatzer *et al.*, 2006) and  
297 HopAH2-2 (PSPTO\_3293) (Lovelace *et al.*, 2018, Schechter *et al.*, 2006). A major function of  
298 virulence proteins is the suppression of PTI (Block & Alfano, 2011). To test whether *Pto*  
299 DC3000 EVs could suppress a prototypic PTI response, we pre-treated leaves with EVs from  
300 cultured bacteria 24 h before eliciting a ROS burst with MAMPs (flg22, elf18). EV pre-  
301 treatments neither significantly reduced nor increased the MAMP-induced ROS production  
302 (Fig. 6A). This suggests that under the tested conditions, *Pto* DC3000 EVs are not  
303 predominantly involved in immune inhibition and/or further enhancing MAMP responses.

304



**Figure 5. Proteomic analysis identifies 369 proteins enriched in *Pto* DC3000 EVs. A)** Comparison of proteins detected in *Pto* DC3000 whole cell lysate (WC), outer membrane (OM) and extracellular vesicles (EV). **B)** Principal Component Analysis (PCA) analysis of

identified proteins C) Volcano plot comparing EV and WC proteomes. EV-enriched proteins were defined in two categories: I) fold change EV/WC > 2 & FDR < 0.05 (t-test); II) measured in three replicates in EV but not in WC. In addition, the mean intensity in EV protein needs to be in the top 50% of all proteins, so only high intensity proteins in EV are selected. Four types of proteins enriched or unique in EVs were highlighted: proteins related to virulence; proteins related to siderophore transport; candidate EV biomarkers, and proteins highly enriched in EVs compared with WC.

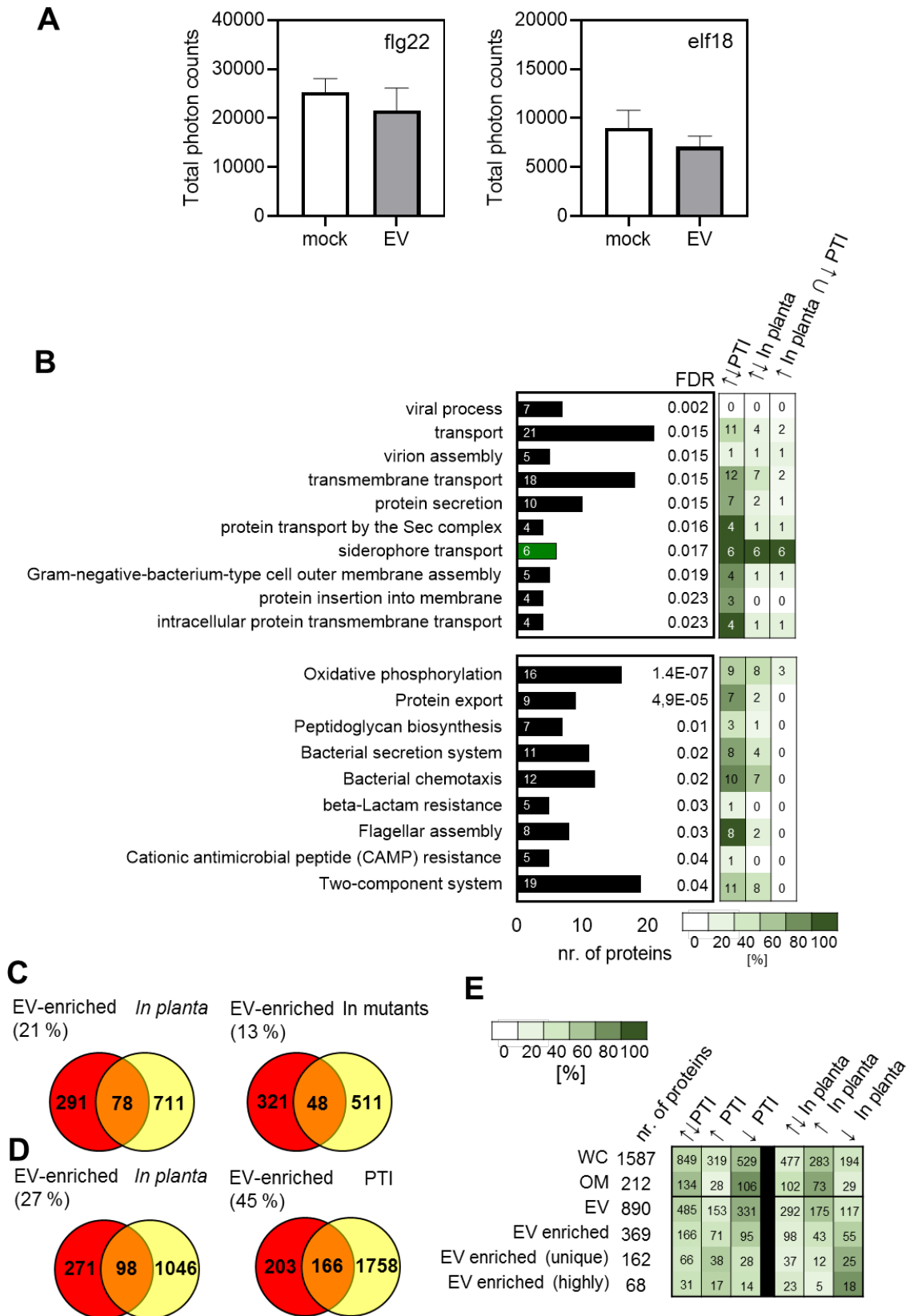
305

306 Next, we performed a gene set analysis on the 369 EV-enriched proteins to examine the  
307 biological processes (from gene ontology (GO) (Ashburner *et al.*, 2000, The Gene Ontology,  
308 2019) and pathways (KEGG (Kanehisa *et al.*, 2010)), in which these proteins are involved. In  
309 total ten GO biological processes were significantly enriched (FDR < 0,05; DAVID  
310 bioinformatics resources (Huang da *et al.*, 2009a, Huang da *et al.*, 2009b)), out of which six  
311 were connected with the general process “transport”, including transmembrane transport,  
312 intracellular transmembrane transport, protein secretion, siderophore transport and protein  
313 transport by the Sec complex (Fig. 6B; Table S2). Nine KEGG categories, including cationic  
314 antimicrobial peptide resistance,  $\beta$ -lactam resistance and bacterial secretion system are  
315 significantly enriched in EVs (Fig. 6B, Table S2).

316

317 We used available *Pto* DC3000 proteome and transcriptome data to examine the *in planta*  
318 responses of the EV-enriched proteins (Nobori *et al.*, 2018, Nobori *et al.*, 2020). Comparison  
319 with the proteome data showed that of the 369 EV-enriched proteins 78 (21 %) are modulated  
320 *in planta* and 48 (13 %) are modulated in immune deficiency mutants (Fig. 6C, Table S3). Of  
321 the 369 genes coding for EV-enriched proteins, 98 genes (27 %) were differentially transcribed  
322 *in planta* compared to cultured bacteria and 166 genes (45 %) responded to the induction of PTI  
323 (Fig. 6D, Table S3). Most EV-unique and EV-highly enriched proteins were transcriptionally  
324 upregulated *in planta* whereas the majority of the genes of all identified proteins (WC, OM and  
325 EV) were downregulated *in planta* (Fig. 6E). When focussing on GO terms, we found that genes  
326 connected with the general process “transport” responded strongly to *in planta* conditions upon

327 PTI activation (Fig. 6B). Interestingly, all genes connected with siderophore transport were  
328 strongly upregulated in response to *in planta* conditions, but downregulated *in planta* upon  
329 activation of PTI (Fig. 6B, Table 1). Thus, during successful infection the significant enrichment  
330 of siderophore transport proteins at EVs may suggest a role for EVs in iron or other metal ion  
331 acquisition (Fig. 5C, orange labelling). When focussing on KEGG pathways, genes connected  
332 with protein export (seven out of nine), secretion systems (eight out of eleven), chemotaxis (ten  
333 out of twelve) and flagellar assembly (eight out of eight) were affected *in planta* in response to  
334 PTI (Fig. 6B).  
335



**Figure 6. Protein profiles of EVs suggest functions other than classical immune suppression.** **A**) Quantification of MAMP-induced ROS in leaves treated without and with EVs (concentration  $\approx 1.10^{10}$ ) for 24 h. The bars represent mean and error bars represents SEM from  $n = 12$ . **B**) Enriched proteins in GO biological processes and KEGG categories in EV-

enriched proteins. The heat-map represents the transcriptional profile based on the comparison of our data with transcriptomic data from (Nobori *et al.*, 2018) of genes encoding proteins identified by GO enrichment analysis. The intensity of green colour represents the percentage of affected genes and numbers in boxes represent the exact number of affected proteins. The arrows indicate transcriptional up- and downregulation. **C)** The Venn diagram represents the number of genes encoding EV-enriched proteins that are transcriptionally regulated *in planta* and responding to immune-deficient *in planta* conditions (Nobori *et al.* 2020). **D)** Transcriptional regulation of *Pto* DC3000 genes encoding EV-enriched proteins *in planta* and responding to PTI (Nobori *et al.*, 2018). **E)** The heat-map represents the transcriptional regulation of genes encoding identified proteins by proteome analysis *in planta* and responding to PTI (Nobori *et al.*, 2018).

336

**Table 1** *In planta* transcription<sup>1</sup> of genes coding proteins enriched in EVs belonging to GO:siderophore transport

Locus tag	Subcellular Localization	Product Description	Gene transcription		other
			<i>In planta</i> *	PTI**	
PSPTO_1207	Outer Membrane	iron(III) dicitrate transport protein fecA	↑	↓	EV marker
PSPTO_1610	Outer Membrane	TonB-dependent siderophore receptor	↑	↓	EV unique
PSPTO_2152	Outer Membrane	TonB-dependent siderophore receptor	↑	↓	
PSPTO_2846	Outer Membrane	TonB-dependent siderophore receptor	↑	↓	EV unique
PSPTO_3294	Outer Membrane	TonB-dependent siderophore receptor	↑	↓	EV marker
PSPTO_3574	Outer Membrane	TonB-dependent siderophore receptor	↑	↓	

<sup>1</sup> Nobori *et al.* 2018. Increased (↑) or decreased (↓) gene transcription *in planta* vs *in vitro*\* or under induced PTI *in planta* vs non-induced PTI *in planta*\*\*.

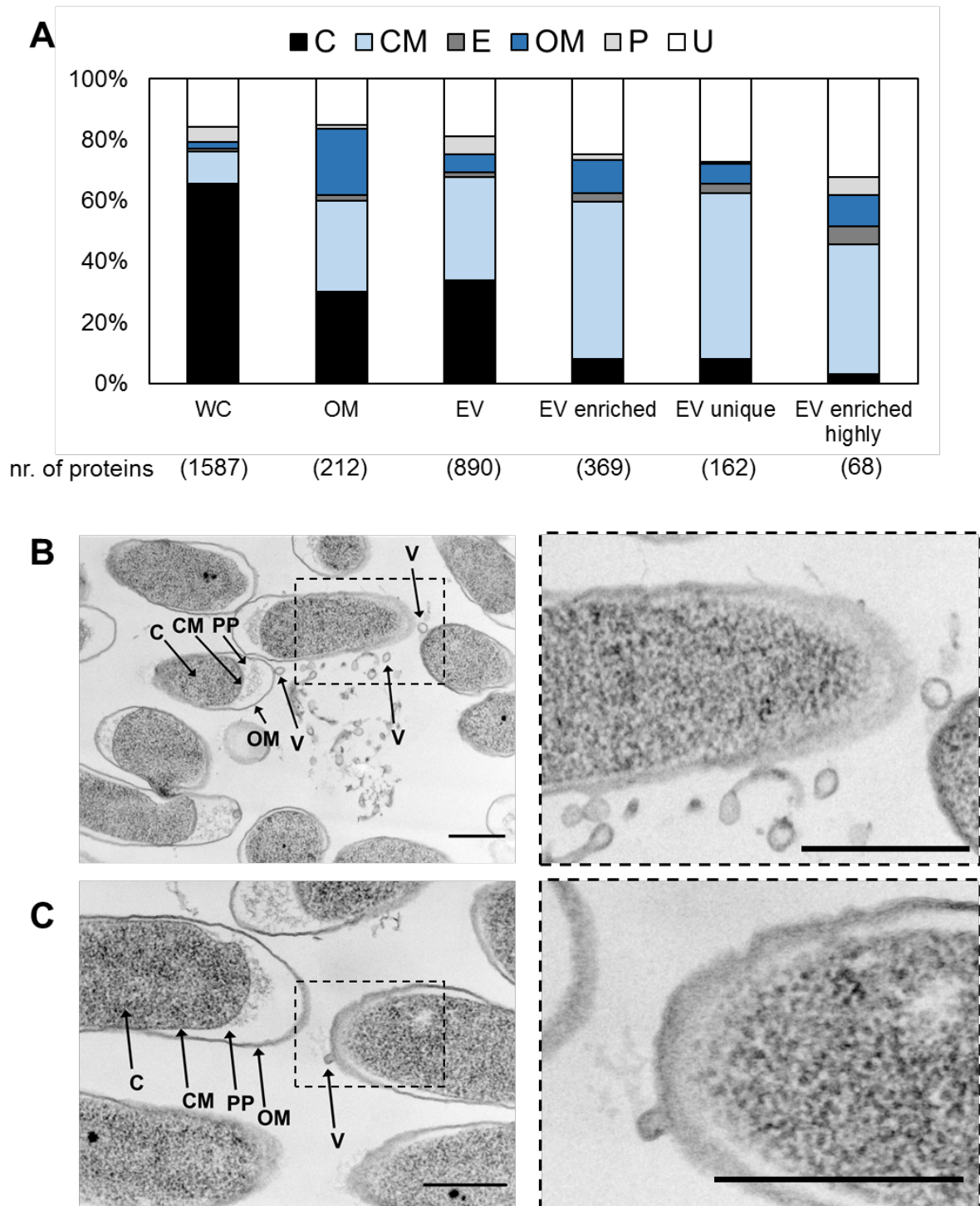
337

### 338 ***Pto* DC3000 appears to release EVs in the form of OMVs and OIMVs**

339 Classification of the proteins enriched in EVs by putative subcellular localization revealed  
 340 distinct localization profiles compared with WC and OM proteins. While 66 % of WC proteins  
 341 were cytoplasmic, about half (51 %) of the EV-enriched proteins were cytoplasmic membrane-  
 342 associated, with the next largest known class being OM-associated (11 %) (Fig. 7A). Yet, being  
 343 putative localizations, we cannot exclude the possibility of other/additional localizations of the  
 344 proteins as currently predicted, in particular for anchor-less proteins. Because the localization  
 345 profile of the EV-enriched protein suggested that *Pto* DC3000 produces EVs in the form of  
 346 OIMVs (Perez-Cruz *et al.*, 2015), we performed additional transmission electron microscopy  
 347 (TEM) analysis of *Pto* DC3000 bacteria. Micrographs of the sectioned samples showed several  
 348 structures reminiscent of budding vesicles from the bacterial outer membrane (Fig. 7B, 7C).  
 349 Combining the data from proteomics and TEM, it may suggest that *Pto* DC3000 produces EVs



350 in the form of both OMVs and OIMVs, as previously described for the closely related species  
 351 *Pseudomonas aeruginosa* (Toyofuku *et al.*, 2019).



**Figure 7. Localization profiles of EV proteins and TEM analysis suggest the release of *Pto* DC3000 EVs in the form of OMVs and OIMVs. A) Predicted protein localization of identified in whole cell lysate (WC), outer membrane (OM), EVs, EV-enriched, EV unique (the proteins identified only in EVs not in WC) and EV enriched – highly (protein which  $FDR < 0.005$  and  $EV/WC > 20$ ) in [%]. B-C) The left panel shows representative TEM micrographs from planktonic *Pto* DC3000 cultures ( $OD_{600} = 3-4$ ). C, cytoplasm; CM,**

cytoplasmic membrane; OM, outer membrane; PP, periplasm; V, vesicle. All scale bars = 500 nm. **B**) A lot of smaller and larger vesicles in proximity to cells. It is important to note that the larger vesicle-like structures could also represent debris of death cells. **C**) Budding vesicle in the right part of the micrograph. Dashed boxes indicate enlarged regions of the micrographs shown in the right panel.

352

### 353 **Comparative analysis of proteomic data revealed 20 candidates for *Pto* DC3000 EVs** 354 **markers**

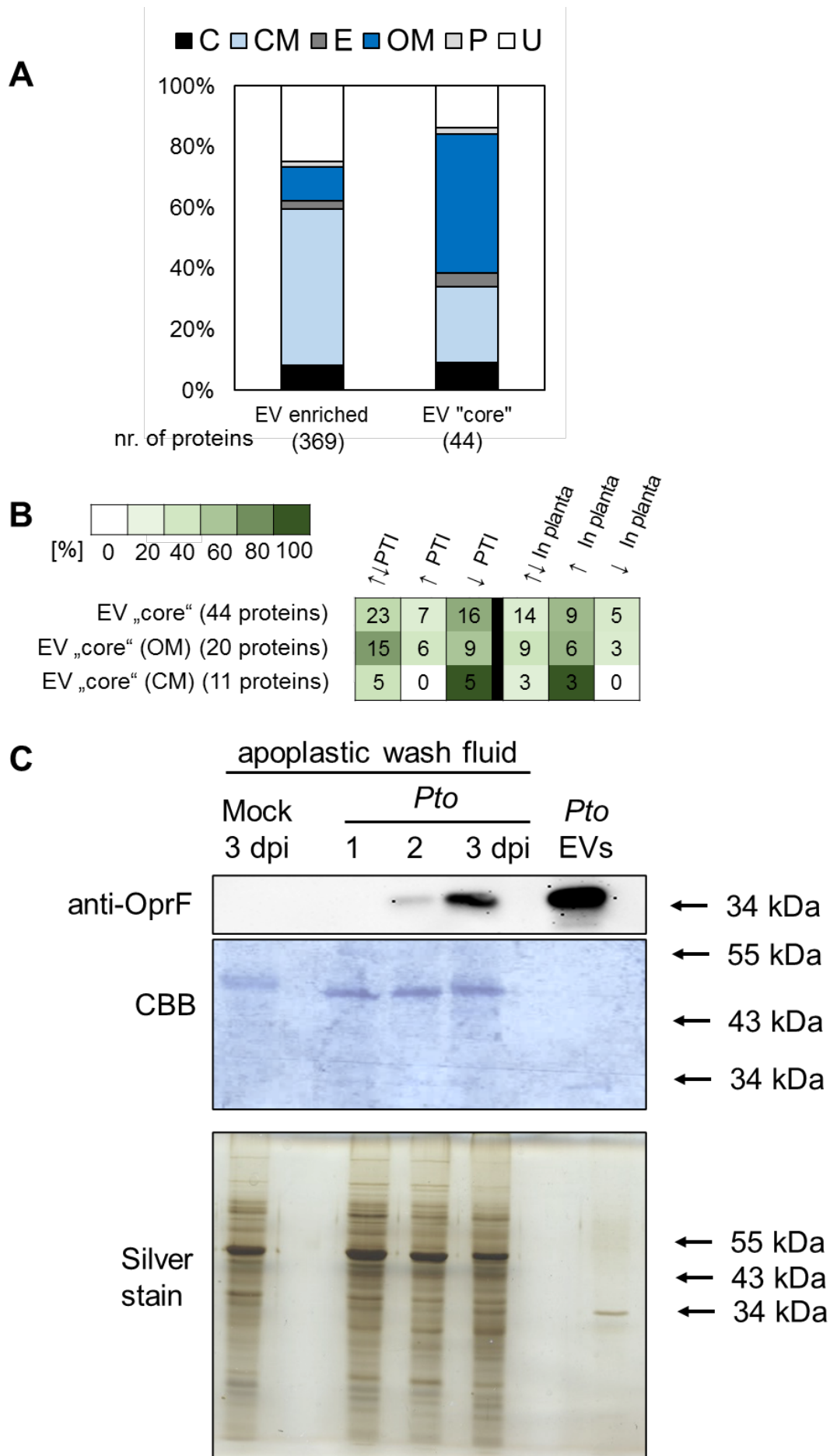
355 Since a number of EV proteomes have been reported from *P. aeruginosa* (Couto N. *et al.*, 2015,  
356 Choi *et al.*, 2011, Reales-Calderon *et al.*, 2015), we addressed whether the protein composition  
357 of EVs from *Pto* DC3000 and *P. aeruginosa* PAO1 (*Pa* PAO1) share similarities. We focussed  
358 on three published *Pa* PAO1 EV proteomes and found that 103 proteins were identified in the  
359 EV proteomes across the three reports (Couto N. *et al.*, 2015, Choi *et al.*, 2011, Reales-Calderon  
360 *et al.*, 2015). Of the 103 shared EV proteins from *Pa* PAO1, we could identify 100 orthologous  
361 proteins encoded in the *Pto* DC3000 genome and 44 proteins were enriched in *Pto* DC3000  
362 EVs (Table S4). We refer to these as the EV “core”. These proteins were highly enriched in  
363 localization to the outer membrane (44 %) and cytoplasmic membrane (26 %) (Fig. 8A),  
364 consistent with EVs released in the form of OMVs (Fig. 7B, 7C). From these 44 proteins, 20  
365 were putative outer membrane-localized proteins and thus represent good candidate biomarkers  
366 for the detection of EVs (Table 2, S4; Fig. 5C blue labelling). Interestingly, 20 out of 31 proteins  
367 with predicted membrane localization (20 out of 31) were transcriptionally regulated *in planta*  
368 in response to PTI activation (Nobori *et al.*, 2018), of which 14 showed downregulation.  
369 Overall, twelve of the 31 proteins responded transcriptionally to the *in planta* condition, with  
370 nine showing upregulation (Fig. 8B).

371

372 One of the predicted EV markers is OprF (Outer membrane porin OprF; Fig. 5C black  
373 labelling), which we used for immunodection of *Pto* DC3000 EVs *in planta* and purified from  
374 *Pto* DC3000 cultures. Using *anti*-OprF antibodies, we identified specific bands in filtered

375 apoplastic fluids of *A. thaliana* leaves infected with *Pto* DC3000 at two and three days post  
376 infection but not in control treated plants (Fig. 8C). This provides additional evidence that *Pto*  
377 DC3000 releases EVs *in planta* during infection.

378



**Figure 8. Prediction of *Pseudomonas* “core” EVs proteins identifies biomarkers for the detection of *Pto* DC3000 EVs in planta.** **A)** Predicted protein localization of the *Pto* DC3000 EV enriched proteins whose orthologs are found in *P. aeruginosa* EV proteomes [%] total number of identified proteins is 44. **B)** Heat-map representing transcriptional changes in genes encoding *Pseudomonas* “core” EV proteins, EV “core” proteins localized on outer membrane (OM) and EV “core” proteins localized on cytoplasmic membrane (CM). The intensity of green colour represents percentage of affected genes and the numbers in boxes show the exact number of affected genes. **C)** Immunoblot monitoring OprF, a predicted *Pto* DC3000 EV biomarker, in EVs collected from apoplastic fluids. OprF antibodies detect bands in apoplastic fluids from *A. thaliana* infected with *Pto* DC3000 and in gradient enriched EVs. Coomassie Brilliant Blue (CBB) and silver staining are shown as loading control.

379

380

**Table 2** *In planta* transcription<sup>1</sup> of genes coding proteins suggested as promising candidates to be EV markers

Locus Tag	UniProt ID	Subcellular Localization	Name	Gene expression		In planta**	other
				PTI*			
PSPTO_0554	Q88A43	Outer Membrane	organic solvent tolerance protein	↑		↑	
PSPTO_0569	Q88A28	Outer Membrane	autotransporting lipase, GDSL family	↑		n.s.	EV unique
PSPTO_1207	Q887S9	Outer Membrane	iron(III) dicitrate transport protein fecA	↑		↑	Siderophore transport
PSPTO_1296	Q887J6	Outer Membrane	porin B	↑		n.s.	
PSPTO_1437	Q886Y7	Outer Membrane	lysyl-tRNA synthetase	↑		n.s.	
PSPTO_1542	Q886N5	Outer Membrane	outer membrane protein	↑		n.s.	
PSPTO_1720	Q885W1	Outer Membrane	outer membrane protein	↑		↓	
PSPTO_2272	Q883S8	Outer Membrane	outer membrane lipoprotein OprI	n.s.		↓	
PSPTO_2299	Q883Q1	Outer Membrane	outer membrane porin OprF	n.s.		↑	
PSPTO_3229	Q880E1	Outer Membrane	filamentous hemagglutinin, intein-containing	↑		↓	
PSPTO_3294	Q87ZX8	Outer Membrane	TonB-dependent siderophore receptor	↑		↑	Siderophore transport
PSPTO_3971	Q87Y41	Outer Membrane	peptidoglycan-associated lipoprotein	n.s.		n.s.	
PSPTO_3987	Q87Y25	Outer Membrane	porin D	n.s.		n.s.	beta-Lactam resistance
PSPTO_4115	Q87XR1	Outer Membrane	lipoprotein SlyB	↑		n.s.	
PSPTO_4366	Q87X24	Outer Membrane	iron-regulated protein A	↑		↑	
PSPTO_4839	Q87VU6	Outer Membrane	hypothetical protein	↑		n.s.	
PSPTO_4940	Q87VJ6	Outer Membrane	hfk protein	↑		n.s.	
PSPTO_4977	Q87VG0	Outer Membrane	outer membrane efflux protein ToIC	↑		n.s.	Bacterial secretion system; Lactam resistance;
PSPTO_5031	Q87VA8	Outer Membrane	type IV pilus biogenesis protein PilJ	n.s.		n.s.	antimicrobial peptide (CAMP) resistance
PSPTO_5391	Q87UB4	Outer Membrane	outer membrane porin, OprD family	↑		↑	EV unique

<sup>1</sup> Nobori et al. 2018. Increased (↑) or decreased (↓) gene transcription *in planta* vs *in vitro*\* or under induced PTI *in planta* vs non-induced PTI *in planta*\*\* . n.s. = non-significant

## 381 **Discussion**

382 Bacterial EVs have been widely studied for their content and biological importance in various  
383 human diseases. Until recently, however, EV production by phytopathogenic bacteria has been  
384 mostly disregarded (Rybak & Robatzek, 2019). In the past few years, the attention in plant-  
385 microbe interactions has turned slowly towards EV signalling. EV production by  
386 phytopathogenic bacteria was shown for *A. tumefaciens* C58, *Xanthomonas campestris* pv  
387 *campestris* (*Xcc*), *X. campestris* pv *vesicatoria*, *Pto* T1, *Pto* DC3000 and *Xyllela fastidiosa*  
388 subsp. *fastidiosa* Temecula-1, subsp. *pauca* 9a5c and subsp. Fb7 (Bahar *et al.*, 2016, Feitosa-  
389 Junior *et al.*, 2019, Chowdhury & Jagannadham, 2013, Ionescu *et al.*, 2014, Knoke *et al.*, 2020,  
390 McMillan *et al.*, 2020, Sidhu *et al.*, 2008, Sole *et al.*, 2015). In this study, we provide several  
391 pieces of evidence for the role of EVs in the interaction of *Pto* DC3000 with plants: i) the  
392 bacteria produce EVs *in planta* during infection; ii) proteins responding to the plant  
393 environment are enriched in EVs; iii) known MAMPs and effectors are associated with EVs;  
394 and iv) plants respond to EVs with prototypic PTI reactions.

395  
396 We show that *Pto* DC3000 produces spheres bulging from its outer membrane and releases  
397 spherical vesicles into the environment (Fig. 1A, 7B, 7C). Our data indicate the production of  
398 EVs predominantly in the form of OMVs albeit the predicted localization profiles of the EV-  
399 enriched proteins also suggest OIMV production (Fig. 7A, 7B, 7C, 8A), both representing well-  
400 established routes of vesicle release in Gram-negative bacteria (Toyofuku *et al.*, 2019). The  
401 more polydisperse size seen for EVs recovered from apoplastic fluids of susceptible *Pto*  
402 DC3000 infected plants could not only represent a mixed pool of plant and bacterial derived  
403 EVs, but also suggests that *Pto* DC3000 might release EVs from different biogenesis routes  
404 including membrane blebbing and explosive cell lysis.

405

406 Our results suggest the regulated production of EVs and the specific enrichment of proteins to  
407 EVs. *Pto* DC3000 cultures produced more EVs with increasing bacterial density during  
408 exponential-phase growth, showing a correlation between bacteria and EV numbers (Fig. 1E).  
409 The production of EVs by *Pto* DC3000 was slightly responsive to bacterial growth style and  
410 isolation technique. EVs produced from biofilm *Pto* DC3000 and fluid planktonic culture were  
411 smaller and more negatively charged than gradient enriched planktonic EVs. Moreover, heat  
412 inactivation of bacteria increased vesicle numbers and size (Fig. 1D, 1F). Since EVs from heat  
413 inactivated bacteria did not differ in charge profiles compared to untreated EVs (Fig. S6D), it  
414 is possible that explosive cell lysis contributes to the higher EV numbers (Toyofuku *et al.*,  
415 2019). Turnbull *et al.* demonstrated that explosive cell lysis of a sub-population of cells from *P.*  
416 *aeruginosa* biofilms results in the generation of bacterial EVs (Turnbull *et al.*, 2016).  
417 Furthermore, heat shock stimulates the release of OMVs, most likely a result of high levels of  
418 un- and misfolded proteins accumulating in heat-stressed cells (Macdonald & Kuehn, 2013,  
419 McBroom & Kuehn, 2007). In this context it is interesting to note that the biophysical  
420 characteristics of *Pto* DC3000 EVs remained largely unchanged under disruptive treatment  
421 conditions except boiling (Fig. 2). Thus, the EVs from *Pto* DC3000 are stable and can maintain  
422 functionality as reported previously for other bacteria (Alves *et al.*, 2016, Arigita *et al.*, 2004,  
423 Frank *et al.*, 2018).

424

425 A range of activities has been associated with bacterial EVs. This includes modulation of host  
426 immunity i.e. through EVs presenting MAMPs and delivering effector molecules (Bahar *et al.*,  
427 2016, Schwechheimer & Kuehn, 2015). We found that EVs from *Pto* DC3000 cultures elicited  
428 a robust induction of the *FRK1* defence marker gene (Fig. 4B, 4C, 4E). Compared with EVs  
429 from *Acidovorax* and *Xanthomonas* bacteria, *Pto* DC3000 EVs provoked a modest induction of  
430 defence gene expression (Bahar *et al.*, 2016). Despite this, the *Pto* DC3000 EVs did not trigger



431 a growth arrest in *Arabidopsis* seedlings (Fig. 4D). These results suggest that the immunogenic  
432 activities of the EVs, but not a longer-term trade-off with growth, may be relevant in their  
433 interaction with plants. In contrast to our results, McMillan et al. reported significant seedling  
434 growth repression in response to *Pto* DC3000 EVs (McMillan *et al.*, 2020). This disparity in  
435 results may be due to several factors, including differences in the growth conditions of both the  
436 bacterial cultures and the *A. thaliana* seedlings, the type of biochemical isolation of EVs and  
437 vesicle dose. That EVs can serve as protective vaccines has been reported for many bacteria but  
438 not phytopathogens. We show here that plants pre-treated with *Pto* DC3000 EVs were modest,  
439 yet significantly protected against subsequent infection with *Pto* DC3000 bacteria (Fig. 4A).  
440 Consistently, EV pre-treatments did not inhibit a MAMP-induced ROS burst (Fig. 6A). The  
441 stronger protective immune response observed by McMillan et al. may be due to differences in  
442 experimental procedures, as noted above (McMillan *et al.*, 2020). In addition, McMillan et al.  
443 showed that pre-treatment with bacterial EVs provided protection against subsequent oomycete  
444 infection (McMillan *et al.*, 2020). The potential for *Pto* DC3000 EVs to induce broad-spectrum  
445 resistance supports a role for PTI.

446  
447 PTI responses to *Pto* DC3000 involve recognition by FLS2, EFR and LIPO-  
448 OLIGOSACCHARIDE-SPECIFIC REDUCED ELICITATION (LORE), which detect  
449 immunogenic flg22, elf18 and 3-OH-FAs (Wan *et al.*, 2019). EVs from bacterial  
450 phytopathogens are enriched in EF-Tu and LPS (Bahar *et al.*, 2016, Feitosa-Junior *et al.*, 2019,  
451 Sidhu *et al.*, 2008), suggesting the presence of elf18 and 3-OH-FAs. Bahar *et al.* demonstrated  
452 that BRI1-ASSOCIATED KINASE 1 (BAK1) and SUPPRESSOR OF BIR 1 (SOBIR1),  
453 interacting co-receptors of PRRs, mediate the immunogenic perception of EVs from *X.*  
454 *campestris* pv. *campestris* (Bahar et al., 2016). We show that our vesicle samples from *Pto*  
455 DC3000 elicit immune responses that are dependent on FLS2 (Fig. 4E). Despite depleting

456 extracellular components from EV samples by density gradient centrifugation, it is possible that  
457 flagella co-purify with the *Pto* DC3000 EV samples, since filamentous structures were observed  
458 in SEM analysis (Fig. 1B). Contamination of flagella in EVs was reported to contribute to the  
459 detection of FliC in EVs from *P. aeruginosa* (Bauman & Kuehn, 2006). However, flagella  
460 proteins such as FliC have a specific affinity for EVs and are involved in EV production in  
461 *Escherichia coli* (Manabe et al., 2013). We cannot exclude that flagella proteins play roles in  
462 EV production in *Pto* DC3000, evidenced by the finding that six flagella-associated proteins  
463 were enriched in *Pto* DC3000 EVs (Table S1).

464

465 Why *Pto* DC3000 produces EVs during infection is unclear. It is evident that the plant apoplast  
466 represents a stressful environment for its colonizing bacteria. Bacteria respond to environmental  
467 stress with the production of EVs, which allows for cell surface remodelling, secretion of  
468 degraded and damaged cargo, and uptake of nutrients in bacterial communities i.e. by packaging  
469 transporters in EVs (Schwechheimer & Kuehn, 2015, Toyofuku *et al.*, 2019, Zingl *et al.*, 2020).  
470 As the growth state of bacteria determines the nutrient availability, the production of EVs could  
471 support the growth of *Pto* DC3000 in culture and *in planta*. Proteomics analysis of gradient  
472 enriched *Pto* DC3000 EVs identified 890 vesicle-associated proteins, of which 369 were  
473 enriched in the EVs relative to the cellular proteome (Fig. 5, Table S1). The mechanisms by  
474 which this enrichment occurs suggests a selective delivery of cargo into the EVs and should be  
475 investigated in the future. Of the EV-enriched proteins, six out of ten GO biological process  
476 categories were related to transport mechanisms (Fig. 6B). Proteins involved in siderophore  
477 transport were enriched in the EVs and have recently been shown to play a role in *Pto* DC3000  
478 infection success (Nobori *et al.*, 2018). Interestingly, the expression of genes coding for all  
479 siderophore transport proteins enriched in EVs was upregulated *in planta* compared to *in vitro*  
480 conditions as well as downregulated upon induction of PTI (Fig. 6B; Table 1) (Nobori *et al.*,

481 2018). Thus, regulation of siderophore transport proteins can be considered as an adaptive  
482 response of *Pto* DC3000 to iron/metal ion availability, and secretion into EVs may allow  
483 improved acquisition of iron, analogous to EV secretion of the siderophore mycobactin in  
484 *Mycobacterium tuberculosis* (Prados-Rosales et al., 2014). The plant's apoplast, which is the  
485 niche colonized by *Pto* DC3000 represents an environment where bacteria are challenged with  
486 iron acquisition and plant defence molecules (Nobori et al., 2018). The role of bacterial EVs in  
487 metal acquisition is not restricted to iron. *Neisseria meningitidis* produces OMVs, which are  
488 enriched in zinc acquisition proteins (Lappann et al., 2013), and zinc regulates siderophore  
489 biosynthesis genes in *Pseudomonas fluorescens* (Rossbach et al., 2000). It is thus feasible that  
490 *Pto* DC3000 produces EVs to help it adapt to metal conditions in the environment including the  
491 plant's apoplast.

492  
493 The hypothesis that *Pto* DC3000 uses EVs to adapt to the growth environment is supported by  
494 our finding that *Pto* DC3000 EVs are enriched in proteins related to the KEGG categories  $\beta$ -  
495 lactam resistance and cationic antimicrobial peptide resistance (Fig. 6B). Several studies  
496 demonstrated that EVs can improve bacterial survival during antibiotic exposure.  
497 *Stenotrophomonas maltophilia* produced more EVs upon treatment with the  $\beta$ -lactam antibiotic  
498 imipenem (Devos et al., 2015, Devos et al., 2017). Its EVs contained  $\beta$ -lactamase and increased  
499 *S. maltophilia* survival in the presence of antibiotics (Devos et al., 2017). Plants defend  
500 infection by upregulation of many defence-related gene including genes coding for  
501 antimicrobial peptides (Campos et al., 2018). It is possible that *Pto* DC3000 produces EVs to  
502 counter the action of plant-derived antimicrobial peptides. Collectively, we propose that *Pto*  
503 DC3000 produces EVs to improve its growth capacity both in culture and *in planta*. These  
504 findings should stimulate further studies on the role of EVs in the interaction of bacteria with  
505 plants, for example identifying the composition of EVs *in planta* using biomarkers.

506

## 507 **Experimental procedures**

### 508 **Bacterial strains and growth**

509 *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto* DC3000) used in this study were routinely  
510 cultured at 28 °C in King's B medium containing 50 µg/mL Rifampicin at 180 rpm and on plates  
511 with 1% agar without agitation. Planktonic growth was performed in 500 mL and growth rates  
512 were measured over time as OD<sub>600</sub>. Biofilm growth was measured after 24 h on plate,  
513 transferring all bacteria per plate in 10 mL 0.85% saline.

514

### 515 **Plant material and growth conditions**

516 *Arabidopsis thaliana* ecotype Columbia (Col-0), *pFRK1::GUS* (Kunze et al. 2004), *fls2c* (Zipfel  
517 et al. 2004) and *efr-1* (Zipfel et al. 2006) mutants were used in this study. For bacterial infections  
518 and ROS assays, Col-0 plants were soil-grown at 21–22 °C and 8 h photoperiod. For GUS  
519 assays, RT-qPCR analysis and induced growth arrest, seedlings were sterile grown on  
520 Murashige and Skoog (MS) plates supplemented with 1% sucrose and 1.5% gelrite (Duchefa,  
521 Netherlands) pH 5.8 for four days (after 2-4 days stratification in the dark at 4 °C), then  
522 transferred to 96-well plates containing 150 µL ½ MS medium supplemented with 1% sucrose  
523 per well and grown for eleven to twelve days in at 22 °C and 16 h photoperiod (120 – 150 µE.m<sup>-2</sup>.s<sup>-1</sup>).  
524

525

### 526 **Extraction and purification of bacterial EVs**

527 EVs were routinely isolated from planktonic cultures at early-logarithmic to late-stationary  
528 phases as well as biofilm cultures (Fig. S2B, S2D). 100 mL of planktonic grown bacteria and  
529 10 mL of biofilm grown bacteria, respectively, were pelleted at 4,500 x g for 2 x 20 min, the  
530 supernatant was decanted and passed through a 0.22 µm membrane (fluid samples; Fig. S2A).

531 Particles were pelleted from the cell-free supernatant at 100,000 x g for 1.5 h. The pellet was  
532 resuspended in 1.7 mL 1mM EDTA and loaded on sucrose density step-gradient (1.7 mL of  
533 sucrose 25%, 35%, 45%, 50%, 55%) and centrifuged at 160,000 x g for 18 h. 2 mL samples  
534 were collected from each of the sucrose density steps and diluted with 1 mM EDTA to 30 mL.  
535 Particles were pelleted at 100,000 x g for 2 h and the pellets were each resuspended in 0.16 mL  
536 1 mM EDTA (gradient enriched samples; Fig. S2A). EV samples were immediately frozen in  
537 liquid nitrogen. Since most EVs migrated to the 55% density fraction (Fig. S6A), we then  
538 collected EVs across fractions 3 to 5, which were less variable in  $\zeta$ -potential and size compared  
539 to fractions 1 and 2 (Fig. S6B, S6C).

540

#### 541 **Extraction of leaf apoplastic fluids**

542 Apoplastic fluids were collected from leaves of six to seven weeks old plants. The leaves were  
543 cut of the rosette and vacuum infiltrated with particle-free 1 mM EDTA. After removing excess  
544 buffer, infiltrated leaves were placed into 20 mL syringes and centrifuged in 50 mL conical  
545 tubes at 900 x g for 20 minutes at 4°C. The resulting apoplastic wash was passed through a 0.22  
546  $\mu$ m membrane (apoplastic fluid samples).

547

#### 548 **EV quantification, size and charge measurements**

549 EVs were quantified, size and charge measured by Nanoparticle Tracking Analysis (NTA)  
550 using ZetaView® BASIC PMX-120 (Particle Metrix, Germany) at room temperature. To detect  
551 EVs, we used the manufacturer's default settings for liposomes. Particle quantification and size  
552 measurements were performed by scanning eleven cell positions each and capturing 30 frames  
553 per position with the following settings: Focus: autofocus; Camera sensitivity for all samples:  
554 85; Shutter: 100; Scattering Intensity: detected automatically. After capture, the videos were  
555 analysed by the in-built ZetaView Software 8.05.11 [ZNTA] with the following specific

556 analysis parameters: Maximum area: 1000, Minimum area 5, Minimum brightness: 25,  
557 Tracelength: 15 ms. Hardware: embedded laser: 40 mW at 488 nm; camera: CMOS. For particle  
558 charge measurements, the same settings were used except Minimum brightness: 30. Statistical  
559 analysis was performed using either One-way ANOVA with Tukey post hoc test or Welsch's  
560 ANOVA with Dunnett's T3 multiple comparisons post hoc test.

561

562 All samples were diluted in particle-free 1 mM EDTA buffer, checked with NTA.  
563 Unconditioned King's B medium contained up to  $1.4 \times 10^9$  particles (Fig. 1D). *Pto* DC3000  
564 cultures contained increasing particles numbers with cultivation time:  $\approx 2.8 \times 10^9$  particles at  
565  $OD_{600} = 3-4$  (50% of influence);  $\approx 3.7 \times 10^9$  particles at  $OD_{600} = 4.5-5.5$  (37% of influence),  $\approx$   
566  $7 \times 10^9$  particles at  $OD_{600} 7.5-9$  (20% of influence), and  $\approx 1.1 \times 10^{10}$  particles at  $OD_{600} 10-11$   
567 (13% of influence) (Fig. 1D). We therefore focused our measurements on samples collected  
568 from  $OD_{600} > 7.5$ , which shows lower than 20% influence of particles from the medium (Fig.  
569 1D), as well as calculated EV concentrations to the colony forming units (CFU) of the bacterial  
570 cultures.

571

## 572 **Scanning electron microscopy**

573 Planktonic grown bacteria at  $OD_{600} = 3-4$  and gradient enriched EVs ( $0.5$  to  $1.5 \times 10^{10}$  particles)  
574 were used for scanning electron microscopy (SEM). The cells were chemically fixed using 2.5%  
575 glutaraldehyde in 50 mM cacodylate buffer (pH 7.0) containing 2 mM  $MgCl_2$ . Then the cells  
576 were applied to a glass slide, covered with a cover slip and plunge frozen in liquid nitrogen.  
577 After this, the cover slip was removed and the cells were placed in fixation buffer again. After  
578 washing 4 times with buffer, post-fixation was carried out with 1%  $OsO_4$  for 15 min. Two  
579 additional washing steps with buffer were followed by three times washing with double distilled  
580 water. The samples were dehydrated in a graded acetone series, critical-point-dried and

581 mounted on an aluminium stub. To enhance conductivity, the samples were sputter-coated with  
582 platinum. Microscopy was carried out using a Zeiss Auriga Crossbeam workstation at 2 kV  
583 (Zeiss, Oberkochen, Germany). The vesicle size was manually measured across five randomly  
584 selected SEM micrographs using Fiji software (Schindelin *et al.*, 2012).

585

### 586 **Transmission electron microscopy**

587 Planktonic grown *Pto* DC3000 at  $OD_{600} = 3-4$  were used for ultrathin sectioning and subsequent  
588 transmission electron microscopy (TEM). The cells were concentrated by centrifugation and  
589 the cells were high-pressure frozen using a Leica HPM100 (Leica Microsystems, Wetzlar,  
590 Germany). This was followed by freeze-substitution with 0.2% osmium tetroxide, 0.1% uranyl  
591 acetate, 9.3% water in water-free acetone in a Leica AFS 2 (Leica Microsystems, Wetzlar,  
592 Germany) as described previously (Flechsler *et al.*, 2020). After embedding in Epon 812  
593 substitute resin (Fluka Chemie AG, Buchs Switzerland), the cells were ultrathin sectioned (50  
594 to 100 nm thickness) and post-stained for 1 min with lead citrate. Transmission electron  
595 microscopy of ultrathin sections was carried out with a JEOL F200 cryo-S(TEM), which was  
596 operated at 200 kV and at room temperature in the TEM mode. Images were acquired using a  
597 bottom-mounted XAROSA 20 mega pixel CMOS camera (EMSIS, Münster, Germany).

598

### 599 ***Pto* DC3000 infection assay**

600 Overnight plate-grown *Pto* DC3000 cells were resuspended in 10 mM  $MgCl_2$  and diluted to  
601  $OD_{600} = 0.0006$ . Using a needle-less syringe, the bacterial suspension was infiltrated into mature  
602 leaves of five to six weeks old plants, three leaves per plant. For pre-treatments, , gradient  
603 enriched EVs from planktonic *Pto* DC3000 (concentration  $\approx 1.10^{10}$ ), and 0.02 mM EDTA as a  
604 negative control and 100 nM flg22 (EZbiolabs) as a positive control were syringe-infiltrated  
605 into leaves 24 h prior *Pto* DC3000 inoculation. Discs of the infected leaves (one disc per leaf,

606 0.6 cm diameter) were excised at one, two- or three-days post infection (dpi). The three leaf  
607 discs from each plant were pooled and ground in 1 mL 10 mM MgCl<sub>2</sub>. Serial dilutions were  
608 plated on LB medium with rifampicin (50 µg/mL) and bacterial colonies were counted one day  
609 after incubation at 28 °C. Statistical analysis was performed using two tailed Welsch's t-test.

610

### 611 **Histochemical β-glucuronidase (GUS) staining**

612 The histochemical GUS assay was performed with eleven day old seedlings. Seedlings were  
613 treated with gradient enriched *Pto* DC3000 EVs (concentration  $\approx 1.10^{10}$ ), 100 nM flg22  
614 (EZbiolabs) or as a control with 0.02 mM EDTA for 18 h. Treated seedlings were immersed in  
615 X-Gluc buffer [2 mM X-Gluc (Biosynth), 50 mM NaPO<sub>4</sub>, pH 7, 0.5 % (v/v) Triton-X100, 0.5  
616 mM K-ferricyanide] for 16 h at 37 °C. Chlorophyll was removed by repeated washing in 80 %  
617 (v/v) ethanol. Observations were made on a WHX 6000 digital microscopy (Krckova *et al.*,  
618 2018).

619

### 620 **Fluorimetric GUS assay**

621 For fluorimetric GUS assays, eleven to twelve days old seedlings were treated with gradient  
622 enriched *Pto* DC3000 EVs (concentration  $\approx 1.10^{10}$ ) or with 100 nM flg22 (EZbiolabs) or as a  
623 control with 0.02 mM EDTA for 18 h. Treated seedlings were frozen in liquid nitrogen in 2 mL  
624 conical tubes containing 2 clean sterile glass beads and liquid nitrogen. The frozen samples  
625 were dry homogenized using a Retch mixer mill (Retch). Homogenized samples were kept on  
626 ice and cold (4 °C). For total protein extraction, GUS extraction buffer was added as described  
627 (Andriankaja *et al.*, 2007) [50 mM sodium phosphate (pH 7); 10 mM 2-mercaptoethanol; 10  
628 mM Na<sub>2</sub>EDTA; 0.1% Triton X-100; 0.1% sodium lauryl-sarcosine and PPIC]. GUS activities  
629 were measured fluorimetrically in reaction buffer (see below) using Methylumbelliferyl-β-D-  
630 glucuronic acid dihydrate (MUG), (Biosynth) as a substrate. Reaction buffer was the same



631 solution as extraction buffer with one modification: PPIC was replaced by 1 mM MUG. The  
632 fluorescence was measured using TECAN fluorimeter at excitation 360 nm and emission 465  
633 nm. The enzymatic activity of the sample was calculated to protein concentration measured by  
634 Bradford protein assay. The absorbance was measured using TECAN spectrometer absorbance  
635 at 595 nm. Statistical analysis was performed using One-way ANOVA with Tukey post hoc  
636 test.

637

### 638 **RNA extraction and RT-qPCR analysis**

639 Gene transcription analysis was performed with twelve days old seedlings. The seedlings were  
640 treated with gradient enriched EVs (concentration  $1 \cdot 10^{10}$ ) and 0.02 mM EDTA as control for 3  
641 h, frozen in liquid nitrogen and ground with 2.5-mm diameter silica beads using a homogenizer  
642 (Retch, Germany). Total RNA was isolated using a TRIzol® reagent (Invitrogen, USA)  
643 according to the manufacturer's protocol. The extracted RNA was treated with a DNA-free kit  
644 (Ambion, USA). Subsequently, 1 µg of RNA was converted into cDNA with M-MLV RNase  
645 H- Point Mutant reverse transcriptase (Promega Corp., USA) and an anchored oligo dT21  
646 primer (Metabion, Germany). Gene transcription was quantified by qPCR using a LightCycler  
647 480 SYBR Green I Master kit and LightCycler 480 (Roche, Switzerland). The PCR conditions  
648 were 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s, 55 °C for 20 s, and 72 °C for  
649 20 s. Melting curve analyses were then carried out. Relative transcription was normalized to the  
650 housekeeping gene *AtTIP41* (Czechowski *et al.*, 2005). Primers were designed using PerlPrimer  
651 v1.1.21 (Marshall, 2004). The primers used are *AtFRK1\_FP*, GCCAACGGAGACATTAGAG;  
652 *AtFRK1\_RP*, CCATAACGACCTGACTCATC. Statistical analysis was performed using One-  
653 way ANOVA with Tukey post hoc test.

654

### 655 **Seedling growth analysis**

656 Four days old seedlings were transferred from MS solid media into the liquid MS media in  
657 transparent 96-well microplates. Each well contained 100  $\mu$ L of media either containing 0.02  
658 mM EDTA as a control or gradient enriched *Pto* DC3000 EVs (concentration  $\approx 1.10^{10}$ ) or with  
659 100 nM flg22 (EZbiolabs) as a positive control. After eight days, the treated seedlings were  
660 dried using paper towel and then the fresh weight was measured. Based on the weight of each  
661 seedling relative seedlings growth [%] to control seedlings was calculated. Statistical analysis  
662 was performed using Welsch's ANOVA with Dunnett's T3 multiple comparisons post hoc test  
663 two tailed Student t-test.

664

### 665 **ROS measurements**

666 ROS production was determined using the luminol-based assay as previously described  
667 (Mersmann *et al.*, 2010). Briefly, leaves of five to six weeks old *A. thaliana* plants were  
668 infiltrated with gradient enriched EVs (concentration  $\approx 1.10^{10}$ ). After 2 h, discs were excised  
669 from the infiltrated leaves and 24 h incubated in ddH<sub>2</sub>O at 22 °C. Then, the leaf discs were  
670 treated with 100 nM flg22 or 100 nM elf18 (EZbiolabs) to induce the production of ROS. The  
671 total photon count was collected for 45 min using a TECAN luminometer. Statistical analysis  
672 was performed using two tailed Student t-test.

673

### 674 **Proteomics**

675 We isolated proteins from *Pto* DC3000 whole cell lysates (WC) (Park *et al.*, 2014) and outer  
676 membrane (OM) (Choi *et al.*, 2011) as previously described. Briefly, WC and OM isolated from  
677 *Pto* DC3000 liquid culture ( $OD_{600} = 3-4$ ). The cells were pelleted via centrifugation (12,000 x  
678 g for 10 min). For WC the pellet was resuspended in 1 mL of 20 mM Tris-HCl (pH 8.0), frozen  
679 in liquid nitrogen, three times thawing-freezing, and three times sonicated for 10 min at 4 °C.  
680 The samples were centrifuged at 6,000 x g for 10 min at 4 °C and supernatants were collected

681 and frozen in liquid nitrogen. For OM preparations, the pellet was resuspended in 1 mL 20 mM  
682 Tris-HCl (pH 8.0), sucrose (20%), followed by adding 5  $\mu$ L Lysozyme (15 mg/mL) and 10  $\mu$ L  
683 0.5 M EDTA, incubation for 40 min on ice and adding 20  $\mu$ L 0.5 M  $MgCl_2$ . After centrifugation  
684 at 9,500 x g for 20 min at 4°C, the pellet was resuspended in 1 mL ice-cold 10 mM Tris-HCl  
685 (pH 8.0) followed by sonication three times for 10 min on ice. The samples were then  
686 centrifuged at 8,000 x g for 5 min at 4 °C, washed with cold 10 mM Tris-HCl (pH 8.0),  
687 resuspend in cold, sterile MilliQ water followed by three times freezing-thawing in liquid  
688 nitrogen, incubation for 20 min at 25 °C and adding the sarcosyl to final concentration 0.5%.  
689 The samples were then centrifuged at 40,000 x g for 90 min at 4 °C, the pellet was resuspended  
690 in ice-cold 10 mM Tris-HCl (pH 8.0) and frozen in liquid nitrogen. Gradient enriched EVs were  
691 isolated as above described (Fig. S1C). For proteomics, the samples were denatured by addition  
692 of 1 x SDS loading buffer. In-gel trypsin digestion was performed according to standard  
693 procedures (Shevchenko *et al.*, 2006). Briefly, 2  $\mu$ g of EV and OM samples and 20  $\mu$ g of WC  
694 samples were loaded on a NuPAGE 4-12% Bis-Tris Protein gels (ThermoFisher Scientific, US),  
695 and the gels were run for 3 min only. Subsequently, the still not size-separated single protein  
696 band per sample was cut, reduced (50 mM DTT), alkylated (55 mM CAA, chloroacetamid) and  
697 digested overnight with trypsin (trypsin-gold, Promega).

698

### 699 **LC-MS/MS data acquisition**

700 Peptides generated by in-gel trypsin digestion were dried in a vacuum concentrator and  
701 dissolved in 0.1% formic acid (FA). LC-MS/MS measurements were performed on a Fusion  
702 Lumos Tribrid mass spectrometer (Thermo Fisher Scientific) equipped with an Ultimate 3000  
703 RSLCnano system. Peptides were delivered to a trap column (ReproSil-pur C18-AQ, 5  $\mu$ m, Dr  
704 Maisch, 20 mm  $\times$  75  $\mu$ m, self-packed) at a flow rate of 5  $\mu$ L/min in 100% solvent A (0.1%  
705 formic acid in HPLC grade water). After 10 min of loading, peptides were transferred to an

706 analytical column (ReproSil Gold C18-AQ, 3  $\mu\text{m}$ , Dr Maisch, 400 mm  $\times$  75  $\mu\text{m}$ , self-packed)  
707 and separated using a 50 min gradient from 4% to 32% of solvent B (0.1% formic acid in  
708 acetonitrile and 5% (v/v) DMSO) at 300 nL/min flow rate. Both nanoLC solvents contained 5%  
709 (v/v) DMSO.

710

711 The Fusion Lumos Tribrid mass spectrometer was operated in data dependent acquisition and  
712 positive ionization mode. MS1 spectra (360–1300 m/z) were recorded at a resolution of 60,000  
713 using an automatic gain control (AGC) target value of 4e5 and maximum injection time  
714 (maxIT) of 50 ms. After peptide fragmentation using higher energy collision induced  
715 dissociation (HCD), MS2 spectra of up to 20 precursor peptides were acquired at a resolution  
716 of 15,000 with an automatic gain control (AGC) target value of 5e4 and maximum injection  
717 time (maxIT) of 22 ms. The precursor isolation window width was set to 1.3 m/z and normalized  
718 collision energy to 30%. Dynamic exclusion was enabled with 20 s exclusion time (mass  
719 tolerance +/-10 ppm).

720

## 721 **Computational analysis of proteomes**

722 LFQ values were used in the statistical analysis of proteome data. To select EV-enriched  
723 proteins, Welch t-test were used to compare protein intensities between EV and WC samples.  
724 The resulted p-values were corrected using the Benjamini-Hochberg method to control the false  
725 discovery rate (FDR). The proteins with FDR < 0.05 and with the intensity in EV at least twice  
726 higher than in WC were selected as EV-enriched proteins (n = 207). In addition, we selected  
727 proteins that were exclusively identified in at least three (out of four) replicates of EV. (n =  
728 162). A complete list of EV-enriched proteins is given in Table S1. The functional enrichment  
729 analysis of the EV proteins were performed using the DAVID functional annotation tool (Huang

730 da *et al.*, 2009a, Huang da *et al.*, 2009b).

731

## 732 **Database searches**

733 Peptide identification and quantification was performed using MaxQuant (version 1.6.3.4) with  
734 its built-in search engine Andromeda (Cox *et al.*, 2011, Tyanova *et al.*, 2016). MS2 spectra  
735 were searched against a *Pseudomonas syringae pv tomato* protein database (UP000002515,  
736 downloaded from Uniprot 04.05.2020) supplemented with common contaminants (built-in  
737 option in MaxQuant). For all MaxQuant searches default parameters were employed. Those  
738 included carbamidomethylation of cysteine as fixed modification and oxidation of methionine  
739 and N-terminal protein acetylation as variable modifications. Trypsin/P was specified as  
740 proteolytic enzyme. Precursor tolerance was set to 4.5 ppm, and fragment ion tolerance to 20  
741 ppm. Results were adjusted to 1% false discovery rate (FDR) on peptide spectrum match (PSM)  
742 and protein level, employing a target-decoy approach using reversed protein sequences. Label-  
743 free quantification (LFQ algorithm) was enabled. The minimal peptide length was defined as 7  
744 amino acids and the “match-between-run” function was not enabled. Each sample type (EV,  
745 OM, WC) was analysed in biological quadruplicates (Table S1).

746

747 We used available localization prediction data at pseudomonas genome database  
748 (pseudomonas.com) (Winsor *et al.*, 2016). Predicted protein localizations are presented as  
749 stacked bar charts (made in MS Excel) as percentage to total number of the proteins in analyzed  
750 sample. We used the available software DAVID bioinformatic resource 6.8  
751 (<https://david.ncifcrf.gov/>) for GO term and KEGG pathway analysis, and the adjusted p-value  
752 cut-off was set to 0.05 (Huang da *et al.*, 2009a, Huang da *et al.*, 2009b). We compared the EV  
753 enriched proteins from *Pto* DC3000 with EV proteomes from planktonic grown *P. aeruginosa*  
754 PAO1 (Choi *et al.*, 2011, Park *et al.*, 2014, Reales-Calderon *et al.*, 2015). We focussed on the

755 proteins that were identified in OMVs from *P. aeruginosa* PAO1 across all three studies and  
756 identified their gene orthologs in *Pto* DC3000 using the pseudomonas genome database  
757 (pseudomonas.com) (Winsor *et al.*, 2016). This set of proteins was compared to the *Pto* DC3000  
758 EV-enriched proteins to predict EV biomarkers. The EV-enriched proteins were also compared  
759 with available *in planta* *Pto* DC3000 transcriptome and proteome data (Nobori *et al.*, 2018)  
760 (Nobori *et al.*, 2020).

761

### 762 **Immunoblot analysis**

763 Standard immunoblot analysis was performed according to Sambrook *et al.* (1989). 10% SDS-  
764 PAGE gels were blotted onto PVDF Immobilon-P membranes (Millipore). *Pto* DC3000 OprF  
765 was detected using 1:2,000 diluted rabbit polyclonal antibody against OprF from *Pseudomonas*  
766 *aeruginosa* (Cusabio Biotech Co.). As secondary antibody, we used a 1:50,000 dilution of the  
767 anti-rabbit IgG-Peroxidase polyclonal antibody (Sigma-Aldrich, A0545). Signal detection was  
768 done using SuperSignal West FemtoMaximum Sensitivity Substrate (Pierce, Thermo  
769 Scientific), according to manufacturer's instructions, and the images were captured using Vilber  
770 Lourmat Peqlab FUSION SL Gel Chemiluminescence Documentation System.

771

### 772 **Statistical analysis**

773 Student *t*-test, Welsch's *t*-test, One-way ANOVA followed by Tukey multiple comparisons test  
774 and Welsch's ANOVA with Dunnett's T3 multiple comparisons post hoc test were performed  
775 using GraphPad Prism version 8.3 for Windows, GraphPad Software, San Diego, California  
776 USA, [www.graphpad.com](http://www.graphpad.com)

777

### 778 **Data availability**

779 The mass spectrometry proteomics data have been deposited to the ProteomeXchange

780 Consortium via the PRIDE (Perez-Riverol *et al.*, 2019) partner repository with the dataset  
781 identifier PXD023971.

782

### 783 **Author contributions**

784 M.J. and S.R. designed research; M.J., C.L., K.R., C.M., L.B., B.S., A.B., A.K. performed  
785 research; M.J., C.L., K.R., C.M., A.B., A.K. and S.R. analysed data; E.S., J.S., F.M., J.M.  
786 developed protocols; M.J. and S.R. wrote the paper with inputs from all authors.

787

### 788 **Acknowledgements**

789 We like to thank members of the Robatzek laboratory for fruitful discussions. We acknowledge  
790 Lucia Grenga and Catriona Thompson (JIC), Franziska Hackbarth and Hermine Kienberger  
791 (BayBioMS) for their laboratory assistance, Miriam Abele for her mass spectrometric support  
792 at the BayBioMS as well as Jennifer Grünert and Cornelia Niemann (LMU Biocenter) for  
793 technical assistance in electron microscopy. This research was supported by the Deutsche  
794 Forschungsgemeinschaft (to S.R.) through a Heisenberg fellowship (RO 3550/14-1) and the  
795 SFB924 “Yield” (TP B15). M.J. was supported by the European Structural and Investment  
796 Funds, OP RDE-funded project ‘CHEMFELLS4UCTP’ (no.  
797 CZ.02.2.69/0.0/0.0/17\_050/0008485).

798

799 **Supplemental Information**

800 **Figure S1. A)** The full-size SEM micrograph used in Fig. 1A of *Pto* DC3000 growth in  
801 planktonic culture ( $OD_{600} = 3-4$ ). **B)** Size profile of EVs from *Pto* DC3000 planktonic cultures  
802 in fluid samples ( $OD_{600} = 7.5-11$ ).

803

804 **Figure S2. Isolation of *Pto* DC3000 EVs. A)** Schematic overview of EVs isolation from  
805 planktonic cultures for fluid sample (1) and gradient enriched sample (2) analysis. **B)** Growth  
806 measurements of planktonic *Pto* DC3000 cultures. Orange indicates EV isolation from early  
807 exponential growth stages ( $OD_{600} = 1-2$ ); green indicates EV isolation from late exponential  
808 growth stages ( $OD_{600} = 3-4$ ). **C)** Schematic overview of EVs isolation from biofilm cultures. **D)**  
809 Growth measurements of biofilm *Pto* DC3000 cultures. The green dot represents the growth  
810 stage from which the bacteria were used for experiments.

811

812 **Figure S3. Biophysical parameters of particles in apoplastic fluids from *A. thaliana* plants**  
813 **infected with *Pto* DC3000. A, D, G)** Particle parameters over days post infection (dpi). **B, E,**  
814 **H)** Particle parameters in response to inoculation with different *Pto* DC3000 densities. **C, F, I)**  
815 Particle parameters in response to inoculation with different *Pto* DC3000 and co-treatment with  
816 flg22. Each dot represents value of independent samples for size and  $\zeta$ -potential it represents  
817 median. 3-12 independent samples were used for each experiment. **J, K)** The profile of  $\zeta$ -  
818 potential for each particle collected from apoplastic fluids of plants treated as indicated and  
819 gradient enriched EVs. Control = 0.2 mM EDTA; flg22 = 100 nM; n.t. = not treated; *Pto*  
820 DC3000  $OD_{600} = 0.0006$ . Each treatment was 3 days long. The dots represent the mean across  
821 the  $\zeta$ -potential values from independent samples: n = 8 (control); n = 10 (*Pto* DC3000); n = 6  
822 (flg22); n = 4 (non-treatment).

823



824 **Figure S4. Pre-treatment with *Pto* DC3000 EVs induces resistance against subsequent *Pto***  
825 **DC3000 infection. A)** Three individual biological repeats of *Pto* DC3000 growth (CFU) after  
826 infection into leaves of *A. thaliana* without and with EV pre-treatment at 3 dpi (control = 0.02  
827 mM EDTA). Each biological repeat consists of 12 independent samples. **B)** *Pto* DC3000 growth  
828 (CFU) after infection (3 dpi) into leaves of *A. thaliana* without and with 100 nM flg22 1 day  
829 pre-treatment (mock = 10 mM MgCl<sub>2</sub>) n = 4. Asterisks represent the statistical difference  
830 between the treated and control samples (two tailed Student t-test p < 0.01).

831  
832 **Figure S5. Characteristics of the proteomic analysis. A)** Barplot shows the number of  
833 identified proteins in each replicate. The solid line indicates the cumulative protein IDs and  
834 dashed line shows the shared protein IDs. **B)** Boxplot shows a comparable distribution of  
835 protein intensities from each replicate.

836  
837 **Figure S6. Biophysical parameters of *Pto* DC3000 EVs across isolation methods. A-C)**  
838 NTA measurements of particle concentration (A),  $\zeta$ -potential (B) and size (C) of *Pto* DC3000  
839 EVs collected from each step of gradient enrichment. **D-E)** NTA analysis of particle  $\zeta$ -potential  
840 (D) and size (E) of *Pto* DC3000 EVs from fluid samples before (live) and after boiling. Each  
841 dot represents an independent sample.

842  
843 **Table S1.** Filtered proteomics data with proteins that were identified in at least in three out of  
844 four biological repeats in at least one variant (WC, OM and EV). Values used for volcano plot:  
845 Highlighted EV-enriched proteins. Subcellar localization of identified proteins. Flagellar  
846 proteins identified in EV enriched proteins.

847

848 **Table S2.** GO analysis of EV-enriched proteins

849

850 **Table S3.** Expression of EV-enriched proteins *in planta*

851

852 **Table S4.** *Pseudomonas* EV “core”

853

## 854 References

- 855 **Alves NJ, Turner KB, Medintz IL, Walper SA.** 2016. Protecting enzymatic function  
856 through directed packaging into bacterial outer membrane vesicles. *Sci Rep* **6**: 24866
- 857 **Andriankaja A, Boisson-Dernier A, Frances L, Sauviac L, Jauneau A, Barker DG, de**  
858 **Carvalho-Niebel F.** 2007. AP2-ERF transcription factors mediate Nod factor dependent  
859 Mt ENOD11 activation in root hairs via a novel cis-regulatory motif. *Plant Cell* **19**: 2866-  
860 85
- 861 **Arigita C, Jiskoot W, Westdijk J, van Ingen C, Hennink WE, Crommelin DJ, Kersten**  
862 **GF.** 2004. Stability of mono- and trivalent meningococcal outer membrane vesicle  
863 vaccines. *Vaccine* **22**: 629-42
- 864 **Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski**  
865 **K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S,**  
866 **Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G.** 2000. Gene ontology:  
867 tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* **25**: 25-9
- 868 **Bahar O, Mordukhovich G, Luu DD, Schwessinger B, Daudi A, Jehle AK, Felix G,**  
869 **Ronald PC.** 2016. Bacterial Outer Membrane Vesicles Induce Plant Immune Responses.  
870 *Molecular Plant-Microbe Interactions* **29**: 374-384
- 871 **Bachurski D, Schuldner M, Nguyen PH, Malz A, Reiners KS, Grenzi PC, Babatz F,**  
872 **Schauss AC, Hansen HP, Hallek M, von Strandmann EP.** 2019. Extracellular vesicle  
873 measurements with nanoparticle tracking analysis - An accuracy and repeatability  
874 comparison between NanoSight NS300 and ZetaView. *Journal of Extracellular Vesicles* **8**
- 875 **Bauman SJ, Kuehn MJ.** 2006. Purification of outer membrane vesicles from *Pseudomonas*  
876 *aeruginosa* and their activation of an IL-8 response. *Microbes Infect* **8**: 2400-8
- 877 **Bielska E, Birch PRJ, Buck AH, Abreu-Goodger C, Innes RW, Jin H, Pfaffl MW,**  
878 **Robatzek S, Regev-Rudzki N, Tisserant C, Wang S, Weiberg A.** 2019. Highlights of the  
879 mini-symposium on extracellular vesicles in inter-organismal communication, held in  
880 Munich, Germany, August 2018. *Journal of Extracellular Vesicles* **8**: 1590116
- 881 **Block A, Alfano JR.** 2011. Plant targets for *Pseudomonas syringae* type III effectors:  
882 virulence targets or guarded decoys? *Curr Opin Microbiol* **14**: 39-46
- 883 **Bredow M, Sementchoukova I, Siegel K, Monaghan J.** 2019. Pattern-Triggered  
884 Oxidative Burst and Seedling Growth Inhibition Assays in *Arabidopsis thaliana*. *J Vis Exp*
- 885 **Buttner D, Bonas U.** 2010. Regulation and secretion of *Xanthomonas* virulence factors.  
886 *FEMS Microbiol Rev* **34**: 107-33
- 887 **Campos ML, de Souza CM, de Oliveira KBS, Dias SC, Franco OL.** 2018. The role of  
888 antimicrobial peptides in plant immunity. *J Exp Bot* **69**: 4997-5011
- 889 **Couto D, Zipfel C.** 2016. Regulation of pattern recognition receptor signalling in plants.  
890 *Nature Reviews Immunology* **16**: 537-52
- 891 **Couto N, Schooling SR, Dutcher JR, Barber J.** 2015. Proteome Profiles of Outer  
892 Membrane Vesicles and Extracellular Matrix of *Pseudomonas aeruginosa* Biofilms. *J*  
893 *Proteome Res* **14**: 4207-22
- 894 **Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M.** 2011. Andromeda:  
895 a peptide search engine integrated into the MaxQuant environment. *Journal of Proteome*  
896 *Research* **10**: 1794-805
- 897 **Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR.** 2005. Genome-wide  
898 identification and testing of superior reference genes for transcript normalization in  
899 *Arabidopsis*. *Plant Physiol* **139**: 5-17
- 900 **Devos S, Van Oudenhove L, Stremersch S, Van Putte W, De Rycke R, Van Driessche G,**  
901 **Vitse J, Raemdonck K, Devreese B.** 2015. The effect of imipenem and diffusible signaling

- 902 factors on the secretion of outer membrane vesicles and associated Ax21 proteins in  
903 *Stenotrophomonas maltophilia*. *Front Microbiol* **6**: 298
- 904 **Devos S, Van Putte W, Vitse J, Van Driessche G, Stremersch S, Van Den Broek W,**  
905 **Raemdonck K, Braeckmans K, Stahlberg H, Kudryashev M, Savvides SN, Devreese B.**  
906 2017. Membrane vesicle secretion and prophage induction in multidrug-resistant  
907 *Stenotrophomonas maltophilia* in response to ciprofloxacin stress. *Environ Microbiol* **19**:  
908 3930-3937
- 909 **Dodds PN, Rathjen JP.** 2010. Plant immunity: towards an integrated view of plant-  
910 pathogen interactions. *Nat Rev Genet* **11**: 539-48
- 911 **Feitosa-Junior OR, Stefanello E, Zaini PA, Nascimento R, Pierry PM, Dandekar AM,**  
912 **Lindow SE, da Silva AM.** 2019. Proteomic and Metabolomic Analyses of *Xylella fastidiosa*  
913 OMV-Enriched Fractions Reveal Association with Virulence Factors and Signaling  
914 Molecules of the DSF Family. *Phytopathology* **109**: 1344-1353
- 915 **Figaj D, Ambroziak P, Przepiora T, Skorko-Glonek J.** 2019. The Role of Proteases in the  
916 Virulence of Plant Pathogenic Bacteria. *Int J Mol Sci* **20**
- 917 **Flechsler J, Heimerl T, Pickl C, Rachel R, Stierhof YD, Klingl A.** 2020. 2D and 3D  
918 immunogold localization on (epoxy) ultrathin sections with and without osmium  
919 tetroxide. *Microsc Res Tech* **83**: 691-705
- 920 **Frank J, Richter M, de Rossi C, Lehr CM, Fuhrmann K, Fuhrmann G.** 2018. Extracellular  
921 vesicles protect glucuronidase model enzymes during freeze-drying. *Sci Rep* **8**: 12377
- 922 **Hall BM, Breidenstein EBM, de la Fuente-Nunez C, Reffuveille F, Mawla GD, Hancock**  
923 **REW, Baker TA.** 2017. Two Isoforms of Clp Peptidase in *Pseudomonas aeruginosa*  
924 Control Distinct Aspects of Cellular Physiology. *Journal of Bacteriology* **199**
- 925 **Huang da W, Sherman BT, Lempicki RA.** 2009a. Bioinformatics enrichment tools: paths  
926 toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* **37**: 1-  
927 13
- 928 **Huang da W, Sherman BT, Lempicki RA.** 2009b. Systematic and integrative analysis of  
929 large gene lists using DAVID bioinformatics resources. *Nature Protocols* **4**: 44-57
- 930 **Choi DS, Kim DK, Choi SJ, Lee J, Choi JP, Rho S, Park SH, Kim YK, Hwang D, Gho YS.**  
931 2011. Proteomic analysis of outer membrane vesicles derived from *Pseudomonas*  
932 *aeruginosa*. *Proteomics* **11**: 3424-9
- 933 **Chowdhury C, Jagannadham MV.** 2013. Virulence factors are released in association  
934 with outer membrane vesicles of *Pseudomonas syringae* pv. tomato T1 during normal  
935 growth. *Biochim Biophys Acta* **1834**: 231-9
- 936 **Ionescu M, Zaini PA, Baccari C, Tran S, da Silva AM, Lindow SE.** 2014. *Xylella fastidiosa*  
937 outer membrane vesicles modulate plant colonization by blocking attachment to surfaces.  
938 *Proceedings of the National Academy of Sciences of the United States of America* **111**:  
939 E3910-E3918
- 940 **Kanehisa M, Goto S, Furumichi M, Tanabe M, Hirakawa M.** 2010. KEGG for  
941 representation and analysis of molecular networks involving diseases and drugs. *Nucleic*  
942 *Acids Res* **38**: D355-60
- 943 **Kaparakis-Liaskos M, Ferrero RL.** 2015. Immune modulation by bacterial outer  
944 membrane vesicles. *Nature Reviews Immunology* **15**: 375-387
- 945 **Klimentova J, Stulik J.** 2015. Methods of isolation and purification of outer membrane  
946 vesicles from gram-negative bacteria. *Microbiol Res* **170**: 1-9
- 947 **Knoke LR, Abad Herrera S, Gotz K, Justesen BH, Gunther Pomorski T, Fritz C,**  
948 **Schakermann S, Bandow JE, Aktas M.** 2020. Agrobacterium tumefaciens Small  
949 Lipoprotein Atu8019 Is Involved in Selective Outer Membrane Vesicle (OMV) Docking to  
950 Bacterial Cells. *Front Microbiol* **11**: 1228

- 951 **Krckova Z, Kocourkova D, Danek M, Brouzdova J, Pejchar P, Janda M, Pokotylo I, Ott**  
952 **PG, Valentova O, Martinec J.** 2018. The Arabidopsis thaliana non-specific phospholipase  
953 C2 is involved in the response to Pseudomonas syringae attack. *Annals of Botany* **121**:  
954 297-310
- 955 **Kunze G, Zipfel C, Robatzek S, Niehaus K, Boller T, Felix G.** 2004. The N terminus of  
956 bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. *Plant Cell* **16**:  
957 3496-3507
- 958 **Kvitko BH, Park DH, Velasquez AC, Wei CF, Russell AB, Martin GB, Schneider DJ,**  
959 **Collmer A.** 2009. Deletions in the repertoire of Pseudomonas syringae pv. tomato DC3000  
960 type III secretion effector genes reveal functional overlap among effectors. *Plos Pathogens*  
961 **5**: e1000388
- 962 **Lappann M, Otto A, Becher D, Vogel U.** 2013. Comparative proteome analysis of  
963 spontaneous outer membrane vesicles and purified outer membranes of Neisseria  
964 meningitidis. *Journal of Bacteriology* **195**: 4425-35
- 965 **Lovelace AH, Smith A, Kvitko BH.** 2018. Pattern-Triggered Immunity Alters the  
966 Transcriptional Regulation of Virulence-Associated Genes and Induces the Sulfur  
967 Starvation Response in Pseudomonas syringae pv. tomato DC3000. *Mol Plant Microbe*  
968 *Interact* **31**: 750-765
- 969 **Macdonald IA, Kuehn MJ.** 2013. Stress-induced outer membrane vesicle production by  
970 Pseudomonas aeruginosa. *Journal of Bacteriology* **195**: 2971-81
- 971 **Manabe T, Kato M, Ueno T, Kawasaki K.** 2013. Flagella proteins contribute to the  
972 production of outer membrane vesicles from Escherichia coli W3110. *Biochem Biophys*  
973 *Res Commun* **441**: 151-6
- 974 **Mansfield J, Genin S, Magori S, Citovsky V, Sriariyanum M, Ronald P, Dow M, Verdier**  
975 **V, Beer SV, Machado MA, Toth I, Salmond G, Foster GD.** 2012. Top 10 plant pathogenic  
976 bacteria in molecular plant pathology. *Molecular Plant Pathology* **13**: 614-629
- 977 **McBroom AJ, Johnson AP, Vemulapalli S, Kuehn MJ.** 2006. Outer membrane vesicle  
978 production by Escherichia coli is independent of membrane instability. *Journal of*  
979 *Bacteriology* **188**: 5385-5392
- 980 **McBroom AJ, Kuehn MJ.** 2007. Release of outer membrane vesicles by Gram-negative  
981 bacteria is a novel envelope stress response. *Mol Microbiol* **63**: 545-58
- 982 **McCaig WD, Koller A, Thanassi DG.** 2013. Production of outer membrane vesicles and  
983 outer membrane tubes by Francisella novicida. *Journal of Bacteriology* **195**: 1120-32
- 984 **McMillan HM, Zebell SG, Ristaino JB, Dong X, Kuehn MJ.** 2020. Protective Plant Immune  
985 Responses are Elicited by Bacterial Outer Membrane Vesicles. *BioRxiv*
- 986 **Melotto M, Underwood W, Koczan J, Nomura K, He SY.** 2006. Plant stomata function in  
987 innate immunity against bacterial invasion. *Cell* **126**: 969-80
- 988 **Mersmann S, Bourdais G, Rietz S, Robatzek S.** 2010. Ethylene signaling regulates  
989 accumulation of the FLS2 receptor and is required for the oxidative burst contributing to  
990 plant immunity. *Plant Physiology* **154**: 391-400
- 991 **Metruccio MM, Evans DJ, Gabriel MM, Kadurugamuwa JL, Fleiszig SM.** 2016.  
992 Pseudomonas aeruginosa Outer Membrane Vesicles Triggered by Human Mucosal Fluid  
993 and Lysozyme Can Prime Host Tissue Surfaces for Bacterial Adhesion. *Front Microbiol* **7**:  
994 871
- 995 **Nascimento R, Gouran H, Chakraborty S, Gillespie HW, Almeida-Souza HO, Tu A, Rao**  
996 **BJ, Feldstein PA, Bruening G, Goulart LR, Dandekar AM.** 2016. The Type II Secreted  
997 Lipase/Esterase LesA is a Key Virulence Factor Required for Xylella fastidiosa  
998 Pathogenesis in Grapevines. *Scientific Reports* **6**

- 999 **Nobori T, Velasquez AC, Wu JN, Kvitko BH, Kremer JM, Wang Y, He SY, Tsuda K.** 2018.  
1000 Transcriptome landscape of a bacterial pathogen under plant immunity. *Proceedings of*  
1001 *the National Academy of Sciences of the United States of America* **115**: E3055-E3064
- 1002 **Nobori T, Wang Y, Wu J, Stolze SC, Tsuda Y, Finkemeier I, Nakagami H, Tsuda K.** 2020.  
1003 Multidimensional gene regulatory landscape of a bacterial pathogen in plants. *Nat Plants*  
1004 **Nobori T, Yiming W, Jingni W, Christina SS, Yayoi T, Iris F, Hirofumi N, Kenichi T.**  
1005 2019. In planta bacterial multi-omics analysis illuminates regulatory principles  
1006 underlying plant-pathogen interactions. *BioRxiv*
- 1007 **Nomura K, Debroy S, Lee YH, Pumplin N, Jones J, He SY.** 2006. A bacterial virulence  
1008 protein suppresses host innate immunity to cause plant disease. *Science* **313**: 220-3
- 1009 **Park AJ, Murphy K, Krieger JR, Brewer D, Taylor P, Habash M, Khursigara CM.** 2014.  
1010 A temporal examination of the planktonic and biofilm proteome of whole cell  
1011 *Pseudomonas aeruginosa* PAO1 using quantitative mass spectrometry. *Mol Cell*  
1012 *Proteomics* **13**: 1095-105
- 1013 **Perez-Cruz C, Delgado L, Lopez-Iglesias C, Mercade E.** 2015. Outer-inner membrane  
1014 vesicles naturally secreted by gram-negative pathogenic bacteria. *Plos One* **10**: e0116896
- 1015 **Perez-Riverol Y, Csordas A, Bai J, Bernal-Llinares M, Hewapathirana S, Kundu DJ,**  
1016 **Inuganti A, Griss J, Mayer G, Eisenacher M, Perez E, Uszkoreit J, Pfeuffer J,**  
1017 **Sachsenberg T, Yilmaz S, Tiwary S, Cox J, Audain E, Walzer M, Jarnuczak AF et al.**  
1018 2019. The PRIDE database and related tools and resources in 2019: improving support  
1019 for quantification data. *Nucleic Acids Res* **47**: D442-D450
- 1020 **Prados-Rosales R, Weinrick BC, Pique DG, Jacobs WR, Jr., Casadevall A, Rodriguez**  
1021 **GM.** 2014. Role for Mycobacterium tuberculosis membrane vesicles in iron acquisition.  
1022 *Journal of Bacteriology* **196**: 1250-6
- 1023 **Raposo G, Stoorvogel W.** 2013. Extracellular vesicles: exosomes, microvesicles, and  
1024 friends. *J Cell Biol* **200**: 373-83
- 1025 **Reales-Calderon JA, Corona F, Monteoliva L, Gil C, Martinez JL.** 2015. Quantitative  
1026 proteomics unravels that the post-transcriptional regulator Crc modulates the generation  
1027 of vesicles and secreted virulence determinants of *Pseudomonas aeruginosa*. *Data Brief*  
1028 **4**: 450-3
- 1029 **Roier S, Zingl FG, Cakar F, Durakovic S, Kohl P, Eichmann TO, Klug L, Gadermaier B,**  
1030 **Weinzerl K, Prassl R, Lass A, Daum G, Reidl J, Feldman MF, Schild S.** 2016. A novel  
1031 mechanism for the biogenesis of outer membrane vesicles in Gram-negative bacteria.  
1032 *Nature Communications* **7**: 10515
- 1033 **Roszbach S, Wilson TL, Kukuk ML, Carty HA.** 2000. Elevated zinc induces siderophore  
1034 biosynthesis genes and a zntA-like gene in *Pseudomonas fluorescens*. *FEMS Microbiol Lett*  
1035 **191**: 61-70
- 1036 **Roszkowiak J, Jajor P, Gula G, Gubernator J, Zak A, Drulis-Kawa Z, Augustyniak D.**  
1037 2019. Interspecies Outer Membrane Vesicles (OMVs) Modulate the Sensitivity of  
1038 Pathogenic Bacteria and Pathogenic Yeasts to Cationic Peptides and Serum Complement.  
1039 *Int J Mol Sci* **20**
- 1040 **Rutter BD, Innes RW.** 2017. Extracellular Vesicles Isolated from the Leaf Apoplast Carry  
1041 Stress-Response Proteins. *Plant Physiology* **173**: 728-741
- 1042 **Rybak K, Robatzek S.** 2019. Functions of Extracellular Vesicles in Immunity and  
1043 Virulence. *Plant Physiology* **179**: 1236-1247
- 1044 **Sampath V, McCaig WD, Thanassi DG.** 2018. Amino acid deprivation and central carbon  
1045 metabolism regulate the production of outer membrane vesicles and tubes by *Francisella*.  
1046 *Mol Microbiol* **107**: 523-541

- 1047 **Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M.** 2006. In-gel digestion for mass  
1048 spectrometric characterization of proteins and proteomes. *Nat Protoc* **1**: 2856-60
- 1049 **Schechter LM, Vencato M, Jordan KL, Schneider SE, Schneider DJ, Collmer A.** 2006.  
1050 Multiple approaches to a complete inventory of *Pseudomonas syringae* pv. tomato  
1051 DC3000 type III secretion system effector proteins. *Mol Plant Microbe Interact* **19**: 1180-  
1052 92
- 1053 **Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch  
1054 S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K,  
1055 Tomancak P, Cardona A.** 2012. Fiji: an open-source platform for biological-image  
1056 analysis. *Nat Methods* **9**: 676-82
- 1057 **Schwechheimer C, Kuehn MJ.** 2015. Outer-membrane vesicles from Gram-negative  
1058 bacteria: biogenesis and functions. *Nature Reviews Microbiology* **13**: 605-619
- 1059 **Sidhu VK, Vorholter FJ, Niehaus K, Watt SA.** 2008. Analysis of outer membrane vesicle  
1060 associated proteins isolated from the plant pathogenic bacterium *Xanthomonas*  
1061 *campestris* pv. *campestris*. *Bmc Microbiology* **8**
- 1062 **Sole M, Scheibner F, Hoffmeister AK, Hartmann N, Hause G, Rother A, Jordan M,  
1063 Lautier M, Arlat M, Buttner D.** 2015. *Xanthomonas campestris* pv. *vesicatoria* Secretes  
1064 Proteases and Xylanases via the Xps Type II Secretion System and Outer Membrane  
1065 Vesicles. *Journal of Bacteriology* **197**: 2879-2893
- 1066 **The Gene Ontology C.** 2019. The Gene Ontology Resource: 20 years and still GOing  
1067 strong. *Nucleic Acids Res* **47**: D330-D338
- 1068 **Toyofuku M, Nomura N, Eberl L.** 2019. Types and origins of bacterial membrane  
1069 vesicles. *Nature Reviews Microbiology* **17**: 13-24
- 1070 **Turnbull L, Toyofuku M, Hynen AL, Kurosawa M, Pessi G, Petty NK, Osvath SR,  
1071 Carcamo-Oyarce G, Gloag ES, Shimoni R, Omasits U, Ito S, Yap X, Monahan LG,  
1072 Cavaliere R, Ahrens CH, Charles IG, Nomura N, Eberl L, Whitchurch CB.** 2016.  
1073 Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and  
1074 biofilms. *Nature Communications* **7**: 11220
- 1075 **Tyanova S, Temu T, Cox J.** 2016. The MaxQuant computational platform for mass  
1076 spectrometry-based shotgun proteomics. *Nat Protoc* **11**: 2301-2319
- 1077 **Vinatzer BA, Teitzel GM, Lee MW, Jelenska J, Hotton S, Fairfax K, Jenrette J,  
1078 Greenberg JT.** 2006. The type III effector repertoire of *Pseudomonas syringae* pv.  
1079 *syringae* B728a and its role in survival and disease on host and non-host plants. *Mol*  
1080 *Microbiol* **62**: 26-44
- 1081 **Wan WL, Frohlich K, Pruitt RN, Nurnberger T, Zhang L.** 2019. Plant cell surface  
1082 immune receptor complex signaling. *Current Opinion in Plant Biology* **50**: 18-28
- 1083 **Wang Y, Garrido-Oter R, Wu J, Winkelmuller TM, Agler M, Colby T, Nobori T, Kemen  
1084 E, Tsuda K.** 2019. Site-specific cleavage of bacterial MucD by secreted proteases mediates  
1085 antibacterial resistance in *Arabidopsis*. *Nature Communications* **10**: 2853
- 1086 **Wei HL, Zhang W, Collmer A.** 2018. Modular Study of the Type III Effector Repertoire in  
1087 *Pseudomonas syringae* pv. tomato DC3000 Reveals a Matrix of Effector Interplay in  
1088 Pathogenesis. *Cell Rep* **23**: 1630-1638
- 1089 **Wilson M, Campbell HL, Ji P, Jones JB, Cuppels DA.** 2002. Biological control of bacterial  
1090 speck of tomato under field conditions at several locations in north america.  
1091 *Phytopathology* **92**: 1284-92
- 1092 **Winsor GL, Griffiths EJ, Lo R, Dhillon BK, Shay JA, Brinkman FS.** 2016. Enhanced  
1093 annotations and features for comparing thousands of *Pseudomonas* genomes in the  
1094 *Pseudomonas* genome database. *Nucleic Acids Res* **44**: D646-53

- 1095 **Xin XF, He SY.** 2013. *Pseudomonas syringae* pv. tomato DC3000: a model pathogen for  
1096 probing disease susceptibility and hormone signaling in plants. *Annu Rev Phytopathol* **51**:  
1097 473-98
- 1098 **Xin XF, Kvitko B, He SY.** 2018. *Pseudomonas syringae*: what it takes to be a pathogen.  
1099 *Nature Reviews Microbiology* **16**: 316-328
- 1100 **Zingl FG, Kohl P, Cakar F, Leitner DR, Mitterer F, Bonnington KE, Rechberger GN,**  
1101 **Kuehn MJ, Guan Z, Reidl J, Schild S.** 2020. Outer Membrane Vesiculation Facilitates  
1102 Surface Exchange and In Vivo Adaptation of *Vibrio cholerae*. *Cell Host & Microbe* **27**: 225-  
1103 237 e8
- 1104 **Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JD, Boller T, Felix G.** 2006. Perception  
1105 of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated  
1106 transformation. *Cell* **125**: 749-60
- 1107 **Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JD, Felix G, Boller T.** 2004. Bacterial  
1108 disease resistance in *Arabidopsis* through flagellin perception. *Nature* **428**: 764-7  
1109