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1 # Virulence of *Erwinia amylovora*, a prevalent apple pathogen: outer membrane proteins and type III secreted effectors increase fitness and compromise plant defenses

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25 **ABSTRACT**

26 Until now, no data are available on the outer membrane (OM) proteome of *Erwinia*
27 *amylovora*, a Gram-negative plant pathogen, causing fire blight in most members of the
28 *Rosaceae* family. Since the OM forms the interface between the bacterial cell and its
29 environment it is in direct contact with the host. Additionally, the type III secretion system,
30 embedded in the OM, is a pathogenicity factor of *E. amylovora*. To assess the influence of
31 the OM composition and the secretion behavior on virulence, a 2-D DIGE analysis and gene
32 expression profiling were performed on a high and lower virulent strain, both *in vitro* and *in*
33 *planta*. Proteome data showed an increase in flagellin for the lower virulent strain *in vitro*,
34 whereas, *in planta* several interesting proteins were identified as being differently expressed
35 between both strains. Further, gene expression of nearly all type III secreted effectors was
36 elevated for the higher virulent strain, both *in vitro* and *in planta*. As a first, we report that
37 several characteristics of virulence can be assigned to the outer membrane proteome.
38 Moreover, we demonstrate that secreted proteins prove to be important factors determining
39 differences in virulence between strains, otherwise regarded as homogeneous on a genome
40 level.

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53 **STATEMENT OF SIGNIFICANCE OF THE STUDY**

54 During this research our attention was directed towards the proteome of the outer membrane
55 of *E. amylovora*. This because the outer membrane proteins are the first to interact with the
56 environment and will be the first to be submitted to host defence responses. Two strains of
57 this pathogen differing in virulence were used and two conditions, *in vitro* and *in planta*, were
58 considered. The proteome of the outer membrane was investigated by 2-D differential gel
59 electrophoresis combined with mass spectrometry. The variations in protein expression
60 profile between both strains of *E. amylovora in vitro* and *in planta* were studied. Furthermore,
61 the secreting behaviour of the pathogen was also investigated by gene expression profiling
62 by RT-qPCR of the genes corresponding to secreted proteins. Again, this was perfomed for
63 both *in vitro* and *in planta* conditions. Thereby, this research provides valuable insights into
64 the outer membrane proteome and the secreting behaviour of *E. amylovora* in relation to
65 virulence.

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77 **INTRODUCTION**

78 *Erwinia amylovora* is a Gram-negative enterobacterium that causes fire blight, a necrotic
79 disease of rosaceous species. Among the hosts, are apple and pear the most important from
80 an economic point of view. Existing control mechanisms have proven inadequate and due to
81 its destructive character, this plant pathogen can cause major economic losses. The cell wall
82 of Gram-negative bacteria is composed of three morphologically defined layers, the inner
83 membrane (IM), the periplasm containing peptidoglycan and the outer membrane (OM) [1-3].
84 The OM functions as a selective barrier and protects the bacteria from the environment by
85 preventing entry of many toxic molecules into the cell [1, 3]. It is highly asymmetric, the inner
86 leaflet is composed of phospholipids while the outer leaflet is mainly composed of
87 lipopolysaccharides (LPS) [1, 3]. Outer membrane proteins (OMPs) are the key molecules
88 that interface the cell with the environment. Two types of proteins can be found in the OM,
89 lipoproteins and integral OMPs. Lipoproteins are anchored to the inner leaflet of the outer
90 membrane by lipid modifications of the N-terminal cysteine residue of their mature form [4].
91 Integral OMPs are generally folded into cylindrical β-barrels with a hydrophilic interior
92 composed of antiparallel amphipathic β-strands [5]. This barrel conformation allows the
93 proteins to function as channels which are of high importance for the intake of nutrients and
94 the excretion of waste products [6]. OmpA is one of the major outer membrane proteins of
95 Gram-negative *Enterobacteriaceae* and it has a function in the structural integrity of the
96 bacterial cell surface [5].

97 Moreover, the OM is the anchorage of surface organelles such as pili, type II and type VI
98 secretion systems [7, 8] as well as type III secretion systems (T3SSs) which are important for
99 virulence of *E. amylovora* [9, 10]. Further, the T3SS is involved in the elicitation of a
100 hypersensitive reaction (HR) in non-hosts [11] and the oxidative burst during compatible
101 interaction between pathogen and host [12]. It forms a specialized syringe structure by which
102 extracellular bacteria inject virulence proteins into the cytosol of their host [13, 14]. These
103 virulence proteins are called type III effectors (T3Es) and are delivered into the cytosol of the
104 host through a complex process [15]. The T3Es can suppress plant immunity or can be

105 recognized by the plant, thereby triggering an effector-induced immunity [16, 17]. Secreted,
106 translocated and structural components of the T3SS are encoded by the hypersensitive
107 response and pathogenicity (*hrp*) genes, which are located on the 62-kb chromosomal
108 pathogenicity island (PAI) [9, 10]. The expression of the *hrp* genes in *E. amylovora* is
109 dependent on environmental stimuli and under conditions of low nutrients and low pH but
110 also in a well-defined culture medium thought to mimic the conditions of the plant's apoplast
111 [18]. Moreover, recently it was suggested that *E. amylovora* utilizes the bacterial alarmone
112 ppGpp as an internal messenger to sense environmental/nutritional stimuli for regulation of
113 the T3SS and virulence [19]. In *E. amylovora*, several T3-secreted proteins have been
114 reported to date [20-22]. DspA/E is considered one of the best-studied and most important
115 effectors. It is homologous to the T3E AvrE of *Pseudomonas syringae* [23] and is required for
116 pathogenicity in apple and pear [23, 24]. The N-terminus of DspA/E interacts with four similar
117 putative leucine-rich repeat (LRR) receptor-like serine/threonine kinases (RLK) from apple
118 [25] and the C-terminus interacts with a pre-ferredoxin, which is an electron carrier in
119 photosystem I [26]. Thereby DspA/E may inhibit signal transduction and photosynthesis in
120 the host.
121 Virulence has been well studied in *E. amylovora*, and several genes have been identified for
122 their importance in virulence in this rather homogeneous species. However, the reason why
123 some strains exhibit a higher virulent ability in nature remains unclear. In previous works, the
124 total proteome of a low and high virulent strain grown *in vitro* [27] and *in planta* [28] has been
125 investigated. In this part of the research, the focus is now on the proteome of the outer
126 membrane, which forms the first line of contact between the bacteria and their surrounding
127 and the translocation of effectors secreted by the T3SS. In order to simulate a natural
128 infection, we included results from an artificial infection model. Using this approach, we were
129 able to draw more realistic conclusions regarding the function of the outer membrane and
130 secretome in the virulence of *E. amylovora*.

131 **MATERIALS AND METHODS**

132 **Bacterial strains**

133 Throughout this research two wild type strains of *E. amylovora,* with differential virulent
134 abilities, were used including a low virulent strain LMG2024 and a high virulent strain PFB5
135 [27, 29]. Strains were stored at -80 °C in 10% glycerol and cultured on yeast peptone
136 glucose agar (YPGA) plates at 24 °C.

137 ***In vitro* bacterial growth and isolation**

138 For the extraction of the OMPs of *E. amylovora*, bacteria were grown overnight in MM2 liquid
139 medium supplemented with 1% sorbitol [30], shaking at 100 rpm at 24 °C. The bacteria were
140 grown until exponential phase (OD_{600nm}=0.8) was reached. Cells from a 250 ml culture were
141 used.

142 To investigate the T3Es, bacterial cultures were first grown overnight in fluid LB medium at
143 28°C with shaking at 120 rpm. Next, the bacteria were washed twice with *hrp*-inducing
144 minimal medium (HrpMM) [31] and resuspended in 100 ml of this HrpMM (OD_{600} = 0.1 –
145 0.15). Then the suspensions were grown overnight at 18°C with shaking at 180 rpm until
146 approximately $OD_{600} \approx 0.7 – 0.8$ before samples were taken.

147 ***In planta* bacterial growth and isolation**

148 The extraction of viable bacterial cells from plant tissue was performed according the
149 previously described procedure [28]. In short, apple rootstocks (Malling 9 clone T337) were
150 chosen as a host and were infected using the scissors method. Thereby the two youngest
151 leaves of the shoots were cut perpendicular with scissors dipped in a bacterial suspension.
152 After approximately 10 to 14 days, depending on the strain, samples were taken. Four
153 biological replicates were included for these experiments.

154 **Isolation of outer membranes using N-lauroylsarcosine**

155 Extraction of the outer membranes was done according to the protocol of Hobb *et al.* (2009)
156 with small modifications. Samples containing cells collected from plant tissue or *in vitro*
157 culture were washed three times with sterile PBS. After the third wash, cells were

158 resuspended in 7 ml of 10 mM HEPES, pH 7.4 and cells were lysed by sonication on ice (4 x
159 10 s with 10 s intervals between the pulses) using a microtip (Labsonic P) at 80% amplitude.
160 Next, lysed cells were centrifugated (10 000 x g, 10 min, 4 °C) to remove cell debris and
161 unlysed cells. The membranes were collected by ultracentrifugation of the supernatants at
162 100 000 x g for 1h at 4 °C (Beckman LE80, Ti70 rotor). The pellet was resuspended in 10
163 mM HEPES, pH 7.4 and again ultracentrifuged at the same conditions as before. Afterwards,
164 the pellet was resuspended in 1% (w/v) N-lauroylsarcosine in 10 mM HEPES, pH 7.4 and
165 shaken (120 rpm) for 30 min at 37 °C. After treatment with N-lauroylsarcosine, the
166 membranes were spun again (100 000 x g, 1 h, 4 °C) and the pellet was washed with 10 mM
167 HEPES pH 7.4. Hereafter, the samples were ultracentrifuged for the last time (100 000 x g, 1
168 h, 4 °C) and the pellet was resuspended in 200 µl of sample solution (7 M urea, 2 M thio-urea
169 and 4% (w/v) CHAPS). Samples from the *in planta* experiments were treated with the 2-D
170 Clean-Up Kit (GE Healthcare) according to the manufacturer's instructions. After the pH was
171 adjusted to 8.5 (using 100 mM NaOH), the protein concentration was determined using the
172 2-D Quant kit (GE Healthcare).

173 **CyDye labeling and 2-D gel electrophoresis**

174 Protein samples were assessed using a 2D DIGE technique as described in Holtappels *et*
175 *al.,* 2015 [27, 28]. Protein samples were labeled using cyanine-derived fluorophores (3 Dyes
176 2-D CYanine Labeling kit From Proteomics Consult) and four biological replicates were
177 considered for both strains. Separation in first dimension was performed using precast
178 immobilized pH gradient (IPG) strips (pH 3-10, 24 cm) and separation in second dimension
179 was done with an HPE-FlatTop Tower (SERVA) using precast, plastic-backed 10-15%
180 polyacrylamide gradient gels. Following second dimension, the gels were scanned at a
181 resolution of 100 µm (pixel size) using an Ettan DIGE Imager (GE Healthcare) and the
182 protein abundance was determined using Progenesis SameSpots (version 4.6). Spots with at
183 least 1.5-fold change in volume ($P < 0.05$) in one condition after normalization, were
184 considered. After this analysis, spots of interest were excised and processed for mass

185 spectrometry [32], which was performed as previously described [27] (See supporting
186 Information Material).

187 **RNA extraction and quantitative RT-PCR**

188 Bacteria grown in HrpMM and bacteria taken from the infected plant material were
189 supplemented with 2 volumes of RNAprotect Bacteria Reagent (Qiagen, Venlo, The
190 Netherlands) and RNA was extracted as described previously [27]. The RNA samples taken
191 from the bacteria extracted from the plant tissue were further purified and concentrated by a
192 Na-Acetate and ethanol precipitation. For both conditions, four biological replicates were
193 considered. Primer3 (Whitehead Institue/MIT Center for Genome Research) was used to
194 develop gene-specific primers based on proteins of interest (Supplementary table S.1.). The
195 normalization factor was based on the expression of reference genes *rpoD*, *recA* and *rpoS*
196 as determined by using the GrayNorm algorithm and both normalized and non-normalized
197 data were presented according to Remans *et al.* (2014) [33]. Quantitative PCR (qPCR)
198 parameters were measured and determined according to the Minimum Information for
199 publication of qPCR Experiments (MIQE) précis checklist derived from Bustin *et al.* (2010)
200 [34] (Supplementary Table S.2).

201 **RESULTS AND DISCUSSION**

202 **Predicted and identified OMPs of *E. amylovora***

203 Uniprot was used to retrieve all proteins annotated as OMPs and other proteins related to the
204 outer membrane of *E. amylovora* CFBP1430 (http://www.uniprot.org/proteomes/ - Proteomes
205 UP000001841 *Erwinia amylovora* CFBP 1430). Proteins derived from the plasmids were not
206 considered. Table 1 lists all 75 potential OMPs and other OM related proteins. They are
207 divided into four categories according to their location as depicted by the gene ontology
208 retrieved from Uniprot. These categories include (i) cellular outer membrane, (ii) integral
209 outer membrane proteins, (iii) cell wall and (iv) flagella. Next, proteins were subdivided
210 according to their pI. No proteins were predicted with a pI lower than 4.

211 During this research, for the first time, OMPs of *E. amylovora* were identified and
212 characterized. By the combination of the OMP proteome of two strains of *E. amylovora*
213 differing in virulence (LMG2024 a low virulent strain and PFB5, a high virulent strain), grown
214 both *in vitro* and *in planta,* a more general and representative profile of the OMP proteome
215 was derived (Fig. 1A). Although it must be emphasized that only two strains were used
216 throughout the experiments. Mass spec analysis of the in total 121 differently abundant spots
217 (Supplementary table S.5) resulted in 97 confidently identified proteins or 30 unique proteins
218 considering redundancy (Table 1). OMPs were isolated using a reliable method based on the
219 use of Sarkosyl, which resulted in a pure OM fraction [35]. Besides the Dps proteins (DNA
220 protection during starvation), no cytoplasmic proteins were identified which emphasized the
221 purity of the extraction method.

222 When comparing the predicted proteins with the identified proteins there was an overlap of
223 around 48% (Table 1). Although the used technique is very sensitive some of the missed
224 proteins may be of low abundance. Furthermore, it has been known that large molecular
225 mass proteins (> 80 kDa) can be missed [36]. Moreover, the chance of identifying proteins
226 with a low molecular mass (< 10 kDa) or highly acidic/basic proteins is very low. Another
227 reason may be the loss of proteins during extraction, labeling or separation in first dimension.
228 In addition, the annotated proteins are only known from their DNA sequence. These proteins
229 may not be expressed in the used minimal medium or in an *in planta* environment or they are
230 only lowly expressed, thereby escaping detection.

231 The dominant OMPs were recovered including OmpA, OmpX, OmpF and OmpC. The
232 identified proteins were classified according to their subcellular location (Table 1). Many
233 spots were identified as OmpA, a protein that is known to be abundantly present in the outer
234 membrane of *E. coli* (10^5 copies per cell) [36]. Two proteins involved in LPS-assembly were
235 identified, LptD and LptE. The most abundant siderophore receptor, FoxR [37, 38], and TolC
236 and its precursor were also identified. These proteins are involved in the formation of efflux
237 pumps in the OM [39, 40]. Further, the protease IcsP/SopA and three proteins involved in the

238 structure of the flagellum (the flagellar hook (FlgE), the flagellar hook-associated protein
239 (FlgL) and flagellin (FliC)) were identified. The precursor of the protein required for assembly
240 of β-barrel proteins, YaeT [41], and the protein thought to be involved in amylovoran
241 translocation across the OM, AmsH [42], were both detected. Three precursors were
242 identified of the uncharacterized lipoprotein YjbH, a heme/hemopexin utilization protein B
243 and a putative protein YnfB. Finally three hypothetical proteins were found.

244 **Flagellar components were more abundant in the low virulent strain *in vitro***

245 A comparison was made between the outer membrane proteome of 2 WT strains of *E.*
246 *amylovora*, which differ in virulence, grown *in vitro*. Data analysis by the SameSpots software
247 suggested only 4 differentially abundant spots between both strains that met the previous set
248 requirements (P-value < 0.05; fold change > 1.5; power > 80% and q-value < 0.05) (Fig. 1B).
249 All four proteins were observed more abundantly in LMG2024 in comparison with the higher
250 virulent strain PFB5 including the outer membrane A precursor, the outer membrane protein
251 F (OmpF), and also two proteins identified as structural components of the bacterial
252 flagellum, FlgL and FliC (Table S.3). The normalized log abundances for these spots are
253 shown in figure 2, indicating the difference in abundance between both strains. The
254 conserved domain from flagellin functions as a pathogen-associated molecular pattern
255 (PAMP) that is recognized by FLS2, a transmembrane pattern recognition receptor (PRR) of
256 the plant [17, 43] playing a role in inducing an immunity response in the host, called the
257 PAMP-triggered immunity (PTI) which can block the pathogen [17]. Thereby, these results
258 confirmed the findings previously reported [27], were we showed that lower virulent strains
259 have more flagellin and motility associated proteins that can lead to an early activation of
260 plant defense mechanisms.

261 **Several proteins showed differences in abundance between both strains *in planta***

262 LMG2024 and PFB5 were grown *in planta* and re-isolated, 10 to 14 days after infection
263 (depending on the strain). OMPs were extracted and four biological replicates were used for
264 both strains. In total 10 gel spots appeared more abundant in LMG2024 in which the

265 following 6 proteins were identified: OmpF and its precursor, OmpX and OmpA, FlgL, a
266 flagellar hook-associated protein, and the protease complex IscP/SopA (Fig. 1B, 3 and Table
267 S.4). The outer membrane protease IcsP/SopA is a member of the enterobacterial omptin
268 family of proteases. As reported for *Shigella flexneri*, this protease is important in virulence
269 by the cleavage of the OM protein IcsA, which is located at the pole of the cell [44]. Within its
270 host cell cytoplasm, *S. flexneri* spreads by the directional assembly and accumulation of
271 actin filaments at the pole were IcsA is located [45-47]. However, a function for IcsP/SopA
272 related to virulence in *E. amylovora* has not yet been identified.

273 In total 16 spots showed a higher abundance for PFB5 in comparison with LMG2024. Mass
274 spectrometry led to the identification of 15 proteins of which OmpA was the most prominent
275 one, being identified in 11 spots (Fig. 1C). The occurrence of these OmpA isoforms may be
276 addressed to post translational modifications, as it is one of the major outer membrane
277 proteins of *E. coli* (10^5 copies per cell) [36] and it plays a role in the structural integrity of the
278 bacterial cell surface [5]. Also has overexpression of OmpA been linked to biofilm formation
279 in *E. coli* [48]. Further, because the large pore size (1 nm) of this barrel, it is has been
280 proposed that this pore conducts large organic molecules [49]. In regards of biofilm
281 formation, OmpA could facilitate the export of extracellular matrix components during biofilm
282 formation [48]. This further supports our hypothesis that this higher virulent strain produces
283 higher amounts of amylovoran for evading of the host defense mechanisms [28]. The other 4
284 spots contained one of the following proteins: an OmpA precursor, an outer membrane
285 channel protein identified as TolC and its precursor and finally a precursor of YaeT. In figure
286 3 all spots with exception to the ones identified as OmpA are depicted. Standardized log
287 abundance of the spot volumes for all biological replicates are indicated for both strains.

288 Results showed that PFB5 expresses higher amounts of precursors of the assembly factor
289 YaeT. Studies in *E. coli* have indicated that this protein is required in the assembly of β-barrel
290 proteins [41]. Expression of TolC and its precursor are both up-regulated in PFB5. Together
291 with AcrA and AcrB, TolC forms an intermembrane multidrug efflux pump in *E. coli* [39, 40].
292 A possible role of TolC, together with AcrAB, in resistance against phytoalexins in *E.*

293 *amylovora* has been suggested [50]. Phytoalexins are secondary metabolites that are
294 synthesized *de novo* by the plant as response upon biotic and abiotic stress [21, 51]. The
295 higher abundance of the previous mentioned proteins in the higher virulent strain PFB5 may
296 explain the overall increased fitness of this strain exhibiting higher virulence [52].
297
298 **Gene expression profiling shows that nearly all T3Es are more expressed in the high**
299 **virulent strain both *in vitro* as *in planta***
300 As a second part of this research, gene expression profiling was performed on the genes
301 corresponding to proteins secreted by the T3SS, which is embedded in the OM. This gene
302 expression profiling was done for both strains grown *in vitro* (Fig. 4A) and *in planta* (Fig. 4B).
303 Results were expressed, relative to LMG2024 (low virulent strain).

304 Relative gene expression of the genes *hrpW*, *hrpK*, *eop2*, *orfB* and *eop3 in vitro* showed a
305 significant up-regulation of these genes in PFB5 (high virulent) (Fig. 4A). Further, for *dspA/E*,
306 *hrpN* and *traF*, a significant up-regulation was observed for the absolute data for PFB5. For
307 the corresponding normalized data, only a trend could be observed, again indicating a higher
308 expression of these genes in PFB5. For *flgL3*, a significantly higher expression could be
309 observed in LMG2024. Only for *hrpJ* and *hrpA*, no significant difference could be
310 demonstrated between LMG2024 and PFB5, although a clear trend is visible, especially for
311 *hrpJ*.

312 *In planta* results demonstrate that with exception of *hrpA*, all genes were up regulated in the
313 high virulent strain PFB5 in comparison with LMG2024 (Fig. 4B). For both *traF* and *eop3*,
314 significant differences were observed in both normalized and non-normalized data. For the
315 expression of genes *hrpW*, *orfB*, *hrpJ* and *hrpN*, a significant difference was observed for the
316 non-normalized data. A clear trend was visual for the normalized data. For all the other
317 genes including *dspA/E*, *eop2*, *flgL3* and *hrpK* a clear trend was seen for the up-regulation of
318 these genes in PFB5, the high virulent strain.

319 **Gene expression of *flgL3* showed to be different between both strains *in vitro* and *in***
320 ***planta***

321 Both set-ups exhibited the same trends with exception of *flgL3*. This gene encodes for a
322 protein homologue to the flagellar protein FlgE [20]. This protein is the major component of
323 the flagellar hook [53, 54] and important for motility. As previously reported, LMG2024 is
324 more motile in comparison with the other strain in an *in vitro* environment [27]. Although
325 motility is important in pathogenic bacteria through chemotaxis, adhesion and invasion,
326 components of the bacterial flagella contain a pathogen-associated molecular pattern
327 (PAMP), which may be recognized by the plant [55-57]. This recognition may lead to PAMP-
328 triggered immunity (PTI) and can induce the production of reactive oxygen species (ROS),
329 ethylene and the induction of the general plant defense [55]. Due to this higher abundance of
330 PAMPs, the likelihood that the host will recognize this strain more rapidly is much higher. In
331 contradiction, *flgL3* was up regulated in the higher virulent strain *in planta* (Fig. 4B). Although
332 this protein can induce PTI, previous research demonstrated a higher amylovoran production
333 for the higher virulent strain both *in vitro* as *in planta*, which may prevent recognition of
334 PAMPs by masking them [27]. Further, amylovoran is important for biofilm formation, which
335 encases the bacteria in an extracellular matrix that protects the bacteria against detrimental
336 environmental factors [58].

337 **The high virulent strain expresses more T3Es which may be important to interfere with**
338 **plant defense mechanisms**

339 Three T3Es have been shown to be important in pathogenicity of *E. amylovora*, DspA/E [23,
340 24], HrpN [59, 60] and HrpJ [20] and all three showed a higher expression in PFB5 (Fig. 4A
341 and B). DspA/E, a homolog of AvrE in *P. syringae*, contributes to disease development by
342 several actions. First, it interacts with RLKs of the plant to interfere with PTI and to induce
343 effector-triggered immunity (ETI). Moreover, two groups of target proteins of DspA/E have
344 been identified in apple, from which the first includes four kinases named DIPM1-4 (DspE-
345 interacting proteins from Malus 1 to 4) [25]. With their extracytoplasmic LRR domains, these

346 putative kinases resemble to an RLK family that exerts functions in defense responses,
347 developmental processes and phytohormone perception [55, 61, 62]. Secondly,
348 preferredoxin, which is converted to ferredoxin serving as an electron carrier in photosystem
349 I, was found to interact with DspA/E in young leaves. Thereby, the import of preferredoxin
350 into developing chloroplasts is blocked, leading to an inhibition of photosynthesis [26]. The
351 ectopic expression of DspA/E in yeast and plants has proven to be toxic indicating that this
352 protein rather targets a cellular process conserved in eukaryotic cells [63, 64]. Moreover,
353 investigation in yeast has proven that DspA/E exerts several functions in eukaryotic cells.
354 First, this protein has a function in growth inhibition associated with perturbations of the actin
355 cytoskeleton and endocytosis and it alters cellular trafficking [63]. Also, DspA/E has been
356 proven to interfere with the regulation of the sphingolipid pathway [65]. Next, DspA/E
357 contributes to disease development by inhibition of the salicylic acid (SA) dependent innate
358 immunity [66]. SA is a signal molecule of which the levels are increased following pathogen
359 infection. This leads to immune responses including systemic acquired resistance, basal
360 resistance and even gene-for-gene resistance [67, 68]. Moreover, down-regulation of the
361 jasmonic acid (JA) pathway by DspA/E has also proven important in the infection process of
362 *E. amylovora* [69].

363 Following these findings, we can hypothesize that the higher virulent strain, which showed a
364 higher expression of the gene encoding DspA/E may have the ability to interfere with the
365 immune system of the plant, leading to successful infection.

366 **PFB5 has a higher expression of *hrpN* and *eop3*, interfering with several important**
367 **mechanisms in the plant**

368 HrpN belongs to the class of harpins which are unlike the T3Es, secreted and targeted to the
369 intercellular spaces of plant tissues [70]. Moreover, HrpN is only secreted and localized in the
370 plants apoplast during infection [71]. Besides its function in elicitation of a HR response,
371 HrpN has also a function in the induction of the SA- and JA-dependent pathways in
372 *Arabidopsis thaliana* [72]. Furthermore, combined action of DspA/E and HrpN has been

373 proven to cause an oxidative burst in host plants [12]. Consequently it can be stated that
374 higher virulent strains are more capable of inducing an oxidative burst in host plants, which
375 leads to a higher mortality of plant cell by the lethal action of the oxidative burst [73]. This
376 uncontrolled cell death (necrosis) will provide the bacterial cells with nutrients necessary for
377 their growth, multiplication and further colonization of the host. Moreover, gene expression of
378 the gene corresponding to Eop3 was tested. This protein is homologues to AvrPhE, a protein
379 belonging to the HopX family of effector proteins [20, 74]. HopX has been shown to suppress
380 programmed cell death by the inhibition of the pro-apoptotic protein Bax [75]. Since the
381 expression of *eop3* in the higher virulent strain was up regulated in a great extent both *in*
382 *vitro* (Fig. 4A) as *in planta* (Fig. 4B), we may hypothesize that this protein can have a function
383 in the inhibition of programmed cell death. Thereby it may help the higher virulent strain to
384 secure its supply of nutrients, which are obtained during necrosis.

385 **Concluding remarks**

386 *In vitro* results confirmed previous findings of our research group [34] since a higher
387 abundance of flagellin of the lower virulent strain was observed. This flagellin can function as
388 a trigger to induce immunity in the host. In planta results indicated a higher abundance of
389 OmpA, YaeT and TolC in the higher virulent strain PFB5. These proteins contribute to the
390 overall fitness of this strain and help to overcome plant defense mechanisms. Moreover,
391 PFB5 may possess a better-armed OM indicating a better survival strategy in comparison
392 with the low virulent strain. Further, by gene expression profiling, we showed that the higher
393 virulent strain, exhibited a higher expression of the gene encoding DspA/E, both *in vitro* and
394 *in planta*. This protein functions as an inhibitor of both JA- as SA-dependent pathways of
395 immune response of the plant. Moreover, in apple RLKs are identified to which this effector
396 can bind in order to prohibit PTI. Together with HrpN, DspA/E induces an oxidative burst,
397 necessary for *E. amylovora* to kill plant cells to gain nutrients. Furthermore, it has been
398 suggested that the *hrp* genes are expressed *in planta* when nutrients and pH are low [25],
399 indicating the importance of this system to acquire nutrients. And finally, the higher virulent
400 strain produces higher amount of amylovoran [34] and [28] which may protect the cell.

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409 **CONFLICT OF INTEREST STATEMENT**

410 The authors have declared no conflict of interest.

411

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620 **TABLE LEGEND**

621 **Table 1:** List of OMPs of *E. amylovora* derived from two strains (LMG2024 and PFB5) grown
622 in two conditions (*in vitro* and *in planta*). Seventy-five proteins were annotated as outer
623 membrane proteins according UniprotKB. The list is subdivided both according the
624 subcellular location: cell outer membrane, cell wall, flagella and integral OMP and according
625 pI. Proteins identified during this research are indicated by their spot number. With exception
626 of DNA protection during starvation, all proteins are identified as being located in the OM.

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632 **Table 1**

Accession number	Description	Theoretical pI	Theoretical MW (kDA)	Subcellular location	Spot number	Protein ID
Outer membrane proteins pI < 7						
D4HWX4	Outer membrane protein 1B	4.73	40.01	Cell outer membrane		
/	Outer membrane protein precursor (Porin)	4.89	40.37	Cell outer membrane	**20, 29, 30, 32, 96**	gi|312171969, gi|635555548
D4HYC0	Outer membrane protein assembly factor BamB	4.91	42.33	Cell outer membrane		
D4HY74	Outer membrane protein assembly factor BamC	4.99	37.64	Cell outer membrane		
D4HZ27	Nucleoside-specific channel-forming protein tsx	5.16	32.68	Cell outer membrane		
D4HVP2	Type III secretion system outer membrane pore HrcC	5.41	74.06	Cell outer membrane		
D4I3V5	Type I secretion system, TolC-family protein	5.52	49	Cell outer membrane		
D4I248	Ferrioxamine E receptor	5.61	77.83	Cell outer membrane	**4, 6**	gi|312173883, gi|635553360
/	Putative ferrioxamine receptor	5.61	77.83	Cell outer membrane	**5**	gi|635553360
D4I324	Protein transport protein hofQ	5.64	45.79	Cell outer membrane		
D4HVA2	Outer membrane protein tolC	5.7	52.27	Cell outer membrane	**11, 12, 13, 16, 17**	gi|312170991, gi|635555086
/	Outer membrane channel protein	5.7	52.27	Cell outer membrane	**15**	gi|490258006
D4I0G2	LPS-assembly protein LptD	5.73	90.23	Cell outer membrane	**2, 3**	gi|490274699, gi|502800886 gi|566688220, gi|635554220 gi|312173556
D4HX37	Protein mxiC	5.75	42.6	Cell outer membrane		
D4I348	TonB-dependent copper receptor	5.78	72.45	Cell outer membrane		
A0RZH5	Outer membrane protein Omp-EA	5.82	41.63	Cell outer membrane	**36**	gi|566688646
D4HX36	Type III secretory protein InvG	5.84	64.19	Cell outer membrane		
D4HYF5	Membrane-bound lytic murein transglycosylase F	5.84	54.67	Cell outer membrane		
D4I1N7	Type III secretory protein InvG	5.85	65.5	Cell outer membrane		
D4HZX1	Type II secretion system protein outD	5.95	69.76	Cell outer membrane		
D4HZU9	IcsP/SopA	6.11	34.93	Cell outer membrane	**84, 87**	gi|292489312, gi|312174384 gi|502800896, gi|635554287
D4I2K5	TonB-dependent receptor protein	6.49	87.08	Cell outer membrane		
D4HYQ5	Probable tonB-dependent receptor yncD	6.58	74.05	Cell outer membrane		
D4HYU8	LPS-assembly lipoprotein LptE	6.91	21.99	Cell outer membrane	**100**	gi|490273064, gi|50280096 gi|635554843

Outer membrane proteins pI > 7						
D4I1N8	Invasion protein InvE	7.07	42.63	Cell outer membrane		
D4I4B8	Cellulose synthase operon protein C	7.66	146.81	Cell outer membrane		
D4I2L4	Lipoprotein E	8.72	28.32	Cell outer membrane		
D4HUR8	Outer membrane protease	8.91	35.21	Cell outer membrane		
D4I2E6	Outer membrane lipoprotein slyB	8.97	15.63	Cell outer membrane		
D4I1Z0	Major outer membrane lipoprotein	8.98	83.69	Cell outer membrane		
D4HX91	Outer membrane protein assembly factor BamD	9.01	27.42	Cell outer membrane		
D4I1U6	Osmotically-inducible lipoprotein E	9.03	12.26	Cell outer membrane		
D4I1R4	Outer-membrane lipoprotein LolB	9.04	23.99	Cell outer membrane		
D4HYI6	Outer membrane protein assembly factor BamE	9.06	12.81	Cell outer membrane		
D4HVS4	Membrane-bound lytic murein transglycosylase C	9.07	41.68	Cell outer membrane		
D4I1F6	Uncharacterized protein ytfM	9.15	64.57	Cell outer membrane		
D4I117	Uncharacterized protein ycfJ	9.17	18.42	Cell outer membrane		
D4I418	Outer membrane protein slp	9.38	21.65	Cell outer membrane		
D4HWJ0	Membrane-bound lytic murein transglycosylase A	9.44	41.8	Cell outer membrane		
D4HVM8	Hypersensitivity response secretion protein HrpJ	9.61	42.4	Cell outer membrane		
D4I3C2	Osmotically-inducible lipoprotein B	10.92	6.92	Cell outer membrane		
D4HWH8	D-alanyl-D-alanine dipeptidase	5.87	23.02	Cell wall		
D4HZ50	Methionine import ATP-binding protein MetN	7.12	40.67	Cell wall		
D4I0D0	Cell division inhibitor SulA	8.76	18.76	Cell wall		
D4HZ40	Multidrug resistance protein A	9.09	42.39	Cell wall		
D4I149	Lipoprotein releasing system, transmembrane protein	9.75	44.55	Cell wall		
D4HUJ9	4-alpha-L-fucosyltransferase	9.75	40.94	Cell wall		
E5B4B4	Flagellar hook protein FlgE	4.87	41.62	Flagella	**24**	gi|310767290, gi|490273389 gi|635555628
E5B7T4	Flagellar hook-associated protein FlgL	4.90	34.08	Flagella	**61**	gi|635555635
D4HVZ2	Flagellin	5.34	29.61	Flagella	**83**	gi|635555329
D4HYY3	Flagellar P-ring protein (Basal body P-ring protein)	9.02	39.2	Flagella		
D4HYY4	Flagellar L-ring protein	9.1	24.89	Flagella		
D4I0Y5	Flagellar L-ring protein (Basal body L-ring protein)	9.5	38.26	Flagella		
Integral outer membrane proteins pI < 7						
D4I7B3	Outer membrane protein C	4.95	40.37	Integral OMP	**27, 28, 33, 34,**	gi|635555548

					35, 37, 38	
D4HZN4	Outer membrane protein X (precursor)	5.08	18.1	Integral OMP	**55, 90, 102, 104, 107, 108, 110, 113, 114**	gi|566689000, gi|635553591
D4I285	Uncharacterized protein yfaZ	5.42	18.99	Integral OMP		
D4I3F0	Outer membrane protein W (precursor)	5.45	23.08	Integral OMP	**95, 96, 97, 98, 100, 101**	gi|490273851, gi|490276757 gi|635554552
4HZ73	Outer membrane protein assembly factor BamA	5.48	88.3	Integral OMP		
E5B4U3	Outer membrane protein F	5.51	38.73	Integral OMP	**56, 57, 58, 59, 60**	gi|635555787
4HZ73	Outer membrane protein assembly factor yaeT precursor	5.56	88.31	Integral OMP	**1**	gi|312173380, gi|490274571 gi|490277172, gi|635553124
D4HVE4	Outer membrane usher protein fimD	5.83	87.49	Integral OMP		
D4I3V6	Type I secretion system, membrane-fusion protein	5.94	48.87	Integral OMP		
Q9AQ56	Major outer membrane protein OmpA, partial	6.79	16.08	Integral OMP	**10, 49, 51, 53, 54, 69, 70, 75, 101, 107**	gi|12382107
D4I0C9	Outer membrane protein A	6.84	38.26	Integral OMP	**8, 9, 23, 26, 41, 42, 43, 46, 47, 48, 50, 51, 53, 54, 62, 64-79, 95, 100, 110**	gi|635555568
D4I0C9	Outer membrane protein A precursor	6.84	38.26	Integral OMP	**19, 33, 36, 37, 38, 39, 40, 45, 49, 81, 82, 89, 94, 96**	gi|490273323, gi|635555568
D4HWE3	Virulence membrane protein pagC	6.9	20.37	Integral OMP		
Integral outer membrane proteins pI > 7						
D4I331	Putative membrane protein igaA homolog	7.69	79.73	Integral OMP		
D4HYT3	Lipid A palmitoyltransferase PagP	7.83	21.9	Integral OMP		
D4HUR2	Outer membrane usher protein fimD	8.32	85.77	Integral OMP		
D4I277	Putative PUP family transporter	8.79	47.55	Integral OMP		
D4HYB6	Quinate/shikimate dehydrogenase	9.18	93.12	Integral OMP		
D4I470	Lipid A biosynthesis (KDO)2-(Lauroyl)-lipid IVA acyltransferase	9.55	37.1	Integral OMP		
D4HUJ0	UDP-GlcNAc:undecaprenylphosphate GlcNAc-1-phosphate transferase	9.69	40.95	Integral OMP		
D4I0V8	Lipid A biosynthesis lauroyl acyltransferase	9.7	35.09	Integral OMP		

D4HUT6	Lipid A biosynthesis lauroyl acyltransferase	10.17	35.46	Integral OMP		
Other classified proteins						
/	Hypothetical protein EAIL5_3404	5.17	44.08	Unknown	**21, 24, 25**	gi|312173970, gi|490275075 gi|490277474, gi|635552822
D4HZN2	DNA protection during starvation protein	5.18	18.82	Cytoplasmic	**104**	gi|562747432, gi|635553593
/	Hypothetical protein EAM01S_18_00570	5.42	18.99	Unknown	**52, 85, 86,90 102, 114**	gi|490275041 gi|635553392
Q46629	Amylovoran export outer membrane protein AmsH Precursor	6.00	41.50	Membrane	**23**	gi|384872315, gi|566689865 gi|635555426
E5B9M3	Uncharacterized lipoprotein yjbH precursor	6.00	78.23	OM Lipoprotein	**7**	gi|490277482, gi|635552812
E5B8Q3	Heme/hemopexin utilization protein B precursor	6.09	64.02	Probably integral OMP	**10**	gi|566688262, gi|635554179
D4I2I9	Putative protein ynfB precursor	9.10	13.23	Signal	**118**	gi|566689423
/	Hypothetical protein EAM01S_18_00570	5.42	18.99	Unknown	**52, 85, 86,90 102, 114**	gi|490275041 gi|635553392

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642 **FIGURE LEGEND**

643 **Figure 1:** A. 2-DE analysis of the proteome of the outer membrane of *E. amylovora* including
644 two strains (LMG2024 and PFB5) grown *in vitro* and *in planta*. Image represents scan from
645 the internal standard so every protein is present. All picked spots are indicated. B. Indicated
646 spots are the proteins identified as differentially expressed during *in vitro* comparison of
647 LMG2024 and PFB5. C. Indicated spots are the proteins identified as differentially expressed
648 during the *in planta* comparison between LMG2024 and PFB5.

649 **Figure 2:** A graphical view of the selected differential protein spots from two strains of *E.*
650 *amylovora*, LMG2024 and PFB5 differing in virulence, grown *in vitro*. Each symbol
651 represents one of the four biological replicates. A. Spot 36 (OmpA), B. Spot 59 (OmpF), C.
652 Spot 61 (FlgL), D. Spot 83 (FliC).

653 **Figure 3:** A graphical view of the selected differential protein spots from two strains of *E.*
654 *amylovora*, LMG2024 and PFB5 differing in virulence, grown *in planta*. Each symbol
655 represents one of the four biological replicates. A. Spot 55 (OmpX), B. Spot 56 (OmpF), C.
656 Spot 57 (OmpF), D. Spot 61 (FlgL), E. Spot 84 (IcsP/SopA), F. Spot 1 (YaeT), G. Spot 12
657 (TolC) and H. Spot 15 (TolC).

658 **Figure 4:** A. Relative gene expression measured by RT-qPCR of *hrpW*, *dspA/E*, *eop2*, *orfB*,
659 *hrpJ*, *traF, eop3*, *flgL3*, *hrpK*, *hrpN* and *hrpA* from *in vitro* samples B. Relative gene
660 expression of the same set of genes measured on *in planta* samples. Normalized data are
661 represented in white and the non-normalized data in black. Up- or down regulations are
662 represented on a $\log_2$ scale y-axis relative to the least virulent strain, LMG2024. Columns
663 represent data from four biological replicates ± standard errors. Asterisks indicate statistical
664 differences ($P < 0.05$, one-way ANOVA after testing normality with Shapiro-Wilk test).

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