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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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ABSTRACT

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

STATEMENT OF SIGNIFICANCE OF THE STUDY

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

INTRODUCTION

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

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

recognized by the plant, thereby triggering an effector-induced immunity [16, 17]. Secreted, translocated and structural components of the T3SS are encoded by the hypersensitive response and pathogenicity (*hrp*) genes, which are located on the 62-kb chromosomal pathogenicity island (PAI) [9, 10]. The expression of the *hrp* genes in *E. amylovora* is dependent on environmental stimuli and under conditions of low nutrients and low pH but also in a well-defined culture medium thought to mimic the conditions of the plant's apoplast [18]. Moreover, recently it was suggested that *E. amylovora* utilizes the bacterial alarmone ppGpp as an internal messenger to sense environmental/nutritional stimuli for regulation of the T3SS and virulence [19]. In *E. amylovora*, several T3-secreted proteins have been reported to date [20-22]. DspA/E is considered one of the best-studied and most important effectors. It is homologous to the T3E AvrE of *Pseudomonas syringae* [23] and is required for pathogenicity in apple and pear [23, 24]. The N-terminus of DspA/E interacts with four similar putative leucine-rich repeat (LRR) receptor-like serine/threonine kinases (RLK) from apple [25] and the C-terminus interacts with a pre-ferredoxin, which is an electron carrier in photosystem I [26]. Thereby DspA/E may inhibit signal transduction and photosynthesis in the host.

Virulence has been well studied in *E. amylovora*, and several genes have been identified for their importance in virulence in this rather homogeneous species. However, the reason why some strains exhibit a higher virulent ability in nature remains unclear. In previous works, the total proteome of a low and high virulent strain grown *in vitro* [27] and *in planta* [28] has been investigated. In this part of the research, the focus is now on the proteome of the outer membrane, which forms the first line of contact between the bacteria and their surrounding and the translocation of effectors secreted by the T3SS. In order to simulate a natural infection, we included results from an artificial infection model. Using this approach, we were able to draw more realistic conclusions regarding the function of the outer membrane and secretome in the virulence of *E. amylovora*.

MATERIALS AND METHODS

Bacterial strains

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

In vitro bacterial growth and isolation

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

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

In planta bacterial growth and isolation

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

Isolation of outer membranes using N-lauroylsarcosine

Extraction of the outer membranes was done according to the protocol of Hobb *et al.* (2009) with small modifications. Samples containing cells collected from plant tissue or *in vitro* 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

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

RNA extraction and quantitative RT-PCR

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

RESULTS AND DISCUSSION

Predicted and identified OMPs of *E. amylovora*

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

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

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

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

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

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

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

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

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

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

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

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

amylovora has been suggested [50]. Phytoalexins are secondary metabolites that are synthesized *de novo* by the plant as response upon biotic and abiotic stress [21, 51]. The higher abundance of the previous mentioned proteins in the higher virulent strain PFB5 may explain the overall increased fitness of this strain exhibiting higher virulence [52].

Gene expression profiling shows that nearly all T3Es are more expressed in the high virulent strain both *in vitro* as *in planta*

As a second part of this research, gene expression profiling was performed on the genes corresponding to proteins secreted by the T3SS, which is embedded in the OM. This gene expression profiling was done for both strains grown *in vitro* (Fig. 4A) and *in planta* (Fig. 4B). Results were expressed, relative to LMG2024 (low virulent strain).

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

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

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

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

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

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

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

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

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

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

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

Concluding remarks

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

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CONFLICT OF INTEREST STATEMENT

The authors have declared no conflict of interest.

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TABLE LEGEND

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

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

FIGURE LEGEND

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

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

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

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