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DOCTORAL DISSERTATION

Virulence of the plant pathogen *Erwinia amylovora*: a comparative proteome analysis

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"The important thing is not to stop questioning. Curiosity has its own reason for existing."

Albert Einstein

Summary

Samenvatting

Summary

Erwinia amylovora is a Gram-negative plant pathogen that is classified as member of the *Enterobacteriaceae* which makes it closely related to many important human and animal pathogens such as *Escherichia coli*, *Salmonella* spp., *Shigella* spp. and *Yersinia* spp. *E. amylovora* causes the destructive disease fire blight which affects most members of the *Rosaceae* family of which apple (*Malus* spp.) and pear (*Pyrus* spp.) are economically the most important species. Other hosts include quince, blackberry, raspberry and many wild and cultivated ornamentals including *Cotoneaster* and *Pyracantha* spp. This devastating disease is spread by wind, insects, birds and human activity. The absence of effective control mechanisms and its destructive character enable *E. amylovora* to disperse rapidly both within susceptible plants and between trees in orchards which could lead to great economic losses. Further, fire blight will become an even greater threat for the fruit production in Europe in the near future because of the expected rise in average global temperature, the growing of cultivars on susceptible rootstocks and the introduction of susceptible cultivars.

Independent research has suggested that *E. amylovora* is a homogeneous species based on physiological, biochemical, phylogenetic and genetic analysis. Moreover, a low diversity within this pathogen has been reported following the comparison of strains locally separated leading to the hypothesis that minimal evolution has occurred since the global dispersion of this pathogen. Contradictory, differences in virulence have been observed between *E. amylovora* strains isolated from nature. Different factors have been identified as being crucial for virulence in *E. amylovora* including a functional type III secretion system (T3SS) to inject effector proteins into the cytosol of the host, exopolysaccharides (EPS) including amylovoran and levan, the sorbitol metabolism, the siderophore desferrioxamine, metalloproteases and two-component signal transduction systems (TCSTs).

To date, an abundance of research is published based on genomic experiments although no conclusive definition has been provided to explain the difference in virulence between different isolates of *E. amylovora*. Because of the rather limited knowledge of the proteome of this plant pathogen, the conducted

research is dedicated to the proteome of *E. amylovora* by comparing four strains exhibiting differences in their virulent ability. For an in-depth comparison, two strains which exhibited the most differences both during artificial infections and during the analysis of the proteome data, were used.

The main goal of the current study was to identify the proteins leading to this differential virulence between different isolates of *E. amylovora*. We wanted to expand our knowledge concerning the proteome of *E. amylovora*. Therefore, a proteomics analysis by two-dimensional differential in gel electrophoresis (2D-DIGE) for *E. amylovora* has been optimized.

First, a comparative proteome study was performed on four strains grown *in vitro* (Chapter 3). Thereby a comparison between strains differing in virulence, grown in a minimal medium, was made. Results showed a higher flagellin amount for the low virulent strain and a higher abundance of amylovoran production in the higher virulent strain. This may suggest that the low virulent strain has a higher chance to be recognized by the plant since flagellin may function as a pathogen-associated molecular pattern (PAMP) which can lead to the induction of PAMP-initiated immunity in the host. Since the higher virulent strain produces higher amounts of amylovoran and lower amounts of flagellin, this strain is better covered and is less likely to be recognized by the host and induce the defense pathways in the plant.

Following these interesting results *in vitro*, a procedure was optimized and employed to extract viable bacterial cells from plant tissue after artificial infection (Chapter 4). Proteins were extracted from these samples, thereby enabling us to make a comparison between the proteome of strains of *E. amylovora* differing in virulence grown inside a host. To date, knowledge concerning the proteome of plant pathogens inside its host is rather limited.

Results suggested fundamental differences between the high and low virulent strain including differences in carbohydrate, amino acid and fatty acid metabolism, in the stress response exhibited by both strains and in the function of RNA processing. A more efficient sorbitol metabolism in the higher virulent strain induced a higher production of building blocks important for amylovoran synthesis. This amylovoran plays an important role in defense against recognition of the pathogen by the host by masking cell surface components and in protection of the cell against rough environmental conditions both by the

protection against reactive oxygen species (ROS) as in the formation of biofilms. Another protective function in the higher virulent strain can be assigned to the lipopolysaccharides (LPS). Results showed that this strain had a higher abundance of two proteins involved in LPS-biosynthesis. Further, evidence was provided for the involvement of components of the RNA degradosome, including Pnp or PNPase and Hfq, in virulence. Both proteome and gene expression results support this hypothesis by a higher abundance of these proteins and a higher expression of the corresponding genes in the higher virulent strain. Finally, important differences were found in the response against stress between both strains. Moreover, results showed that the lower virulent strain has a higher abundance of cold shock proteins, while the higher virulent strains produces more heat shock proteins. Literature suggests that cold shock proteins, like flagellin, may be recognized as a PAMP by the host leading to a defense response.

The outer membrane and its proteins form the interface between the pathogen and its environment, comprising the first line of defense of the pathogen against resistance mechanisms of the plant. In chapter 5, the composition of the outer membrane of *E. amylovora* is described and compared with the predicted outer membrane proteome based on genetic information. When comparing the outer membrane proteome *in vitro* between two strains differing in virulence, structural proteins of the flagella were identified as more abundant in the lower virulent strain, again indicating a role for these proteins in pathogen recognition by its host. When comparing the outer membrane proteins of both strains grown *in planta*, TolC was more abundant in the higher virulent strain indicating a better overall fitness of this strain and a higher resistance against phytoalexins produced by the plant. A higher abundance of OmpA was also found in the higher virulent strain. This protein has an important structural role by maintaining cell integrity.

Lastly, this dissertation includes a gene expression profiling by RT-qPCR of genes corresponding to important type III secreted proteins (Chapter 6). Like many other Gram-negative pathogens, *E. amylovora* uses a T3SS to inject effectors into the cytosol of its host to cause disease and interfere with defense mechanisms of the plant. Eleven genes corresponding to the most important

type III secreted effectors were considered and were measured for both bacteria grown *in vitro* and grown *in planta*.

Following these results it can be suggested that the higher virulent strain produces a higher amount of effectors to inject into the host leading to disease development by the production of ROS in order to kill plant cells and acquire nutrients. Moreover, some of these effectors, including DspA/E are involved in the suppression of defense of the plant leading to successful infection.

In conclusion, all results suggest a higher recognition of the lower virulent strain by the host due to the higher presence of PAMPs and the lower amylovoran production to mask these cell surface structures. Elongation factor Tu, cold shock proteins and flagellin were identified as higher abundant in the lower virulent strain leading to a higher recognition by the host which could lead to PAMP triggered immunity in the plant. The higher virulent strain grows faster in the presence of sorbitol, the main transport sugar in apple and pear which may lead to a higher amylovoran production. Besides a function in masking cell surface components, amylovoran has a function in protecting the pathogen against ROS produced by the plant and it has a function in biofilm formation. Further, the high virulent strain produces higher amounts of lipopolysaccharides to protect itself against ROS and it produces higher amount of heat shock proteins to deal with the hostile environment inside the plant. A higher expression of genes corresponding to the main type III effectors in the high virulent strain suggest that this strain has a higher ability to interfere with the defense mechanism of the host by blocking signaling pathways normally leading to resistance. These results indicate that the interplay between different virulence factors is necessary for the higher virulent strain to start and sustain a successful infection in which the lower virulent strain fails.

Samenvatting

Erwinia amylovora is een Gram-negatieve plant-pathogeen die behoort tot de familie van de *Enterobacteriaceae*. Hierdoor is *E. amylovora* nauw verwant met vele belangrijke menselijke en dierlijke pathogenen zoals onder andere *Escherichia. coli*, *Salmonella* spp., *Shigella* spp. and *Yersinia* spp. *E. amylovora* veroorzaakt bacterievuur oftewel perenvuur, een destructieve ziekte die de meeste leden van de *Rosaceae* familie treft waaronder appel (*Malus* spp.) en peer (*Pyrus* spp.) die vanuit economisch standpunt de belangrijkste gastheren zijn. Tot de andere waardplanten behoren de kweepeer, de braam, de framboos en vele wilde maar ook gecultiveerde sierplanten waaronder de soorten *Cotoneaster* en *Pyracantha*. Deze ziekte wordt verspreid via de wind, insecten, vogels en menselijke activiteit. Door het ontbreken van effectieve bestrijdingsmiddelen en door het destructieve karakter, is *E. amylovora* in staat om zich snel te verspreiden zowel in als tussen gevoelige waardplanten en tussen bomen in de boomgaarden hetgeen kan leiden tot grote verliezen voor de telers. Verder kan bacterievuur in de toekomst nog een grotere impact uitoefenen op de fruitproductie door de verwachtte stijging in globale temperatuur, het groeien van cultivars op gevoelige onderstammen en eveneens de introductie van gevoelige cultivars.

Onafhankelijk onderzoek heeft aangetoond dat *E. amylovora* een relatief homogene soort is gebaseerd op fysiologische, biochemische, fylogenetische en genetische analyses. Onderzoek op stammen van *E. amylovora* die fysisch gescheiden zijn sinds de globale verspreiding van de pathogeen, heeft geleid tot de hypothese dat er minimale evolutie heeft plaatsgevonden binnen deze soort. In contrast met deze stelling, worden er in de natuur stammen gevonden die grote verschillen in virulentie vertonen. Verschillende factoren zijn geïdentificeerd die noodzakelijk zijn voor de virulentie van *E. amylovora*, waaronder een functioneel type III secretiesysteem (T3SS) om effectorproteïnen te injecteren in het cytosol van de gastheer, exopolysacharides (EPS) waarvan amylovoran en levan deel uit maken, het sorbitol metabolisme, de siderofoor desferrioxamine, metalloproteasen en "two-component signal transduction" systemen (TCST).

Op genetisch vlak is er veel onderzoek gedaan op dit pathogeen, hoewel geen duidelijke conclusie is geformuleerd om de verschillen in virulentie tussen verschillende stammen van *E. amylovora* te verklaren. Omwille van de eerder gelimiteerde kennis omtrent het proteoom van *E. amylovora*, werd dit werk volledig gewijd aan deze tak van onderzoek. Eveneens konden er uitspaken gedaan worden omtrent de virulentie omdat er gedurende de experimenten gebruik gemaakt werd van vier stammen van *E. amylovora* die verschillen vertonen in hun virulentiegraad. Voor de gedetailleerde vergelijkingen, werd er gebruik gemaakt van twee stammen die de meeste verschillen vertoonden, zowel tijdens artificiële infecties als gedurende de analyse van de proteoomresultaten.

Het voornaamste doel van het huidige werk was om de proteïnen te identificeren die zorgen voor dit verschil in virulentie tussen verschillende stammen van *E. amylovora*. We wilden bijdragen tot de kennis omtrent het proteoom van dit pathogeen en hiervoor werd een proteoomtechniek op basis van 2D "differential in gel electrophoresis" (DIGE) geoptimaliseerd voor *E. amylovora*.

Er werd gestart met een vergelijkende proteoomstudie van de vier stammen, opgegroeid *in vitro* (Hoofdstuk 3). Hiervoor werd er een vergelijking gemaakt tussen de stammen, verschillend in virulentie, gegroeid in een standaard minimaal medium. Resultaten van deze experimenten toonden aan dat er meer flagelline aanwezig was bij de minst virulente stam en een hogere hoeveelheid amylovoran geproduceerd werd door de meest virulente stam. Deze bevindingen geven indicaties dat de minst virulente stam een grotere kans heeft om sneller herkend te worden door de plant omdat flagelline herkend wordt als "pathogen-associated molecular pattern" (PAMP), hetgeen kan leiden tot de activatie van defensiemechanismen van de plant, namelijk de "PAMP-triggered immunity" (PTI) in de gastheer. De meer virulente stam aan de andere kant, produceert een grotere hoeveelheid amylovoran en bezit minder flagelline, wat kan betekenen dat deze stam beter gemaskeerd is door de functie van amylovoran. Hierdoor vermindert eveneens de kans om de verdedigingsmechanismen in de plant te activeren.

Omwille van deze interessante bevindingen *in vitro*, wilden we dit extrapoleren naar een meer toegepast model. Hiervoor werd een techniek geoptimaliseerd waarbij we in staat waren om bacteriële cellen te isoleren uit het plantenweefsel

na artificiële infecties (Hoofdstuk 4). Proteïnen werden geëxtraheerd uit deze stalen wat het mogelijk maakte om de vergelijking te maken tussen stammen, die verschillen in virulentie, gegroeid in een gastheer. Tot op heden is er weinig data beschikbaar in verband met het proteoom van een plant-pathogeen geïsoleerd uit plantenweefsels.

De resultaten van deze experimenten toonden aan dat er fundamentele verschillen zijn tussen de meer en minder virulente stammen waaronder verschillen in het koolhydraat-, aminozuur- en vetzuurmetabolisme, in de stress respons die vertoond werd door beide stammen en in de functie van de "RNA processing". Een meer efficiënt sorbitolmetabolisme werd waargenomen voor de meer virulente stam wat kan leiden tot een hogere productie van bouwstenen voor de opbouw van amylovoran. Dit exopolysacharide speelt een belangrijke rol in de verdediging tegen herkenning van de pathogeen door de gastheer enerzijds, maar speelt ook een belangrijke rol in de verdediging tegen oxidatieve stress anderzijds. De verdediging tegen oxidatieve stress kan opgedeeld worden in twee niveaus, namelijk de bescherming van de cel op zich door de vorming van een capsule maar ook door de formatie van een biofilm, hetgeen volledige kolonies bacteriële cellen kan beschermen. Ook produceert deze stam meer lipopolysacharides (LPS), waarvoor bewezen is dat deze bescherming kan bieden tegen oxidatieve stress. Onze resultaten geven indicaties van een hogere aanwezigheid van twee proteïnen betrokken in LPS-biosynthese. Verder is er ook bewijs voor de invloed van een aantal componenten van het RNA degradosoom op de virulentie. Deze omvatten de proteïnen Pnp oftewel PNPase en Hfq. Zowel de data van het proteoomexperiment als de resultaten van de genexpressie ondersteunen deze hypothese door een hogere abundantie van een aantal proteïnen en een hogere expressie van de genen overeenkomstig met deze proteïnen in de meer virulente stam. Ten laatste zijn er belangrijke verschillen aan het licht gekomen in verband met de stress respons van beide stammen. Resultaten toonden namelijk dat de minder virulente stam, een hogere abundantie heeft van "cold shock" proteïnen terwijl de meer virulente stam meer "heat shock" proteïnen heeft geproduceerd tijdens het infectieproces. Uit de literatuur werd duidelijk dat deze "cold shock" proteïnen net als flagelline, herkent kunnen worden als PAMPs wat kan leiden tot activatie van verdedigingsmechanismen van de plant.

Het buitenmembraan vormt samen met zijn proteïnen de scheiding tussen de bacterie en zijn omgeving en vormt daarbij de eerste lijn van bescherming tegen nefaste invloeden van de omgeving of tegen verdedigingsmechanismen van de plant. In hoofdstuk 5 wordt de compositie van het buitenmembraan van *E. amylovora* beschreven en vergeleken met het voorspelde proteoom van het buitenmembraan gebaseerd op genetische informatie. Wanneer het proteoom van het buitenmembraan *in vitro* vergeleken werd tussen de twee stammen, werden structurele componenten van de flagellen geïdentificeerd als meer voorkomend in de minst virulente stam. Hierdoor werd opnieuw de link gelegd tussen een minder virulente stam en de aanwezigheid van flagelline, hetgeen kan leiden tot herkenning van de pathogeen door de gastheer. Voor dit deel van het werk, werd er eveneens gewerkt met bacteriële cellen die uit het plantenweefsel werden geïsoleerd en hierbij werd ook het proteoom van het buitenmembraan van bacteriën gegroeid *in planta,* bestudeerd. Deze resultaten duidden op een hogere hoeveelheid van het proteïne TolC in de meer virulente stam. Dit proteïne speelt een rol in de algemene fitheid van de bacteriën en het is eveneens betrokken bij de resistentie tegen fytoalexines die geproduceerd worden door de gastheer als defensie mechanisme. Ook werd er een grotere hoeveelheid OmpA gevonden voor de meer virulente stam, hetgeen belangrijk is voor structurele integriteit van de cel.

Als laatste werd er onderzoek gedaan naar de expressie van genen corresponderend met belangrijke type III gesecreteerde proteïnen van *E. amylovora* (Hoofdstuk 6). Net als vele andere Gram-negatieve bacteriën, gebruikt *E. amylovora* een type III secretiesysteem om belangrijke effectoren tot in het cytosol van de gastheer te brengen. Deze hebben een rol in zowel de ontwikkeling van de ziekte als in het interfereren met bepaalde signaaltransductiewegen die leiden tot verdedigingsmechanismen van de plant. Elf genen, overeenkomend met de elf belangrijkste gesecreteerde proteïnen door *E. amylovora* werden gemeten voor beide stammen gegroeid in zowel een standaard minimaal medium als in de plant zelf. Deze resultaten geven aan dat de meest virulente stam in staat is om meer van deze effectoren te produceren in vergelijking met de minder virulente stam. Deze bevindingen leiden tot de hypothese dat de meer virulente stam meer effectoren injecteert in de gastheercel en hierdoor meer cellen kan afdoden om nutriënten te vergaren om

te overleven in de plant. Daarenboven is het reeds geweten dat sommige van deze effectoren, waaronder DspA/E, betrokken zijn bij de onderdrukking van verdedigingsmechanismen van de plant waardoor een succesvolle infectie word bereikt.

Ter conclusie kunnen we stellen dat alle resultaten wijzen op het feit dat de minder virulente stam meer componenten, namelijk PAMPs, heeft die herkent kunnen worden door de gastheer en minder amylovoran produceert om deze componenten te maskeren. Zowel de "elongation factor Tu", "cold shock" proteïnen als flagelline komen meer voor in de minder virulente stam wat leidt tot een hogere herkenningskans door de plant. De meer virulente stam aan de andere kant, groeit sneller in een medium met sorbitol, de voornaamste transportsuiker in appel en peer, hetgeen ook een verhogende functie heeft op de amylovoransynthese. Buiten de maskerende functie van cel componenten, speelt amylovoran ook een rol in de bescherming tegen reactieve zuurstofspecies (ROS) die door de plant geproduceerd worden en heeft het een functie in biofilmformatie. Overigens produceert de meer virulente stam ook LPS om zich te beschermen tegen ROS en deze produceert "heat shock" proteïnen om te overleven in een vijandige omgeving in de gastheer. Een hogere expressie van alle genen overeenkomstig met de belangrijkste type III effectoren in de meer virulente stam, suggereert dat deze stam een betere mogelijkheid heeft om te interfereren met de verdedigingsmechanismen van de gastheer door bepaalde signalisatiewegen van de plant te blokkeren die zouden leiden tot resistentie tegen de pathogenen. Deze resultaten geven aan dat de wisselwerking tussen de verschillende virulentiefactoren de meer virulente stam in staat stelt om een succesvolle infectie en kolonisatie van de plant te bereiken, hetgeen voor de minder virulente stam onmogelijk lijkt.

Table of contents

Table of contents

List of abbreviations

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2-DE	Two-Dimensional Electrophoresis
A	Ampere
ADP	Adenosine DiPhosphate
AMP	AntiMicrobial Peptides
Ams	Amylovoran synthesing
ANOVA	Analysis Of Variance
ATP	Adenosine TriPhosphate
Avr	Avirulence protein
CCW	Counterclockwise
cDNA	complementary DNA
CFU	Colony Forming Units
CID	Collision Induced Dissociation
CPC	Cetylpyrimidinium chloride (CPC)
CW	Clockwise
DFO	DesFerriOxamine
DIGE	Differential In-Gel Electrophoresis
DNA	DeoxyriboNucleic Acid
Dpi	Days post infection
Dsp	Disease specific
DTT	Dithiothreitol
E. amylovora	*Erwinia amylovora*
E. coli	*Escherichia coli*
ECF	Extra Cytoplasmatic Functions
EF	Elongation Factor

eLRR	extracellular LRR
Eop	Erwinia outer protein
EPS	ExoPolySaccharides
ETI	Effector-Triggered Immunity
ETS	Effector-Triggered Susceptibility
Gal	Galactose
Glc A	Glucuronic Acid
H_2O_2	Hydrogen peroxide
ha	hectare
HAE	Hrp-Associated Enzymes
HEE	Hrp-Effectors and Elicitors
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
Hfq	Host factor for phage Q beta RNA replication
HR	Hypersensitive Response
Hrc	Hrp-conserved
Hrp	Hypersensitive response and pathogenicity
HrpMM	*hrp*-inducing minimal medium
Hsv	Hrp-associated systemic virulence
ID	Internal Diameter
IM	Inner membrane
IPG	Immobilized pH Gradient
IT	Island Transfer
JA	Jasmonic Acid
kb	kilobase
kDa	kilodalton

LB	Luria-Bertani
LC	Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
LPS	Lipopolysaccharides
LRR	Leucine-Rich Repeats
LTQ	Linear Trap Quadrupole
MAMP	Microbial-Associated Molecular Pattern
MAPK	Mitogen-Activated Protein Kinase
MBMA	Modified Basal Medium A
Mbp	Millions of base pairs
MIQE	Minimum Information for publication of qPCR Experiments
MM2	Minimal Medium 2
mRNA	messenger RNA
MS	Mass Spectrometry
NaOH	Sodium Hydroxide
NB	Nucleotide Binding
NB-LRR	Nucleotide Binding Leucine-Rich Repeats
$O_2^{\cdot -}$	Superoxide radical
OD	Optical Density
$OH^{\cdot}$	Hydroxyl radical
OM	Outer membrane
OMP	Outer membrane protein
P. syringae	*Pseudomonas syringae*
PAI	Pathogenicity Island

PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate-Buffered Saline
PC	Principal Component
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
pI	Isoelectric Point
PL	Phospholipid
ppm	parts per million
PR	Pathogenisis-related
PRR	Pattern Recognition Receptor
PTI	PAMP-Triggered Immunity
Pyr	Pyruvate
QS	Quorum Sensing
R	Resistance
RLK	Receptor-Like Kinase
RNA	RiboNucleic Acid
ROS	Reactive Oxygen Species
rpm	revolutions per minute
RSLC	Rapid Separation Liquid Chromatography
RT-qPCR	Reverse Transcription-quantitative PCR
S. flexneri	*Shigella flexneri*
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SDS-PAGE	Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis

sRNA	small RNA
T3E	Type III Effector
T3SS	Type III Secretion System
TCST	Two-Component Signal Transduction
TE	Tris-HCl-EDTA
tRNA	transfer RNA
UDP	Uridine DiPhosphate
UTP	Uridine TriPhosphate
V	Volt
VPE	Vacuolar Processing Enzyme
W	Watt
WT	Wild Type
YPGA	Yeast Peptone Glucose Agar

1

General introduction

1.1 The plant pathogen *Erwinia amylovora*

1.1.1 Morphology and physiology

Erwinia amylovora is the causal agent of the destructive disease fire blight of many rosaceous plants including apple, pear, quince, blackberry, raspberry and many wild and cultivated ornamentals belonging to this family (Vanneste, 2000a). First it was described as *Micrococcus amylovorus* and afterwards as *Bacillus amylovorus*. In the early 1900s it was renamed *Erwinia amylovora* (Burril) Winslow *et al*. It was the first bacterium identified as a plant pathogen and it was first discovered in North America (Mansfield *et al.*, 2012; van der Zwet *et al.*, 2012). Cells of this pathogenic bacterium are rod-shaped with an average length of 1 – 3 µm and a width of 0.3 – 1.2 µm (Figure 1.1) (Bubán & Orosz-Kovács, 2003). *E. amylovora* is Gram-negative and is classified as member of the family of the *Enterobacteriaceae* which makes it closely related to many important human and animal pathogens such as *Escherichia coli*, *Salmonella* spp., *Shigella* spp. and *Yersinia* spp.

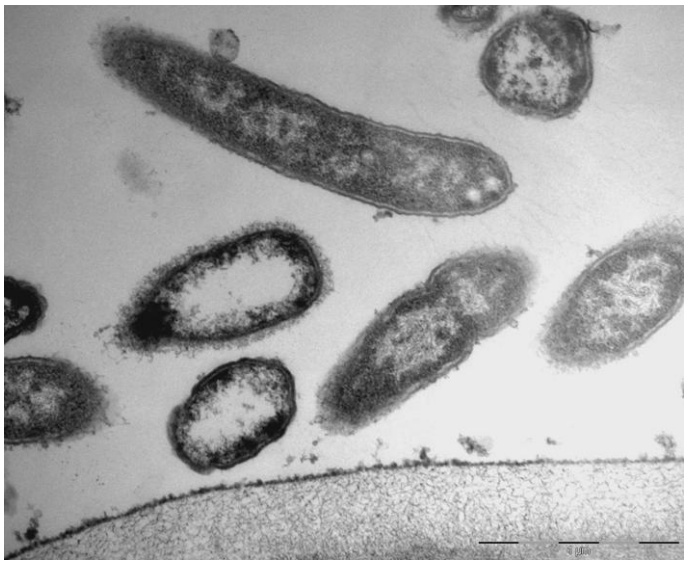

Figure 1.1: *Bacterial cells of E. amylovora grown in planta with internal structures. Leaf samples were taken 14 days post-infection (dpi). Bar corresponds with 1 µm.*

E. amylovora is facultative anaerobic which enables the bacteria to switch from an aerobic to an anaerobic metabolism depending on the environment. Further, this bacterium is not able to reduce nitrate to nitrite (Vanneste, 2000a).
Compared to other enterobacteria sequenced so far, *E. amylovora* contains one of the smallest genomes, around 3.8 Mbp (Toth *et al.*, 2006). Currently, genomes of fifteen strains of *E. amylovora* are published (Sebaihia *et al.*, 2010; Smits *et al.*, 2010; Powney *et al.*, 2011; Mann *et al.*, 2013; Smits *et al.*, 2014). These strains include *E. amylovora* ATCC 49946 isolated from apple in New York (Sebaihia *et al.*, 2010), *E. amylovora* CFBP1430 isolated from *Crataegus* in France (Smits *et al.*, 2010) and *E. amylovora* ATCC BAA-2158 isolated from thornless blackberry in Illinois (Powney *et al.*, 2011). These genome sequences provide nearly the complete genomic information of *E. amylovora*. A comparative genomic study of *E. amylovora* CFBP1430 and ATCC 49946 demonstrated a 99.99% identity of these two genomes at the nucleotide level, emphasizing low diversity within this pathogen (Smits *et al.*, 2010). These indications confirm previous findings that *E. amylovora* is a relatively homogeneous species (Vanneste, 2000a; Triplett *et al.*, 2006; Smits *et al.*, 2010). Although, a distinction must be made between *Spiraeoideae*- and *Rubus* infecting strains, since greater genetic diversity was observed between these strains (Mann *et al.*, 2013; Smits *et al.*, 2014).

1.1.2 Topology of the outer membrane of Gram-negative bacteria

The cell envelope of Gram-negative bacteria is composed of three morphologically distinct layers, the inner membrane (IM), the periplasm containing peptidoglycan and the outer membrane (OM) (Figure 1.2) (Glauert & Thornley, 1969; Lugtenberg & Van Alphen, 1983; Bos *et al.*, 2007; Ruiz *et al.*, 2009). The IM is a bilayer composed of phospholipids and proteins. Two types of IM proteins can be identified, integral IM that span the IM α-helical transmembrane domains, and lipoproteins that are anchored to the outer leaflet of the IM (Ruiz *et al.*, 2006). The periplasm is an oxidizing environment, containing enzymes that catalyze the formation of disulfide bonds (Nakamoto & Bardwell, 2004). Furthermore, it contains the peptidoglycan layer which serves as an extracytoplasmic cytoskeleton that contributes to the cell shape. It is

composed of glycan chains that are cross-linked by oligopeptides (Vollmer & Holtje, 2004).

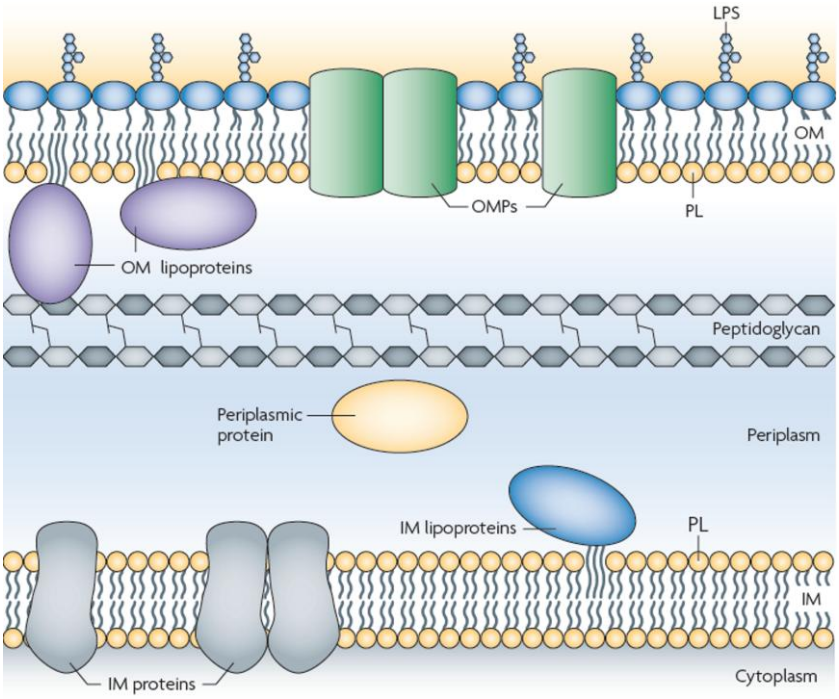

Figure 1.2: *Structure of the Gram-negative cell envelope. First the cytoplasm is surrounded by the inner membrane (IM) composed of a phospholipid (PL) bilayer containing proteins. Next there is the periplasm, an aqueous compartment containing the peptidoglycan layer and soluble proteins. The outer membrane (OM) is anchored to the cell by proteins, covalently bound to the peptidoglycan. The OM is an asymmetric membrane, the inner leaflet is composed of phospholipids (PL) while the outer leaflet is primarily composed of lipopolysaccharides (LPS). Further the OM contains two types of proteins: the integral outer membrane proteins (OMPs) and lipoproteins (Ruiz et al., 2009).*

The OM functions as a selective barrier and protects the bacteria from the environment. It is highly asymmetric and the inner leaflet is composed of phospholipids while the outer leaflet is mainly composed of lipopolysaccharides (LPS) (Glauert & Thornley, 1969; Bos *et al.*, 2007). LPS consist of three structural units: lipid A, which consists of a hydrophobic domain, a core oligosaccharide and a distal polysaccharide, the O-antigen (Raetz & Whitfield, 2002). Two types of proteins are 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 (Tokuda & Matsuyama,

2004). Integral OMPs are generally folded into cylindric β-barrels with a hydrophilic interior composed of antiparallel amphipathic β-strands (Koebnik et al., 2000). This barrel conformation allows the bacteria to function as channels which are of high importance for the intake of nutrients and the excretion of waste products (Ruiz et al., 2006).

1.1.3 Structure of flagella and motility of E. amylovora

Many bacterial species move by rotating thin helical filaments called flagella. These flagella are driven at their base by a reversible rotary motor embedded in the cell envelope and movement is powered by an ion flux (Berg, 2003). Bacterial flagella rotate (Silverman & Simon, 1974) which make the cells swim in a direction parallel to its long side axis. The flagellar filaments form a bundle and spin counterclockwise (CCW), a movement that pushes the cell forwards (Berg, 2003). To change its direction, one or more filaments leave the bundle and spin clockwise (CW) which makes the cell tumble and change its course (Macnab & Ornston, 1977; Turner *et al.*, 2000).

The structure of a bacterial flagellum is shown in figure 1.3. The basal body comprises a rod and four rings including an MS-ring (FliF) which forms an integral membrane ring, a periplasmic P-ring (FlgI), a lipopolysaccharide L-ring (FlgH) and a cytoplasmic C-ring (FliM and FliN) (Berg, 2003; Macnab, 2003). The flagellar motor contains a rotor, consisting of multiple copies of FliG, noncovalently attached to the MS ring and a stator which consists of multiple copies of an integral membrane structure build of MotA and MotB, arranged around the basal body. The stator is attached noncovalently to the peptidoglycan layer (Macnab, 2003). MotA and MotB form a complex that functions as a torque-generating unit (Berg, 2003). The hook (FlgE) and the filament or flagellin (FliC) are single polypeptide polymers (Berg, 2003) and are separated by two short junction proteins (Ikeda *et al.*, 1987) which are named FlgK and FlgL in *E. coli* (Berg, 2003). The filament cap (FliD) is positioned at the tip of the growing filament (Iino, 1969; Berg, 2003). The transport apparatus consisting of multiple proteins (FlhA, FlhB, FliH, FliI, FliO, FliP, FliQ and FliR) passes components for the other structures through a channel at the center of the MS-ring (Berg, 2003).

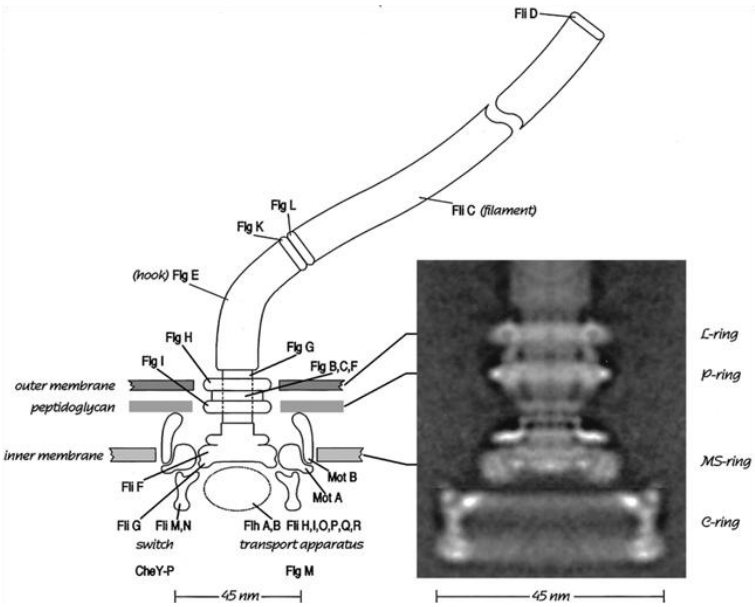

Figure 1.3: *A schematic diagram of the flagellar motor. CheY-P is the chemotaxis signaling molecule that binds to FliM, and FlgM is the anti-sigma factor pumped out of the cell by the transport apparatus. The general morphological features are C-ring, MS-ring, P-ring, L-ring, hook, hook-associated proteins (which include the distal cap), and filament (Berg, 2003).*

Both an electrical as chemical transmembrane protonmotive force is used by the rotary motor of the flagellum (Manson *et al.*, 1977; Matsura *et al.*, 1977). When the protons cross the membrane they bind to a specific aspartate residue of MotB which induces a conformational change in the stator that drives the rotor. Next, the aspartate residue is deprotonated leading to the release of the proton in the cytoplasm and the stator return to its original state (Kojima & Blair, 2001).

Bacteria can sense changes in their environment by chemoreceptors located in their cytoplasmic membrane and they can respond to these changes. Chemotaxis is the process by which bacteria approach environments with favorable chemical compositions and avoid those with non-favorable conditions (Adler, 1966). A key process in bacterial chemotaxis is the interaction between CheY and the switch of the flagellar rotor (Barak & Eisenbach, 1996). When phosphorylated by its kinase CheA, CheY-P binds to FliM (Welch *et al.*, 1993). This interaction is needed to capture CheY-P and the subsequent interaction with

FliN leads to a change in direction of the flagellar rotation from CCW to CW (Sarkar *et al.*, 2010).

E. amylovora is motile by means of 2 to 7 peritrichous flagella (Billing *et al.*, 1961; Huang & Goodman, 1970). The motility of *E. amylovora* is dependent on temperature, pH and other environmental factors (Raymundo & Ries, 1980b). Motility seems to be of aid during the invasion of apple blossoms (Bayot & Ries, 1986), however, they seem nonmotile after their entry into the apoplast (Raymundo & Ries, 1980b). A regulator for flagella biosynthesis, motility and chemotaxis has been identified for *E. coli*, namely the master regulator FlhDC. This master regulator is negatively regulated by the RcsCDB phosphorelay system in *E. coli* (Francez-Charlot *et al.*, 2003). For *E. amylovora*, the Rcs phosphorelay system has been identified as being important in virulence and survival in immature pear fruit (Wang *et al.*, 2009). Further, negative (GrrS/A) and positive (EnvZ/OmpR) regulators of swarming motility have been identified (Zhao *et al.*, 2009b). The GrrS/GrrA system, also known as GacS/GacA and BarA/UvrY, is widly distributed and well-studied in γ-proteobacteria (Lapouge *et al.*, 2008; Zhao *et al.*, 2009b).

1.2 Plant-pathogen interactions

1.2.1 Primary infection and disease cycle

E. amylovora is spread by wind, insects, birds and human activity. Infection is primarily initiated by the entry of this bacterium through nectarthodes in flowers, stomata or wounds in succulent tissue. *E. amylovora* is characterized by its fast multiplication and rapid dispersion throughout the plant via the vascular tissue and causes necrosis of the plant tissue or it can reside in symptomless tissue (Vanneste, 2000b; Malnoy *et al.*, 2012). All parts of the plant can be infected including flowers, leaves, branches, stems, fruits and roots, causing blossom blight, shoot blight and rootstock blight (Vanneste, 2000a). Infection is mostly initiated by entry in flowers. Flower stigmas, colonized by *E. amylovora* form a constant source of inoculum for blossom blight epidemics (Thomson, 1986). Furthermore, it was observed that *E. amylovora* populations can multiply very rapidly to high numbers in an epiphytic phase on some floral parts (Johnson & Stockwell, 1998).

When the stigma comes into the receptive phase, the papillae become wet and a secretion is formed. This stigmatic secretion is perfect for the bacteria to colonize, grow and multiplicate (Figure 1.4) (Hattingh *et al.*, 1986). Rain and dew facilitate the movement of the bacteria from the stigma to the hypanthium but the bacteria can also use their flagella to direct their movement (Thomson, 2000; Bubán & Orosz-Kovács, 2003). Moreover, positive chemotaxis is observed for *E. amylovora* towards apple nectar (Raymundo & Ries, 1980a).

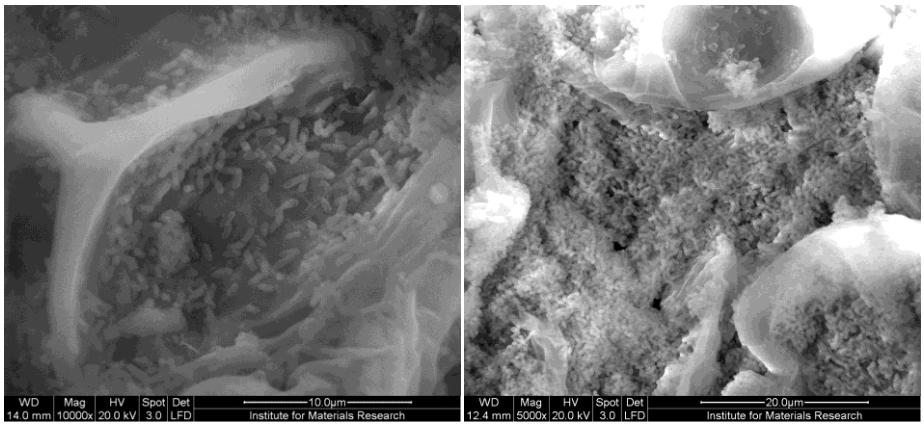

Figure 1.4: *Scanning electron micrograph of apple stigma infected with E. amylovora. Papillae are collapsed and completely covered and surrounded by bacterial cells (left) 1 dpi, (right) 8 dpi.*

The disease cycle of fire blight is depicted in figure 1.5. In winter bacterial cells survive in the bark and in cankers. When temperature rises in spring, bacteria multiply rapidly and cankers become active again and produce bacterial ooze, a characteristic sign of fire blight (Thomson, 2000). This ooze is composed of bacteria, polysaccharides and plant sap and is produced in infection sites (Geider, 2000; Oh & Beer, 2005). Bacteria are transported from this ooze to open flowers and other parts of the plant that are wounded by insects, rain, wind and hail. Following flower infections, bacteria are transported endophytically to other parts of the plant.

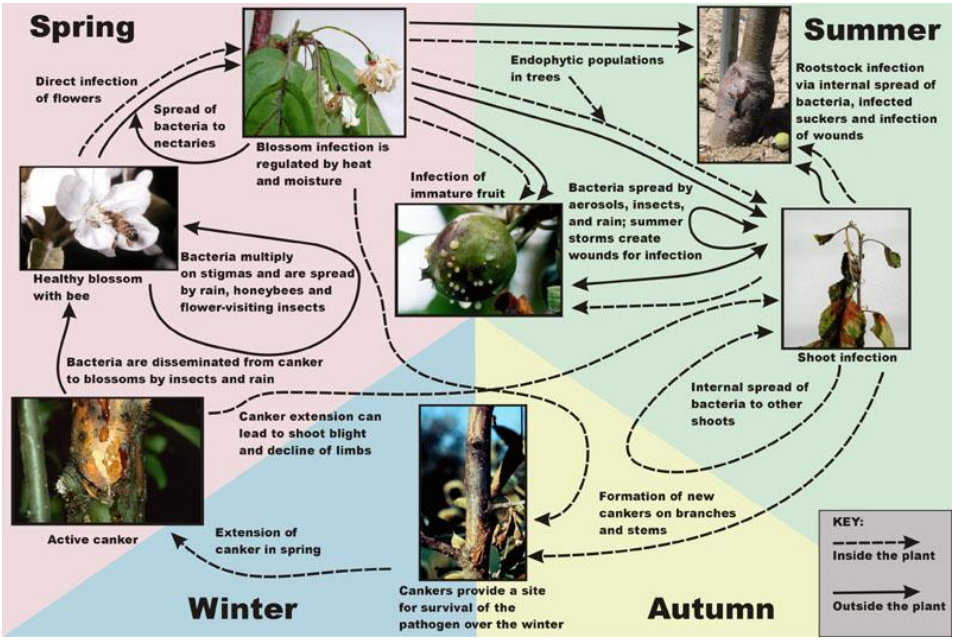

Figure 1.5: *Disease cycle of fire blight caused by E. amylovora. Solid lines represent movement of the pathogen outside the plant while dashed lines represent movement of the bacteria inside the host. Modified from disease cycle of Sherman Thomson (Thomson, 2000) by (Norelli et al., 2003).*

E. amylovora has the ability to migrate in host tissues of a sensitive host, when climatic conditions are favorable. After killing the blossom, bacteria can move rapidly into the pedicel and to twigs further infecting leaves. When infection gets walled off, cankers can be produced or infection might reach the main branch and eventually proceed down the trunk, killing the entire tree (Vanneste, 2000b; van der Zwet *et al.*, 2012). Once established, bacteria can move within the vascular system of the plant. Migration of this pathogen can be followed since necrosis is the direct consequence of progression in the plant. Besides necrosis, fire blight is characterized by water soaking and wilting (Vanneste, 2000b).

Primary infection may become a source for secondary inoculum by the production of ooze drops on flowers, leaves, shoots and stems and dry strands of bacteria that are present on infected pedistels, fruits, shoots or stems. Bacteria from these secondary inoculums can further be dispersed by rain, wind, insects, birds and humans (Thomson, 2000; van der Zwet *et al.*, 2012).

1.2.2 Pathogenesis and survival inside the host: virulence factors of E. amylovora

Early studies on *E. amylovora* showed that this pathogen, unlike many other plant pathogens, does not secrete pectolytic or cellulolytic enzymes (Seemuller & Beer, 1976), nor phytotoxic metabolites (Eastgate, 2000). Two factors have been identified as being essential for pathogenesis in *E. amylovora*, including the *hrp/dsp* genes and the extracellular polysaccharides (Steinberger & Beer, 1988; Barny *et al.*, 1990; Bellemann & Geider, 1992). More recent research has identified other factors that are not directly involved in pathogenesis but help the bacteria to survive inside its host (Eastgate, 2000).

1.2.2.1 The type III secretion system

Pathogenesis in *E. amylovora* is dependent on a functional type III secretion system (T3SS) (Oh & Beer, 2005) which forms a specialized syringe structure by which extracellular bacteria inject virulence proteins into the cytosol of its host (He *et al.*, 2004; Buttner & He, 2009). Both structural components of the T3SS and T3SS related proteins are encoded by the hypersensitive response and pathogenicity (*hrp*) genes which are located on the 62-kb chromosomal pathogenicity island (PAI). This island can be subdivided in four distinct regions including the *hrp/hrc* region, the Hrp effectors and elicitors (HEE) region, the Hrp-associated enzymes (HAE) region and the island transfer (IT) region (Figure 1.6) (Oh & Beer, 2005; Mann *et al.*, 2012). Up to date, two factors have been identified as being important in the expression of the *hrp* genes in *E. amylovora.* The expression is dependent on environmental stimuli, moreover, they are expressed *in planta* under conditions of low nutrients and low pH and in a well defined culture medium thought to mimic the conditions of the plants apoplast (Wei *et al.*, 1992b). Secondly, transcription of *hrp* genes is also regulated by HrpL, a sigma factor of the ECF (extra cytoplasmatic functions) subfamily. HrpL recognizes a conserved promoter motif, the *hrp* box (Wei & Beer, 1995). On the other hand, HrpS, a NtrC-family σ^{54} enhancer, is required for *hrpL* transcription in *E. amylovora* (Wei *et al.*, 2000).

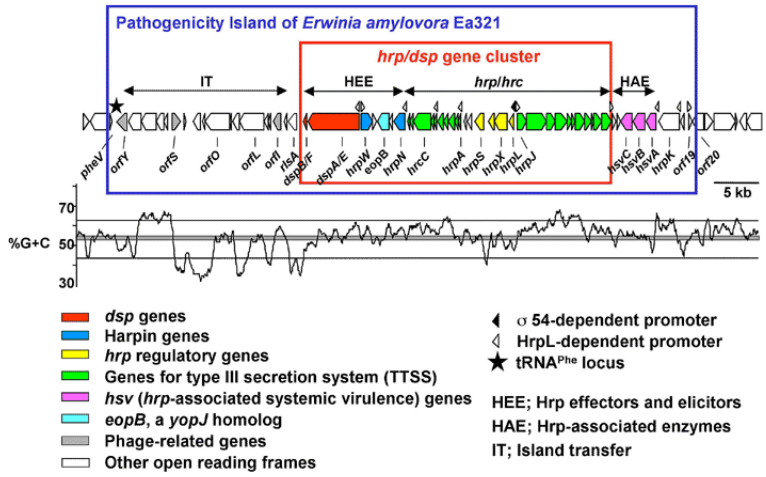

Figure 1.6: *The Hrp pathogenicity island of E. amylovora strain Ea321. It contains four DNA regions including the hrp/hrc regions, the HEE region, the HAE region and the IT region (Oh & Beer, 2005).*

Nine *hrc* genes (HR and conserved) which are located together with the *hrp* genes on the PAI, are believed to be involved in the structure of the T3SS (Oh & Beer, 2005). The structure and morphology of the T3SS has not yet been identified for *E. amylovora* although much research has already been done on the structural assembly of this injectosome in many Gram-negative plant and animal pathogens (Cornelis & Van Gijsegem, 2000).

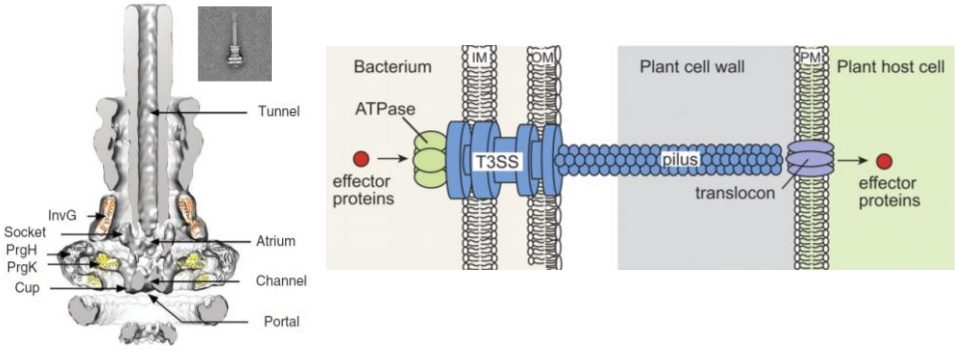

Figure 1.7: *Structure of a type III secretion system (left), longitudinal section through the structure of a needle complex, indicating the different spaces of different sizes that define the secretion path within the injectosome (Radics et al., 2014). (right) Schematic representation of the T3SS from plant-pathogenic bacteria. The T3SS which spans the bacterial membranes is connected to an extracellular pilus that spans the plant cell wall and serves as transport channel for the effector proteins that are transported into the cytosol of the host cell (Buttner & He, 2009).*

The T3SS forms a specialized syringe structure by which extracellular bacteria inject virulence proteins into the cytosol of its host (Figure 1.7) (He *et al.*, 2004; Buttner & He, 2009). These virulence proteins are called type III effectors (T3Es) and are delivered into the host cytosol through a complex and ordered process (Buttner, 2012). The T3Es can suppress plant immunity or they can be recognized by the plant and hereby trigger an effector-triggered immunity (Jones & Dangl, 2006; Feng & Zhou, 2012).

Eleven T3-secreted proteins have been identified for *E. amylovora* (Nissinen *et al.*, 2007). DspA (Gaudriault *et al.*, 1997) and DspE (Bogdanove *et al.*, 1998), also known as DspA/E, a homolog of AvrE of *Pseudomonas syringae* (Gaudriault *et al.*, 1997), is known as a pathogenicity factor. Moreover, this large protein (198 kDa) is required for pathogenicity in apple and pear (Gaudriault *et al.*, 1997; Bogdanove *et al.*, 1998). DspA/E interacts with the intracellular domains of host plant receptor kinases (Meng *et al.*, 2006) and pre-ferredoxin(Bonasera, 2006). Furthermore it has been suggested that DspA/E is involved in disease development by the inhibition of the salycilic acid dependent innate immunity of the plant (DebRoy *et al.*, 2004). DspB/F is a small protein suggested to function as a chaperone during the secretion of DspA/E (Gaudriault *et al.*, 2002). Further, two harpins, HrpN and HrpW, are secreted by *E. amylovora*. These are glycine-rich, lack cysteine, are heat stable and both are involved in the induction of the hypersensitive response (HR) in nonhost plants (Wei *et al.*, 1992a; Kim & Beer, 1998). In contradiction with other T3Es that are translocated to the cytoplasm of the plant, harpins are targeted to the intercellular spaces of plant tissues (Alfano & Collmer, 2004). Mutants in the *hrpN* gene showed to be non-pathogenic (Wei *et al.*, 1992a; Barny, 1995), while HrpW on the other hand is not required for virulence (Kim & Beer, 1998). Furthermore, both HrpN and DspA/E have proven important factors in the elicitation of an oxidative burst in compatible host plants (Venisse *et al.*, 2003). HrpA which forms a Hrp pilin is also secreted. This protein extends outside the bacterial cell which may reach the host cell (Kim *et al.*, 1997; Jin *et al.*, 2001). Mutants of the *hrpA* gene are not able to cause HR in non host plants nor cause disease in hosts. HrpA is also important for the secretion of the effector proteins HrpW and DspA/E (Jin *et al.*, 2001). Another secreted protein required for pathogenesis is HrpJ, homologues to the protein YopN of *Yersinia spp*. (Bogdanove *et al.*, 1996). This protein plays a major role

in HR in nonhosts and it was suggested to be important in the accumulation of extracellular harpins (Nissinen *et al.*, 2007). Although HrpK from *E. amylovora* is distantly related to the same protein of *P. syringae* (Alfano & Collmer, 2004), a *hrpK* mutant of *E. amylovora* was not restricted in virulence (Oh *et al.*, 2005) as was a *hrpK* mutant in *P. syringae* (Petnicki-Ocwieja *et al.*, 2005), so the function of this effector in *E. amylovora* is still to be determined. The gene product of *orfB*, Eop1, also called EopB (*Erwinia* outer protein B) (Oh & Beer, 2005), is a member of the YopJ/AvrRxv/HopZ family of protease effectors which are homologues and conserved among plant and animal pathogens (Nissinen *et al.*, 2007). Eop2 and Eop3 were also secreted. Eop2 is homologous to the helper protein HopAK1 of *P. syringae* which induces a HR in tobacco (Alfano & Collmer, 2004; Nissinen *et al.*, 2007) and resembles HrpW (Nissinen *et al.*, 2007), still the function in *E. amylovora* remains unknown. The Eop3 effector is homologues to members of the HopX family which are common in *P. syringae* strains (Nissinen *et al.*, 2007). TraF is similar to proteins involved in plasmid transfer and pilus formation (Haase & Lanka, 1997; Nissinen *et al.*, 2007). It also possesses a signal peptide for type II secretion (Nissinen *et al.*, 2007). *E. amylovora* secretes also a homologue of the flagellar protein FlgE, namely FlgL (Nissinen *et al.*, 2007).

1.2.2.2 Exopolysaccharides

The importance of exopolysaccharides (EPS) in pathogenesis of *E. amylovora* was demonstrated since EPS-deficient mutants were found to be non-pathogenic (Steinberger & Beer, 1988; Bellemann & Geider, 1992). Moreover, they are not able to migrate through plant vessels (Bogs *et al.*, 1998) and they do not multiply inside the host (Bellemann & Geider, 1992). Several functions for EPS have been suggested including bypassing the defense mechanism of the plant by masking of cell surface components, inducing wilt by blocking water movement in the xylem, the capability of retaining water and nutrient and a protective function (Eastgate, 2000; Geider, 2000; Ordax *et al.*, 2010). The major component of EPS of *E. amylovora* is amylovoran, which is also the major component of bacterial ooze. Amylovoran is an acidic heteropolysaccharide with pentasaccharide repeating units primarily consisting of four galactose residues

and one glucuronic acid residue (Figure 1.8) (Nimtz *et al.*, 1996; Maes *et al.*, 2001).

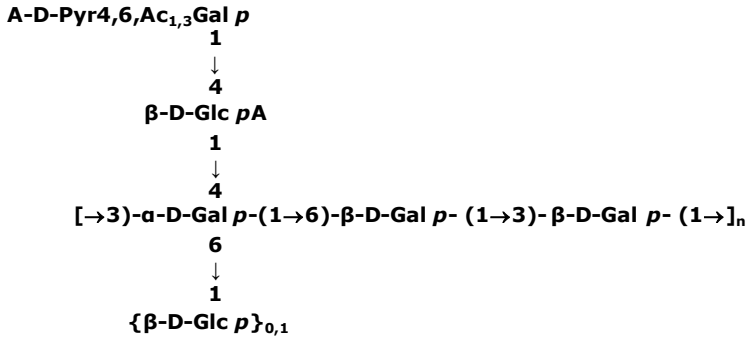

Figure 1.8: *The structure of a repeating unit of amylovoran with Gal, galactose; Glc A, glucuronic acid; Pyr, pyruvate with keto-group; α and β, sugar linkages; D, sugar configuration; p, pyranoside and n, the level of polymerization (Nimtz et al., 1996).*

Biosynthesis of amylovoran requires a large number of genes because of its complex sugar linkages. Most of the structural genes necessary for amylovoran biosynthesis are found on the *ams* gene cluster located on the chromosome of *E. amylovora.* This operon is 16 kb in length and consists of 12 genes, *amsA-amsL* (Bernhard *et al.*, 1993; Bugert & Geider, 1995). AmsA shows a tyrosine kinase activity (Ilan *et al.*, 1999) while AmsC, AmsH and AmsL may be involved in oligosaccharide transport and assembly (Langlotz *et al.*, 2011). Further AmsB, AmsD, AmsE, AmsG, AmsJ and AmsK are thought to have sugar tranferase activities (Bugert & Geider, 1995; Geider, 2000; Langlotz *et al.*, 2011). AmsI has the function of an acid phosphatase (Bugert & Geider, 1997). On the right of the *ams* gene cluster, two genes *galE* and *galF*, are located which are important in precursor formation, UDP-galactose and UDP-glucose respectively (Metzger *et al.*, 1994; Bugert & Geider, 1995).

A second component of EPS in *E. amylovora* is levan, a homopolymer of fructose residues. Levan is synthesized extracellularly from sucrose by levansucrase, encoded by the *lsc* gene (Gross *et al.*, 1992; Geier & Geider, 1993). Mutants deficient in levan production are defected in their virulence (Geier & Geider, 1993). Levan biosynthesis is positively regulated by RlsA, encoded by the *rlsA* gene (Kelm *et al.*, 1997), which is located next to *dspB/F* on the PAI.

Koczan *et al.,* (2009) suggested that EPS in *E. amylovora* are involved in biofilm formation and also indicated that this process plays an important role in

pathogenesis of *E. amylovora*. It was shown that amylovoran is necessary for biofilm formation and levan contributes to this process. Furthermore, it was also concluded that the quantity of amylovoran produced by individual *E. amylovora* strains is correlated with the degree of virulence (Koczan *et al.*, 2009). Biofilms are multicellular communities in which cells are embedded in a matrix of extracellular compounds attached to a surface (Branda *et al.*, 2005). The formation of biofilms provides protection for the bacteria from deleterious conditions (Davey & O'Toole, 2000) and is important for survival under stress conditions (Ordax *et al.*, 2010). Major components of a biofilm include water, bacterial cells and exopolysaccharides (Sutherland, 2001). These EPS of the matrix provide a physical barrier against the diffusion of compounds produced by the host and a protection against environmental stress factors (Flemming, 1993; Gilbert *et al.*, 1997). The process of biofilm formation has been described for *Pseudomonas aeruginosa* and can be divided in five distinct phases including reversible attachment, irreversible attachment, maturation 1, maturation 2 and the dispersion phase (Sauer *et al.*, 2002). The cues that trigger biofilm formation are largely unknown but it is suggested that biofilms are formed in response to environmental triggers (Davey & O'Toole, 2000) and quorum sensing signals (Sauer *et al.*, 2002). Quorum sensing (QS) allows bacteria to communicate with each other inside of the biofilm by the secretion of signal molecules. Research has shown that *Erwinia* species produce two types of QS molecules including N-acyl homoserine lactones and AI-2-type signaling molecules (Barnard & Salmond, 2007). Contradictory, studies in *E. amylovora* have indicated a non-quorum sensing role for the autoinducer-2 *luxS* gene due to the lack of genomic evidence for the existence of autoinducer-2 receptors (Rezzonico & Duffy, 2008; Smits *et al.*, 2010; Rezzonico *et al.*, 2012).

1.2.2.3 The sorbitol metabolism

In contradiction with many other plants that use sucrose for carbohydrate transport, rosaceous plants use sorbitol as the dominant sugar alcohol (Aldridge *et al.*, 1997; Oh & Beer, 2005). Therefore, it is of great importance that *E. amylovora* is able to utilize this carbon source. The *srl* operon, necessary for the sorbitol metabolism, consists of six genes (Figure 1.9). Three genes are important for the uptake of sorbitol (*srlA*, *srlB* and *srlE*), *srlD* encodes for a

dehydrogenase which is necessary for the conversion of sorbitol to fructose and the last two genes are regulatory (*srlM* and *srlR*) (Aldridge *et al.*, 1997; Geider, 2000).

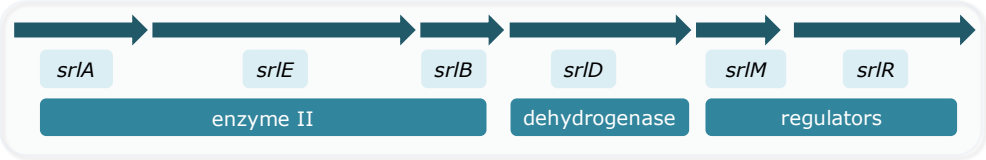

Figure 1.9: *Genetic map of the sorbitol operon. Enzyme II is the specific component for sugar uptake (modified from Geider, 2000).*

Mutations in *srlD* showed that this protein is necessary for symptom formation on apple seedlings. Furthermore, this operon is repressed by the presence of glucose and is induced by sorbitol (Aldridge *et al.*, 1997). Sorbitol provides the pathogen with a good carbon source to produce amylovoran (Bennett & Billing, 1978) and increases EPS biosynthesis (Bellemann *et al.*, 1994).

1.2.2.4 Desferrioxamine and protease

To cope with low iron availability in plant tissues, *E. amylovora* produces and secretes siderophores (Dellagi *et al.*, 1998). These siderophores bind to Fe^{3+} under iron-limiting conditions and are taken-up through specific receptors (Oh & Beer, 2005). *E. amylovora* secretes siderophores of the hydroxamate type which belong to the cyclic desferrioxamines (DFOs) consisting of alternating diamine and dicarboxylic acid building blocks linked by amide bonds (Smits & Duffy, 2011). The most important siderophore of *E. amylovora* is DFO-E and its specific receptor, FoxR (Kachadourian *et al.*, 1996; Dellagi *et al.*, 1998). A *foxR* mutant was not able to induce the same amount of necrotic symptoms on both apple seedlings and flowers and showed less bacterial growth in comparison with the wild-type strain (Dellagi *et al.*, 1998). Secondly, it has been suggested that desferrioxamine provides protection for *E. amylovora* against oxidative conditions (Venisse *et al.*, 2003).

In minimal medium, *E. amylovora* secretes a 48 kDa metalloprotease, PrtA. This protein is secreted by the type I secretion system composed of three structural proteins including PrtD, PrtE and PrtF. A mutant in one of these structural genes,

prtD, is unable to secrete this metalloprotease and leads to a reduced colonization of apple leaves (Zhang *et al.*, 1999).

1.2.2.5 Two-component signal transduction (TCST) systems

It has been known for many years that pathogenic bacteria use two-component signal transduction (TCST) systems to control the expression of virulence factors required for infection. TCSTs consist of a histidine kinase and a response regulator. They are signal transduction devices that play a critical role in sensing and responding to environmental cues through changes in gene expression (Beier & Gross, 2006; Mole *et al.*, 2007). Several TCSTs have already been identified in *E. amylovora* for regulation of the T3SS and for amylovoran production (Zhao *et al.*, 2009b). Wei *et al.* (2000) have shown that the structural components of the T3SS encoded by the *hrp* genes are regulated by the TCST consisting of HrpX and HrpY. This system regulates the expression of HrpS, a sigma 54 enhancer-binding protein. Together, HrpS and HrpY activate the expression of HrpL which regulates various genes and operons from the *hrp* gene cluster (Wei *et al.*, 2000). Another known TCST of *E. amylovora* is called the RcsCDB phosphorelay system which regulates the biosynthesis of amylovoran (Kelm *et al.*, 1997; Wang *et al.*, 2011b). The expression of the *ams* gene cluster is regulated by RcsA and RcsB, which are conserved regulatory proteins (Kelm *et al.*, 1997). RcsC is an environmental sensor that phosphorylates RcsB (Wehland *et al.*, 1999). Next, this phosphorylated RcsB binds to RcsA which on its turn binds to the *ams* promoter, thereby stimulating transcription (Kelm *et al.*, 1997; Wehland *et al.*, 1999). Supplemenetary, two other TCSTs have been identified as being important in regulation of amylovoran production, namely GrrS/GrrA, also called GacS/GacA and EnvZ/OmpR. When both were inactivated in *E. amylovora*, amylovoran production was significantly higher, indicating a role for these regulators in amylovoran synthesis (Li *et al.*, 2014).

1.2.3 Plant defense mechanisms in response to pathogens

Plants are constantly exposed to microbes and unlike mammals, they neither possess an immune system comprising lymphocytes nor a somatic adaptive immune system. Instead, the defense mechanism of plants relies on two different systems. First there is the innate immunity of each cell and secondly there are systemic signals emerging from infection sites.

The immune system of the plant can be divided in two main levels which can be represented as a four phased model (Figure 1.10) (Jones & Dangl, 2006).

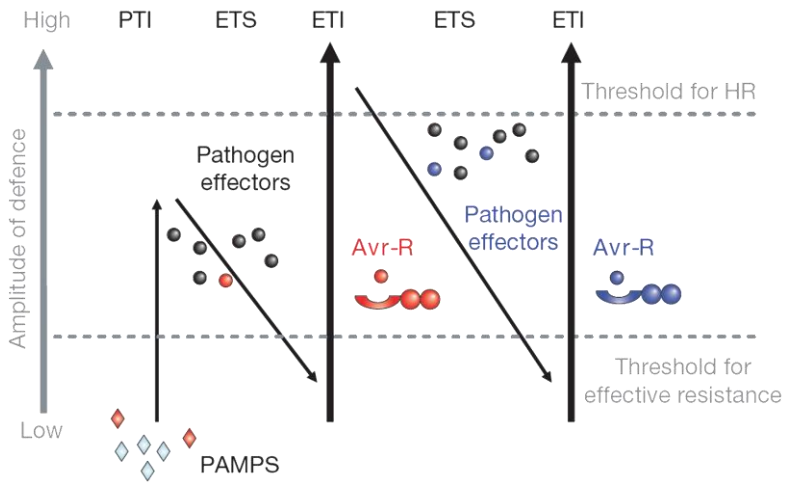

Figure 1.10: *Zigzag model that illustrates the quantitative output of the plant immune system. In phase 1, the plants detect microbial- or pathogen-associated molecular patterns (MAMPs/PAMPs, red diamonds) via PRRs to trigger PAMP-triggered immunity (PTI). In phase 2, successful pathogens deliver effectors that interfere with PTI, or otherwise enable pathogen nutrition and dispersal, resulting in effector-triggered susceptibility (ETS). In phase 3, one effector (indicated in red) is recognized by an NB-LRR protein, activating effector-triggered immunity (ETI), an amplified version of PTI that often passes a threshold for induction of hypersensitive cell death (HR). In phase 4, pathogen isolates are selected that have lost the red effector, and perhaps gained new effectors through horizontal gene flow (in blue); these can help pathogens to suppress ETI. Selection favors new plant NB-LRR alleles that can recognize one of the newly acquired effectors, resulting again in ETI (Jones & Dangl, 2006).*

Once microbes have reached the interior of the plant, they have to face the plant cell wall which forms a rigid, cellulose-based enclosure around each cell. When present in the apoplast, the microbes encounter extracellular surface receptors specialized in recognition of microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs). These are conserved microbial elicitors

that are recognized by pattern recognition receptors (PRRs) (Janeway & Medzhitov, 2002; Zipfel & Felix, 2005). Perception of a pathogen at the host surface triggers PAMP-initiated immunity (PTI). This process usually halts infection preliminary (Dangl & Jones, 2001). Multiple cell surface components of Gram-negative bacteria function as PAMPs including LPS (Dow *et al.*, 2000; Gerber *et al.*, 2004), the major constituent of the OM, elongation factor Tu (EF-Tu), cold-shock proteins (Bittel & Robatzek, 2007), peptidoglycans (Gust *et al.*, 2007) and flagellin, the major constituent of the filament of the flagella. Moreover, the plant recognizes a specific, conserved part of the flagellin polypeptide, which corresponds to a 22 amino acid peptide (flg22) (Felix *et al.*, 1999). *Arabidopsis* produces a flagellin receptor, FLS2 (Flagellin Sensing 2), which is a receptor-like kinase (RLK) that consists of extracellular leucine-rich repeats (LRRs) and an intracellular serine/threonine kinase domain (Gomez-Gomez & Boller, 2000). Following the recognition of flagellin, a complete MAP kinase cascade and WRKY transcription factors are activated (Asai *et al.*, 2002).

In order to suppress this PTI responses, successful pathogens use effector proteins. Gram-negative pathogenic bacteria are able to produce a T3SS by which they can inject effector proteins into the host cytosol. These effectors promote pathogenicity and the T3SS is essential for the development of disease symptoms in the host. Moreover, these effectors can possess enzyme activity modifying host proteins, inhibit defense responses elicited by PAMP recognition and they can activate transcription in the plant to downregulate host defenses (Chisholm *et al.*, 2006). This interference of effectors with PTI is called effector-triggered susceptibility (ETS) (Jones & Dangl, 2006).

Some pathogens are able to produce small molecule effectors that mimic plant hormones. For example, *Pseudomonas syringae* is able to produce coronatine which mimics jasmonic acid that contributes to virulence by suppressing salicylic acid-mediated host responses (Zhao *et al.*, 2003; Brooks *et al.*, 2005). Salicylic acid (SA), jasmonic acid (JA) and ethylene are three plant signaling molecules that regulate plant defense against microbial attack (Thomma *et al.*, 2001). SA-dependent signaling is important in establishing local and systemic bacterial resistance, while JA-dependent signaling is induced by mechanical wounding and herbivore predation. Furthermore, the SA and JA defense pathways are mutually antagonistic which is readily exploited by bacterial pathogens. Comparison of the

defense responses of susceptible and resistant cultivars of *Malus* against *E. amylovora* infection, showed that resistant plants had higher basic SA levels (Milčevičová *et al.*, 2010). Following infection, a strong down-regulation of the JA pathway is observed in the susceptible cultivar. Further, after treatment of the plants with methyl-jasmonate, susceptible plants showed an increased resistance against *E. amylovora* (Duge De Bernonville *et al.*, 2012). Ethylene dependent signaling is important for response against pathogens and wounding (Thomma *et al.*, 2001; Kunkel & Brooks, 2002). Upon infection with *E. amylovora*, two year old apple trees produced different volatiles including ethylene, 2,3-butanediole, isoprene-ozone and 3-hexenal as a defense mechanism (Spinelli *et al.*, 2011). Moreover, ethylene together with ROS and the vacuolar processing enzyme (VPE) play a role in cell death during the HR through a signal transduction cascade (Iakimova *et al.*, 2013).

The second level of plant immunity is characterized as the effector-triggered immunity (ETI) which involves resistance (R) genes encoding for NB-LRR proteins that recognizes a specific effector or avirulence protein (Avr). Moreover, ETI is stronger than PTI and often leads to a HR (Dangl & Jones, 2001; Jones & Dangl, 2006). To date numerous R genes have been cloned from a wide range of plant species, however only two main classes can be distinguished. The largest class of resistance genes encodes for proteins containing a nucleotide binding (NB) site and leucine-rich repeat (LRR) domains. (Dangl & Jones, 2001) and secondly, there are the extracellular LRR (eLRR) proteins (Fritz-Laylin *et al.*, 2005). Recently, Vogt *et al.* (2013) identified a possible gene-for-gene relationship between *E. amylovora* and its host *Malus* x *robusta* 5 based on the importance of the effector AvrRpt2 for resistance in the host.

When entering its host plant, *E. amylovora* is detected as an incompatible pathogen and the plant initiates a first line of defense. Reactive oxygen species (ROS) are produced including $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$, to kill the infected plant cells (Torres *et al.*, 2006). Hereby a stress response is evoked in the host and leads to lipid peroxidation, electrolyte leakage and modulation in the antioxidant status (Venisse *et al.*, 2001; Venisse *et al.*, 2003). Unlike other pathogens, *E. amylovora* profits by these oxidative bursts and even uses them for a successful colonization (Venisse *et al.*, 2001). Moreover, *E. amylovora* produces the effectors HrpN and DspA/E to elicit an oxidative burst (Venisse *et al.*, 2003).

1.3 Economic importance and management of fire blight

Fire blight has been reported in 40 countries around the world. It has spread from North America through the North American continent to the Pacific Rim, Europe and the Middle East (Bonn & van der Zwet, 2000). The first reports of the disease in Belgium appeared in 1972 near the coast. The most susceptible pear cultivars in Belgium proved to be 'Durondeau' followed by 'Doyenne du Comice' and 'Conference' (Deckers, 1996). A function in dispersion of the disease to fruit orchards was also found for both the common hawthorn hedges and the very susceptible *Cotoneaster salicifolius floccus* (Deckers, 1996). Infection with *E. amylovora* is reported every year in Belgium, both on apple and pear but also on ornamental host plants.

The total area in Belgium for cultivation of apple and pear in 2012 was estimated around 15000 ha of which 6398 ha was assigned to apple trees and 8318 ha was used for pear production. Moreover, the production of apples in 2014 was estimated on 327400 ton and pear production reached the amount of 393900 ton (www.eurostat.eu).

Due to its destructive character and the lack of effective control mechanisms, *E. amylovora* is capable of dispersing rapidly both within susceptible plants and between trees in orchards which could result in great economic losses. Because of the expected rise in average global temperatures, the growing of cultivars on susceptible rootstocks (M.26 and M.9) and the introduction of susceptible cultivars, fire blight will become an even greater threat for the fruit production in Europe in the near future (Vanneste, 2000a; Deckers & Schoofs, 2008). In recent years several new apple cultivars have successfully been introduced, including 'Braeburn', 'Fuji', 'Gala', 'Jonagold', and 'Pink Lady', which have shown to be more susceptible to fire blight than most older cultivars (Norelli *et al.*, 2003).

Most fire blight control measures are based on prevention. This includes pruning and removal of infected plant parts and the use of chemical and bio-control agents.

When cutting away diseased plant parts, a disruption of the equilibrium between vegetative and reproductive growth can be created which can result in loss of fruit production (Khan *et al.*, 2012). Chemical control can be divided in two

groups including copper derivates and antibiotics. Streptomycin, an antibiotic is considered the most effective control method against fire blight, but excessive use has lead to the emerge of streptomycin-resistant strains of *E. amylovora* in the US and in other countries, including Canada, Isreal and New Zealand (McManus *et al.*, 2002; McGhee *et al.*, 2011). Moreover, due to the contribution to environmental and health concerns and the emerging of resistant strains in the USA, use of this antibiotic is prohibited in many countries, including Belgium (McManus *et al.*, 2002; Khan *et al.*, 2012). Due to their possible phytotoxic effects on skin of fruits, the bacteriostatic copper compounds may be less attractive for common use (Geider, 1999; Norelli *et al.*, 2003; Khan *et al.*, 2012).

Following these implications, other products and methods are available in the defense against fire blight. New developed products contain compounds capable to potentiate the defense mechanisms of the plant including benzothiadiazole (Actigard®, Bion®, Blockade® and Boost®), prohexadione-Ca (Apogee® and Regalis®), phosetyl-Al (Aliette®), laminarin (Vacciplant®) and harpin protein (Employ® and Harp-N-Tek®).

Another method, using microbial antagonist has received much attention the last years. The antagonists include bacteria (*Pseudomonas fluorescens, Bacillus subtilis, Pantoea agglomerans,* …) and the yeast *Aureobasisium pullulans* (Beer & Rundle, 1983; Giddens *et al.*, 2003; Broggini *et al.*, 2005; Cabrefiga *et al.*, 2007; Loncaric *et al.*, 2008). The mechanism of these antagonists is dependent on antibiotics production to kill bacterial *E. amylovora* cells and on to competition with *E. amylovora* for space and nutrients. Further, the use of phage therapy has also been suggested for the control of fire blight (Nagy *et al.*, 2012).

Antimicrobial peptides (AMPs) have proven to be an interesting candidate for plant protection. Already a pseudopeptide, pantocine, produced by *Pantoea agglomerans* has been identified to inhibit *E. amylovora* (Montesinos, 2007).

Another method consists of introducing resistance in hosts using hybridization schemes. However, this has proven difficult due to the polygenic nature of resistance mechanisms (Khan *et al.*, 2012). Although, there have been some successful reports on fire blight resistant apples by the introduction of an attacin E encoding gene into apple rootstocks. Attacin is an antimicrobial protein that is

produced by the pupae of *Hyalophora cecropia* or the giant silk moth as response to bacterial infection (Ko *et al.*, 2002; Borejsza-Wysocka *et al.*, 2010). But until now, there are no transgenic lines commercially available with resistance towards fire blight (Gessler & Patocchi, 2007). However, transgenic plants are not yet allowed in many European countries due to environmental concerns. Therefore, more efficacious and environmentally friendly strategies to control fire blight are needed.

2

Objectives

Objectives

Erwinia amylovora is a Gram-negative plant pathogen that causes the destructive disease fire blight. This disease affect most members of the *Rosaceae* family of which apple and pear are economically the most important. Bacteria can be transported by wind, insects, birds and human activities. *E. amylovora* is capable of a rapid dispersion, both by its destructive character and the lack of efficacious and environmentally friendly strategies to control this disease. Moreover, the expected rise in average global temperatures, the breeding of cultivars on susceptible rootstocks and the introduction of susceptible cultivars, makes fire blight an even greater threat for the fruit production in Europe in the near future (Deckers & Schoofs, 2008).

E. amylovora is considered to be a homogeneous species based on physiological, biochemical, phylogenetic and genetic analysis (Vanneste, 2000a; Smits *et al.*, 2010; Wang *et al.*, 2010; Zhao & Qi, 2011). Moreover, a comparative genomic study of *E. amylovora* CFBP1430 and ATCC 49946 demonstrated a 99.99% similarity of these two genomes at the nucleotide level, emphasizing low diversity within this pathogen (Smits *et al.*, 2010). Although it must be mentioned that a distinction has been found between *Spiraeoideae*- and *Rubus* infecting strains, since greater genetic diversity is observed between these strains (Mann *et al.*, 2013; Smits *et al.*, 2014). Many different virulence factors have been characterized by genetic studies, but the proteins corresponding to these genes have not been described. In addition, it has been reported that strains of *E. amylovora* isolated from nature, exhibit differences in virulence (Cabrefiga & Montesinos, 2005; Lee *et al.*, 2010; Wang *et al.*, 2010) but genetic studies seemed insufficient to explain these differences.

In contrary to the huge amount of genetic data available, the proteome is barely known. Research concerning the proteome of this pathogen is limited to the extracellular proteome of a raspberry and an apple isolate of *E. amylovora* grown in a *hrp*-inducing medium (Braun & Hildebrand, 2005), the identification of the type III secreted proteins (Nissinen *et al.*, 2007) and the lysine acetylome of *E. amylovora* (Wu *et al.*, 2013). The latter suggested that protein lysine acetylation or other post-translational modifications might be involved in the differential virulence between strains of *E. amylovora* (Wu *et al.*, 2013).

Therefore, the main goal of this work was to identify the different proteins and metabolic processes that enable this organism to survive and colonize the plant. Furthermore, by using different strains of *E. amylovora*, exhibiting differential virulence, we were able to widen our research and investigate the proteins involved in virulence in *E. amylovora*.

During this research, two wild-type strains of *E. amylovora* which are considered as high virulent (PFB5 and BG16) and two hardly virulent (LMG2024 and PD437) strains were used (Chapter 3).

In this regard, the following research questions have been put forward in the outline of this dissertation:

2.1 Can the difference in virulence between strains grown *in vitro* be correlated to differences in proteome patterns?

Because of the limited knowledge of the proteome of *E. amylovora*, first, a comparative proteomics study was performed on four WT strains (LMG2024, PD437, BG16 and PFB5), grown *in vitro* in a standardized and controlled environment. Initially a virulence assay was performed to confirm differential virulence between the strains. The experimental design in Chapter 3 included (i) a general comparative proteome analysis of the four strains and (ii) an in-depth proteome analysis of two strains (LMG2024 and PFB5).

2.2 Which proteins may be involved in difference in virulence between different strains when grown *in planta*?

Artificial infections on shoots of apple rootstocks were used to investigate the differences in virulence between the strains (Chapter 4). A similar experimental approach as the *in vitro* study (Chapter 3) was used to study the proteome of the four mentioned strains. This experimental set-up allowed us to identify proteins and even metabolic processes that were used by this pathogen inside its host. Further, a comparison was made between both strains to gain insights in the different pathogenic strategies they use to start and even more important, to sustain infection. Research and conclusions were strengthened by gene expression by means of RT-qPCR.

2.3 Which of the proteins of the outer membrane could be involved in the difference in virulence between strains?

E. amylovora is a Gram-negative bacterium, implicating that it has a specialized outer membrane. This outer membrane and its proteins form the interface of the pathogen and its environment. Furthermore, they form the first protective barrier against the defense mechanisms of the host. In order to investigate the function of outer membrane proteins in virulence, the four strains were compared *in vitro.* Furthermore, LMG2024 and PFB5 were selected to identify differences in protein composition of the outer membrane of both strains grown *in planta*.

2.4 What could be the role of type III secreted proteins in virulence?

During infection, *E. amylovora* secretes effector proteins into the host cells. These effector proteins are transported and injected into the cytosol of the host by the specialized type III secretion system (He *et al.*, 2004; Buttner & He, 2009). In total eleven proteins were identified as being secreted by *E. amylovora* (Nissinen *et al.*, 2007). In order to identify which of these secreted proteins are involved in and could explain differences in virulence between different strains, primers were developed for the secreted proteins. Using RT-qPCR, the gene expression of these genes of four strains grown *in vitro* and of two strains grown *in planta* were analyzed and correlated with the virulence.

3

A comparative proteome analysis reveals flagellin, chemotaxis regulated proteins and amylovoran to be involved in virulence differences between *Erwinia amylovora* strains

Research article – Published

A comparative proteome analysis reveals flagellin, chemotaxis regulated proteins and amylovoran to be involved in virulence differences between *Erwinia amylovora* strains

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3.1 Abstract

Erwinia amylovora is a Gram-negative bacterium that causes the destructive disease fire blight affecting most members of the *Rosaceae* family, of which apple and pear are economically the most important hosts. *E. amylovora* has been considered as a homogeneous species in whole, although significant differences in virulence patterns have been observed. However, the underlying causes of the differences in virulence remain to be discovered. In a first-time comparative proteomic approach using *E. amylovora*, 2D differential in-gel electrophoresis (DIGE) was used to identify proteins that could explain the gradual difference in virulence between four different strains. Two important proteins were identified, FliC and CheY, both involved in flagella structure, motility and chemotaxis, which were more abundant in the least virulent strain. In the highly virulent strains the protein GalF, involved in amylovoran production, was more abundant, which was consistent with the higher expression of the gene and the higher amylovoran content in this strain *in vitro*. Together, these results confirm the involvement of amylovoran in virulence, but also imply an indirect role of flagellin in virulence as elicitor of plant defense.

3.2 Introduction

Fire blight, caused by the Gram-negative bacterium *Erwinia amylovora*, is a destructive disease which affects most members of the *Rosaceae* family of which apple and pear are economically the most important species. *E. amylovora* is classified as member of the family of the *Enterobacteriaceae* which makes it closely related to many important human and animal pathogens such as *Escherichia coli*, *Salmonella* spp., *Shigella* spp. and *Yersinia* spp. Due to its destructive character and the lack of effective control mechanisms, *E. amylovora* is capable of dispersing rapidly both within susceptible plants and between trees in orchards which could result in great economic losses. Because of the expected rise in average global temperatures, the breeding of cultivars on susceptible rootstocks and the introduction of susceptible cultivars, fire blight will become an even greater threat for the fruit production in Europe in the near future (Deckers & Schoofs, 2008).

The most important virulence factors, necessary for this rapid disease development, have been identified. The first virulence factor is the *hrp* (hypersensitive response and pathogenicity) gene cluster, which is closely associated with the pathogenicity in host species and the hypersensitive response in non-host plants (Steinberger & Beer, 1988). Certain *hrp* genes encode the type III secretion pathway (Alfano & Collmer, 1997) and a pilus-like structure is formed which delivers virulence-associated molecules directly into the plant cells which is called the type III secretion system (T3SS) (Bogdanove *et al.*, 1996). Two of these effector proteins, HrpN and DspA/E, are responsible for the elicitation of oxidative stress responses in the host (Venisse *et al.*, 2003). In apple, DspA/E interacts with four similar kinases in the host cytoplasma (Meng *et al.*, 2006). A second very important virulence factor is amylovoran. Together with levan, it forms the main substance of exopolysaccharides (EPS) of *E. amylovora*. Indication for this virulence factor came from the observation that mutants defective in the formation of amylovoran lack pathogenic ability (Bellemann & Geider, 1992) and further research on this topic pointed out that also levan is involved in virulence (Gross *et al.*, 1992). Amylovoran is an acidic heteropolysaccharide composed of a branched repeating unit consisting of galactose, glucuronic acid and pyruvate residues (Nimtz *et al.*, 1996). The *ams* gene cluster is required for the biosynthesis of amylovoran and is located on the *E. amylovora* chromosome (Bugert & Geider, 1995). The biosynthesis of the other exopolysaccharide levan is controlled by levansucrase, encoded by the *lsc* gene (Geier & Geider, 1993). Recently Koczan *et al.* (2009) have provided evidence that amylovoran also plays a significant role in biofilm formation and that this biofilm formation has a part in the pathogenesis of *E. amylovora* (Koczan *et al.*, 2009). Two other virulence characteristics include sorbitol-regulated genes (Aldridge *et al.*, 1997) and the cyclic, iron-binding desferrioxamines (Dellagi *et al.*, 1998).

It has been known for many years that pathogenic bacteria use two-component systems (TCST) to control the expression of virulence factors required for infection. TCSTs consist of a histidine kinase and a response regulator. They are signal transduction devices that play a critical role in sensing and responding to environmental cues through changes in gene expression (Beier & Gross, 2006; Mole *et al.*, 2007). Two such TCSTs have already been identified in *E. amylovora*

for the regulation of the T3SS and for amylovoran production. Wei et al. (2000) have shown that the structural components of the T3SS encoded by the *hrp* genes are regulated by the TCST consisting of HrpX and HrpY. This system regulates the expression of HrpS, a sigma 54 enhancer-binding protein. Together, HrpS and HrpY activate the expression of HrpL which regulates various genes and operons from the *hrp* gene cluster (Wei *et al.*, 2000). The other known TCST of *E. amylovora* is called RcsCDB phosphorelay system which regulates the biosynthesis of amylovoran (Wang *et al.*, 2011a).

E. amylovora is motile by means of peritrichous flagella (Billing *et al.*, 1961; Huang & Goodman, 1970). The motility is dependent on temperature, pH and other environmental factors (Raymundo & Ries, 1980b). Motility seems to be of aid during the invasion of apple blossoms (Bayot & Ries, 1986), however, they seem nonmotile after their entry into the apoplast (Raymundo & Ries, 1980b). A regulator for flagella biosynthesis, motility and chemotaxis has been identified for *E. coli*, the master regulator FlhDC. This master regulator is negatively regulated by the RcsCDB phosphorelay system in *E. coli* (Francez-Charlot *et al.*, 2003). For *E. amylovora*, negative (GrrSA) and positive (EnvZ/OmpR) regulators of swarming motility have been identified (Zhao *et al.*, 2009b).

E. amylovora is considered to be a homogeneous species based on physiological, biochemical, phylogenetic and genetic analyses (Vanneste, 2000a; Smits *et al.*, 2010; Wang *et al.*, 2010; Zhao & Qi, 2011). However, differences in virulence have been observed in strains of *E. amylovora* isolated from nature (Cabrefiga & Montesinos, 2005; Lee *et al.*, 2010; Wang *et al.*, 2010), but the proteins underlying these differences in virulence have not been described. For the first time, we used a comparative proteomic approach on *E. amylovora*, and identified proteins that may be responsible for the difference in virulence between four strains of *E. amylovora* that show a clear difference in virulence (Maes *et al.*, 2001). The experiments were done in an *in vitro* model to enable the identification of differentially expressed proteins under standardized and controlled conditions. Furthermore, phenotypic studies and reverse transcription-quantitative PCR (RT-qPCR) were performed to validate the results found in the proteomic approach.

3.3 Experimental Procedures

3.3.1 *Bacterial strains*

For the experiments four wild type (WT) strains of *E. amylovora* were used, LMG2024 isolated from *Pyrus communis* (Belgian coordinated collections, Ghent university), strain PD437 also isolated from *P. communis* (Plantenziektenkundige dienst, Wageningen, The Netherlands), strain PFB5, isolated from *Prunus salicina* (S.K. Mohan, Idaho, USA) and BG16 isolated from *Malus sylvestris* (Bulgaria) with collection number SGB 225/12. LMG2024 and PD437 are normal virulent strains while PFB5 and BG16 are highly pathogenic strains (Maes *et al.*, 2001). The genome of *E. amylovora* has been sequenced (Sebaihia *et al.*, 2010; Smits *et al.*, 2010; Powney *et al.*, 2011) making it possible to identify individual 2-DE protein spots.

3.3.2 Bacterial growth and isolation

For the 2D-electrophoresis and RT-qPCR experiments, the bacteria were grown overnight in MM_2 liquid medium supplemented with 1% sorbitol (Bellemann *et al.*, 1994), and shaken at 100 rpm at 24 °C. The bacteria were grown until the exponential phase (OD_{600nm}=0.8) was reached. These overnight cultures were then pelleted and washed three times in phosphate-buffered saline (PBS). Cultures grown in LB broth were used for the other experiments including swarming assay and amylovoran determination.

3.3.3 Virulence assay

The virulence of the four strains was tested using a pear shoot assay (cultivar Conférence). Briefly, the youngest pear leaves were inoculated by cutting the two youngest leaves perpendicularly to the midvein using scissors dipped in the bacterial suspension. The cell suspension was made from overnight bacterial cultures on YPGA, in phosphate buffered saline (PBS) with a density of 1 x 10^8 CFU/ml. The progression of symptoms was observed at 6, 8, 11 and 14 days post-inoculation. Five replicates were used for each strain. Progression of necrosis was recorded using a visual scale and the necrosis severity index (NSI) was calculated as described previously (Wang *et al.*, 2010).

3.3.4 Protein extraction

The washed pellet was lysed using a sample solution containing 7 M urea, 2 M thio-urea and 4% (w/v) CHAPS. The lysate was sonicated for 1 min on ice using a microtip and further incubated for 30 min on ice. The homogenate was centrifuged (76 000 xg for 90 min) to remove the cellular debris and the supernatant containing the protein fraction was used for further analysis. After the pH was adjusted to 8.5 with 100 mM sodium hydroxide, the total protein concentration was determined using the 2-D Quant kit (GE Healthcare) according to the instructions of the manufacturer.

3.3.5 CyDye labeling

The extracted proteins were then analyzed by differential in-gel electrophoresis (DIGE) and were minimally labeled with cyanine-derived fluors (3 Dyes 2-D Cyanine Labeling kit from Proteomics Consult) containing a N-hydroxysuccinimide ester-reactive group. The protein samples were labeled using Cy3 and Cy5 and a pooled internal standard representing equal amounts of both strains was labeled with Cy2. For each labeling reaction, 25 µg of protein was incubated with 200 pmol CyDye, at room temperature for 30 min in the dark. The reactions were quenched by incubation for 10 min with 1 µl of 10 mM lysine on ice in the dark. The individual Cy3, Cy5 and Cy2 labeled samples were mixed according to the experimental design including a dye swap. Each gel was loaded with 75 µg proteins, 25 µg from each sample and 25 µg from the internal standard. For this experiment, 4 biological replicates were considered for each strain.

3.3.6 2-D gel electrophoresis

The mixed, labeled protein samples were diluted with lysis buffer (0.005% bromophenol blue, 90 mM DTT and 2% IPG-Ampholyte mix (SERVA)) to 120 µl. For separation in the first dimension, precast immobilized pH gradient (IPG) strips (SERVA; pH 3-10, 24 cm) were first rehydrated in IPG-enriched (5 µl/ml) Destreak (GE Healthcare) for at least 8h. The first dimension was performed using an IPGphor isoelectric focusing apparatus (GE Healthcare). The protein samples were loaded onto the IPG strips via anodic cuploading using the

following settings: 250 V for 1 h, 1000 V (gradient) for 7 h, 8000 V for 3 h and 8000 V (gradient) for 3 h and 45 min for a total of 49.2 kVh (50 µA/strip, 20 °C). IPG strips were hereafter stored at -20 °C.

For separation in the second dimension, thawed IPG strips were first equilibrated for 15 min at room temperature, while shaken in an equilibration buffer (SERVA) with DTT followed by an equilibration for another 15 min in an equilibration buffer (SERVA) containing iodoacetamide following the manufacturer's protocol. The second dimension was performed at 18 °C with an HPE-FlatTop Tower (SERVA) using precast, plastic-backed 10-15% polyacrylamide gels (2D-Large-Gel Flatbed NF 10-15% gradient gels) according to the manufacturer's instructions. The electrophoresis conditions were 30 min at 100 V with 7 mA/gel and 1 W/gel; 30 min at 200 V with 13 mA/gel and 3 W/gel; 10 min at 300 V with 20 mA/gel and 5 W/gel; 4 h and 50 min at 1500 V with 40 mA/gel and 30 W/gel and finally 50 min at 1500 V with 45 mA/gel and 40 W/gel. Four gels were run simultaneously and after completion of the run, gels were fixed overnight in 1% citric acid and 15% ethanol.

3.3.7 Gel imaging and data analysis

After SDS-PAGE, the fluorescent labeled proteins were visualized directly by scanning using an Ettan DIGE Imager (GE Healthcare). All gels were scanned at 100 µm (pixel size) resolution.

Determination of spot abundance and statistical analysis were performed using the Progenesis SameSpots software 4.5. The quality of the spot patterns was checked by using the quality control feature of the software. Statistical analysis of protein abundance was performed on four gels for each condition. Differences between conditions and strains were validated by Principal Component Analysis (PCA) to determine if samples had the groupings expected or if there were any outliers in the data. Differences among matched spot intensities were statistically validated by performing an ANOVA at a 5% significance level. The P-values were refined by a q-value to eliminate the false positives. The SameSpot software is also capable of the calculation of a power analysis which represents the probability of finding a significant expression change where it exists. With a target power of 0.8, it was possible to choose the fold change below 2.0 without

increasing the risk of including false positive spots. The spots considered, were spots with at least 1.5-fold changes in volume ($P<0.05$) in one condition after normalization.

3.3.8 Protein identification

The spots of interest were excised from the 2-D gels in 1.5 mm diameter gel plugs using a semi-automated Screen picker (made by Proteomics Consult). Hereafter the plugs were processed for mass spectrometry according the protocol of (Shevchenko *et al.*, 1996).

3.3.9 LC-MS/MS analysis

An Easy-nLC 1000 liquid chromatograph (Thermo Scientific) was on-line coupled to a mass calibrated LTQ-Orbitrap Velos Pro (Thermo Scientific) via a Nanospray Flex ion source (Thermo Scientific) using sleeved 30 µm ID stainless steel emitters (spray voltage +2.3 kV, capillary temperature: 200 °C). The SpeedVac dried tryptic peptide mixture was dissolved in 20 µl buffer A (0.1% v/v formic acid in Milli-Q water) of which half was loaded, concentrated and desalted on a trapping pre-column (Acclaim PepMap 100 C_{18}, 75 µm ID × 2 cm nanoViper, 3 µm, 100 Å, Thermo Scientific) at a buffer A flow rate of 5 µl/min for 5 min. The peptide mixture was separated on an Acclaim PepMap RSLC C_{18} column (50 µm ID x 15 cm nanoViper, 2 µm, 100 Å, Thermo Scientific) at a flow rate of 250 nL/min with a linear gradient in 40 min of 0 to 70% buffer B (0.1% v/v formic acid in acetonitrile) in buffer A.

MS data were acquired in a data-dependent mode under direct control of the Xcalibur software (version 2.2.SP1.48), selecting the fragmentation events based on the top six precursor abundances in the survey scan (350–2000 Th). The resolution of the full scan was 30000 at 400 Th with a target value of 1 x 10^6 ions and one microscan. CID MS/MS spectra were acquired with a target value of 10000 and the maximum injection time was 100 ms. Dynamic exclusion was 30 s and early expiration was disabled. The isolation window for MS/MS fragmentation was set to 2 Th and the normalised collision energy, Q-value and activation time were 30%, 0.25 and 10 ms, respectively. Helium was used as the collision gas.

3.3.10 Data analysis

The analysis of the mass spectrometric raw data was carried out using Proteome Discoverer software v.1.2 (Thermo Scientific) with build-in Sequest v.1.3.0339 and interfaced with an in-house Mascot v.2.4 server (Matrix Science). MS/MS spectra were searched against the *Erwinia* protein collection extracted from NCBI database (query '*Erwinia*' on March 13th 2013; 104711 entries) and peptide scoring for identification was based on the following search criteria: enzyme trypsin, maximum missed cleavages 2, precursor mass tolerance 10 ppm and fragment mass tolerance 0.5 Da. Carbamidomethylation of cysteine and oxidation of methionine were set as fixed and as dynamic modifications, respectively.

Result files of both search engines were uploaded and automatically evaluated in Scaffold v.3.6.1 (Proteome Software) using the Peptide Prophet and Protein Prophet algorithm with a preset minimal peptide and protein identification probability of 95% and 99%, respectively.

3.3.11 RNA extraction and quantitative RT-PCR

Cell cultures, grown until mid-exponential phase, were supplemented with 2 volumes of RNAprotect Bacteria Reagent (Qiagen, Venlo, The Netherlands). After an incubation period of 5 min, bacteria were collected by centrifugation (5000 g, 10 min) and RNA was extracted using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Further, The TURBO DNA-free kit (Ambion) was used to remove DNA and final reverse transcription was carried out from 1 µg of DNase-treated total RNA using the PrimeScript RT Reagens Kit (Takara). The cDNA samples were ten-fold diluted using 1/10 diluted TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored at -20 °C until use. Quantitative PCR (qPCR) was performed using Fast SYBR Green chemistry according to the manufacturer's instructions on an ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems, Belgium). Relative gene expression was calculated as $2^{-\Delta Cq}$ and was normalised with a normalisation factor based on the expression of the following reference genes: *rpsL*, *rpoD* and *gyrA*. Reference genes were tested using the GrayNorm algorithm and both normalised and non-normalised data were presented as an accuracy interval

according to Remans *et al.* (2014). Gene-specific primers based on proteins of interest indicated by the proteomic study were developed using Primer3 (Whitehead Institute/MIT Center for Genome Research). 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.* (2009) (Table S3).

3.3.12 Motility assay

To investigate differences in motility between strains, the bacteria were cultured overnight in LB broth until approximately 2 x 10^8 CFU/ml. The bacteria were pelleted and washed three times with PBS. Hereafter each sample was resuspended to 0.2 OD_{600} in PBS. Then 5 µl of the diluted bacterial suspension was plated onto the center of swarming agar plates (10 g tryptone, 5 g NaCl, 3 g agar per liter of water). Swarming diameters were measured after 18, 24, 36 and 48 h at 28 °C. For this assay, four biological replicates were performed with 10 plates per strain per experiment.

3.3.13 Cetylpyridinium chloride (CPC) assay for the determination of amylovoran concentration

A turbidity assay was used to determine the amylovoran concentration in the supernatants of the cell suspension of the four strains of *E. amylovora* (Bellemann & Geider, 1992; Maes *et al.*, 2001; Hildebrand *et al.*, 2006). The bacteria cultures were grown overnight in LB broth. The cells were pelleted and washed three times with PBS. After the third wash, the pellet was resuspended in 200 µl of PBS. From this liquid suspension, 100 µl was inoculated in 10 ml of sterile MBMA medium supplemented with 1% sorbitol. This cell suspension was incubated at 28 °C with shaking for 2 to 3 days before the cells were pelleted by centrifugation (20 000 xg, 20 min). Hereafter 50 µl of CPC at 50 mg/ml was added to 1 ml of supernatants and the mixture was incubated for 10 min at room temperature. Then the amylovoran concentration was determined by measuring the turbidity at OD_{600}. The final concentration of amylovoran production was normalised for a cell density of 1.0. For both strains, the experiment was repeated four times, with 9 replicates per strain.

3.3.14 Statistical analysis

For the virulence assay, amylovoran determination and swarming assays, a Kruskal-Wallis test and a pairwise Wilcox comparison were performed since the data were not distributed normally. A one-way ANOVA was performed on the data of the RT-qPCR experiments with Tukey's pairwise comparisons and a transformation to appropriate normality if necessary. All statistics were performed using R version 3.0.3.

3.4 Results

3.4.1 Virulence assay of the four strains

In accordance with previous research (Maes *et al.*, 2001), the four strains used in this research, LMG2024, PD437, BG16 and PFB5 show differences in their virulence when inoculating pear shoots cultivar Conférence (Figure 3.1). Percentage necrosis was measured at 6, 8, 11 and 14 days post-inoculation (dpi) (Figure 3.1B). These results demonstrated a difference in migration in the host. Figure 3.1A shows the infection pattern after 14 dpi. For LMG2024, infection was limited to the upper leaves while for PD437, a migration throughout the shoot was observed although infection here was also limited to the upper leaves. For the higher virulent strains BG16 and PFB5, the infection was systemic after 14 dpi and the pathogen had migrated throughout the entire plant and almost all leaves were infected.

3.4.2 2-D gel electrophoresis

Four selected WT isolates of *E. amylovora* with known virulence differences (Maes *et al.*, 2001) (Figure 3.1) were studied at the protein level by 2D DIGE PCA analysis (Figure 3.2). The 2D DIGE protein profiles illustrated the clustering of the replicate profiles obtained for each of the four bacterial isolates, when grown *in vitro* in liquid MM2 medium, mimicking the internal plant environment. The PC1 axis indicated a variation of 48.16% while the PC2 axis explained 14.95% of the variation (Figure 3.2). Out of these, the highly virulent PFB5 isolate and the lowly virulent strain LMG2024 were selected for an in-depth proteomic analysis.

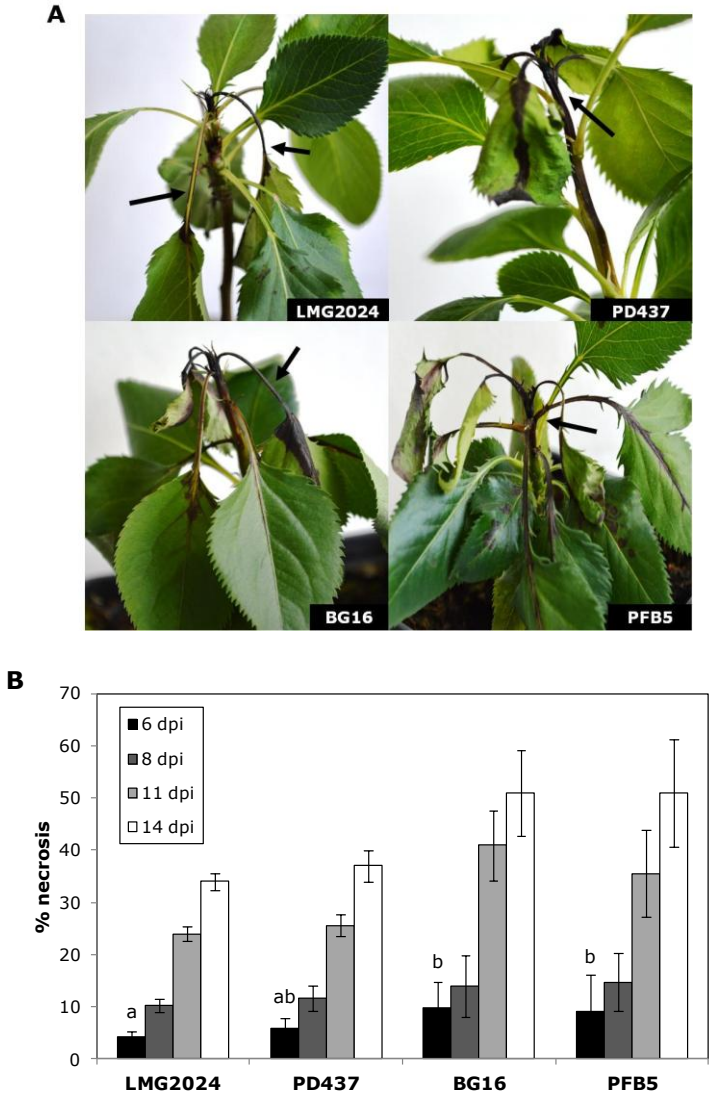


Figure 3.1: *Virulence assay to characterize differences in virulence between four strains of E. amylovora, LMG2024, PD437, BG16 and PFB5. A. Virulence of the strains in pear shoots, at 14 dpi. Arrows denote the disease symptoms. B. The percentage necrosis was observed at 6, 8, 11 and 14 dpi. Bars represent the average of five shoots ± standard errors. Letters indicate significantly differing results (P < 0.05, Kruskal-Wallis test with a pairwise Wilcox comparison test) from strains at the same dpi.*

2D gel image analysis using SameSpots enabled the selection of 94 significantly altered spots ($P < 0.05$) with an abundance ratio fold change cutoff of > 1.5, a q-value < 0.05 and a power > 80%.

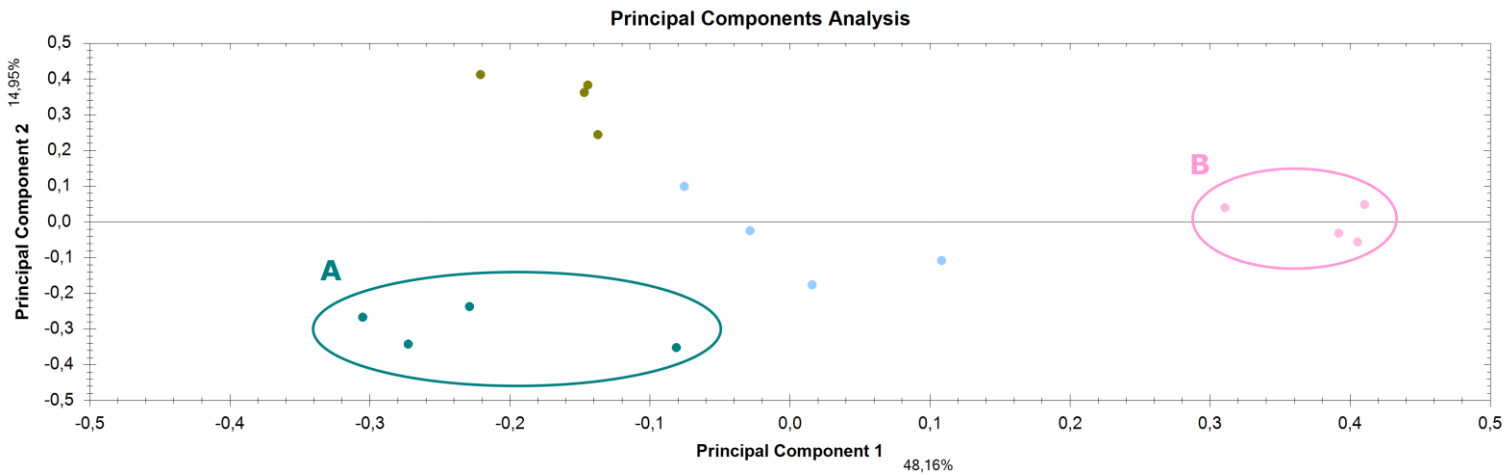


Figure 3.2: *Principal Component Analysis performed on the complete data set of the gels including the differentially abundant spots identified by the SameSpots software. The axis PC1 explains 48,16% of the variation and the axis PC2 14,95%: A: PFB5, B: LMG2024.*

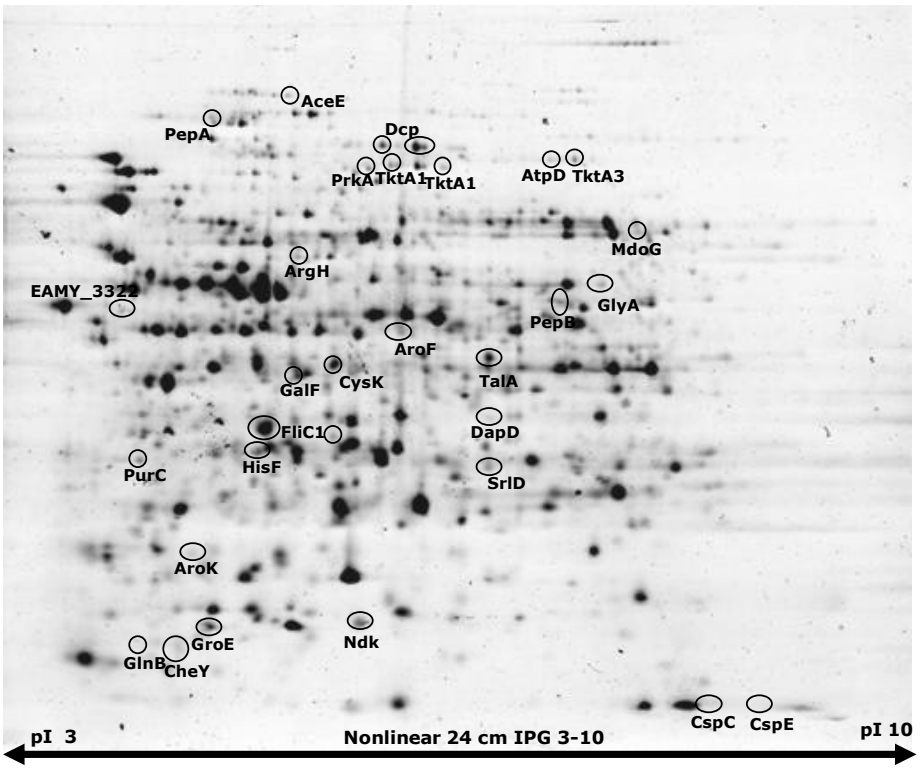


Figure 3.3: *2-DE analysis of the proteome of E. amylovora. The image represents scan from the internal standard so every protein is present. All identified proteins are indicated.*

Subsequent mass spectrometric analysis resulted for LMG2024 and PFB5 in 16 and 13, respectively non-redundant confidently identified proteins in 29 gel spots fulfilling the aforementioned cutoff criteria (Figure 3.3). An overview of the identified proteins and their classification into categories according to their biological function, as described in the protein knowledge database UniProt, is presented in Table 3.1 and Figure 3.4.

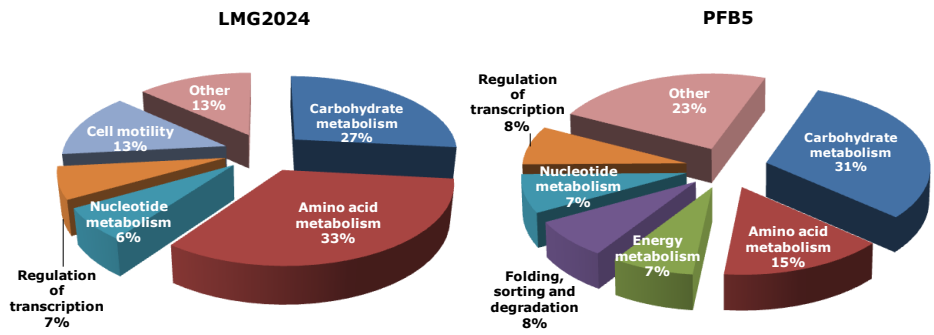


Figure 3.4: *Pie charts representing the biological functions of the identified proteins in LMG2024 and PFB5 that were differentially expressed. Biological functions were assigned according to the information provided by gene ontology tools at UniProtKB.*

These results were limited to spots that were translated in only one protein during identification. The proteins differentially expressed in LMG2024 are involved in different metabolic pathways: (i) four proteins in aerobic glycolysis and/or carbohydrate metabolism (pyruvate dehydrogenase E1 component (AceE), sorbitol-6-phosphate dehydrogenase (SrlD), glucans biosynthesis protein G (MdoG) and transketolase (TktA1)), (ii) five proteins in amino acid metabolism (argininosuccinate lyase (ArgH), serine hydroxymethyltransferase (GlyA), phospho-2-dehydro-3-deoxyheptonate aldolase (AroF), 2,3,4,5-tetrahydropyridine-2,6-carboxylate N-succinyltransferase (DapD) and shikimate kinase I (AroK)), (iii) one in the nucleotide metabolism (PurC), (iv) two in regulation of transcription (cold shock-like protein (CspC)) and cold shock protein (CspE)), (v) two involved in cell motility (two spots were identified as flagellin (FliC1) and chemotaxis regulatory protein (CheY)) and (vi) two classified as other proteins (aminopeptidase B (PepB), and aminopeptidase N (PepN)). One protein involved in virulence was found more abundantly in the low virulent strain in comparison with the high virulent strain, namely sorbitol-6-phosphate dehydrogenase (SrlD). This protein plays an important role in the sorbitol metabolism and is 1.6 times more abundant in LMG2024 in comparison with PFB5.

Interestingly two proteins involved in cell motility were more abundant in the low virulent strain compared to the more virulent one. Both flagellin (FliC1) and the chemotaxis regulatory protein (CheY) are upregulated by 3.2 times and 2.3 times, respectively (Figure 3.5). In *Bacillus subtilis*, CheY and CheA form a two-

component regulatory system involved in signal transduction of chemotaxis (Garrity & Ordal, 1995). CheA becomes autophosporylated by the binding of attractants to transmembrane receptors (Garrity & Ordal, 1997). Phosphate transfer from CheA to the response regulator CheY (Bischoff *et al.*, 1993; Garrity & Ordal, 1997) and subsequent interaction of the modified CheY with the flagellar motor switch complex induces a counterclockwise rotation resulting in a smooth swimming motility (Bischoff *et al.*, 1993; Bren & Eisenbach, 1998). This could imply a better motility of LMG2024.

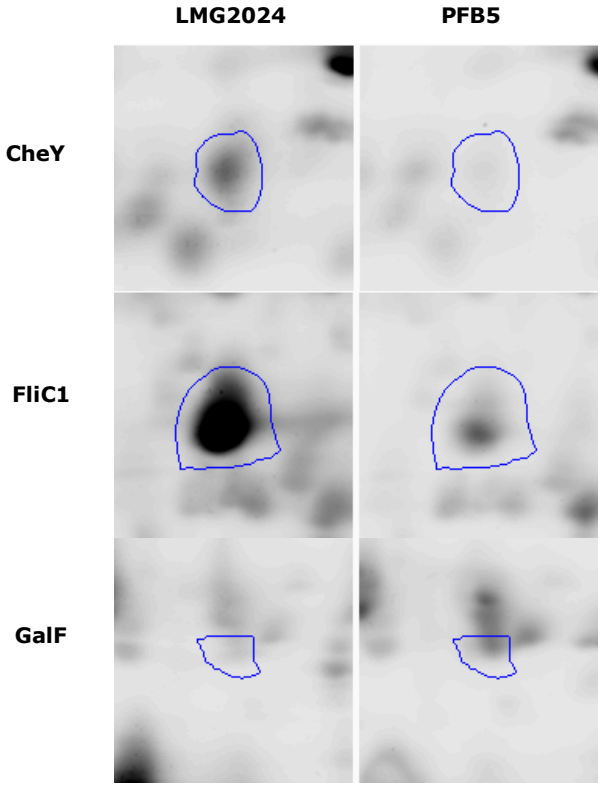


Figure 3.5: *Up-expression profile of three proteins possibly involved in virulence.*

The proteins that were differentially expressed in PFB5 were also involved in different metabolic pathways: (i) four in the carbohydrate metabolism (transaldolase (TalA), transketolases (TktA1 and TktA3) and UTP-glucose-1-phosphate uridylyltransferase (GalF)), (ii) two in the amino acid metabolism (cysteine synthase A (CysA) and imidazole glycerol phosphate synthase subunit (HisF)), (iii) one in the energy metabolism (F_0F_1 ATP synthase subunit beta

(AtpD)), (iv) one involved in folding, sorting and degradation (10 kDa chaperonin (Cpn10)), (v) one in the nucleotide metabolism (nucleoside diphosphate kinase (Ndk)), (vi) one in the regulation of transcription (nitrogen regulatory protein P-II 1 (GlnB)), (vii) three proteins categorized as other (two spots were identified as dipeptidyl carboxypeptidase II (Dcp), serine kinase (PrkA)) and (viii) one hypothetical protein EAMY_3322. Former research has implied that *galF* is homologous to *amsM*. This gene is located on the right side of the *ams* cluster and the enzyme is involved in the formation of UDP-glucose from α-D-glucose-1P which is essential for amylovoran production (Geider, 2000). This protein was found to be 1.8 times more abundant in the high virulent strain in comparison with the low virulent one (Figure 3.5).

Table 3.1: *Identification table of differentially regulated proteins between LMG2024 and PFB5.*

Spot number	Protein ID	Name	Gene name	Theoretical MW(kDa)/pI	ANOVA P-value	Fold change
Carbohydrate metabolism						
Upregulated proteins in LMG2024						
49	gi|292489305	pyruvate dehydrogenase E1 component	*aceE*	99.3/5.4	0.00761	2.9
151	gi|292487104	transketolase	*tktA1*	72.3/5.65	0.00203	2.4
259	gi|292487912	glucans biosynthesis protein G	*mdoG*	59.0 /8.78	0.00134	1.6
602	gi|292489538	sorbitol-6-phosphate dehydrogenase	*srlD*	27.8/5.67	0.0112	1.6
Upregulated proteins in PFB5						
142	gi|292488973	transketolase	*tktA3*	72.6/5.96	0.00104	4.7
149	gi|292487104	transketolase	*tktA1*	72.3/5.65	0.00337	1.6
444	gi|292488972	transaldolase A	*talA*	35.5/5.87	3.973e-005	3.2
475	gi|292488712 gi|292899868	UTP-glucose-1-phosphate uridylyltransferase	*galF*	37.0/5.83	0.00875	1.8
Amino acid metabolism						
Upregulated proteins in LMG2024						
299	gi|292486634	argininosuccinate lyase	*argH*	50.2/5.47	0.0103	1.9
341	gi|292489072	serine hydroxymethyltransferase	*glyA*	45.6/6.02	0.0027	3.9
416	gi|292487312	phospho-2-dehydro-3-deoxyheptonate aldolase	*aroF*	39.5/5.52	6.095e-005	2.1
534	gi|292489231	2,3,4,5-tetrahydropyridine-2,6-carboxylate N-succinyltransferase	*dapD*	29.7/5.66	0.00704	2.7
718	gi|292489894	shikimate kinase I	*aroK*	19.5/5.06	0.0058	1.7

Table 3.1 (continued): *Identification table of differentially regulated proteins between LMG2024 and PFB5.*

Spot number	Protein ID	Name	Gene name	Theoretical MW(kDa)/pI	ANOVA P-value	Fold change
Upregulated proteins in PFB5						
476	gi|292488955	cysteine synthase A	*cysK*	34.6/5.46	0.00386	1.9
571	gi|292488697	imidazole glycerol phosphate synthase subunit	*hisF*	28.5/5.31	0.00333	1.5
Energy metabolism						
Upregulated proteins in PFB5						
141	gi|292490160	F0F1 ATP synthase subunit beta	*atpD*	51.4/4.80	0.0126	3.4
Folding, sorting and degradation						
Upregulated proteins in PFB5						
788	gi|292489643	10 kDa chaperonin (protein Cpn10)	*groE*	10.3/5.82	0.0113	1.7
Nucleotide metabolism						
Upregulated proteins in LMG2024						
591	gi|292488997	phosphoribosylaminoimidazolesuccinocarboxamide synthase	*purC*	26.9/4.94	9.011e-004	1.8
Upregulated proteins in PFB5						
784	gi|292489051	nucleoside diphosphate kinase	*ndk*	15.5/5.54	2.135e-004	2.6
Regulation of transcription						
Upregulated proteins in LMG2024						
839	gi|292488490	cold shock-like protein	cspC	7.4/6.54	0.006	2.2
847	gi|292487595	cold shock protein	*cspE*	7.4/6.55	0.00158	3.6
Upregulated proteins in PFB5						
800	gi|292489074	nitrogen regulatory protein P-II 1	*glnB*	12.5/5.04	0.012	1.7

Table 3.1 (continued): *Identification table of differentially regulated proteins between LMG2024 and PFB5.*

Spot number	Protein ID	Name	Gene name	Theoretical MW(kDa)/pI	ANOVA P-value	Fold change
Cell motility						
Upregulated proteins in LMG2024						
546	gi|292488615	flagellin, filament structural protein FliC	*fliC1*	29.6/5.34	0.00514	3.2
552	gi|292488615	flagellin, filament structural protein FliC	*fliC1*	29.6/5.34	0.00288	1.7
801	gi|292488561 gi|292489166	chemotaxis regulatory protein CheY	*cheY*	14.3/5.06	0.00251	2.3
Other						
Upregulated proteins in LMG2024						
55	gi|292486492	aminopeptidase N	*pepN*	98.6/5.14	0.00302	3.9
368	gi|292489064	aminopeptidase B	*pepB*	46.1/5.95	0.00719	1.6
Upregulated proteins in PFB5						
122	gi|292488225	dipeptidyl carboxypeptidase II	*dcp*	79.9/5.78	0.000373	3.8
124	gi|292488225	dipeptidyl carboxypeptidase II	*dcp*	79.9/5.78	0.00215	2.8
160	gi|292488457	PrkA serine kinase	*prkA*	74.2/5.99	0.000373	2.3
378	gi|292489785	hypothetical protein EAMY_3322	/	44.1/5.17	0.0045	2.2

3.4.3 The four strains showed a difference in the amount of amylovoran production and exhibited different swarming motility phenotypes

The amylovoran production in the supernatant of the bacterial cultures was tested by means of a turbidity assay using CPC (Bellemann & Geider, 1992; Maes *et al.*, 2001; Hildebrand *et al.*, 2006). The strains showed a significant difference in the production of these EPS (Figure 3.6A). We measured levels of amylovoran that were in accordance with their supposed virulence (Maes *et al.*, 2001): PD437 produces significantly more amylovoran than LMG2024. BG16 and PFB5 produce a significantly higher concentration of these EPS in comparison with the lower virulent strains, LMG2024 and PD437. Additionally, a swarming assay was performed and a regular circular swarming motility was observed for all strains (Figure 3.6B). Swarming diameters were measured after 18, 24, 36 and 48 h. At all time points bacterial swarming motility was significantly reduced in PFB5 compared with the other strains. The least virulent strain shows the highest swarming potential at all time points, which could confirm the findings of the higher concentrations of flagellin and chemotaxis regulatory proteins.

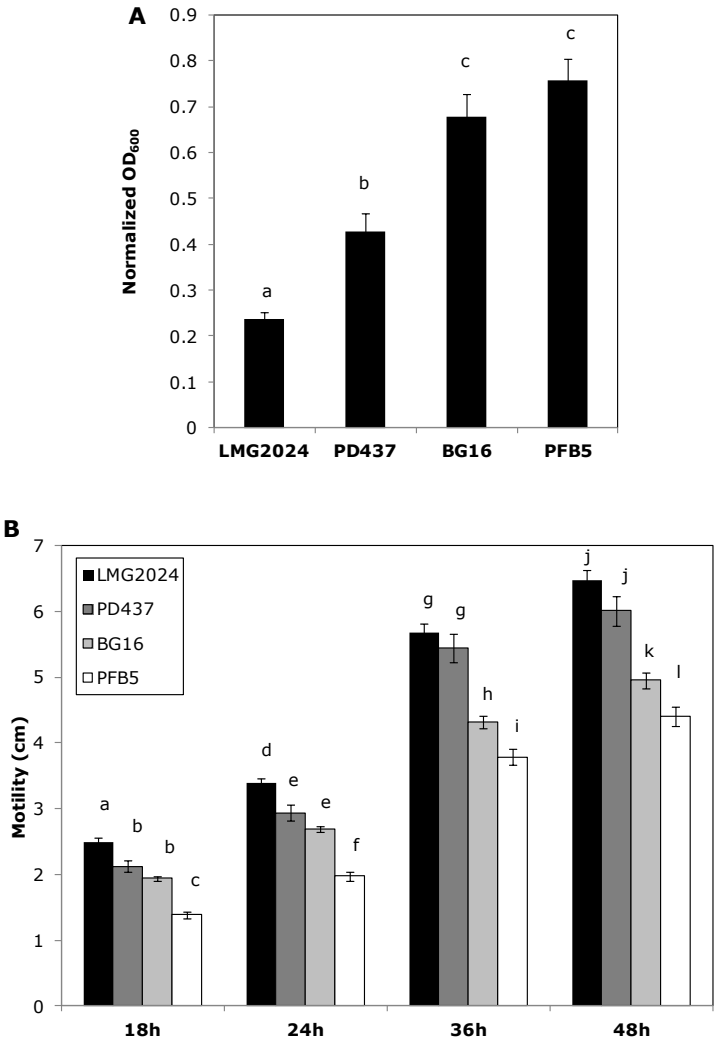


Figure 3.6: *Phenotypic experiments in comparison of LMG2024, PD437, BG16 and PFB5 A. The amount of amylovoran produced was normalised to a cell density of 1. Bars represent the average of four replicates ± standard errors. The letters indicate a statistically significant difference between strains (P < 0.001, Kruskal-Wallis test with a pairwise Wilcox comparison test) B. Comparison of the swarming capacity of the four strains. Swarming diameters were measured after 18, 24, 36 and 48 h. Data points represent for each test mean of four replicates ± standard errors. The letters indicate a statistically significant difference between strains at one time point (P < 0.005, Kruskal-Wallis test with a pairwise Wilcox comparison test).*

3.4.4 Verification of observed differential protein levels at the gene expression level using RT-qPCR

To validate the protein expression patterns observed in our proteomic analysis, genes encoding interesting proteins were selected for gene expression profiling (Table 3.2).

Table 3.2: *Used primers for RT-qPCR.*

Genes	Gene ID	Locus tag	Left primer	Right primer	Amplicon size	Primer efficiency
fliC1	8911752	EAMY_2141	CGGTACAGAACCGTCTGGAT	TCAGTGGCGTAGTCAGCATC	103	102.4%
cheY1	8912565	EAMY_2087	AACATGGACGGTCTGGAACT	TTTTTGCTTCAGCCGTTACC	91	100.0%
galE	8913771	EAMY_2240	GGTCCCCTTCGAAGAAAGTC	CGATGACGTGGTGATACTGG	104	93.9%
galF	8912924	EAMY_2241	CGTTCATGATCGTCACACCT	TACGAACTGGAAGCCTTGCT	93	99.2%
cheA1	8912561	EAMY_2095	GGTGAAAGCTCACTGGAAGC	AAATGGATCTGCGATTCGTC	98	102.0%
srlD	8914018	EAMY_3073	CAACCTTGCCGGATTTAGAA	TGGGCTATTTCCTCTGTTCG	99	115.7%
rpsL	8951948	EAM_3203	AACTCCGCACTGCGTAAAGT	GGATCAGGATCACGGAGTGT	112	98.07%
rpoD	8914668	EAMY_RS19375	TCCGGTGCATATGATTGAGA	CGTTCGGCTAACTCTTCTGG	102	100.69%
gyrA	8911809	EAMY_RS28155	CGAGCACGTTCATAGCGTAA	TATGCAATGTCGGTCATCGT	97	97.49%

RNA was extracted from the four strains grown in MM_2 medium until the mid-exponential phase. Proteomic results showed a higher concentration of the proteins FliC and CheY for LMG2024. Therefore, the corresponding genes were measured for all strains by RT-qPCR. Both genes showed a higher expression level in LMG2024 and PD437 in comparison with BG16 and PFB5 (Figure 3.7A, B). For flagellin (FliC), significant differences could be seen between LMG2024 and BG16 and between LMG2024 and PFB5. PD437 had a significantly higher expression of flagellin than PFB5. The expression of genes encoding chemotaxis regulatory proteins CheY1 and CheA1 was significantly higher in LMG2024 compared to PFB5 (Figure 3.7B, C), which was in accordance with the proteomic results. The other two strains formed an intermediate between LMG2024 and PFB5. On the other hand, two genes important in amylovoran synthesis, *galF* and *galE*, showed a higher expression in the more virulent strain BG16 (Figure 3.7D, E). For *galF*, a significant difference was shown between LMG2024 and BG16, and between PD437 and BG16 (Figure 3.7D). Results for *galE* were similar but here there was only a statistical difference between BG16 and the other strains (Figure 3.7E). Since proteomic results suggested an upregulation of SrlD in LMG2024, the expression of the gene *srlD* was tested. Results showed no significant difference between strains (Figure 3.7F). Overall, the ranking of the strains in terms of gene expression was in accordance with the virulence assays.

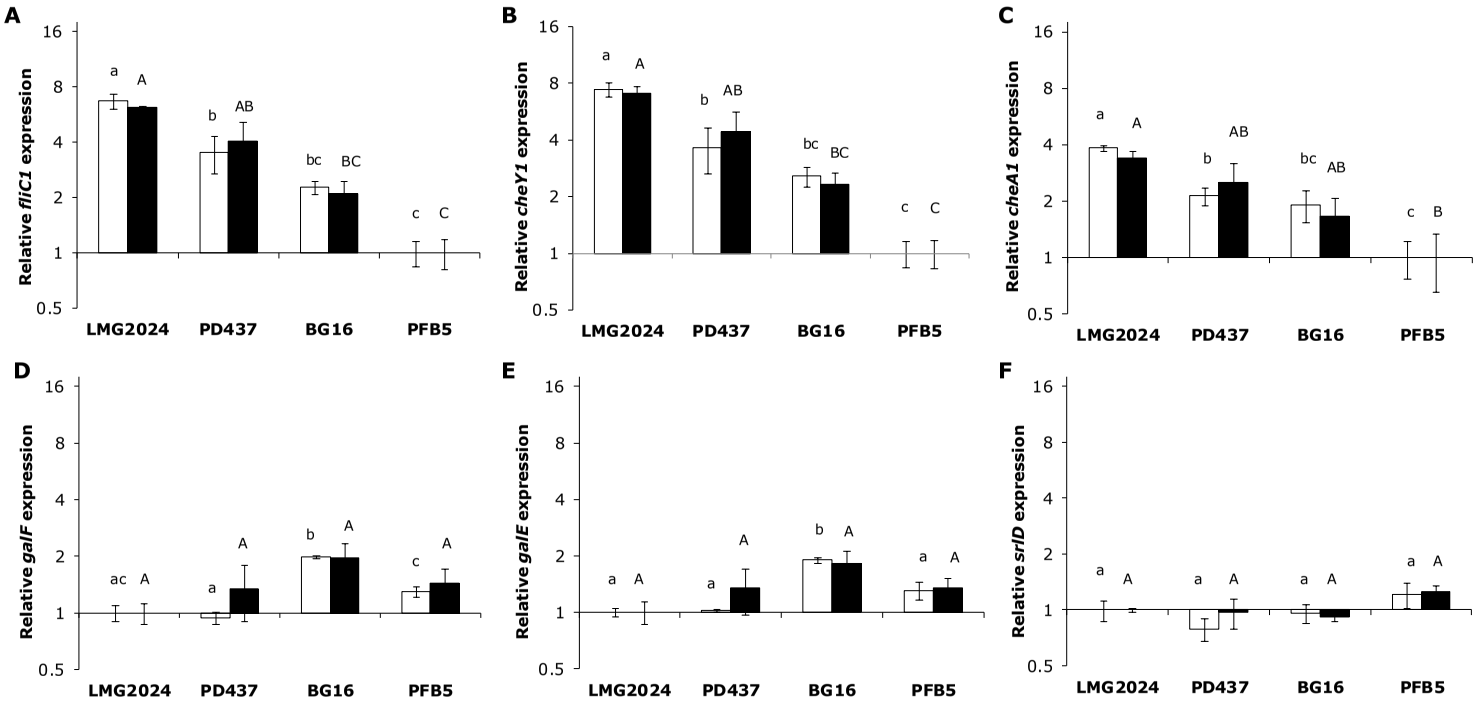


Figure 3.7: *Relative gene expression measured by RT-qPCR. Normalised data are represented in white and the non-normalised data in black. Up- or downregulations are represented on a* log_2 *scale y axis relative to the most virulent strain (A, B, C) or the least virulent strain (D and E). Columns represent the data from four biological replicates ± standard errors. Letters indicate statistical differences (P < 0.05, one-way ANOVA after testing normality with Shapiro-Wilk test). Relative expression of flagellin (A. fliC1), chemotaxis regulatory protein (B. cheY1, and C. cheA1), UTP-glucose-1-phosphate uridylyltransferase (D. galF), UDP-glucose 4-epimerase (E. galE) and sorbitol-6-phosphate dehydrogenase (F. srlD).*

3.5 Discussion

E. amylovora has been indicated as a homogeneous species in whole (Vanneste, 2000a; Smits *et al.*, 2010; Wang *et al.*, 2010), although differences have been reported in virulence between strains. Furthermore, *E. amylovora* strains exhibiting differences in virulence have been isolated from nature and have been characterized (Lee *et al.*, 2010; Wang *et al.*, 2010). Wang *et al.* (2010) have reported that the pathogen may adapt to different hosts, thereby eliciting different levels of disease on different host plants. Differences in the disease development and virulence had previously been observed for the four strains used in our study: LMG2024, PD437, BG16 and PFB5 (Maes *et al.*, 2001). These differences were here confirmed in our experimental set-up using controlled, standardized conditions. Subsequent proteomic and phenotypic experiments delivered more insight in the metabolic behavior of two strains, LMG2024 and PFB5, and its relation with their virulence. Indeed, besides the differences in the production and synthesis of factors possibly involved in virulence, also differences in other metabolic processes were observed (Figure 3.8). The metabolism fuels the cellular activities with building blocks and energy. Figure 3.8 shows an overview representing the two strains used for the proteomic analysis which proved to be the most different. It shows that both LMG2024 and PFB5 have an upregulation of specifically defined metabolic pathways depending on the demand of end products. In LMG2024 there is an upregulation in the glycine, serine and threonine metabolism and in the phenylalanine, tyrosine and tryptophan biosynthesis. For PFB5 on the other hand, an upregulation is found for the histidine, cysteine and methionine metabolism. This indicates that both strains are in demand of different amino acids as building blocks for their proteins. In PFB5, TalA, a protein important for the balance of the metabolites in the pentose phosphate pathway, is more abundant. This protein forms the connection between the pentose phosphate pathway, glycolysis and the galactose metabolism on one side and the connection between the pentose phosphate pathway and the histidine metabolism on the other side. The connection to the galactose metabolism leads to the production of GalF, important in the amylovoran production.

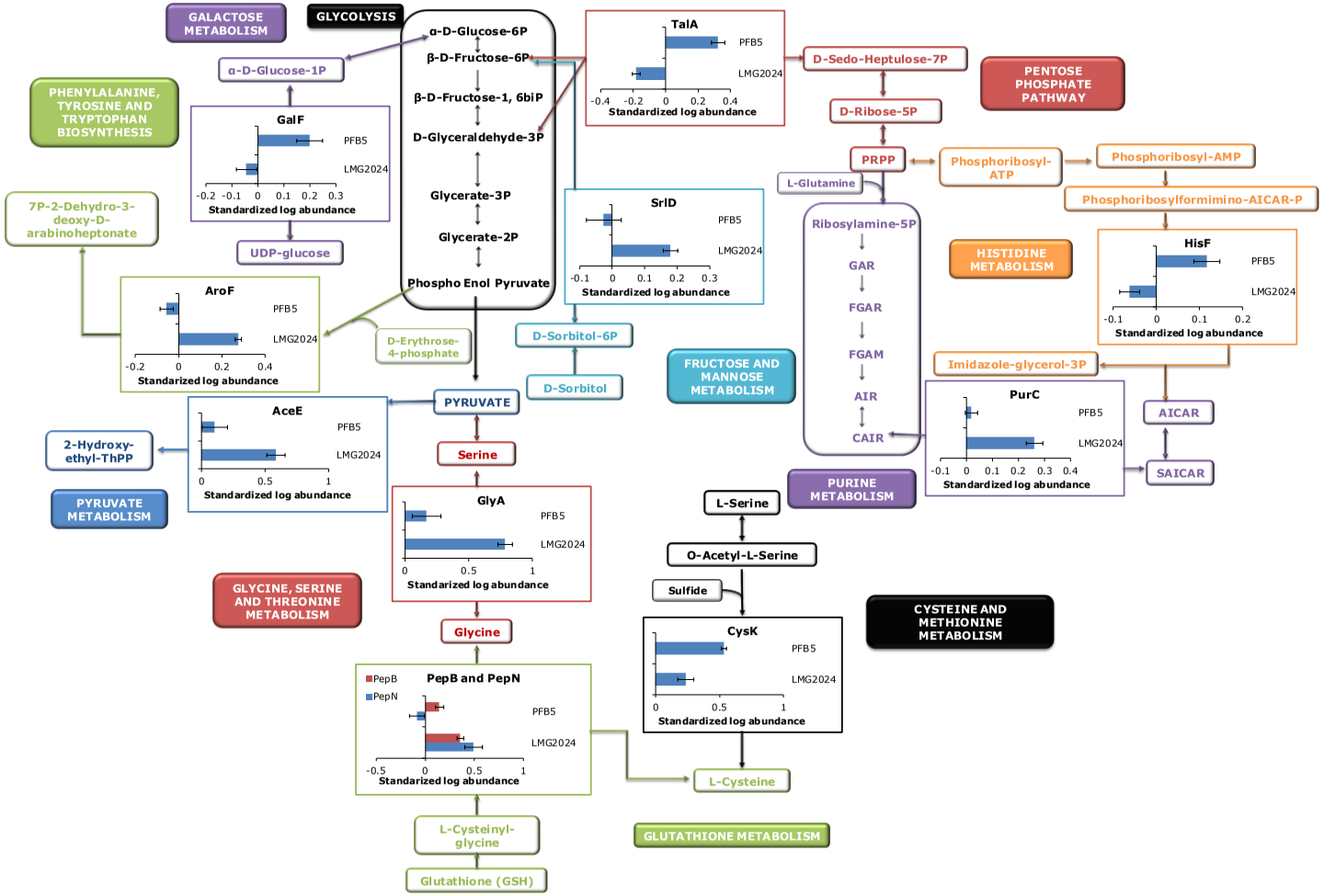

GALACTOSE METABOLISM
GLYCOLYSIS
α-D-Glucose-6P
β-D-Fructose-6P
β-D-Fructose-1, 6biP
D-Glyceraldehyde-3P
Glycerate-3P
Glycerate-2P
Phospho Enol Pyruvate
α-D-Glucose-1P
PHENYLALANINE, TYROSINE AND TRYPTOPHAN BIOSYNTHESIS
GalF
PFB5
LMG2024
Standardized log abundance
UDP-glucose
7P-2-Dehydro-3-deoxy-D-arabinoheptonate
AroF
Standarized log abundance
D-Erythrose-4-phosphate
TalA
D-Sedo-Heptulose-7P
D-Ribose-5P
PENTOSE PHOSPHATE PATHWAY
PRPP
Phosphoribosyl-ATP
Phosphoribosyl-AMP
Phosphoribosylformimino-AICAR-P
L-Glutamine
Ribosylamine-5P
GAR
FGAR
FGAM
AIR
CAIR
HISTIDINE METABOLISM
HisF
Imidazole-glycerol-3P
AICAR
SAICAR
PurC
PURINE METABOLISM
SrlD
D-Sorbitol-6P
D-Sorbitol
FRUCTOSE AND MANNOSE METABOLISM
PYRUVATE
AceE
2-Hydroxy-ethyl-ThPP
PYRUVATE METABOLISM
Serine
GlyA
GLYCINE, SERINE AND THREONINE METABOLISM
Glycine
PepB and PepN
PepB
PepN
L-Cysteinyl-glycine
Glutathione (GSH)
GLUTATHIONE METABOLISM
L-Serine
O-Acetyl-L-Serine
Sulfide
CysK
L-Cysteine
CYSTEINE AND METHIONINE METABOLISM

Figure 3.8: *Schematic metabolic pathway chart containing identified proteins exhibiting differential expression between strains. Diagram bars represent the protein expression levels displayed as normalised log spot volumes. Results represent the mean values from 4 replicate gels with standard errors.*

SrlD, a protein involved in the fructose and mannose metabolism, is upregulated in LMG2024. This protein, involved in the sorbitol metabolism, is important for *E. amylovora* for surviving inside the host and therefore important for virulence. Since sorbitol is the main transport sugar in apple and pear, disruption of the sorbitol uptake results in loss of pathogenicity in apple seedlings (Aldridge *et al.*, 1997). It forms the connection between the fructose and mannose metabolism, the glycolysis, and several pathways involved in biosynthesis of amino acids. These results were not reflected in the results obtained from the RT-qPCR. The latter showed no significant difference between the four strains.

The proteomic results suggested that the less virulent strain, LMG2024, had a higher production of flagellin than the more virulent strain. FliC, which is the subunit of the flagellar filament, had been identified in multiple spots and in spot 546 even with a fold change of 3.2 in comparison with PFB5. The identification of the same protein in multiple spots can be explained by the fact that proteins become degraded, processed or post-translationally modified. Such modifications lead to products of the same protein but with different pI and/or molecular weights. In pathogenic bacteria, flagella contribute to virulence through chemotaxis, adhesion and invasion of host surfaces. In contradiction, other studies state that this flagellin contains a pathogen-associated molecular pattern (PAMP), namely the flg22 epitope that is recognized by the flagellin-sensitive 2 (FLS2) receptor kinase, which functions as pattern recognition receptor (PRR) (Gomez-Gomez & Boller, 2000; Boller & Felix, 2009; Sun *et al.*, 2013). Treatment of *Arabidopsis* with flagellin leads to the production of ROS, ethylene and the induction of the general defense of the plant (Gomez-Gomez & Boller, 2000). Normally, interaction between *E. amylovora* and a host plant results in an incompatible reaction leading to ROS production which seems necessary for a successful bacterial infection (Venisse *et al.*, 2001; Vrancken *et al.*, 2013b). This oxidative burst is elicited by the injection of both HrpN and DspA/E by the pathogen (Venisse *et al.*, 2003). Both HrpN and DspA/E together with HrpJ are type III effectors (T3Es) secreted by the T3SS. These T3Es have been identified as being necessary for pathogenicity in host plants (Gaudriault *et*

al., 1997; Bogdanove *et al.*, 1998; Nissinen *et al.*, 2007; Lee *et al.*, 2010). Furthermore, the expression of DspA/E has been proven to be important for virulence between different isolates of *E. amylovora* (Lee *et al.*, 2010). As LMG2024 seemed less capable in starting and sustaining a strong infection, a hypothesis could be that the flagellin acts as a nonspecific elicitor of the defense mechanisms of the host (Felix *et al.*, 1999; Venisse *et al.*, 2002), causing a faster recognition of the pathogen in comparison with the higher virulent strain, PFB5. This poses a problem for the pathogen since its presence is already noticed before they could induce the ROS production and the plant defenses are already switched on. Because of the interesting findings concerning this flagellin, a swarming motility assay and RT-qPCR on the corresponding gene were performed. Swarming is a form of multicellular swimming and during this process, hyper flagellated swarm cells are formed (Fraser & Hughes, 1999). Results from both experiments confirm a better swarming capacity and a higher expression of the flagellin gene in the lowest virulent strain.

Another protein involved in motility, CheY, was found to be more abundant in the lowly virulent strain in both proteomic and gene expression analyses. This protein is located near the flagellar motor and can change the direction of the flagellar rotation (Bischoff *et al.*, 1993; Bren & Eisenbach, 1998; Sarkar *et al.*, 2010). Together with CheA, CheY forms a two-component regulatory system in *B. subtilis* which is involved in signal transduction of chemotaxis and interacts with the flagellar motor complex to cause a counterclockwise rotation of the flagella (Bischoff *et al.*, 1993; Garrity & Ordal, 1995; Garrity & Ordal, 1997). The gene expression of both, *cheY1* and *cheA1*, suggests a higher transcription for both of these genes in LMG2024, which again implies of a better motility of the lowest virulent strain. CheY has also been identified as repressor of adherence and invasion in *Campylobacter jejuni* (Yao *et al.*, 1997) which could also contribute to the lower virulent ability of LMG2024.

Here we showed in a classical turbidity assay that amylovoran production of PFB5 and BG16 was much higher than that of LMG2024 and PD437, a feature also reflected in a higher expression of GalF in the highly virulent strain. Results from the gene expression showed a significant difference in UDP-glucose production between the less virulent strains and BG16. No significant difference could be found between LMG2024 and PFB5 which could be the result of a

diminished need of UDP-glucose in this strain in comparison with BG16. Another gene important in amylovoran production, UDP-glucose 4-epimerase or *galE*, was also tested. Results were similar as for *galF*. Since both strains produce comparable amounts of amylovoran (Figure 3.6A), other proteins could be involved in this process not identified by proteomics, which were therefore not tested by RT-qPCR. Results show that LMG2024 was not able to produce high amounts of amylovoran, so it seems plausible that there must be an underlying mechanism responsible for the better swarm capability of LMG2024 compared to PFB5. As recently been found, both positive (EnvZ/OmpR) and negative (GrrSA) regulators can have an influence on swarming motility in these two strains (Zhao *et al.*, 2009b) although these proteins were not identified during the protein identifications. Romeiro has suggested that EPS are barely immunogenic, allowing the bacteria to elude host recognition and escape host defenses (Romeiro *et al.*, 1981) and it also has been suggested that amylovoran masks cell surface components that could induce plant defenses (Eastgate, 2000). This can also give an indication why PFB5, which produces higher amounts amylovoran than LMG2024, is more equipped to evade the defense mechanisms of the plant. However, this is in contradiction to the hypothesis that swarmer cells require cellular components such as EPS for their mass migration (McCarter, 2006). The recent study by Wu *et al.* (2013) should also be mentioned here. Although they did not give an explanation to the differential virulence between strains of *E. amylovora*, they suggested that protein lysine acetylation or other post-translational modifications can be involved in this phenomenon (Wu *et al.*, 2013).

3.6 Conclusions

During this research, strains of *E. amylovora* with a difference in virulence were compared in a standardized and controlled environment. This approach allows us to identify differences expressed before interactions with the host and lost after infection. Since *E. amylovora* is nonmotile after entrance in the plant apoplast (Raymundo & Ries, 1980b), no information can be deducted after an infection about motility. Our results give an overview of differences between these strains before interaction with a host has occurred. A higher flagellin content has been found in the less virulent strains. This could imply a better motility of these

strains since flagella are necessary for invasion of apple blossom (Bayot & Ries, 1986). In contrast, flagella contain conserved domains which are recognized by the plant defense system (Gomez-Gomez & Boller, 2000; Boller & Felix, 2009; Sun *et al.*, 2013). Gene expression and phenotypic characterization confirm these results which state that more flagellin is produced in LMG2024 and this can be one of the main reasons for the lower virulent capability of this strain. This in combination with a lower amylovoran content that would normally mask cell surface components, makes LMG2024 a target for recognition by the host defense mechanisms before it is able to inject its effectors into the plant cells. Differences in metabolic demands lead to upregulation of different metabolic pathways which can also be important in virulence. The next step in this research will be to investigate how strains of *E. amylovora* differing in virulence behave in *in planta* conditions. A similar comparative analysis of the proteome of these four strains is in progress.

3.7 Supplementary data

Table S.3.1: *All identified spots which were more abundant for LMG2024 in comparison with PFB5.*

Protein spot number	Name
Spot 17	hypothetical protein ECA3659
	type III secretion apparatus
Spot 49	pyruvate dehydrogenase E1 component
Spot 55	aminopeptidase N
Spot 132	dipeptidyl carboxypeptidase II
	ferrioxamine receptor
Spot 135	molecular chaperone DnaK
	chemotaxis protein CheA
	transketolase
Spot 151	transketolase
Spot 190	glucosamine--fructose-6-phosphate aminotransferase
	sulfite reductase (NADPH) flavoprotein alpha-component
Spot 259	glucans biosynthesis protein G
Spot 270	ketol-acid reductoisomerase
	ATP synthase F1 subunit alpha
Spot 299	argininosuccinate lyase
Spot 340	phosphopyruvate hydratase
	argininosuccinate synthase
	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
	elongation factor Tu
Spot 341	serine hydroxymethyltransferase
Spot 368	aminopeptidase B
Spot 405	phosphoglycerate kinase
	hypothetical protein EpC_15080
	DNA-directed RNA polymerase subunit alpha
Spot 416	phospho-2-dehydro-3-deoxyheptonate aldolase
Spot 424	hypothetical protein EAMY_1364
	phosphoribosylformylglycinamidine cyclo-ligase
Spot 472	glyceraldehyde 3-phosphate dehydrogenase A
	Octaprenyl diphosphate synthase
	thioredoxin-like protein
Spot 485	elongation factor EF-Ts
	transaldolase B
	glyceraldehyde 3-phosphate dehydrogenase A
	chemotaxis signal transduction protein
Spot 490	cysteine synthase A
	ATP phosphoribosyltransferase
	glyceraldehyde 3-phosphate dehydrogenase A
	glucokinase
Spot 534	2,3,4,5-tetrahydropyridine-2,6-carboxylate N-succinyltransferase

Table S.3.1 (continued): *All identified spots which were more abundant for LMG2024 in comparison with PFB5.*

Protein spot number	Name
Spot 546	flagellin, filament structural protein FliC
Spot 550	N5-glutamine S-adenosyl-L-methionine-dependent methyltransferase
	cell division inhibitor MinD
	FeS assembly ATPase SufC
Spot 552	flagellin, filament structural protein FliC
Spot 591	phosphoribosylaminoimidazolesuccinocarboxamide synthase
Spot 602	sorbitol-6-phosphate dehydrogenase
Spot 630	heat shock protein GrpE
	FKBP-type peptidyl-prolyl cis-trans isomerase
Spot 653	hypothetical protein EAMY_0645
	ribose 5-phosphate isomerase A
Spot 718	shikimate kinase I
Spot 801	chemotaxis regulatory protein CheY
Spot 839	cold shock-like protein CspC
	cold shock protein CspE
Spot 844	cold shock protein CspE
	cold shock-like protein CspC
Spot 846	cold shock protein CspE
	cold shock-like protein CspC
Spot 847	cold shock protein CspE
Spot 851	cold shock-like protein cspE (CSP-E)
	50S ribosomal protein L33
	cold shock protein CspE

Table S.3.2: *All identified spots which were more abundant for PFB5 in comparison with LMG2024.*

Protein spot number	Name
Spot 70	ATP-dependent Clp protease, ATP-binding subunit ClpB
	hypothetical protein EAMY_2751
Spot 122	dipeptidyl carboxypeptidase II
Spot 124	dipeptidyl carboxypeptidase II
Spot 141	F0F1 ATP synthase subunit beta
Spot 142	transketolase
Spot 149	transketolase
Spot 160	PrkA serine kinase
Spot 213	phosphoenolpyruvate carboxykinase
	molecular chaperone GroEL
Spot 216	molecular chaperone GroEL
	phosphoenolpyruvate carboxykinase
Spot 221	glutamine synthetase
	serine/threonine protein kinase
	phosphoglucomutase
Spot 223	hypothetical protein ETA_06180
	phosphoglucomutase
	glutamine synthetase
	serine/threonine protein kinase
Spot 271	HrpW protein
	dihydrolipoamide dehydrogenase
Spot 354	hrp-associated systemic virulence protein HsvB
	isocitrate dehydrogenase
	protein chain elongation factor EF-Tu
	ribonucleotide-diphosphate reductase subunit beta
Spot 378	hypothetical protein EAMY_3322
Spot 387	3-phosphoserine aminotransferase
	fructose-bisphosphate aldolase
Spot 415	succinyl-CoA synthetase subunit beta
	aspartate aminotransferase
	phosphoglycerate kinase
	N-succinyl-diaminopimelate deacylase
	UDP-glucose 4-epimerase
Spot 444	transaldolase A
Spot 474	cysteine synthase A
	UTP-glucose-1-phosphate uridylyltransferase
	transaldolase B
	glutathione synthetase
Spot 475	UTP-glucose-1-phosphate uridylyltransferase
Spot 476	cysteine synthase A
Spot 571	imidazole glycerol phosphate synthase subunit
Spot 589	sorbitol-6-phosphate dehydrogenase
	triosephosphate isomerase

Table S.3.2 (continued): *All identified spots which were more abundant for PFB5 in comparison with LMG2024.*

Protein spot number	Name
Spot 766	elongation factor Tu 2
	50S ribosomal protein L9
Spot 788	10 kDa chaperonin (protein Cpn10)
Spot 789	nucleoside diphosphate kinase
Spot 800	nitrogen regulatory protein P-II 1

Table S.3.3: *Quantitative PCR (qPCR) parameters according to the Minimum Information for publication of qPCR Experiments (MIQE) checklist derived from Bustin et al. (2009).*

Sample/Template	
Source	*Erwinia amylovora* cells in liquid MM2 medium
Method of preservation	Fresh samples were taken and immediately processed
Storage time	None
Handling	Liquid supplemented with 2 volumes of RNAprotect Bacteria Reagent* (Qiagen, Venlo, The Netherlands).
Extraction method	RNeasy Mini Kit* (Qiagen, Venlo, The Netherlands)
RNA: DNA-free	TURBO DNA-free Kit* (Ambion, Lennik, Belgium)
Concentration	NanoDrop®: ND-1000 Spectrofotometer (Isogen Life Science, IJsselstein, the Netherlands)
RNA: integrity	Not tested; RNAprotect Bacteria Reagent* (Qiagen, Venlo,The Netherlands).
Assay optimisation/validation	
Accession number	See Table 3.2
Amplicon details	Amplicon size see Table 3.2
Primer sequence	See Table 3.2
In silico	Primer-BLAST
Empirical	Primer concentration (300 nM), annealing temperature (60°C)
Priming conditions	Random hexamer priming
PCR efficiency	Dilution curves
Linear dynamic range	Samples are within the efficiency curve
RT/PCR	
Protocols	See Materials and Methods
Reagents	See Materials and Methods
NTC	C_q & melt curves
Data analysis	
Specialist software	7500 Fast System Sequence Detection Software, version 1.4 (Applied Biosystems, Lennik, Belgium, 2001-2006)
Statistical justification	4 biological replicates, one-way ANOVA
Normalisation	3 reference genes selected using GrayNorm

*All procedures were performed according to the manufacturer's protocol.

4

Proteome analysis of wild type strains of *Erwinia amylovora* grown *in planta*

Research article – in preparation

4.1 Abstract

Erwinia amylovora, a Gram-negative plant pathogen belonging to the *Enterobactericeae*, is responsible for the devastating disease fire blight. This disease affects most members of *Maloideae* of the family *Rosaceae* including apple and pear. Here, a unique *in planta* infection model is introduced to study proteomic changes in a highly virulent *E. amylovora* strain upon interaction with its host as compared to a much lower virulent strain. For this purpose separate shoots of apple rootstocks were wound-infected with one of both strains. Viable bacterial cells were isolated from the plant tissue when infection became systemic and processed in an analytical platform combining 2-D fluorescence difference gel electrophoresis and mass spectrometry. In general, the higher virulent strain showed a higher abundance of proteins involved in carbohydrate, amino acid and fatty acid metabolism. Sorbitol metabolism and amylovoran synthesis were both up-regulated and several features related to protection against the defense mechanisms of the host were identified as well. The lower virulent strain had a higher abundance of cold shock proteins, whereas more heat shock proteins were produced in the most infectious one. Furthermore, we identified a function for different components of the RNA degradosome including PNPase and Hfq in virulence for the higher virulent strain. Finally, changes found in protein abundance showed good accordance at the transcript level, as was verified by quantitative real-time PCR on both bacterial isolates.

In conclusion, this *in planta* infection model allowed the characterization of important changes in the proteome of *E. amylovora* related to differences in virulence. This model may be a valuable tool to study the complexity of plant-pathogen interactions.

4.2 Introduction

Fire blight, caused by the Gram-negative bacterium *Erwinia amylovora*, affects most members of the *Rosaceae* family including apple (*Malus* spp.), pear (*Pyrus* spp.) and ornamentals such as *Cotoneaster* and *Pyracantha* spp. This devastating disease is spread by wind, insects, birds and human activity. In nature, infection is mostly initiated by the entry of this bacterium in flowers. Other infection sites are natural openings such as nectaries, stomata or wounds

on the plant surface. *E. amylovora* is characterized by its fast multiplication and rapid dispersion throughout the plant via the vascular tissue and causes necrosis of the plant tissue or it can reside in symptomless tissue (Vanneste, 2000b; Malnoy *et al.*, 2012). When the pathogen has reached the interior of the plant, they may be recognized by microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs). These PAMPs include lipopolysaccharides (LPS), elongation factor Tu, cold-shock proteins, peptidoglycans and flagellin (Dow *et al.*, 2000; Gerber *et al.*, 2004; Bittel & Robatzek, 2007; Gust *et al.*, 2007). These PAMPs may be recognized by pattern recognition receptors (PRRs) of the host which can lead to an PAMP-initiated immuntity (PTI) (Jones & Dangl, 2006). When *E. amylovora* enters its host plant, the bacteria are detected as incompatible pathogens and the plant initiates a first line of defense consisting of the production of reactive oxygen species (ROS) including $O_2^{\cdot -}$, H_2O_2 and $OH^{\cdot}$ to kill the plant cells, resulting in further lipid peroxidation, electrolyte leakage and modulation in the antioxidant status (Venisse *et al.*, 2001; Venisse *et al.*, 2003). Surrounding plant cells will gain a localized acquired resistance that accounts for a cell wall reinforcement, accumulation of phytoalexins and the activation of a broad spectrum of defense proteins such as antioxidant enzymes and pathogenesis-related (PR) proteins (van Loon *et al.*, 2006; Vrancken *et al.*, 2013a; Vrancken *et al.*, 2013b). When synthesized throughout the plant, these PR proteins can lead to a systemic acquired resistance (SAR) which forms the third line of defense (Venisse *et al.*, 2002). During the plant-pathogen interaction of *E. amylovora* and its host, the Hrp effectors HrpN and DspA/E of the pathogen are responsible for the elicitation of this oxidative burst (Venisse *et al.*, 2003). These Hrp effectors are delivered into the host cytoplasma by a type III secretion system (T3SS) encoded by the hypersensitive response and pathogenicity (*hrp*) genes located within the pathogenicity island (PAI) (Oh & Beer, 2005; Buttner & He, 2009). This T3SS, together with amylovoran, are very important virulence factors of *E. amylovora*. Next, the pathogen seems to cope with this oxidative burst, as it is even exploited by the pathogen for establishing a successful invasion and colonization of the host plant. Until now, only three different elements have been identified as being important in survival of the oxidative burst including the exopolysaccharide (EPS) amylovoran (Venisse *et al.*, 2001; Geider, 2006), the siderophore desferrioxamine (Venisse

et al., 2003) and the major molecular component of the outer membrane of Gram-negative bacteria, lipopolysaccharides (Berry *et al.*, 2009). Amylovoran, the main component of bacterial ooze (Bennett & Billing, 1980), is an important virulence factor. Amylovoran is a hetero-polysaccharide primarily consisting of galactose and glucuronic acid residues (Nimtz *et al.*, 1996; Maes *et al.*, 2001). Most of the structural genes necessary for amylovoran biosynthesis are located on the *ams* gene cluster (Bernhard *et al.*, 1993; Bugert & Geider, 1995). Two genes *galE* and *galF*, located on the right of the *ams* gene cluster, are important in the formation of the precursors, UDP-galactose and UDP-glucose, respectively (Metzger *et al.*, 1994; Bugert & Geider, 1995). Moreover, strains not capable of producing amylovoran appear to be non-pathogenic (Steinberger & Beer, 1988), are not able to migrate through plant vessels (Bogs *et al.*, 1998) and they do not multiply inside the host (Bellemann & Geider, 1992).

On the other hand, siderophores are produced to cope with low iron availability and general nutrient limitations inside the host. The pathogen produces siderophores of the hydroxamate type, belonging to the desferrioxamines (DFOs), primarily DFO E and a specific receptor FoxR (Kachadourian *et al.*, 1996; Dellagi *et al.*, 1998). It has been suggested that desferrioxamine is necessary for the pathogen to survive high levels of hydrogen peroxide (Venisse *et al.*, 2003). Last but not least, lipopolysaccharides (LPS) form the major component of the outer membrane of Gram-negative bacteria and are composed of three structural units: lipid A, which consists of a hydrophobic domain, a core oligosaccharide and a distal polysaccharide, the O-antigen (Raetz & Whitfield, 2002). A mutation in a gene involved in LPS biosynthesis, *waaL*, has proven a function of this LPS in the protection against hydrogen peroxide and thus against oxidative stress (Berry *et al.*, 2009).

Recently, Hfq was identified as being important in virulence of *E. amylovora* by regulation of small RNAs (sRNAs) (Zeng *et al.*, 2013). The sRNAs are regulated by Hfq target specific mRNAs in the cell, thereby exerting a function in the posttranscriptional regulation of gene expression (Chao & Vogel, 2010). Hfq functions as a chaperone to stimulate pairing between the sRNAs and their target mRNAs (Gottesman & Storz, 2011). Many genes functioning in different processes have been found to be controlled by Hfq including stress tolerance, LPS synthesis, bacterial outer membrane proteins, motility and sugar, nitrogen

and fatty acid metabolism (Chao & Vogel, 2010). In *Escherichia coli* for instance, Hfq forms a minor component of the degradosome which plays an important role in RNA processing and decay (Aiba, 2007; Kaberdin & Lin-Chao, 2009).

Many genomic studies have identified different aspects of virulence in *E. amylovora* (Zhao *et al.*, 2005; Berry *et al.*, 2009; Zhao *et al.*, 2009a; Zhao *et al.*, 2009b; McNally *et al.*, 2012). These studies have provided us with evidence that different virulence factors are crucial for this plant pathogen. Although *E. amylovora* is considered a relatively homogeneous species (Vanneste, 2000a; Triplett *et al.*, 2006; Smits *et al.*, 2010; Wang *et al.*, 2010), different strains have been isolated from nature exhibiting differences in virulence (Cabrefiga & Montesinos, 2005; Wang *et al.*, 2010). A comparative proteomic research *in vitro* indicated that hardly virulent strains produce more proteins involved in motility, flagellin (FliC) and a chemotaxis regulatory protein (CheY) in comparison with a higher virulent strain, while this higher virulent strain produces higher amounts of amylovoran (Chapter 3). Until now, no studies are available concerning the proteome of *E. amylovora* when infecting and colonizing a host plant. Furthermore, only limited proteome data as such are available for other plant pathogens grown in an *in planta* model (Mehta & Rosato, 2003; Andrade *et al.*, 2008). We suggest a method to isolate viable cells from plant tissue and a subsequent extraction of proteins from these samples without interference of plant proteins.

4.3 Materials and methods

4.3.1 Bacterial strains, media and growth conditions

The bacterial strains used in this study are listed in Table 4.1. Four strains of *E. amylovora*, differing in virulence, were used (Chapter 3). Strains were stored at -80 °C in 10% glycerol and cultured on yeast peptone glucose agar (YPGA) plates at 24 °C.

Table 4.1: *Bacterial strains used in this study*

Strain	Isolated from	General virulence	Reference
LMG2024	*Pyrus communis*	+	Hauben *et al.,* 1998 ; Chapter 3
PD437	*Pyrus communis*	++	Maes *et al.,* 2001; Chapter 3
BG16	*Malus sp.*	++++	Maes *et al.,* 2001; Chapter 3
PFB5	*Prunus salicina*	++++	Maes *et al.,* 2001; Chapter 3

4.3.2 Bacterial growth and isolation

For the extraction of viable bacterial cells from plant tissue, apple rootstocks were used as host (Malling 9 clone T337) obtained from Carolus Trees (Sint-Truiden, Belgium). Bacteria from overnight cultures on YPGA, were suspended in phosphate buffered saline (PBS) with a density of 1 x 10^8 CFU/ml. The two youngest apple leaves from each shoot were cut perpendicularly to the midvein using scissors dipped in the bacterial suspension. Sampling occurred when infection was systemic, approximately 10 to 14 days after inoculation depending on the used strain. In general, shoots infected with lower virulent strains needed more time before infection became systemic in comparison with higher virulent ones.

The bacteria were extracted from the plant tissue as described by Maes *et al.,* (2009) with slight modifications. From every rootstock, the infected shoots were removed. After removal from the rootstocks, the infected shoots and leaves were thoroughly sterilized by submerging them for 5 sec in 1% sodium hypochlorite. Next, they were rinsed three times using sterile water. The shoots and leaves were cut in 0.5 cm pieces and extracted for 40 - 90 min in 250 ml of a sterile buffer (120 mM phosphate buffer, pH 8, 0.1% w/v sodium pyrophosphate, 0.1% v/v Tween-20, 25% w/v polyvinylpolypyrrolidone), while shaken (200 rpm, room temperature) in the presence of glass beads (0.1 mm – 1 mm). After filtration through 2 Wattman filters (pore size 8 µm), the extract was centrifuged (8000 xg, 15 min, 4 °C) to pellet the bacteria. Hereafter, the bacteria were washed three times with PBS.

4.3.3 Protein extraction

Protein extraction was performed as described previously (Chapter 3). In short, the washed pellet was lysed using a sample solution containing 7 M urea, 2 M thio-urea and 4% (w/v) CHAPS. The lysate was sonicated for 1 min on ice using a microtip and afterwards shaked (120 rpm) for 30 min on ice. The homogenate was centrifuged (76 000 g for 90 min) to remove the cellular debris and the supernatant containing the protein fraction was used for further analysis. After the pH was adjusted to 8.5 using 100mM NaOH, the protein concentration was determined using the 2-D Quant kit (GE Healthcare) according to the instructions of the manufacturer.

4.3.4 CyDye labeling and 2-D gel electrophoresis

The procedures have been described (Chapter 3). In brief, protein samples were minimally labeled using cyanine-derived fluors (3 Dyes 2-D Cyanine Labeling kit From Proteomics Consult). Cy3 and Cy5 were used for labeling 25 µg from each protein sample while Cy2 was used to label the pooled internal standard, representing equal amounts of all protein samples. Individually labeled samples were mixed according the experimental design and each gel was loaded with 75 µg of proteins including 25 µg from the sample labeled with Cy3, 25 µg from the Cy5 labeled sample and 25 µg from the Cy2 labeled internal standard. During this experiment, four biological replicates per strain were considered. Next, the labeled samples were diluted with lysis buffer (0,005% bromophenol blue, 90 mM DTT and 2% IPG-Ampholyte mix (SERVA)) to 120 µl.

Precast immobilized pH gradient (IPG) strips (pH 3-10, 24 cm, SERVA) were used for separation in the first dimension. Before starting, these strips were rehydrated in IPG-enriched (5 µl/ml) Destreak (GE Healthcare) for at least 8h. The protein samples were loaded onto the IPG strips using an IPGphor isoelectric focusing apparatus (GE Healthcare). Proteins were applied on the strips via anodic cuploading using the following settings: 250 V for 1 h, 1000 V (gradient) for 7 h, 8000 V for 3 h and 8000 V (gradient) for 3 h 45 min for a total of 49,2 kVh (50 µA/strip, 20 °C). Strips were frozen at -20 °C until use.

The second dimension was started by equilibration of IPG strips, first the strips were shaken for 15 min in an equilibration buffer (SERVA) containing DTT. Next the solution was replaced by the same equilibration buffer but now containing iodoacetamide and the strips were again shaken for 15 min. For the separation in second dimension a HPE-FlatTop Tower (SERVA) was used. Plastic-backed 10-15% polyacrylamide gels (2D-Large-Gel Flatbed NF 10-15% gradient gels) were used and four gels were run simultaneously according following settings: 30 min at 100 V with 7 mA/gel and 1 W/gel; 30 min at 200 V with 13 mA/gel and 3 W/gel; 10 min at 300 V with 20 mA/gel and 5 W/gel; 4 h 50 min at 1500 V with 40 mA/gel and 30 W/gel and finally 50 min at 1500 V with 45 mA/gel and 40 W/gel. When separation was accomplished, gels were fixed overnight in 1% citric acid and 15% ethanol.

4.3.5 Gel imaging and data analysis

Next, the gels were scanned at 100 µm (pixel size) resolution using an Ettan DIGE Imager (GE Healthcare). Image analysis was accomplished using the Progenesis SameSpots software version 4.5. After the quality of the gel images was secured, spots were automatically detected by the software. Next, automatic alignment was performed followed by a manual editing. Statistical analysis of protein abundance was performed on four gels for each condition. Differences among matched spot intensities were statistically validated by performing an ANOVA at a 5% significance level. By calculating a power analysis, which represents the probability of finding a significant expression change where it exists and using a target power of 0.8, we could choose the fold change below 2.0 without increasing the risk of including false positive spots. The spots considered during this study, were spots with at least 1.5-fold changes in volume ($P < 0.05$) after normalization, a P-value > 80% and a q-value < 0.05. The spots of interest were located and were excised from the gels using a semi-automated Screen picker (made by Proteomics Consult). Hereafter, the gel plugs were processed for mass spectrometry (Shevchenko *et al.*, 1996).

4.3.6 LC-MS/MS analysis

An Easy-nLC 1000 liquid chromatograph (Thermo Scientific) was on-line coupled to a mass calibrated LTQ-Orbitrap Velos Pro (Thermo Scientific) via a Nanospray Flex ion source (Thermo Scientific) using sleeved 30 µm ID stainless steel emitters (spray voltage +2.3 kV, capillary temperature: 200 °C). The SpeedVac dried tryptic peptide mixture was dissolved in 20 µl buffer A (0.1% v/v formic acid in Milli-Q water) of which half was loaded, concentrated and desalted on a trapping pre-column (Acclaim PepMap 100 C_{18}, 75 µm ID × 2 cm nanoViper, 3 µm, 100 Å, Thermo Scientific) at a buffer A flow rate of 5 µl/min for 5 minutes. The peptide mixture was separated on an Acclaim PepMap RSLC C_{18} column (50 µm ID x 15 cm nanoViper, 2 µm, 100 Å, Thermo Scientific) at a flow rate of 250 nL/min with a linear gradient in 40 minutes of 0 to 70% buffer B (0.1% v/v formic acid in acetonitrile) in buffer A.

MS data were acquired in a data-dependent mode under direct control of the Xcalibur software (version 2.2.SP1.48), selecting the fragmentation events based on the top six precursor abundances in the survey scan (350–2000 Th).

The resolution of the full scan was 30000 at 400 Th with a target value of $1x10^6$ ions and one microscan. CID MS/MS spectra were acquired with a target value of 10000 and the maximum injection time was 100 ms. Dynamic exclusion was 30 s and early expiration was disabled. The isolation window for MS/MS fragmentation was set to 2 Th and the normalised collision energy, Q-value and activation time were 30%, 0.25 and 10 ms, respectively. Helium was used as the collision gas.

4.3.7 Data analysis

The analysis of the mass spectrometric raw data was carried out using Proteome Discoverer software v.1.2 (Thermo Scientific) with build-in Sequest v.1.3.0339 and interfaced with an in-house Mascot v.2.4 server (Matrix Science). MS/MS spectra were searched against the *Erwinia* protein collection extracted from NCBI database (query '*Erwinia*' on March 13th 2013; 104711 entries) and peptide scoring for identification was based on following search criteria: enzyme trypsin, maximum missed cleavages 2, precursor mass tolerance 10 ppm and fragment mass tolerance 0.5 Da. Carbamidomethylation of cysteine and oxidation of methionine were set as fixed and dynamic modifications, respectively.

Result files of both search engines were uploaded and automatically evaluated in Scaffold v.3.6.1 (Proteome Software) using the Peptide Prophet and Protein Prophet algorithm with a preset minimal peptide and protein identification probability of 95% and 99%, respectively.

4.3.8 RNA extraction and quantitative RT-PCR

Frozen samples containing bacteria extracted from the plant tissue were thawed and supplemented with 2 volumes of RNAprotect Bacteria Reagent (Qiagen, Venlo, The Netherlands). After an incubation period of 5 min, bacteria were collected by centrifugation (5000 g, 10 min) and RNA was extracted using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Next, RNA was further purified and concentrated by a Na-Acetate and ethanol precipitation. The TURBO DNA-free kit (Ambion) was used to remove DNA and final reverse transcription was carried out from 1 µg of DNase-treated total RNA using the PrimeScript RT Reagens Kit (Takara). The

cDNA samples were ten-fold diluted using 1/10 diluted TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored at -20 °C until use. Quantitative PCR (qPCR) was performed using Fast SYBR Green chemistry according to the manufacturer's instructions on an ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems, Belgium). Relative gene expression was calculated as $2^{-\Delta Cq}$ and was normalised with a normalization factor determined by the GrayNorm algorithm (Remans *et al.*, 2014). In total six reference genes were considered including *recA, gyrA*, *rpoS* (Waleron *et al.*, 2008), *rpoD*, *proC* (Savli *et al.*, 2003) and *rpsL* (Dumas *et al.*, 2006). These reference genes were tested using the GrayNorm algorithm. Following the GrayNorm output, the normalisation factor was based on the expression of the following reference genes: *rpsL*, *rpoD* and *proC*. Both normalised and non-normalised data were presented as an accuracy interval according to Remans *et al.* (2014). Gene-specific primers based on proteins of interest indicated by the proteomics study were developed using Primer3 (Whitehead Institue/MIT Center for Genome Research). Used primers are listed in Table S.4.3. Reverse transcription quantitative PCR (RTqPCR) parameters were measured and determined according to the Minimum Information for publication of RTqPCR Experiments (MIQE) guidelines derived from Bustin *et al.* (2010) (Table S.4.4).

4.3.9 Growth curves

A bacterial suspension of 1 x 10^8 CFU/ml was made in PBS which was diluted 100 times in sterile MM2-medium. Bacteria were grown for 80 h at 28 °C while shaking (120 rpm). Turbidity was measured every 2 hours at 600 nm using a FLUOstar Omega plate reader (BMG Labtech). The experiment was repeated 5 times with 3 technical repetitions.

4.4 Results

4.4.1 DIGE of the proteome of different strains of E. amylovora

Separate shoots of the apple rootstocks were infected with one of four *E. amylovora* WT strains (Table 4.1). Bacteria were isolated from the infected shoots and leaves at the time a systemic infection was reached (10 – 14 days depending on the strain used). The differences between the 2D-electrophoretic profiles of the four different strains were validated by a PCA (Figure 4.1). Three groups can be distinguished. Because the mutual differences between the high virulent strains, PFB5 and BG16, were very small and the most differences were observed between LMG2024 and the high virulent strains we focused the further research on LMG2024 and PFB5. Furthermore, the overall differences observed between infections is more pronounced between these strains and further, these two strains were already used for an *in vitro* comparative proteomics research (Chapter 3). Using the SameSpots software version 4.5, 120 spots were indicated as being differently expressed between LMG2024 and PFB5 (Figure 4.2) of which 83 resulted in identification of 154 proteins by mass spectrometry (Tables S.4.1 and S.4.2). The proteins were classified into categories according to their biological function as described in the protein knowledge database UniProt and visualized by two pie charts (Figure 4.3). When comparing the overall division of categories for both strains, the low virulent strain expressed more proteins involved in stress response and translation and protein transport in comparison with the higher virulent strain. The latter on the other hand had a higher abundance of proteins involved in carbohydrate and amino acid metabolism. Also proteins involved in RNA processing and the fatty acid metabolism were identified for PFB5. These results indicate important differences in the overall metabolism of both strains.

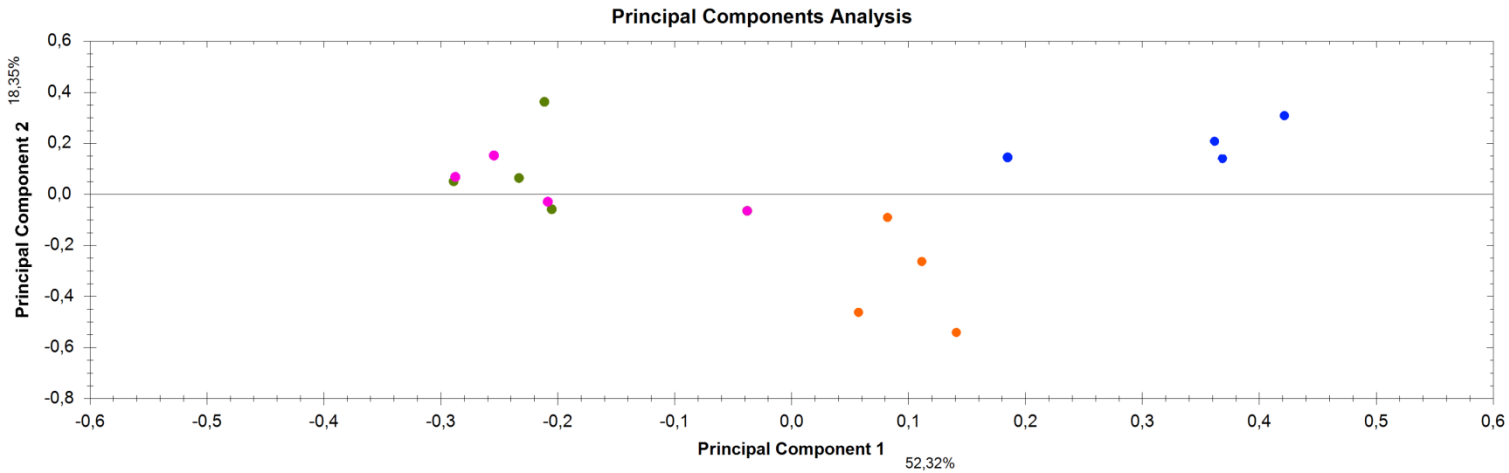


Figure 4.1: *Principal Component Analysis performed on the complete data set of the gels. Different strains are depicted in different colours. Green dots represent PFB5, pink dots BG16, red dots PD437 and blue dots represent LMG2024.*

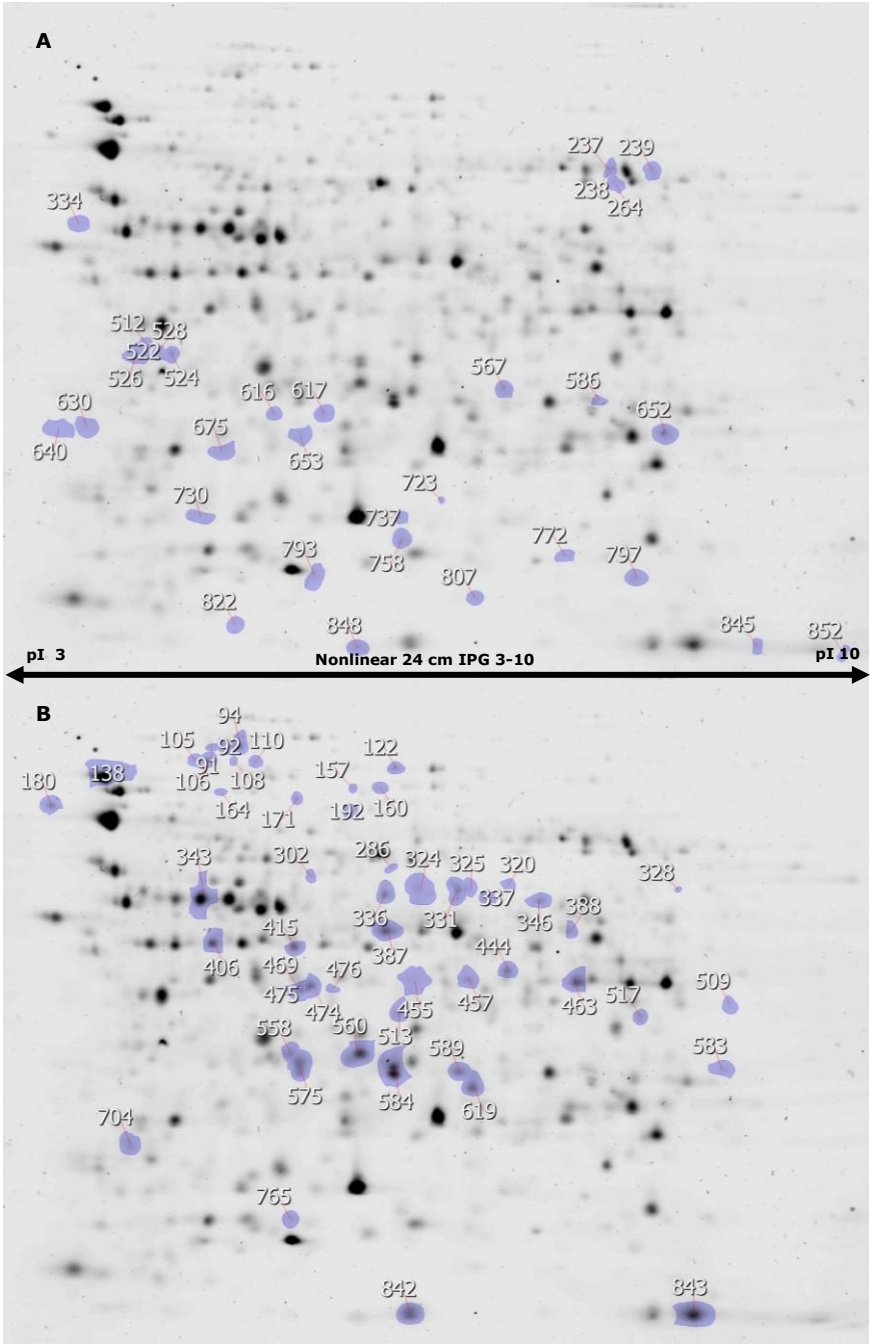


Figure 4.2: *Representative 2-DE gels of E. amylovora proteins. Image represents scan from the internal standard so every protein is present. A. 2-DE gel with spots indicated upregulated in LMG2024 in comparison with PFB5 B. 2-DE gel with spots indicated upregulated in PFB5 in comparison with LMG2024.*

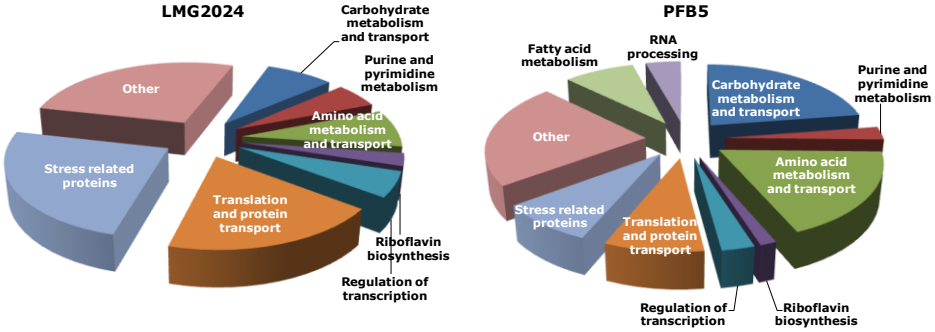


Figure 4.3: *Pie charts representing the biological functions of the identified proteins for both LMG2024 and PFB5. Biological functions were assigned according to information provided by gene ontology tools at UniProtKB.*

4.4.2 Proteins involved in stress response and tolerance

In total 15 proteins involved in stress response were identified as being differently expressed between both strains (Table 4.2), of which 9 were more abundant in LMG2024 and 6 were up-regulated in PFB5. In order to validate these protein expression patterns, genes encoding for these proteins were selected for gene expression profiling (Figure 4.4). For the genes *pspA*, *ahpC*, *yfiA*, *cspE* and *cspA*, the RT-qPCR data confirmed the proteomics data. For the chaperones HtpG, HchA and GrpE the proteomics data suggested an increase in expression of these proteins in the lower virulent strain while gene expression suggested an higher expression of these genes in PFB5. These results indicate that data from proteomics and transcriptomics experiments can be contradictory in some cases. Proteomics data suggested a higher abundance of the chaperone DnaK, the transcription elongation factor (NusA), glutathione oxidoreductase (Gor), glutaredoxin-2 (GrxB), the 10 kDA chaperonin (Cpn10) and the cold shock-like protein (CspC) in PFB5. These results were confirmed by RT-qPCR with exception of *gor* and *cspC* which were more expressed in LMG2024. In *E. coli* DnaK forms a cellular chaperone machinery with GrpE and DnaJ capable of restoring heat-induced protein damage (Schroder *et al.*, 1993) and RT-qPCR results showed upregulation of all three genes for the higher virulent strain. Further research has identified a fourth member cooperating with the DnaK-DnaJ-GrpE complex, ClpB (Zolkiewski, 1999) which is also more abundant in PFB5 in comparison with LMG2024 (Table S.4.1, spot 192). These results showed that PFB5 used primarily heat shock proteins in its defense against

stress while the lower virulent strain exhibits an up-regulation of cold shock proteins CspA, CspE. Although the protein CspC showed a higher abundancy in PFB5 these results are in contradiction with the gene expression results which stated that *cspC* is more expressed in LMG2024.

Table 4.2: *Proteins identified as being involved in stress response*

Spot n°	Protein ID	Name	Gene name	Theoretical pI/MW(kDa)	ANOVA P-value	Fold change
Upregulated proteins in LMG2024						
512[a]	gi|292487505	chaperone protein htpG	*htpG*	5.00 / 71.08	0.003	3.0
522[a]	gi|292487505	chaperone protein htpG	*htpG*	5.00 / 71.08	0.009	2.6
526[a]	gi|292489712	chaperone protein HchA (Hsp31)	*yedU*	5.00 / 30.67	0.002	2.3
616	gi|292488353	phage shock protein A	*pspA*	5.50 / 25.40	7.233e-004	5
617[a]	gi|292488353	phage shock protein A	*pspA*	5.50 / 25.40	1.890e-005	4.6
630[a]	gi|292489107	heat shock protein GrpE	*grpE*	4.66 / 21.67	8.920e-004	1.8
675	gi|291552852	alkyl hydroperoxide reductase subunit C	*ahpC*	5.70 / 22.15	0.004	1.8
723	gi|291552852	alkyl hydroperoxide reductase subunit C	*ahpC*	5.70 / 22.15	0.007	1.8
730	gi|292487760	DNA protection during starvation protein	*dps*	5.18 / 18.82	0.009	2.3
797	gi|188534769	ribosome associated factor, stabilizes ribosomes against dissociation	*yfiA*	6.19 / 12.63	0.003	5.9
845	gi|291553014	cold shock protein CspE	*cspE*	8.09 / 73.80	0.008	1.9
848	gi|292487397	cold shock protein CspG	*cspA*	5.64 / 75.23	0.004	2.9
852[a]	gi|291553014	cold shock protein CspE	*cspE*	8.09 / 73.80	0.002	2.6
Upregulated proteins in PFB5						
138	gi|292489412	molecular chaperone DnaK	*dnaK*	4.82 / 68.66	0.006	1.9
160[a]	gi|292489412	molecular chaperone DnaK	*dnaK*	4.82 / 68.66	0.005	2.7
180	gi|292486836	transcription elongation factor NusA	*nusA*	4.48 / 55.35	0.002	2.3
324[a]	gi|292490004	glutathione oxidoreductase	*gor*	5.71 / 48.86	0.009	1.9
331[a]	gi|292490004	glutathione oxidoreductase	*gor*	5.71 / 48.86	0.002	2.4
619	gi|292487928	glutaredoxin-2	*grxB*	5.73 / 24.0	9.832e-004	2.2
765[a]	gi|291555080	10 kDa chaperonin (protein Cpn10)	*groES*	5.41 / 10.27	0.007	3.0
842[a]	gi|292488490	cold shock-like protein CspC	*cspC*	7.4/ 6.54	1.225e-004	2.6
843	gi|292488490	cold shock-like protein CspC	*cspC*	7.4/ 6.54	0.002	4.6

[a]Indication that multiple proteins were identified in this spot. Complete data set is represented in supplementary data.

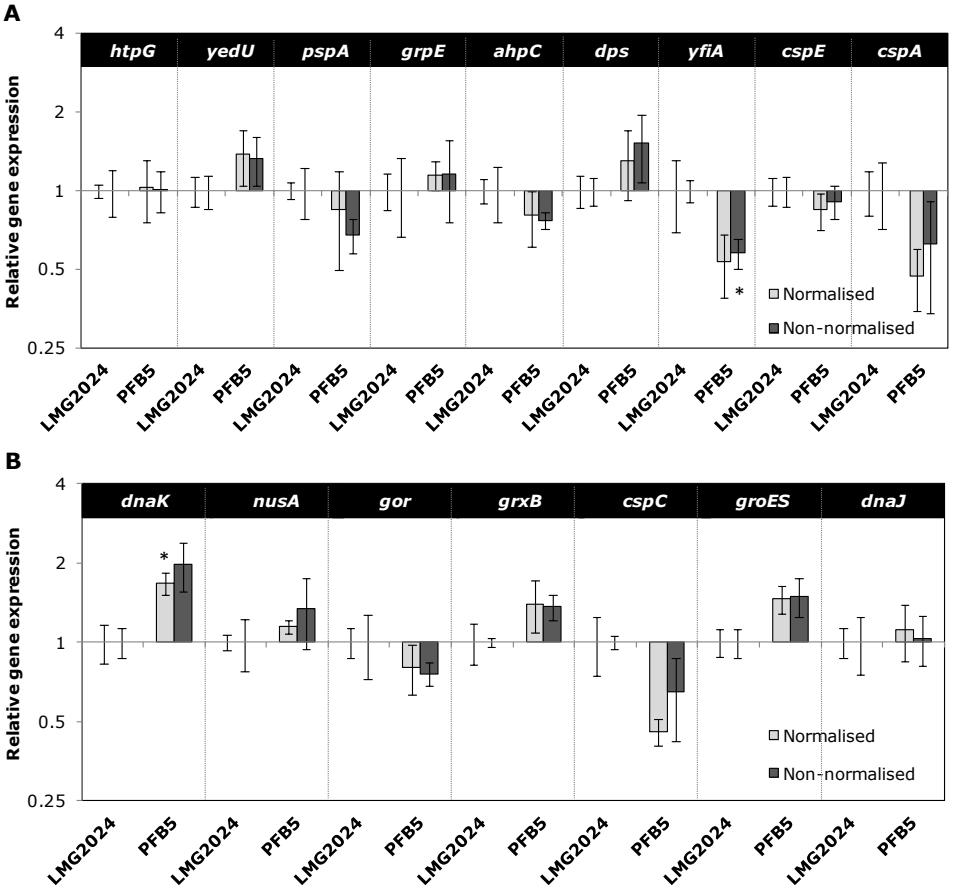


Figure 4.4: *Relative gene expression measured by RT-qPCR for genes involved in stress response. Normalised data are represented in light grey and the non-normalised data in dark grey. Up- or downregulations are represented on a* log_2 *scale y axis relative to the least virulent strain. Columns represent data from four biological replicates ± standard errors. Asterisk (*) indicates a statistically significant difference between strains (P < 0.005, Kruskal-Wallis test with a pairwise Wilcox comparison test). A. Expression of genes corresponding to interesting proteins more abundant in LMG2024. B. Expression of genes corresponding to interesting stress related proteins more abundant in PFB5.*

4.4.3 Proteins involved in RNA processing

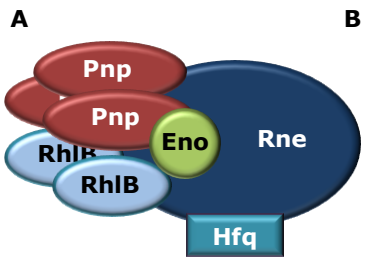
In PFB5, three proteins involved in RNA processing were upregulated, polyribonucleotide nucleotidyltransferase (Pnp), enolase (Eno) and the transcription termination factor Rho (Table 4.3). In *E. coli* and *Rhodobacter*, these proteins are part of the RNA degradosome type A (Eno and Pnp) (Figure 4.5A) and the RNA degradosome type C (Rho), respectively. Two other proteins, endoribonuclease E (RnE) and RNA helicase (RhlB) form together with Eno and

Pnp the RNA degradosome (Carpousis, 2007). Hfq, important in virulence in *E. amylovora* (Zeng *et al.*, 2013; Zeng & Sundin, 2014), forms a minor component of this degradosome (Morita *et al.*, 2005; Kaberdin & Lin-Chao, 2009). Comparison of the binding sequences of enolase (residues 833-850) (Chandran & Luisi, 2006) and polyribonucleotide nucleotidyltransferase (residues 1021-1061) (Nurmohamed *et al.*, 2009) on the RNA degradosome in *E. coli* showed high similarity with these sequences in *E. amylovora* (81% and 71% respectively) (Figure 4.5B). These results may indicate a role for the RNA degradosome in *E. amylovora*. In total, 6 genes involved in degradosome structure and function were tested for both strains (Figure 4.5C). Results showed a higher expression of all genes in PFB5, although no large differences were observed for *rne* and *rhlB* whereas for *eno* and *hfq* significant differences were observed between both strains.

Table 4.3: *Proteins involved in RNA processing up-regulated in PFB5 in comparison with LMG2024.*

Spot n°	Protein ID	Name	Gene name	Theoretical pI/MW(kDa)	ANOVA P-value	Fold change
105[a]	gi|292486841	polyribonucleotide nucleotidyltransferase	*pnp*	5.16 / 76.81	0.004	2.0
106	gi|292486841	polyribonucleotide nucleotidyltransferase	*pnp*	5.16 / 76.81	0.004	1.7
328	gi|292486658	transcription termination factor Rho	*rho*	6.75 / 46.94	0.006	2.4
343[a]	gi|292487224	enolase	*eno*	5.18 / 45.36	0.012	2.2

[a]Indication that multiple proteins were identified in this spot. Complete data set is represented in supplementary data.



B

Enolase binding sequence on RNA degradosome				
Score	Expect	Identities	Positives	Gaps
41.4 bits(90)	2.00E-06	13/16 (81%)	14/16 (87%)	0/16 (0%)

```
Query  1    ASPELASGKVWIRYPI  16
            ASPE ASGKVWI YP+
Sbjct  831  ASPESASGKVWISYPV  846
```

PNPase binding sequence on RNA degradosome				
Score	Expect	Identities	Positives	Gaps
52.8 bits(125)	3.00E-10	24/34 (71%)	26/34 (76%)	0/34 (0%)

```
Query  1     RHSDWQRPTFAFEGKGAAGGHTATHHASAAPARP  34
             RHSDW RP F FEGKGAAG HTA+H A+A   RP
Sbjct  1146  RHSDWVRPAFNFEGKGAAGAHTASHLATAPATRP  1179
```

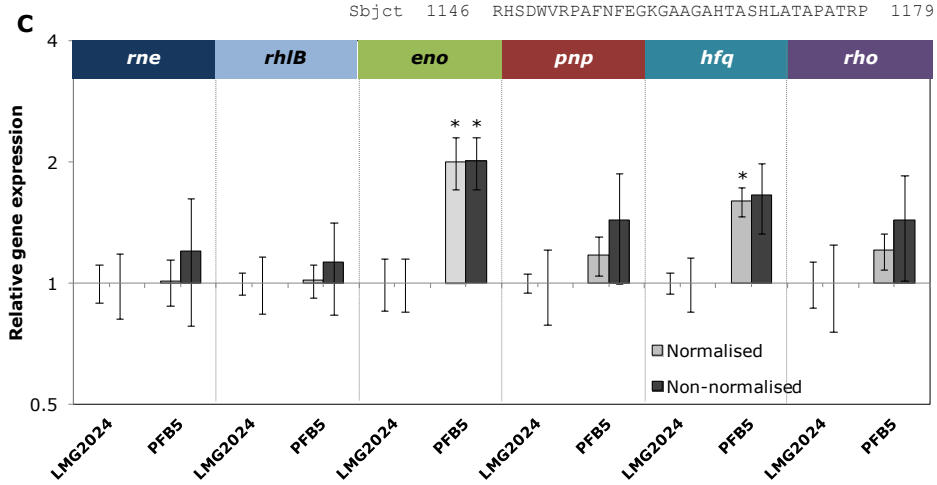


Figure 4.5: *Representation of proteins involved in RNA processing. A. Model of the E. coli RNA degradosome according Kaberdin and Lin-Chao (2009). B. Sequence alignment of the enolase and PNPase binding sequences on the RNA degradomsome of E. coli with E. amylovora. Alignments were prepared using the NCBI Blast Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). C. Relative gene expression measured by RT-qPCR. Normalised data are represented in light grey and the non-normalised data in dark grey. Up- or downregulations are represented on a* $\log_2$ *scale y axis relative to the least virulent strain. Columns represent data from four biological replicates ± standard errors. Asterisk (*) indicates a statistically significant difference between strains (P < 0.005, Kruskal-Wallis test with a pairwise Wilcox comparison test).*

4.4.4 Proteins involved in lipopolysaccharide biosynthesis

Two proteins involved in biosynthesis of lipopolysaccharides (LPS) were identified (Table 4.4). The LPS-assembly protein, LptD together with LptE are involved in the assembly of LPS at the surface of the outer membrane. This LPS-assembly protein LptD is encoded by the gene *imp*. ADP-heptose synthase (RfaE) on the other hand is involved in the synthesis of the LPS core precursor. These results were reflected by the RT-qPCR data which also showed an up-

regulation of both genes in PFB5 (Figure 4.6). Regarding these results, it can be concluded that LPS production is more active in the highly virulent strain.

Table 4.4: *Proteins involved in LPS biosynthesis which were identified as more abundant in PFB5 in comparison with LMG2024.*

Spot n°	Protein ID	Name	Gene name	Theoretical pI/MW(kDa)	ANOVA P-value	Fold change
92[a]	gi|292489391	LPS-assembly protein	*imp*	5.73 / 90.23	5.420e-005	1.9
94[a]	gi|292489391	LPS-assembly protein	*imp*	5.73 / 90.23	2.704e-005	2.1
302[a]	gi|292486914	ADP-heptose synthase	*rfaE*	5.42 / 50.78	0.015	2.0

[a]Indication that multiple proteins were identified in this spot. Complete data set is represented in supplementary data.

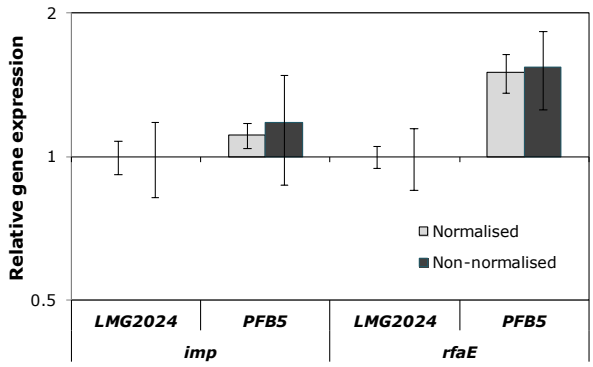


Figure 4.6: *Relative gene expression measured by RT-qPCR for genes involved in LPS biosynthesis. Normalised data are represented in light grey and the non-normalised data in dark grey. Up- or downregulations are represented on a* $\log_2$ *scale y axis relative to the least virulent strain. Columns represent data from four biological replicates ± standard errors. Asterisk (*) indicates a statistically significant difference between strains (P < 0.005, Kruskal-Wallis test with a pairwise Wilcox comparison test).*

4.4.5 Proteins involved in the carbohydrate and fatty acid metabolism

In figure 4.7, the proteins up-regulated in PFB5 involved in the carbohydrate metabolism are depicted. In total 14 proteins involved in this process were identified as being more abundant in PFB5 (Table 4.5). Results were validated by gene expression profiling and proteomics results were confirmed with exception of *ugd* and *talA*. Sorbitol-6-phosphate dehydrogenase (SrlD) is involved in the conversion of sorbitol, the main transport sugar in apple and pear (Aldridge *et al.*, 1997), to β-D-fructose-6P which fuels the glycolysis. Moreover, sorbitol is a good carbon source for amylovoran synthesis (Bennett &

Billing, 1978; Geider, 2000). The results show an up-regulation in the production of two precursors of amylovoran, UDP-glucose and UDP-galactose by GalF and GalE respectively, as well. Thus, both the sorbitol metabolism and the amylovoran synthesis are up-regulated in the higher virulent strain.

Moreover, the higher abundance of these different proteins of the carbohydrate metabolism in PFB5 enables this strain to proceed a higher cell growth and resulting in a higher production of proteins related to the amino acid and fatty acid metabolism. Pyruvate forms the connection between the glycolysis and the fatty acid metabolism via acetyl-CoA which is an important key in the production of fatty acids (Figure 4.8) and was reflected by the higher expression of three proteins important in the formation of acetyl-CoA and malonyl-CoA departing from pyruvate (Figure 4.8). These proteins include phosphate acetyltransferase (Pta), acetate kinase (AckA) and the acetyl-CoA carboxylase biotin carboxylase subunit (AccC). Gene expression analysed by RT-qPCR confirmed these findings (Table 4.6).

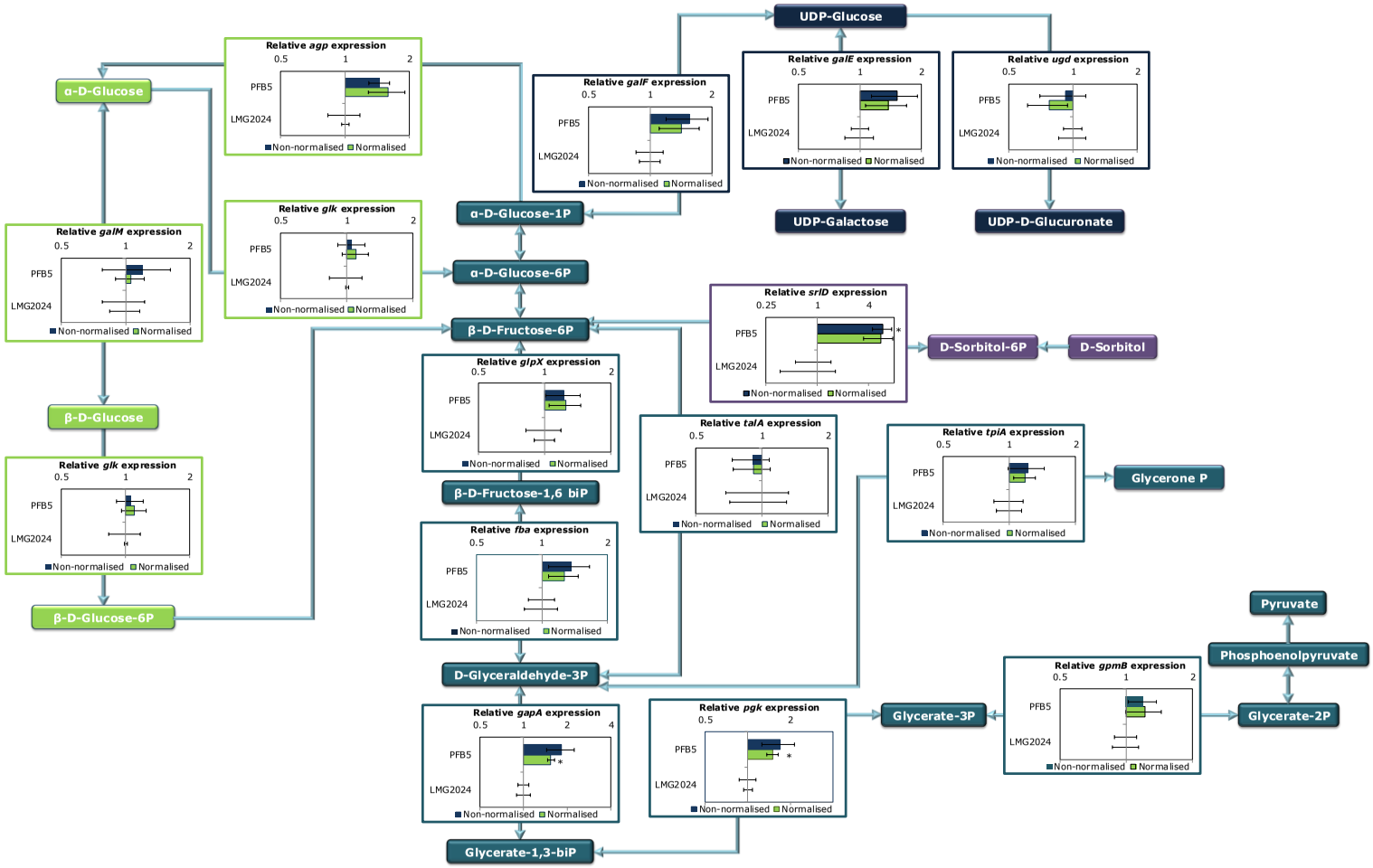

UDP-Glucose
Relative agp expression
α-D-Glucose
Relative galF expression
Relative galE expression
Relative ugd expression
UDP-Galactose
UDP-D-Glucuronate
Relative glk expression
α-D-Glucose-1P
α-D-Glucose-6P
Relative galM expression
β-D-Fructose-6P
Relative srlD expression
D-Sorbitol-6P
D-Sorbitol
Relative glpX expression
β-D-Glucose
Relative talA expression
Relative tpiA expression
Glycerone P
Relative glk expression
β-D-Fructose-1,6 biP
Relative fba expression
β-D-Glucose-6P
Pyruvate
Phosphoenolpyruvate
D-Glyceraldehyde-3P
Relative gpmB expression
Relative gapA expression
Relative pgk expression
Glycerate-3P
Glycerate-2P
Glycerate-1,3-biP
PFB5
LMG2024
Non-normalised
Normalised

Figure 4.7: *Schematic metabolic pathway chart containing identified proteins exhibiting up-regulation in the higher virulent strain. Diagram bars represent relative gene expression measured by RT-qPCR. Normalised data are represented in green and the non-normalised data in blue. Up- or downregulations are represented on a log_2 scale y axis relative to the least virulent strain. Columns represent data from four biological replicates ± standard errors. Asterisk (*) indicates a statistically significant difference between strains (P < 0.005, Kruskal-Wallis test with a pairwise Wilcox comparison test).*

Table 4.5: *Proteins involved in carbohydrate metabolism more abundant in PFB5 in comparison with LMG2024.*

Spot n°	Protein ID	Name	Gene name	Theoretical MW(kDa)/pI	ANOVA P-value	Fold change
105 [a] 108 [a] 110 [a] 164 [a]	gi|292487886	glucose-1-phosphatase	*agp*	65.69/5.69	0.004 0.008 0.005 0.006	2.0 2.2 2.3 1.6
336 387 [a]	gi|292488437 gi|292487107	nucleotide sugar dehydrogenase fructose-bisphosphate aldolase class II	*ugd* *fba*	49.39/5.59 39.2/ 5.62	0.004 0.015	2.9 2.4
406 [a]	gi|292488711 gi|385787229	UDP-glucose 4-epimerase phosphoglycerate kinase	*galE* *pgk*	36.87/ 5.45 41.14/5.10	0.012	2.3
415 [a]	gi|292899867 gi|292487675	UDP-glucose 4-epimerase galactose-1-epimerase	*galE* *galM*	36.87/ 5.45 37.85/5.35	0.006	2.2
444 [a]	gi|292488452 gi|292488972	glyceraldehyde 3-phosphate dehydrogenase A transaldolase A	*gapA* *talA*	35.47/6.40 35.51/ 5.87	0.002	2.0
455 457 [a]	gi|292488452	glyceraldehyde 3-phosphate dehydrogenase A	*gapA*	35.47/6.40	0.014 0.005	1.8 2.3
463 [a]	gi|292488452 gi|292489593	glyceraldehyde 3-phosphate dehydrogenase A fructose-bisphosphatase	*gapA* *glpX*	35.47/6.40 36.65/5.95	0.014 0.014	1.7 1.7
469 [a]	gi|292488939 gi|292488712 gi|292488452	Glucokinase UTP-glucose-1-phosphate uridylyltransferase glyceraldehyde 3-phosphate dehydrogenase A	*glk* *galF* *gapA*	34.77/5.39 37.02/ 5.83 35.47/6.40	0.001	3.0
474 [a]	gi|292488939 gi|292488452	glucokinase glyceraldehyde-3-phosphate dehydrogenase	*glk* *gapA*	34.77/5.39 35.47/6.40	0.004	2.0
475	gi|292488712	UTP-glucose-1-phosphate uridylyltransferase	*galF*	37.02/ 5.83	0.010	1.9
476 [a]	gi|292488939 gi|292488452	glucokinase glyceraldehyde-3-phosphate dehydrogenase	*glk* *gapA*	34.77/5.39 35.47/6.40	1.666e-004	3.3
513 [a]	gi|292489538	sorbitol-6-phosphate dehydrogenase	*srlD*	27.81 /5.67	0.007	1.8
575 [a]	gi|292487674 gi|292489538	phosphoglyceromutase sorbitol-6-phosphate dehydrogenase	*gpmB* *srlD*	28.51/5.62 27.81 /5.67	0.010	2.9
584 [a]	gi|292486606 gi|292489538	triosephosphate isomerase sorbitol-6-phosphate dehydrogenase	*tpiA* *srlD*	26.7/5.98 27.81 /5.67	0.006	4.1
589 [a]	gi|292486606	triosephosphate isomerase	*tpiA*	26.7/5.98	0.013	2.1

[a]Indication that multiple proteins were identified in this spot. Complete data set is represented in supplementary data.

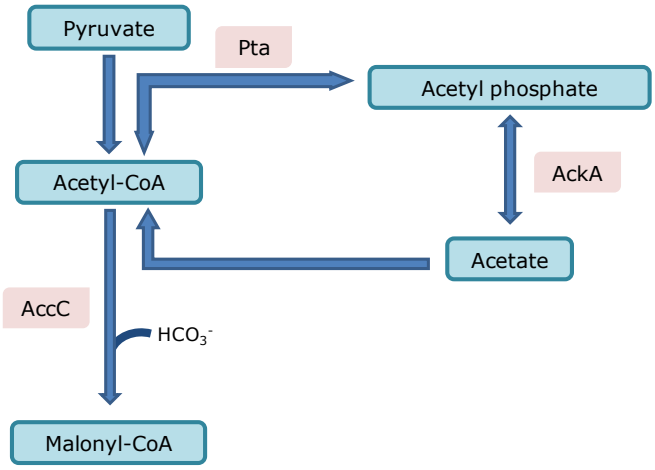


Figure 4.8: *Schematic metabolic pathway of the fatty acid metabolism representing identified proteins up-regulated in PFB5.*

Table 4.6: *Proteins involved in fatty acid metabolism are listed. Corresponding genes were used for a relative gene expression measured by RT-qPCR. Normalised (light green) and non-normalised data (dark green) are represented relative to the low virulent strain, LMG2024. Data represent four biological replicates ± standard errors.*

Protein data						Gene expression		
Spot n°	Protein ID	Name	Theoretical MW(kDa)/ pI	ANOVA P-value	Fold change	Gene	Strain LMG2024	Strain PFB5
108[a]	gi|292488858	phosphate acetyltransferase	76.85/5.31	0.008	2.2	*pta*	1 ± 0.15	1.64 ± 0.48
							1 ± 0.28	1.41 ± 0.29
302[a]	gi|292486764	acetyl-CoA carboxylase biotin carboxylase subunit	49.58/6.30	0.015	2.0	*accC*	1 ± 0.18	1.17 ± 0.18
							1 ± 0.25	1.23 ± 0.41
388[a]	gi|292488856	acetate kinase	43.21/ 6.01	0.011	1.9	*ackA*	1 ± 0.15	1.29 ± 0.15
							1 ± 0.33	1.20 ± 0.24

[a]Indication that multiple proteins were identified in this spot. Complete data set is represented in supplementary data.

4.4.6 *Difference in growth between LMG2024 and PFB5 in the presence of sorbitol*

In order to find out which role sorbitol plays in growth of this pathogen, the bacteria were grown in fluid MM2-medium supplemented with 1% of sorbitol for 80 h at 28 °C. Approximately every two hours, turbidity was measured and growth curves were made (Figure 4.9). The first 24 h, until approximately mid-exponential phase, PFB5 grows much faster than the lower virulent strain.

From 24 h on, LMG2024 seems to have adapted to the environment with sorbitol and was able to accelerate its growth and eventually bypassed PFB5. These graphs showed that PFB5 has the ability to adapt much faster to an environment with low nutrient availability and sorbitol as carbon source.

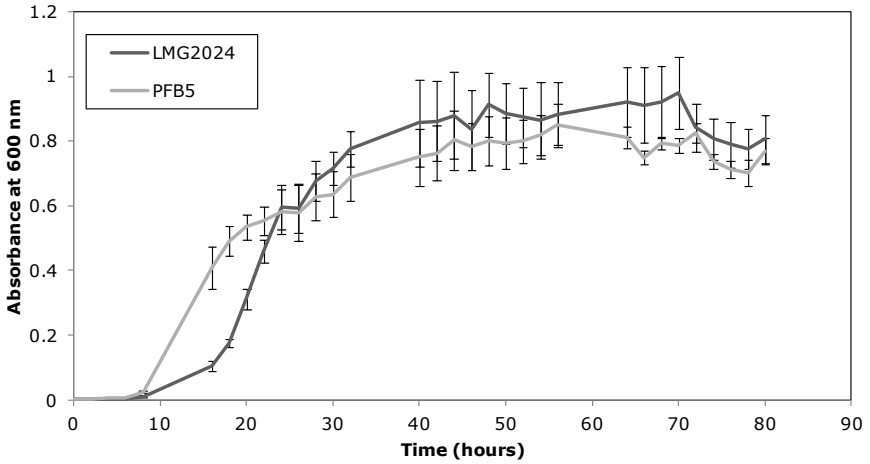


Figure 4.9: *Growth curves of E. amylovora strains LMG2024 and PFB5. Initial bacterial concentration in MM2-medium supplemented with sorbitol were 10^6 cells/ml. Data represent means of five replicates ± standard errors.*

4.5 Discussion

Until now, no study has been published using a proteomic approach to identify the proteins which are expressed differently between strains of *E. amylovora* inside a host plant. When comparing our research with studies performed on other plant pathogens, it becomes clear that this approach is not common used. Moreover, for most plant pathogens, proteome research is rather limited to the proteome of one WT strain *in vitro* (*Xanthomonas oryzae* pv. *oryzae*) (Xu *et al.*, 2013), the proteome of a mutant *in vitro* (*Dickeya dadantii*) (Bouchart *et al.*, 2007) or the proteome of the plant after infection with a pathogen (*Pseudomonas syringae*) (Parker *et al.*, 2013). Only for *Xanthomonas campestris* pv. *campestris*, similar research has been published (Andrade *et al.*, 2008).

During this study, an *in planta* infection model based on shoot infections on apple rootstocks allowed the characterization of important changes in the proteome of *E. amylovora* related to differences in virulence. Protein samples were analyzed by mass spectrometry and identification provided only bacterial

proteins which indicated the effectiveness of this technique. Moreover, in comparison with the technique used to isolate endophytic bacteria (Zinniel *et al.*, 2002; Thijs *et al.*, 2014), the described method provided enough bacteria for protein extraction and does not require further cultivating of the bacteria on selected agar plates.

The present study shows that both strains exhibit different behavior inside the host plant and cope differently with the defense mechanisms of the host. When regarding the categorized proteins in the pie chart of the lower virulent strain LMG2024 (Figure 4.3), a big part is reserved for proteins involved in stress response. This indicates that LMG2024 is primarily trying to cope with oxidative stress produced by the plants defense mechanisms (Venisse *et al.*, 2001). Proteomic data indicate a higher abundance of 9 proteins in LMG2024 (Table 4.2) and both proteomics data as gene expression by RT-qPCR confirm a higher abundance of the proteins/genes including PspA/*pspA*, AhpC/*ahpC*, YfiA/*yfiA*, CspE/*cspE* and CspA/*cspA* (Figure 4.4A). For the higher virulent strain, PFB5, 6 proteins involved in stress response were identified as more abundant (Table 4.2) of which 4 were confirmed by gene expression including the proteins DnaK, NusA, GrxB and Cpn10 (encoded by *groES*) (Figure 4.4B). This indicated that both strains use a different strategy to handle oxidative stress produced by the plant. The contradiction between some results from proteomics and those from the gene expression by RT-qPCR can be explained by the fact that some of the identifications produced multiple proteins present in one single spot (proteins with similar molecular weight and isoelectric point). Therefore, it cannot be predicted which protein caused the difference between both strains.

Gene expression profiling by RT-qPCR demonstrated an up-regulation of following genes encoding cold-shock proteins in LMG2024: *cspA*, *cspC* and *cspE* (Figure 4.4). Cold-shock proteins can be recognized by the plant as PAMPs, thereby triggering an immune defense in the plant (Jones & Dangl, 2006). However, in PFB5 an up-regulation of several genes encoding heat-shock proteins including *yedU*, *grpE*, *dnaK* and *dnaJ* was observed (Figure 4.4). DnaK is a Hsp70 homolog of *E. coli* that has a function in the pathway of protein folding by preventing off pathway aggregation (Becker & Craig, 1994; Hendrick & Hartl, 1995). The complete Hsp70 chaperone system consists not only off the main chaperone DnaK, but also comprises the cochaperones DnaJ and GrpE

(Liberek *et al.*, 1991; Pierpaoli *et al.*, 1997). The chaperone protein HchA or Hsp31 (encoded by *yedU*) has been identified as being an extra cooperative in this multicomplex in order to manage protein folding under severe stress (Mujacic *et al.*, 2004). These results indicate that LMG2024 uses cold shock proteins to overcome stress, although these proteins may induce the immune response of the plant via PTI. The high virulent strain on the other hand, exhibited a higher expression of heat-shock proteins.

Proteins more abundant in PFB5 can mainly be subdivided in major metabolic pathways: carbohydrate metabolism, amino acid metabolism, RNA processing and the fatty acid metabolism. When considering the proteins involved in the carbohydrate metabolism (Figure 4.7 and Table 4.5) we noticed that there was an up-regulation both in gene expression and abundance of the protein sorbitol-6-phosphate dehydrogenase. This protein is involved in the conversion of sorbitol to β-D-fructose-6P which fuels the glycolysis. Sorbitol is the main transport sugar in apple and pear (Aldridge *et al.*, 1997) and *srlD* which encodes for the dehydrogenase SrlD is required for symptom formation on apple seedlings (Geider, 2000). Sorbitol also provides the pathogen with a good carbon source to produce amylovoran (Bennett & Billing, 1978) and increases EPS biosynthesis (Bellemann *et al.*, 1994). This was observed by the production of two proteins and genes involved in the production of two main building blocks of amylovoran, UDP- glucose and –galactose (Geider, 2000). These results indicated a higher production of amylovoran, a very important virulence factor of *E. amylovora* (Bellemann *et al.*, 1994) which has multiple functions in survival and virulence of this pathogen. Amylovoran is important in biofilm formation in the plant xylem (Koczan *et al.*, 2009), forming multi-cellular communities in which cells are embedded in a matrix of extracellular compounds attached to a surface (Branda *et al.*, 2005). Major components of a biofilm include water, bacterial cells and exopolysaccharides (Sutherland, 2001) under which amylovoran and levan are classified in *E. amylovora* (Geier & Geider, 1993; Nimtz *et al.*, 1996). The formation of biofilms provides protection (Davey & O'Toole, 2000) and is important for survival under stress conditions (Ordax *et al.*, 2010).

Moreover, growth curves in medium supplemented with sorbitol (Figure 4.9) showed a slower growth of LMG2024 indicating that it takes longer for LMG2024

to adapt to an environment with this carbon source. As shown in figure 4.7, amylovoran production, powered by sorbitol, is also impaired in LMG2024. These differences implicate great challenges for LMG2024 inside the plant in comparison with PFB5.

Proteomic results also suggested an up-regulation of polyribonucleotide nucleotidyltransferase (Pnp) and enolase (Eno) which are both important components of the RNA degradosome in *E. coli* (Figure 4.5A). The principal components of the RNA degradosome of *E. coli* are RNase E, PNPase, RhlB and enolase (Carpousis, 2007). Results showed an up-regulation of all these components in PFB5 in comparison with LMG2024 with the exception of *rne* and *rhlB* (Figure 4.5C). This multi-enzyme complex plays an important role in RNA processing and decay. Minor components of this degradosome can have major implications on the function of the complex. DnaK has been identified as a minor protein component of the degradosome (Miczak *et al.*, 1996) which has been earlier shown to be involved in stress response in PFB5. DnaK and Eno have been identified as moonlighting proteins, meaning they exhibit more than one function (Henderson & Martin, 2011). Contradictory, some of these minor components can also function in a degradosome-independent manner, including Pnp or PNPase (Andrade & Arraiano, 2008). In *Salmonella enterica*, this protein is identified as a global regulator of virulence (Clements *et al.*, 2002). Furthermore, it has been suggested that PNPase exerts a regulatory role on small RNAs (sRNAs), thereby regulating the expression of several outer membrane proteins (Guillier *et al.*, 2006). Another minor component is the RNA chaperone Hfq which can enhance the efficiency of the RNase E cleavage through interaction with the C-terminal domain of this endoribonuclease (Morita *et al.*, 2005; Kaberdin & Lin-Chao, 2009). Hfq has been identified as being important for virulence in *E. amylovora* (Zeng *et al.*, 2013) and our results show a higher expression of the *hfq* gene in PFB5, the higher virulent strain. Hfq acts as an RNA chaperone by binding sRNAs and mRNAs and so functions in the posttranscriptional regulation of gene expression (Valentin-Hansen *et al.*, 2004; Chao & Vogel, 2010). Genes regulated by Hfq can be classified under varying biological functions including host cell invasion, motility, central metabolism, LPS biosynthesis, two-component regulatory systems and fatty acid metabolism (Chao & Vogel, 2010; Zeng *et al.*, 2013). Our results showed an upregulation of

proteins involved in the LPS biosynthesis (Figure 4.6) and fatty acid metabolism (Figure 4.8) in PFB5 which may indicate a role for Hfq regulated gene expression in *E. amylovora* and a direct function of Hfq in virulence.

E. amylovora is known for its ability to induce a HR response in its host and hereby using the lethal action of ROS to kill the plant cells and acquire nutrients (Venisse *et al.*, 2002) which raised the questions which mechanisms the bacterium uses to overcome this oxidative stress and how they establish infection in such a hostile environment. Our results answer these questions partially by comparing two strains with a difference in virulence. Although the lower virulent strain produces more stress related proteins, some of these proteins function as a PAMP inducing the defense mechanism of the plant. In addition this strain had a higher abundance of the elongation factor Tu which is also identified as PAMP (spot 522, Table S.4.2). Furthermore, this strain is not capable in producing the same amount of amylovoran as PFB5 to mask these cell surface components as shown during this and previous research (Chapter 3). Thereby they are not able to progress throughout the plant and they rather stay stationary. The higher virulent strain on the other hand has a lower share of stress related proteins but induces a couple of factors important for virulence. PFB5 is more efficient at adapting in a new environment and dividing faster. There is an up-regulation of the sorbitol metabolism which leads to a higher production of amylovoran precursors. Amylovoran masks cell surface components that could induce defense responses of the host (Geider, 2000), it forms a first protection against the ROS (Geider, 2006) and is involved in biofilm formation (Koczan *et al.*, 2009). Proteins involved in the biosynthesis of LPS were also upregulated in PFB5 which forms a third form of protection for the pathogen since LPS has been identified as a protective substance against ROS (Berry *et al.*, 2009).

In summary, this proteome study provides new insights in the difference in virulence between two strains of *E. amylovora* exhibiting differential virulency. Both the higher abundance of PAMPs and the lower production of amylovoran may be important characteristics that impair virulency in the low virulent strain. Further, a better sorbitol metabolism and higher amylovoran production enable the high virulent strain to escape host defense mechanism and protect it against plant produced ROS. In addition, the RNA degradosome together with Hfq may

also contribute to this difference in virulence. These results provide possible targets for the production of specific antimicrobial compounds. Proteins identified during this research could for example be used for the development of synthetic antimicrobial peptides (AMPs) (Montesinos, 2007; Montesinos & Bardaji, 2008). These AMPs are oligopeptides with a varying number of amino acids (Bahar & Ren, 2013) and can be de-novo synthesized. In the future, it might be possible to develop an AMP fashioned to interfere with specific proteins identified as being important for virulence in *E. amylovora*.

4.6 Supplementary data

Table S.4.1: *Complete list of all identified proteins found to be more abundant in the lower virulent strain, LMG2024.*

Spot number	Protein ID	Name	Gene name	Theoretical MW(kDa)/pI	ANOVA P-value	Fold change
237	gi|292488413	periplasmic oligopeptide-binding protein	*oppA*	60.84/6.30	0.010	3.4
	gi|292486786	malate dehydrogenase	*mdh*	32.32/6,45		
238	gi|292486786	malate dehydrogenase	*mdh*	32.32/6,45	0.011	3.8
	gi|292488413	oligopeptide ABC transporter substrate-binding protein	*oppA*	60.84/6.30		
239	gi|292488413	periplasmic oligopeptide-binding protein	*oppA*	60.84/6.30	3.033e-004	2.1
264	gi|288225943	OmpA, partial	*ompA*	11.78/5.73	0.011	4.4
	gi|292489036	inosine 5'-monophosphate dehydrogenase	*guaB*	51.46/ 6.34		
	gi|292488413	ABC transporter substrate-binding protein	*oppA*	60.84/6.30		
334	gi|292487466	trigger factor	*tig*	48.22/ 4.77	0.006	2.1
	gi|292488010	hypothetical protein EAMY_1529		42.65/4.64		
512	gi|292487505	chaperone protein htpG	*htpG*	71.08/ 5.00	0.003	3.0
	gi|292488602	hypothetical protein EAMY_2128		41.4/ 5.32		
	gi|292489226	elongation factor EF-Ts	*tsf*	30.33/5.07		
522	gi|292487505	chaperone protein htpG	*htpG*	71.08/ 5.00	0.009	2.6
	gi|292489937	hypothetical protein EAMY_3476		25.74/ 5.11		
	gi|292489851	elongation factor Tu	*tufA1*	43.22/ 5.27		
524	gi|292489273	spermidine synthase	*speE*	31.98/ 5.10	0.001	3.0
	gi|292487095	agmatinase	*speB*	33.5/5.28		
	gi|292489937	hypothetical protein EAMY_3476		25.74/ 5.11		
526	gi|292489712	chaperone protein HchA (Hsp31)	*yedU*	30.67/ 5.00	0.002	2.3
	gi|292489937	hypothetical protein EAMY_3476		25.74/ 5.11		
528	gi|292489273	spermidine synthase	*speE*	31.98/ 5.10	0.010	2.5
	gi|292489937	hypothetical protein EAMY_3476		25.74/ 5.11		
567	gi|292487117	hypothetical protein EAMY_0629		27.22/ 6.12	0.003	1.8

Table S.4.1 (continued): *Complete list of all identified proteins found to be more abundant in the lower virulent strain, LMG2024.*

Spot number	Protein ID	Name	Gene name	Theoretical MW(kDa)/pI	ANOVA P-value	Fold change
586	gi|292486698	uridine phosphorylase	*udp*	27.26/ 6.07	0.007	1.7
	gi|188535332	osmolarity response regulator	*ompR*	27.35/6.04		
616	gi|292488353	phage shock protein A	*pspA*	25.40/5.50	7.233e-004	5
617	gi|292488353	phage shock protein A	*pspA*	25.40/5.50	1.890e-005	4.6
	gi|292487625	phosphatase in N-acetylglucosamine metabolism	*nagD*	27.19/ 5.47		
630	gi|292489107	heat shock protein GrpE	*grpE*	21.67/4.66	8.920e-004	1.8
	gi|292489603	FKBP-type peptidyl-prolyl cis-trans isomerase	*slpA*	22.22/4.75		
640	gi|292487303	16S rRNA-processing protein RimM	*rimM*	20.62/4.55	0.013	1.6
652	gi|292487797	arginine-binding periplasmic protein 1	*artI*	26.74/8,35	0.006	2.0
653	gi|292487133	hypothetical protein EAMY_0645		23.03/5.39	0.003	1.9
	gi|292487119	ribose 5-phosphate isomerase A	*rpiA*	23.16/ 5.36		
675	gi|292487433	alkyl hydroperoxide reductase subunit C	*ahpC*	22.15/5,70	0.004	1.8
723	gi|292487433	alkyl hydroperoxide reductase subunit C	*ahpC*	22.15/5,70	0.007	1.8
730	gi|292487760	DNA protection during starvation protein	*dps*	18.82/5,18	0.009	2.3
737	gi|292486802	PTS system transporter subunit IIA-like nitrogen-regulatory protein PtsN	*ptsN*	17.85/5.77	0.007	2.8
758	gi|292487446	6,7-dimethyl-8-ribityllumazine synthase (riboflavin synthase)	*ribH*	16.13/ 5.57	0.001	2.2
772	gi|292489605	50S ribosomal protein L9	*rplI*	15.83/6.14	3.842e-004	4.0
793	gi|292490033	hypothetical protein EAMY_3574		14.93/5.44	4.043e-004	3.4
797	gi|188534769	ribosome associated factor, stabilizes ribosomes against dissociation	*yfiA*	12.63/ 6.19	0.003	5.9
807	gi|292487481	Nitrogen regulatory protein P-II	*glnB*	12.29/5.84	6.741e-004	4.6
	gi|292489605	50S ribosomal protein L9	*rplI*	15.83/6.14		
822	gi|292487236	hypothetical protein EAMY_0750		10.73/ 5.32	0.004	2.3
845	gi|291553014	cold shock protein CspE	*cspE*	7.38/ 8.09	0.008	1.9
848	gi|292487397	cold shock protein	*cspA*	7.52/ 5.64	0.004	2.9
852	gi|291553014	Cold shock protein CspE	*cspE*	7.38/ 8.09	0.002	2.6
	gi|292487576	hypothetical protein ETA_23740		8.85/7.87		

Table S.4.2: *Complete list of all identified proteins found to be more abundant in the higher virulent strain, PFB5.*

Spot number	Protein ID	Name	Gene name	Theoretical MW(kDa)/pI	ANOVA P-value	Fold change
91	gi|292489852	elongation factor G	*fusA*	77.23/5,21	0.003	2.0
92	gi|292489391	LPS-assembly protein	*imp*	90.23 /5.73	5.420e-005	1.9
	gi|292489852	elongation factor G	*fusA*	77.23/5,21		
94	gi|292489391	LPS-assembly protein	*imp*	90.23 /5.73	2.704e-005	2.1
	gi|292489852	elongation factor G	*fusA*	77.23/5,21		
105	gi|292486841	polyribonucleotide nucleotidyltransferase	*pnp*	76.81/ 5.16	0.004	2.0
	gi|292487886	glucose-1-phosphatase	*agp*	65.69/5.69		
106	gi|292486841	polyribonucleotide nucleotidyltransferase	*pnp*	76.81/ 5.16	0.004	1.7
108	gi|292488858	phosphate acetyltransferase	*pta*	76.85/5.31	0.008	2.2
	gi|292487886	glucose-1-phosphatase	*agp*	65.69/5.69		
110	gi|292488858	phosphate acetyltransferase	*pta*	76.85/5.31	0.005	2.3
	gi|292487886	glucose-1-phosphatase	*agp*	65.69/5.69		
122	gi|292488225	dipeptidyl carboxypeptidase II	*dcp*	79.89/5,78	0.001	2.5
138	gi|292489412	molecular chaperone DnaK	*dnaK*	68.67/4,82	0.006	1.9
157	gi|292488457	PrkA serine kinase	*prkA*	74.24/5,57	0.002	3.2
160	gi|292488457	PrkA serine kinase	*prkA*	74.24/5,57	0.005	2.7
	gi|292489412	molecular chaperone DnaK	*dnaK*	68.67/4,82		
164	gi|292487886	glucose-1-phosphatase	*agp*	65.69/5.69	0.006	1.6
	gi|292489997	Zn-dependent oligopeptidase	*prlC*	77.23/ 5.30		
171	gi|292489201	prolyl-tRNA synthetase	*proS*	63.56/5,38	0.007	1.7
180	gi|292486836	transcription elongation factor NusA	*nusA*	55.35/4,48	0.002	2.3
192	gi|292487223	CTP synthase	*pyrG*	60.49/ 5.50	0.007	1.9
	gi|292487321	ATP-dependent Clp protease, ATP-binding subunit ClpB	*clpB*	95.21/5.33		
286	gi|292488315	carboxypeptidase	*ctaQ*	55.79/5.57	0.001	2.4
	gi|292488827	glucans biosynthesis protein D	*ydcG*	66.39/5.81		
302	gi|292486764	acetyl-CoA carboxylase biotin carboxylase subunit	*accC*	49.58/6.30	0.015	2.0
	gi|292486914	ADP-heptose synthase	*rfaE*	50.78/ 5.42		
	gi|292486634	Argininosuccinate lyase	*argH*	50.24/5.47		

Table S.4.2 (continued): *Complete list of all identified proteins found to be more abundant in the higher virulent strain, PFB5.*

Spot number	Protein ID	Name	Gene name	Theoretical MW(kDa)/pI	ANOVA P-value	Fold change
320	gi|291615530	putative aldehyde dehydrogenase	*aldD*	53.58/6,23	0.008	1.9
324	gi|292489231	2,3,4,5-tetrahydropyridine-2,6-carboxylate N-succinyltransferase	*dapD*	29.74/ 5.66	0.009	1.9
	gi|292486953	diaminobutyrate--2-oxoglutarate aminotransferase	*dat*	50.11/5.78		
	gi|292490004	glutathione oxidoreductase	*gor*	48.86/ 5.71		
325	gi|292489656	histidyl-tRNA synthetase	*hisS3*	49.32/5,78	0.005	2.4
328	gi|292486658	transcription termination factor Rho	*rho*	46.93/6,75	0.006	2.4
331	gi|292489656	histidyl-tRNA synthetase	*hisS3*	49.32/5,78	0.002	2.4
	gi|292490004	glutathione oxidoreductase	*gor*	48.86/ 5.71		
336	gi|292488437	nucleotide sugar dehydrogenase	*ugd*	49.39/5,59	0.004	2.9
337	gi|292488394	bifunctional indole-3-glycerol phosphate synthase/phosphoribosylanthranilate isomerase	*trpC*	49.52/5,78	0.005	2.0
343	gi|292487953	3-oxoacyl-ACP synthase	*fabF*	34.1/ 4.94	0.012	2.2
	gi|292487224	enolase	*eno*	45.36/5.18		
346	gi|292489072	serine hydroxymethyltransferase	*glyA*	45.59/6,02	0.009	1.7
387	gi|292487827	3-phosphoserine aminotransferase	*serC*	39.7/5.77	0.015	2.4
	gi|292487107	fructose-bisphosphate aldolase class II	*fbp*	39.2/ 5.62		
388	gi|292488856	acetate kinase	*ackA*	43.21/ 6.01	0.011	1.9
	gi|292489878	bifunctional acetylornithine delta-aminotransferase/N-succinyldiaminopimelate aminotransferase	*argD*	43.17/5.96		
406	gi|292488711	UDP-glucose 4-epimerase	*galE*	36.87/ 5.45	0.012	2.3
	gi|385787229	phosphoglycerate kinase	*pgk*	41.14/5.10		
415	gi|292487312	phospho-2-dehydro-3-deoxyheptonate aldolase	*aroG*	39.51/5.52	0.006	2.2
	gi|292899867	UDP-glucose 4-epimerase	*galE*	36.87/ 5.45		
	gi|292487675	galactose-1-epimerase	*galM*	37.85/5.35		
444	gi|292487846	aspartate aminotransferase	*aspC*	43.02/ 5.47	0.002	2.0
	gi|292488452	glyceraldehyde 3-phosphate dehydrogenase A	*gapA*	35.47/6.40		
	gi|292488732	oxidoreductase	*mocA*	35.15/ 5.93		
	gi|292488972	transaldolase A	*talA*	35.51/ 5.87		

Table S.4.2 (continued): *Complete list of all identified proteins found to be more abundant in the higher virulent strain, PFB5.*

Spot number	Protein ID	Name	Gene name	Theoretical MW(kDa)/pI	ANOVA P-value	Fold change
455	gi|292488452	glyceraldehyde 3-phosphate dehydrogenase A	*gapA*	35.47/6.40	0.014	1.8
457	gi|292486854	aspartate carbamoyltransferase	*aspC*	34.22/ 5.80	0.005	2.3
	gi|292488452	glyceraldehyde 3-phosphate dehydrogenase A	*gapA*	35.47/6.40		
463	gi|292488732	oxidoreductase	*mocA*	35.15/ 5.93	0.014	1.7
	gi|292489593	fructose-bisphosphatase	*glpX*	36.65/5.95		
	gi|292486786	malate dehydrogenase	*mdh*	32.32/6,45		
	gi|292488300	hypothetical protein EAMY_1824		33.67/7.81		
469	gi|292488452	glyceraldehyde 3-phosphate dehydrogenase A	*gapA*	35.47/6.40	0.001	3.0
	gi|292488452	glyceraldehyde-3-phosphate dehydrogenase	*gapA*	35.47/6.40		
	gi|292488939	Glucokinase	*glk*	34.77/5.39		
	gi|292488712	UTP-glucose-1-phosphate uridylyltransferase	*galF*	37.02/ 5.83		
	gi|292488955	cysteine synthase A	*cys*	34.55/ 5.46		
474	gi|292488452	glyceraldehyde-3-phosphate dehydrogenase	*gapA*	35.47/6.40	0.004	2.0
	gi|292488955	cysteine synthase A	*cys*	34.55/ 5.46		
	gi|292487086	glutathione synthetase	*gshB*	35.28/ 5.46		
	gi|292488863	ABC transporter substrate-binding protein	*ophA*	37.7/5.84		
475	gi|292488939	glucokinase	*glk*	34.77/5.39	0.010	1.9
	gi|292488712	UTP-glucose-1-phosphate uridylyltransferase	*galF*	37.02/ 5.83		
	gi|292488452	glyceraldehyde 3-phosphate dehydrogenase A	*gapA*	35.47/6.40		
476	gi|292488955	cysteine synthase A	*cys*	34.55/ 5.46	1.666e-004	3.3
	gi|292488691	ATP phosphoribosyltransferase	*hisG*	33.42/ 5.49		
509	gi|292488939	glucokinase	*glk*	34.77/5.39	0.003	2.3
	gi|292487616	glutamate/aspartate ABC transporter substrate-binding protein	*gltI*	34.1/9.10		
	gi|292489538	sorbitol-6-phosphate dehydrogenase	*srlD*	27.81 /5.67		
513	gi|292488272	glutaminase	*yneH*	33.7/5.68	0.007	1.8
	gi|292487954	malonyl CoA-acyl carrier protein transacylase	*fabD*	32.29/5.72		
517	gi|292486786	malate dehydrogenase	*mdh*	32.32/6,45	0.008	1.9

Table S.4.2 (continued): *Complete list of all identified proteins found to be more abundant in the higher virulent strain, PFB5.*

Spot number	Protein ID	Name	Gene name	Theoretical MW(kDa)/pI	ANOVA P-value	Fold change
558	gi|292489722	hypothetical protein EAMY_3259		29.06/5.54	0.001	3.0
560	gi|292489722	hypothetical protein EAMY_3259		29.06/5.54	0.012	1.7
575	gi|292487596	aliphatic amidase	*ybeM*	28.36/ 5.42	0.010	2.9
	gi|292487674	phosphoglyceromutase	*gpmB*	28.51/5.62		
	gi|292486980	hypothetical protein EAMY_0492		28.8 /5.97		
583	gi|292489538	sorbitol-6-phosphate dehydrogenase	*srlD*	27.81 /5.67	0.012	1.9
	gi|292489228	30S ribosomal protein S2	*rpsB*	26.86/6.67		
	gi|292486980	hypothetical protein EAMY_0492		28.8 /5.97		
584	gi|292486606	triosephosphate isomerase	*tpiA*	26.7/5.98	0.006	4.1
	gi|292489538	sorbitol-6-phosphate dehydrogenase	*srlD*	27.81 /5.67		
589	gi|292489422	transcriptional regulator Ycf27	*ycf27*	27.24 /5.62	0.013	2.1
	gi|188535332	osmolarity response regulator	*ompR*	27.35/6.04		
	gi|292486606	triosephosphate isomerase	*tpiA*	26.7/5.98		
619	gi|292487928	glutaredoxin-2	*grxB*	24.0/5,73	9.832e-004	2.2
704	gi|292488322	ACP phosphodiesterase	*yajB*	22.55/4,88	0.013	1.7
765	gi|291555080	10 kDa chaperonin (protein Cpn10)	*groE*	10.27/ 5.41	0.007	3.0
	gi|292487446	6,7-dimethyl-8-ribityllumazine synthase (riboflavin synthase)	*ribH*	16.13/ 5.57		
	gi|292898134	hypothetical protein EAM_0410		14.55/ 5.39		
	gi|292489605	50S ribosomal protein L9	*rplI*	15.83/6.14		
842	gi|292488322	ACP phosphodiesterase	*yajB*	22.55/ 4.88	1.225e-004	2.6
	gi|259907813	PTS system phosphohistidinoprotein-hexose phosphotransferase Hpr	*ptsH*	9.09/5.59		
	gi|292488490	cold shock-like protein CspC	*cspC*	7.4/ 6.54		
843	gi|292488490	cold shock-like protein CspC	*cspC*	7.4/ 6.54	0.002	4.6

Table S.4.3: *Table containing all primers used.*

Gene	Gene ID	Locus tag	Left primer	Right primer	Amplicon size	Primer efficiency%
htpG	8913760	EAMY_1019	GGAAGGCCAGGAGAAGATTT	GAACCTCGATGCCTTTCTTG	101	96.44
yedU	8913422	EAMY_3249	TACCCCGGCATAATCACTGT	AATCACCGGATTCTTTGCTG	103	97.11
pspA	8913673	EAMY_1877	AACAAAAGCTGACCGACCTG	CTTTCCAGCTCGCTGATTTC	100	85.02
grpE	8914172	EAMY_2639	CTTTTAGCCACTGCGACCAT	TCGGAAGAAGTCGAGCCTAA	101	84.0
ahpC	8911425	EAMY_0947	AACACAACGGTGGCTTTACC	TCCAGCCCCAGATTTTACTG	102	94.19
dps	8914339	EAMY_1275	CGATATGCGCAAAGCTATCA	TGGACTCGATGAACCACAGA	104	86.88
yfiA	6298075	ETA_26460	TCAAATCGTTAATGGCCGTA	CGAAAGAATTCGTCGCTGAT	99	92.12
cspE	8912600	EAMY_1109	GTCCAAAGGATTCGGTTTCA	GCTGACCTTCAGCCAGAGTT	107	100.31
cspA	8911412	EAMY_0911	AAGGTTTCGGCTTCATCTCC	TGACCTTCTTCCAGCGTCTT	100	97.24
dnaK	8912710	EAMY_2944	GTTAGTCCTGGCCATCGGTA	AGGGATTCAACTGCATCTGG	97	92.17
groE	8913010	EAMY_3180	ACGATCGTGTGATCGTCAAG	GCACTTCACCACGGGTAGAT	102	89.91
nusA	8914674	EAMY_0348	AGCTAAGATCGCCGTGAAAA	ACTGGATACCGCCTGAACAC	94	96.05
gor	8912188	EAMY_3543	GGTCCTGATTACGGCTTTGA	GAGGTATGAATGCGGTCGAT	95	99.51
grxB	8914381	EAMY_1445	ATACAGCTGCCGTCGTCTTT	GCTGGTGGTGATGTTGAATG	96	98.74
cspC	8911702	EAMY_2016	TTTCATCACTCCTGCTGACG	AACTCAACGTTCTGGCCTTC	102	105.0
rne	8912830	EAMY_1466	TCAGTTCGGTACGGTCATCA	TTTATCAGTCTGGCGGGAAG	100	100.01
rhlB	8913004	EAMY_0168	ATGACAGGGTGGCAGAGAAC	ATGTTCGACCTTGGCTTCAT	100	100.3
eno	8913726	EAMY_0738	CGAATCTGACTGGGATGGTT	AGGATCCTGGTGTTGGTCAC	99	97.04
pnp	8913881	EAMY_0353	GTGGTCTCCGTTAACCCTCA	GCACCGATAGGACCGTTAAA	104	89.33
hfq	8913048	EAMY_3157	GCCCTGCAGTTTGATACCAT	TGGCTAAGGGGCAATCATTA	101	103.65
rho	8914133	EAMY_0170	ACTTAACCGCACGCGTACTT	TTGCAGCAGCATGGTTTTAC	101	96.49
imp	8912702	EAMY_2923	GCCGCTACACTATCCTGGAA	TTCATCGCGGTCATGAATAA	101	99.22
rfaE	8912960	EAMY_0426	GGGCGGTGACTATAAACCTG	AAATGCCGTCTTCGAAATTG	95	103.37
pta	8911821	EAMY_2387	AAACAAAGGCATGACCGAAG	ACCAGTCCATCCACTTCACC	99	98.78
ackA	8911820	EAMY_2385	CCATTATCGATCCAGCGAAT	TTTAGCGCCATCAAGTTTCC	101	94.89
accC	8914700	EAMY_0276	ACACTTTATCGCCCATCAGG	CTTCCTCTCCGAAAATGCTG	104	98.43
agp	8912313	EAMY_1403	GGGTTAACGAAACAGGGTGA	CGCCGGTGATAAAGAACTGT	103	93.75
glk	8911854	EAMY_2468	TTGGTTGAAAAAGCCCAGTC	GAACACCAGCAGGATGTCAA	101	98.83

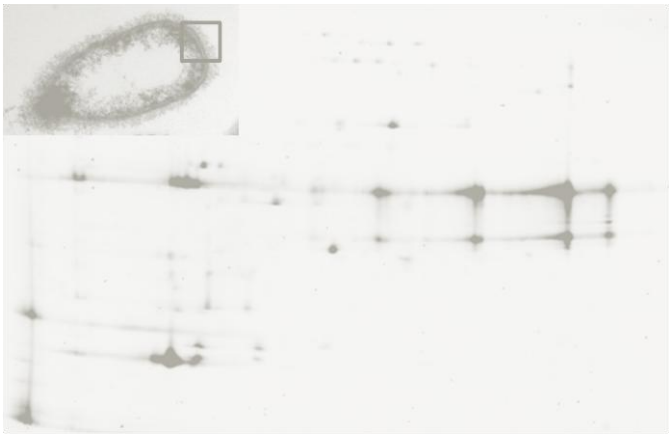
Table S.4.3 (continued): *Table containing all primers used.*

Gene	Gene ID	Locus tag	Left primer	Right primer	Amplicon size	Primer efficiency%
galM	8913002	EAMY_1190	GTGAACACATCCAGCCGTAA	CAGGGTTACGATCACGCTTT	98	99.27
galF	8912924	EAMY_2241	CGTTCATGATCGTCACACCT	TACGAACTGGAAGCCTTGCT	93	94.7
galE	8913771	EAMY_2240	GGTCCCCTTCGAAGAAAGTC	CGATGACGTGGTGATACTGG	104	83.0
ugd	8914111	EAMY_1961	TGTCGACGAAAACAAAGTCG	GACCAGCCTCGTAATTCTGC	98	97.98
fbp	8913356	EAMY_3130	CGGCAAATACGTAGTGCTGA	GATACGGCGGTAAATCGAAA	97	93.76
glpX	8912752	EAMY_0124	CGGATGCAGATTTTCGTCTT	CAATATGGCCACCAGTGAAA	106	99.8
srlD	8914018	EAMY_3073	CAACCTTGCCGGATTTAGAA	TGGGCTATTTCCTCTGTTCG	99	99.15
talA	8911858	EAMY_2501	CTCTACGGAGGCATTCAACC	GCCTTCTGCCAGCTTTTCTA	100	87.61
tpiA	8913358	EAMY_0118	GGTACATGACAGGTGGAGCA	AACAAGCACATGGTCAACGA	97	92.6
gapA	8914474	EAMY_1976	TTCTGGGCTACACCGAAGAT	TTATCGTTCAGGGCGATACC	100	95.1
pgk	8912759	EAMY_0618	GGACTGAAATCGTTGCCAAT	CTTCGATGCCAAACAGGTCT	99	93.15
gpmB	8911968	EAMY_2958	CCTGATAAGCCTGCTGCTCT	GGAGAAACGCTATGGAATGC	88	94.19
rpsL	8951948	EAM_3203	AACTCCGCACTGCGTAAAGT	GGATCAGGATCACGGAGTGT	112	97.24
rpoD	8914668	EAMY_RS19375	TCCGGTGCATATGATTGAGA	CGTTCGGCTAACTCTTCTGG	102	96.74
proC	8949266	EAM_0929	GCCGGCCTATGTGTTTATGT	GATTGCGCTGCAAACTGATA	96	95.89

Table S.4.4: *Quantitative PCR (qPCR) parameters according to the Minimum Information for publication of qPCR Experiments (MIQE) checklist derived from Bustin et al. (2010).*

Sample/Template	
Source	*Erwinia amylovora* cells extracted from plant tissue
Method of preservation	After samples were taken, they were frozen in liquid N_2 and stored at -80°C
Storage time	Three weeks
Handling	Liquid supplemented with 2 volumes of RNAprotect Bacteria Reagent* (Qiagen, Venlo, The Netherlands).
Extraction method	RNeasy Mini Kit* (Qiagen, Venlo, The Netherlands)
RNA: DNA-free	TURBO DNA-free Kit* (Ambion, Lennik, Belgium)
Concentration	NanoDrop®: ND-1000 Spectrofotometer (Isogen Life Science, IJsselstein, the Netherlands)
RNA: integrity	Not tested; RNAprotect Bacteria Reagent* (Qiagen, Venlo,The Netherlands).
Assay optimisation/validation	
Accession number	See Table S.4.3
Amplicon details	Amplicon size see Table 3.2
Primer sequence	See Table 3.2
In silico	Primer-BLAST
Empirical	Primer concentration (300 nM), annealing temperature (60°C)
Priming conditions	Random hexamer priming
PCR efficiency	Dilution curves
Linear dynamic range	Samples are within the efficiency curve
RT/PCR	
Protocols	See Materials and Methods
Reagents	See Materials and Methods
NTC	C_q & melt curves
Data analysis	
Specialist software	7500 Fast System Sequence Detection Software, version 1.4 (Applied Biosystems, Lennik, Belgium, 2001-2006)
Statistical justification	4 biological replicates, one-way ANOVA
Normalisation	3 reference genes selected using GrayNorm

*All procedures were performed according to the manufacturer's protocol.



5

Proteomic analysis of the outer membrane proteome of *Erwinia amylovora* and the implications on virulence

Research article – in preparation

5.1 Abstract

The outer membrane (OM) and its proteins, form the interface between the cell and its environment. Outer membrane proteins (OMPs) are potential targets for a protective immune response of the host. Until now, no data was available on the protein composition of the OM of *Erwinia amylovora*, a plant pathogenic enterobacterium that causes fire blight in most members of the *Rosaceae* family including apple and pear. To gain better insight in the composition of the outer membrane protein components a reliable extraction method to purify the pure OM, based on N-lauroylsarcosine (Sarkosyl), was used. The further processing of the protein composition was done combining DIGE and mass spectrometry. The proteome profile of the OM of two high virulent (PFB5 and BG16) and two less virulent strains (LMG2024 and PD437) grown *in vitro* and of the strains, PFB5 and LMG2024 grown *in planta* was analysed. The identified OMPs are involved in (i) β-barrel type transmembrane proteins and their precusors, (ii) LPS-assembly, (iii) flagella structure, (iv) amylovoran translocation, (v) a protease complex, (vi) a siderophore receptor, (vii) precursors of lipoprotein, utilization and putative proteins and (viii) hypothetical proteins. An in depth comparative analysis of the OM proteome of *in vitro* grown strains showed a higher abundance of proteins involved in the structure of the flagella in the low virulent strain. *In planta*, the abundance of general OMPs, the FlgL and the protease Iscp/SopA was higher in the low virulent strain LMG2024, while OmpA, TolC with its precursor and the precursor of YaeT were more abundant in the high virulent strain PFB5. This work clearly shows a relation between the virulence of *E. amylovora* and the protein composition of the outer membrane.

5.2 Introduction

Fire blight, caused by the Gram-negative plant pathogen *Erwinia amylovora*, is a devastating disease affecting apple, pear and other rosaceous plants. *E. amylvora* is an enterobacterium which makes it closely related to important human and animal pathogens such as *Escherichia coli*, *Salmonella* spp., *Shigella* spp. and *Yersinia* spp. Existing control mechanisms have proven inadequate and due to its destructive character, this plant pathogen is able of rapid dispersion both in and between trees in orchards which leads to great economic losses.

Cell walls of Gram-negative bacteria are composed of three morphologically defined layers, the inner membrane (IM), the periplasm containing peptidoglycan and the outer membrane (OM) (Glauert & Thornley, 1969; Lugtenberg & Van Alphen, 1983; Bos *et al.*, 2007). The OM functions as a selective barrier and protects the bacteria from the environment by preventing entry of many toxic molecules into the cell (Glauert & Thornley, 1969; Bos *et al.*, 2007). It is highly asymmetric and the inner leaflet is composed of phospholipids while the outer leaflet is mainly composed of lipopolysaccharides (LPS) (Glauert & Thornley, 1969; Bos *et al.*, 2007). LPS consist of three structural units: lipid A, which consists of a hydrophobic domain, a core oligosaccharide and a distal polysaccharide, the O-antigen (Raetz & Whitfield, 2002). This LPS is essential for the barrier function of the OM and its structure is modified by environmental conditions (Nikaido, 2003). Outer membrane proteins (OMPs) are the key molecules that interface the cell with the environment. Two types of proteins are 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 (Tokuda & Matsuyama, 2004). Integral OMPs are generally folded into cylindric β-barrels with a hydrophilic interior composed of antiparallel amphipathic β-strands (Koebnik *et al.*, 2000). 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 (Ruiz *et al.*, 2006). In *Escherichia coli*, more than a dozen different lipoproteins have been identified (Blattner *et al.*, 1997). Only a few integral OMPs including OmpA and the general porins are expressed in high levels. OmpA is one of the major outer membrane proteins of *E. coli*. It has a function in the structural integrity of the bacterial cell surface (Koebnik *et al.*, 2000). Its structural composition includes two domains: first a membrane anchor composed of an N-terminal membrane-embedded domain of amino acid residues and a C-terminal domain, located in the periplasmic space. This provides a physical link between the outer membrane and the peptidoglycan layer (Koebnik *et al.*, 2000). OmpX, a small β-barrel membrane protein, belongs to a family of highly conserved proteins that appears to be important for virulence in *E. coli* (Heffernan *et al.*, 1994; Vogt & Schulz, 1999). The matrix porin, OmpF and osmoporin OmpC are general diffusion pores, regulated by osmotic pressure and temperature. These two

porins are highly homologues and are weakly cation selective. Both have a structure consisting of three identical subunits with each subunit consisting of a 16-stranded anti-parallel β-barrel containing a pore (Cowan *et al.*, 1992). YaeT, the homologue of Omp85 of *Neisseria meningitides*, is an essential OMP involved in OM biosynthesis and is essential for viability (Voulhoux *et al.*, 2003; Bos *et al.*, 2007). Depletion studies of YaeT in *E. coli* showed that this protein is also an essential OMP which is required for the assembly of β-barrel proteins. Furthermore, the β-barrel protein YaeT is part of a hetero-oligomeric complex also including three other OM lipoproteins YfgL, YfiO and NlpB (Wu *et al.*, 2005). TolC and its homologues are involved in type I protein secretion and drugs extrusion (Koronakis *et al.*, 2000). It forms a β-barrel in the OM where each monomer contributes four β-strands, while the majority of the protein extends as long α-helices in the periplasm (Koronakis *et al.*, 2000). This OMP is a component of an efflux pump that excretes compounds from the cell preventing their build-up (Fralick, 1996; Tikhonova & Zgurskaya, 2004). Furthermore, research in *E. amylovora* has indicated that TolC has a function in virulence and fitness by the interaction with AcrAB leading to resistance against phytoalexins (Al-Karablieh *et al.*, 2009).

Moreover, the OM is the anchorage of surface organelles such as pili, type II and type VI secretion systems (Smits *et al.*, 2010; De Maayer *et al.*, 2011) and type III secretion systems with a function in pathogenesis (Remaut & Waksman, 2004). This type III secretion is an important virulence characteristic in *E. amylovora* (Oh & Beer, 2005; Mann *et al.*, 2012) and is involved in the elicitation of a hypersensitive reaction (HR) in non-hosts (Baker *et al.*, 1993) and responsible for the oxidative burst during compatible interaction between pathogen and host (Venisse *et al.*, 2003).

In this report a proteomics approach was used to characterize the protein composition of the outer membrane of *E. amylovora*. The outer membranes were isolated using a technique based on ultracentrifugation and N-lauroylsarcosine (Sarkosyl). Next, proteins were separated by 2D DIGE and analysed by mass spectrometry. A general gel image was formed including all spots deducted from four strains (LMG2024, PD437, BG16 and PFB5) grown *in vitro* and two strains (LMG2024 and PFB5) grown *in planta*. In total, 121 spots were obtained of which 80% was identified leading to the identification of 30

unique proteins which were mainly categorized as outer membrane proteins. Further, all predicted outer membrane proteins from Uniprot were listed and a comparison was made between both predicted and experimentally identified outer membrane proteins.

5.3 Materials and methods

5.3.1 Bacterial strains

During this research, in total four wild type strains of *E. amylovora* were used. For the *in vitro* comparison of the outer membrane, four strains were used of which difference in virulence has been previously reported (Maes *et al.,* 2001; Chapter 3) including LMG2024 isolated from *Pyrus communis,* strain PD437, also isolated from *Pyrus communis*, strain PFB5, isolated from *Prunus salicina* and BG16 isolated from *Malus sylvestris* with collection number SGB 225/12. LMG2024 and PD437 are lower virulent strains while PFB5 and BG16 are highly virulent strains. Based on previous results (Chapter 3 and 4) and because of the overall difference in infection between strains, LMG2024 (low virulent) and PFB5 (high virulent) were chosen for the *in planta* experiments.

5.3.2 In vitro bacterial growth and isolation

Bacteria were grown overnight in MM2 liquid medium supplemented with 1% sorbitol (Bellemann *et al.*, 1994), shaking at 100 rpm at 24 °C. The bacteria were grown until the exponential phase (OD_{600nm}=0.8) was reached. Cells from a 250 ml culture were used.

5.3.3 In planta bacterial growth and isolation

For the extraction of viable bacterial cells from plant tissue the protocol was followed as described previously (Chapter 4). In short, apple rootstocks (Maling 9 clone T337) were infected with a cell suspension made in phosphate buffered saline (PBS) with a density of 1 x 10^8 CFU/ml by cutting the two youngest leaves using scissors dipped in the bacterial suspension. Sampling occurred when infection was systemic, approximately 10 to 14 days after inoculation, depending on the used strain. Next, the bacterial cells were extracted from the plant tissue. Shoots and leaves were thoroughly sterilized externally with 1%

sodium hypochlorite by submerging leaves and shoots for 5 seconds. Hereafter, they were washed three times with sterile water. The shoots and leaves were cut in 0.5 cm pieces and extracted for 40 - 90 min in 250 ml of a sterile buffer (120 mM phosphate buffer, pH 8, 0.1% w/v sodium pyrophosphate, 0.1% v/v Tween-20, 25% w/v polyvinylpolypyrrolidone), and shaken (200 rpm, room temperature) in the presence of glass beads (0.1 mm – 1 mm). After filtration through 2 Wattman filters (pore size 8 µm), the extract was centrifuged to pellet the bacteria (8000 x g, 15 min, 4 °C).

5.3.4 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 resuspended in 7 ml of 10 mM HEPES, pH 7.4 and cells were lysed by sonication on ice (4 times 10 s) using a microtip. Next, lysed cells were centrifugated (10 000 x g, 10 min, 4 °C) to remove cell debris and unlysed cells. The membranes were collected by ultracentrifugation of the supernantans at 100 000 g for 1 h at 4 °C (Beckman LE80, Ti70 rotor). The pellet was resuspended in 10 mM HEPES, pH 7.4 and again ultracentrifuged at the same conditions as before. Afterwards, the pellet was resuspended in 1% (w/v) N-lauroylsarcosine in 10 mM HEPES, pH 7.4 and shaked (120 rpm) for 30 min at 37 °C. After treatment with N-lauroylsarcosine, the membranes were spun again (100 000 x g, 1 h, 4 °C) and the pellet was washed with 10 mM HEPES pH 7.4. Hereafter, the samples were ultracentrifuged for the last time (100 000 x g, 1 h, 4 °C) and the pellet was resuspended in 200 µl of sample solution (7 M urea, 2 M thio-urea and 4% (w/v) CHAPS). If necessary, samples were treated with the 2-D Clean-Up Kit (GE Healthcare) according to the manufacturer's instructions. After the pH was adjusted to 8.5 (using 100 mM NaOH), the protein concentration was determined using the 2-D Quant kit (GE Healthcare).

5.3.5 CyDye labeling and 2-D gel electrophoresis

Protein samples were minimally labeled using cyanine-derived fluors (3 Dyes 2-D CYanine Labeling kit From Proteomics Consult) before separating them in two

dimensions. The protein samples were labeled using Cy3 and Cy5 and a pooled internal standard representing equal amounts of all strains were labeled with Cy2. Each gel was loaded with 75 µg proteins, 25 µg from each sample and 25 µg from the internal standard. For this experiment, 4 biological replicates were considered for each strain and each growing condition. The internal standard was applied on each gel for the normalization of spot abundances between gels.

For separation in the first dimension, precast immobilized pH gradient (IPG) strips (pH 3-10, 24 cm) were used and it was performed using an IPGphor isoelectric focusing apparatus (GE Healthcare). The protein samples were loaded onto the IPG strips via anodic cuploading. Isoelectric focussing was conducted for 250 V for 1 h, 1000 V (gradient) for 7 h, 8000 V for 3 h and 8000 V (gradient) for 3 h 45 min for a total of 49,2 kVh (50 µA/strip, 20 °C). Hereafter IPG strips were stored at -20 °C until use.

For the second dimension, the IPG strips were first equilibrated for 15 min at room temperature, while shaking in an equilibration buffer (SERVA) with DTT. And secondly the strips were equilibrated for another 15 min in the same equilibration buffer (SERVA) but this time containing iodoactemide following the manufacturer's protocol. Next, proteins were separated based on molecular weight using an HPE-FlatTop Tower (SERVA) using precast, plastic-backed 10-15% polyacrylamide gels (2D-Large-Gel Flatbed NF 10-15% gradient gels). The running conditions were 30 min at 100 V with 7 mA/gel and 1 W/gel; 30 min at 200 V with 13 mA/gel and 3 W/gel; 10 min at 300 V with 20 mA/gel and 5 W/gel; 4 h 50 min at 1500 V with 40 mA/gel and 30 W/gel and finally 50 min at 1500 V with 45 mA/gel and 40 W/gel. Four gels were run simultaneously and after completion of the run, gels were fixed overnight in 1% citric acid and 15% ethanol. Next gels were scanned at a resolution of 100 µm (pixel size) using an Ettan DIGE Imager (GE Healthcare).

Determination of protein abundance and statistical analysis were performed using the Progenesis SameSpots software version 4.6. The spots considered, were spots with at least 1.5-fold changes in volume ($P < 0.05$) in one condition after normalization. The P-values were refined by a q-value to eliminate the false positives and a target power of 0.8 was set. Spots of interest were excised from the 2-DE gels in 1.5 mm diameter gel plugs using a semi-automated Screen picker (made by Proteomics Consult, Kampenhout, Belgium). Hereafter

the plugs were processed for mass spectrometry according the protocol of Shevchenko *et al.* (1996).

5.3.6 LC-MS/MS analysis

An Easy-nLC 1000 liquid chromatograph (Thermo Scientific) was on-line coupled to a mass calibrated LTQ-Orbitrap Velos Pro (Thermo Scientific) via a Nanospray Flex ion source (Thermo Scientific) using sleeved 30 µm ID stainless steel emitters (spray voltage +2.3 kV, capillary temperature: 200 °C). The SpeedVac dried tryptic peptide mixture was dissolved in 20 µl buffer A (0.1% v/v formic acid in Milli-Q water) of which half was loaded, concentrated and desalted on a trapping pre-column (Acclaim PepMap 100 C_{18}, 75 µm ID × 2 cm nanoViper, 3 µm, 100 Å, Thermo Scientific) at a buffer A flow rate of 5 µl/min for 5 minutes. The peptide mixture was separated on an Acclaim PepMap RSLC C_{18} column (50 µm ID x 15 cm nanoViper, 2 µm, 100 Å, Thermo Scientific) at a flow rate of 250 nL/min with a linear gradient in 40 minutes of 0 to 70% buffer B (0.1% v/v formic acid in acetonitrile) in buffer A.

MS data were acquired in a data-dependent mode under direct control of the Xcalibur software (version 2.2.SP1.48), selecting the fragmentation events based on the top six precursor abundances in the survey scan (350–2000 Th). The resolution of the full scan was 30000 at 400 Th with a target value of $1x10^6$ ions and one microscan. CID MS/MS spectra were acquired with a target value of 10000 and the maximum injection time was 100 ms. Dynamic exclusion was 30 s and early expiration was disabled. The isolation window for MS/MS fragmentation was set to 2 Th and the normalised collision energy, Q-value and activation time were 30%, 0.25 and 10 ms, respectively. Helium was used as the collision gas.

5.3.7 Data analysis

The analysis of the mass spectrometric raw data was carried out using Proteome Discoverer software v.1.2 (Thermo Scientific) with build-in Sequest v.1.3.0339 and interfaced with an in-house Mascot v.2.5 server (Matrix Science). MS/MS spectra were searched against the *Erwinia* protein collection extracted from NCBI database (query '*Erwinia*' on March 13th 2013; 104711 entries) and peptide scoring for identification was based on following search criteria: enzyme

trypsin, maximum missed cleavages 2, precursor mass tolerance 10 ppm and fragment mass tolerance 0.5 Da. Carbamidomethylation of cysteine and oxidation of methionine were set as fixed and dynamic modifications, respectively.

Result files of both search engines were uploaded and automatically evaluated in Scaffold v.4.4.1.1 (Proteome Software) using the Peptide Prophet and Protein Prophet algorithm with a preset minimal peptide and protein identification probability of 95% and 99%, respectively.

5.4 Results

5.4.1 Predicted 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/). Proteins derived from the plasmids were not considered. Table 5.1 lists all 63 potential OMPs and other OM related proteins. They are divided in four categories according to their subcellular location as depicted by the gene ontology retrieved from Uniprot. These categories include (i) cellular outer membrane, (ii) integral membrane proteins, (iii) cell wall and (iv) flagella. Next, proteins are subdivided according to their pI. No proteins are predicted with a pI lower than 4.

Table 5.1: *List of OMPs of E. amylovora. Sixty three 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. Further, a division was made according pI.*

Accession number	Description	Theoretical pI	Theoretical MW (kDA)	Subcellular location
Outer membrane proteins pI < 7				
D4HWX4	Outer membrane protein 1B	4.73	40.01	Cell outer membrane
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
D4I324	Protein transport protein hofQ	5.64	45.79	Cell outer membrane
D4HVA2	Outer membrane protein tolC	5.7	52.27	Cell outer membrane
D4I0G2	LPS-assembly protein LptD	5.73	90.23	Cell outer membrane
D4HX37	Protein mxiC	5.75	42.6	Cell outer membrane
D4I348	TonB-dependent copper receptor	5.78	72.45	Cell outer membrane
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
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

Table 5.1 (continued): *List of OMPs of E. amylovora. Sixty three 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. Further, a division was made according pI.*

Accession number	Description	Theoretical pI	Theoretical MW (kDA)	Subcellular location
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

Table 5.1 (continued): *List of OMPs of E. amylovora. Sixty three 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. Further, a division was made according pI.*

Accession number	Description	Theoretical pI	Theoretical MW (kDA)	Subcellular location
D4HUJ9	4-alpha-L-fucosyltransferase	9.75	40.94	Cell wall
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				
D4HZN4	Outer membrane protein X	5.08	18.1	Integral OMP
D4I285	Uncharacterized protein yfaZ	5.42	18.99	Integral OMP
D4I3F0	Outer membrane protein W	5.45	23.08	Integral OMP
4HZ73	Outer membrane protein assembly factor BamA	5.48	88.3	Integral OMP
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
D4I0C9	Outer membrane protein A	6.84	38.26	Integral OMP
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

5.4.2 Identified OMPs of E. amylovora

During this research, the OMP proteome of *E. amylovora* was analyzed, using a sarcosine-insoluble outer membrane fraction. By the combination of the OMP proteome of four strains of *E. amylovora* grown *in vitro* and two of these strains grown *in planta* a more general and representative profile of the OMP proteome was derived. Moreover, all proteins differently expressed between strains and proteins differently abundant for a low and a virulent strain inside the plant are incorporated in this OMP proteome. This OMP proteome including spots from four strains grown *in vitro* (LMG2024, PD437, BG16 and PFB5) and 2 strains grown *in planta* (LMG2024 and PFB5) is shown in figure 5.1. In total 121 spots were picked of which 97 were identified and 30 unique proteins were found (Table 5.2).

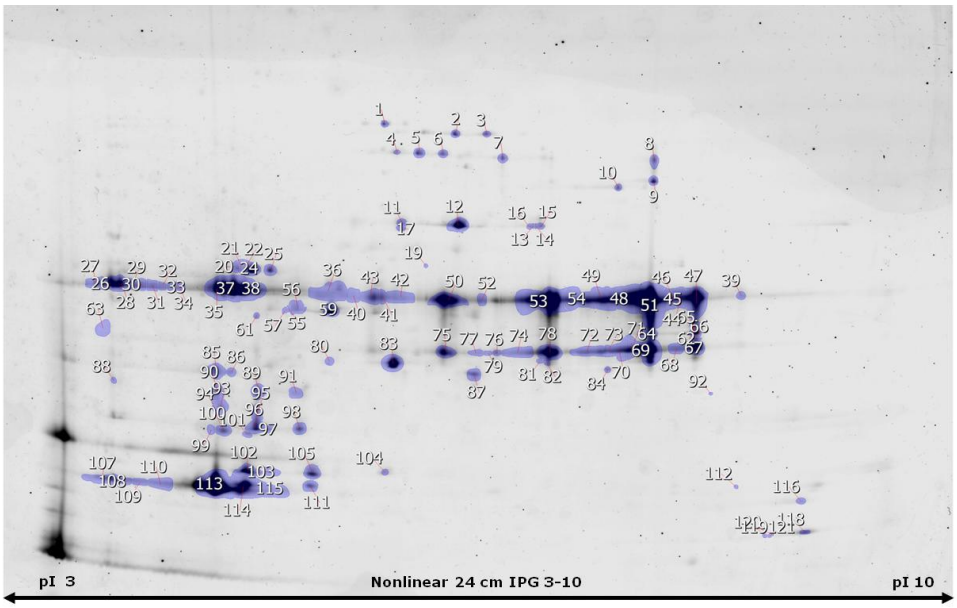


Figure 5.1: *2-DE analysis of the proteome of the outer membrane of E. amylovora. Image represents scan from the internal standard so every protein is present. All picked spots are indicated.*

The dominant OMPs were recovered including OmpA, OmpX, OmpF and OmpC. The identified proteins were classified according to their subcellular location (Table 5.2). All proteins were predicted to be associated with the outer membrane with exception of spot 104 identified as DNA protection during

starvation protein which is annotated to the cytoplasmic proteins. Many spots were identified as OmpA, a protein which is known to be abundantly present in the outer membrane of *E. coli* (10^5 copies per cell) (Molloy *et al.*, 2000). Two proteins involved in LPS-assembly were identified, LptD and LptE. The most abundant siderophore receptor, FoxR (Kachadourian *et al.*, 1996; Dellagi *et al.*, 1998), and TolC and its precursor were also identified. These proteins are involved in the formation of efflux pumps in the OM (Fralick, 1996; Tikhonova & Zgurskaya, 2004). The protease IcsP/SopA was also identified. In *Shigella flexneri*, this protein is responsible for the proteolytic cleavage of IcsA thereby maintaining the highest concentration of IcsA at the pole. *S. flexneri* uses the continual assembly of actin onto one pole of the cell to move within the cytoplasm of infected cells (Steinhauer *et al.*, 1999). Furthermore, three proteins involved in flagella structure were present in the proteome of the OM, these include the flagellar hook (FlgE), the flagellar hook-associated protein (FlgL) and flagellin (FliC). The precursor of the protein required for assembly of β-barrel proteins, YaeT (Wu *et al.*, 2005), and the protein thought to be involved in amylovoran translocation across the OM, AmsH (Bugert & Geider, 1995), were both detected. Three precursors were identified of the uncharacterized lipoprotein YjbH, a heme/hemopexin utilization protein B and a putative protein YnfB and finally three hypothetical proteins were determined.

Table 5.2: *Identified proteins of the OM of E. amylovora. With exception of DNA protection during starvation, all proteins are identified as being located in the OM. Subcellular location is represented.*

Spot number	Accession number	Protein ID	Description	Subcellular location
2,3	D4I0G2	gi|490274699, gi|502800886 gi|566688220, gi|635554220 gi|312173556	LPS-assembly protein LptD precursor	Cell outer membrane
4, 6	D4I248	gi|312173883, gi|635553360	ferrioxamine E receptor	Cell outer membrane
5	/	gi|635553360	putative ferrioxamine receptor	Cell outer membrane
11, 12, 13, 16, 17	D4HVA2	gi|312170991, gi|635555086	outer membrane protein tolC precursor	Cell outer membrane
15		gi|490258006	outer membrane channel protein	Cell outer membrane
20, 29, 30, 32, 96		gi|312171969, gi|635555548	outer membrane protein precursor (Porin)	Cell outer membrane
36	A0RZH5	gi|566688646	outer membrane protein Omp-EA	Cell outer membrane
84, 87	D4HZU9	gi|292489312, gi|312174384 gi|502800896, gi|635554287	IcsP/SopA	Cell outer membrane
100	D4HYU8	gi|490273064, gi|50280096 gi|635554843	LPS-assembly lipoprotein rlpB precursor	Cell outer membrane
104	D4HZN2	gi|562747432, gi|635553593	DNA protection during starvation protein	Cytoplasmic
24	E5B4B4	gi|310767290, gi|490273389 gi|635555628	flagellar hook protein FlgE	Flagella
61	E5B7T4	gi|635555635	flagellar hook-associated protein FlgL	Flagella
83	D4HVZ2	gi|635555329	flagellin	Flagella
1	4HZ73	gi|312173380, gi|490274571 gi|490277172, gi|635553124	outer membrane protein assembly factor yaeT precursor	Integral OMP
8, 9, 23, 26, 41, 42, 43, 46, 47, 48, 50, 51, 53, 54, 62, 64-79, 95, 100, 110	D4I0C9	gi|635555568	outer membrane protein A	Integral OMP

Table 5.2 (continued): *Identified proteins of the OM of E. amylovora. With exception of DNA protection during starvation, all proteins are identified as being located in the OM. Subcellular location is represented.*

Spot number	Accession number	Protein ID	Description	Subcellular location
10, 49, 51, 53, 54, 69, 70, 75, 101, 107	Q9AQ56	gi|12382107	major outer membrane protein OmpA, partial	Integral OMP
19, 33, 36, 37, 38, 39, 40, 45, 49, 81, 82, 89, 94, 96	D4I0C9	gi|490273323, gi|635555568	outer membrane protein A precursor	Integral OMP
27, 28, 33, 34, 35, 37, 38	D4I7B3	gi|635555548	outer membrane protein C	Integral OMP
51	D4I0C9	gi|490259763	membrane protein	Integral OMP
54	Q9AQ56	gi|14578660	major outer membrane protein, partial	Integral OMP
55, 90, 102, 104, 107, 108, 110, 113, 114	D4HZN4	gi|566689000 gi|635553591	outer membrane protein X (precursor)	Integral OMP
56, 57, 58, 59, 60	E5B4U3	gi|635555787	outer membrane protein F	Integral OMP
95, 96, 97, 98, 100, 101	D4I3F0	gi|490273851, gi|490276757 gi|635554552	outer membrane protein W (precursor)	Integral OMP
23	Q46629	gi|384872315, gi|566689865 gi|635555426	amylovoran export outer membrane protein AmsH Precursor	Membrane
7	E5B9M3	gi|490277482, gi|635552812	uncharacterized lipoprotein yjbH precursor	OM Lipoprotein
10	E5B8Q3	gi|566688262, gi|635554179	heme/hemopexin utilization protein B precursor	Probably integral OMP
118	D4I2I9	gi|566689423	putative protein ynfB precursor	Signal
21, 24, 25	/	gi|312173970, gi|490275075 gi|490277474, gi|635552822	hypothetical protein EAIL5_3404	Unknown
52, 102, 114	/	gi|490275041 gi|635553392	hypothetical protein EAM01S_18_00570	Unknown
85, 86, 90	/	gi|490273614, gi|635555806	hypothetical protein	Unknown

5.4.3 Differences in OMP composition between a low and high virulent strain in vitro

Four strains with differential virulence were used for a comparative study *in vitro* including two low virulent strains, LMG2024 and PD437 and two high virulent strains, BG16 and PFB5. Data analysis by the SameSpots software suggested only 4 differently abundant spots between LMG2024 and PFB5 that met the previous set requirements (P-value < 0.05; fold change > 1.5; power > 80% and q-value < 0.05) (Figure 5.2). All four proteins were observed more abundantly in LMG2024 in comparison with the higher virulent strain PFB5. These proteins included the outer membrane A precursor, the outer membrane protein F (OmpF), the flagellar hook associated protein FlgL and flagellin (FliC) (Table 5.3). The normalised log abundances for these spots are shown in figure 5.3, indicating the difference in abundance between both strains.

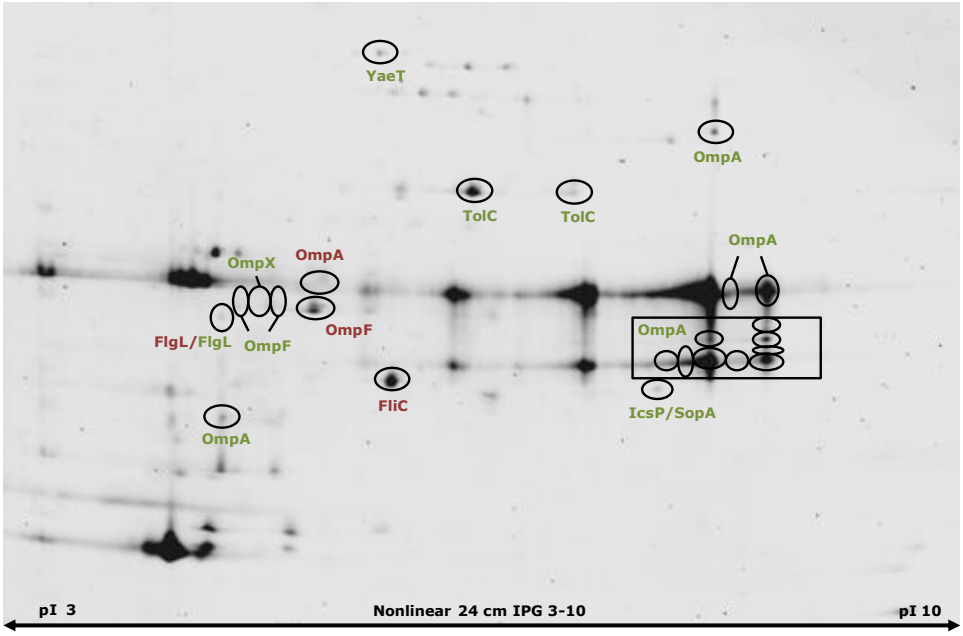


Figure 5.2: *2-DE analysis of the proteome of the outer membrane of E. amylovora. Image represents scan from the internal standard. Spots in red are the proteins identified during in vitro comparison of LMG2024 and PFB5. Spots depicted in green are the proteins identified during the in planta comparison between LMG2024 and PFB5.*

Table 5.3: *Identification table of differentially regulated proteins between LMG2024 and PFB5 in vitro. All proteins were found to be more abundant in LMG2024.*

Spot n°	Protein ID	Name	Gene name	Theoretical pI/MW(kDa)	ANOVA P-value	Fold change
36	gi|490273323 gi|635555568	Outer membrane protein A precursor	*ompA*	6.84 / 38.23	0.008	1.6
59	gi|635555787	Outer membrane protein F	*ompF*	5.51 / 38.74	0.011	3.1
61	gi|635555635	Flagellar hook-associated protein FlgL	*flgL*	4.90 / 34.08	2.588e-004	15.7
83	gi|635555329	Flagellin	*fliC*	5.34 / 29.62	0.002	20.0

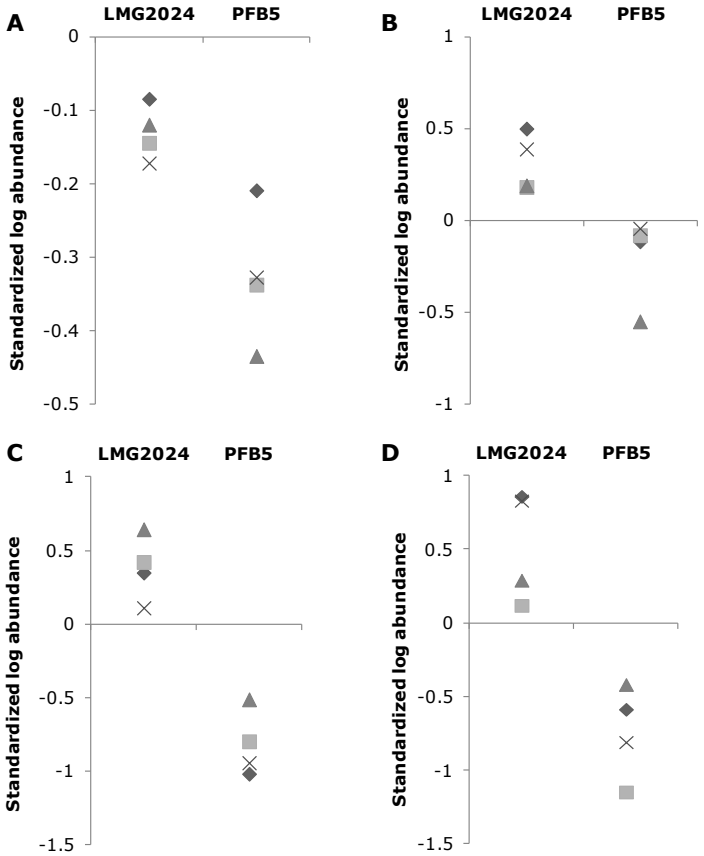


Figure 5.3: *A graphical view of the selected differential protein spots from two strains of E. amylovora, LMG2024 and PFB5 differing in virulence, grown in vitro. Each symbol represents one of the four biological replicates. A. Spot 36 (OmpA), B. Spot 59 (OmpF), C. Spot 61 (FlgL), D. Spot 83 (FliC).*

5.4.4 Differences in OMP composition between a low and high virulent strain in planta

A proteome comparison of the outer membrane was also made for LMG2024 and PFB5 grown *in planta*. When infection appeared to be systemic, after approximately 10-14 days depending on the used strain, samples were taken. OMPs were extracted and four biological replicates were used for both strains. In total 10 spots appeared more abundant in LMG2024 in comparison with PFB5 of which 6 were identified: OmpF and its precursor, OmpX and OmpA, FlgL, a flagellar hook-associated protein, and the protease IscP/SopA (Table 5.4 and Figure 5.4). For PFB5, the software indicated a higher abundance of 16 spots in PFB5 of which 15 were identified (Figure 5.2). From these results, it became clear that OmpA was the most abundant protein since it was identified in 11 spots. The other 4 spots included an OmpA precursor, an outer membrane channel protein identified as TolC and its precursor and finally a precursor of YaeT. In figure 5.4 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.

Table 5.4: *Identification table of differentially regulated proteins between LMG2024 and PFB5 in planta.*

Spot n°	Protein ID	Name	Gene name	Theoretical pI/MW(kDa)	ANOVA P-value	Fold change
Proteins more abundant in LMG2024						
55	gi|566689000 gi|635553591	Outer membrane protein X precursor	*ompX*	5.08 / 18.1	9.62E-02	3.6
56	gi|635555787	outer membrane protein F	*ompF*	5.51 / 38.73	4.28E-01	2.9
57	gi|490273588 gi|635555787	Outer membrane protein F precursor	*ompF*	5.51 / 38.75	0.001	2.8
61	gi|635555635	flagellar hook-associated protein FlgL	*flgL*	4.90 / 34.08	0.002	3
84	gi|292489312 gi|312174384	IcsP/SopA	*ompT3*	6.11/34.93	0.001	13.3
89	gi|490273323 gi|635555568	Outer membrane protein A precursor	*ompA*	6.84 / 38.23	0.002	3
Proteins more abundant in PFB5						
1	gi|312173380 gi|490274571 gi|490277172	Outer membrane protein assembly factor yaeT precursor	*yaeT*	5.56 /88.32	0.005	2
9	gi|635555568	outer membrane protein A	*ompA*	6.84 / 38.26	0.007	1.7
12	gi|312170991 gi|635555086	Outer membrane protein tolC precursor	*tolC*	5.70 / 52.24	0.003	1.9
15	gi|490258006	outer membrane channel protein	*tolC*	5.70 / 52.27	0.005	2.6
45	gi|490273323 gi|635555568	Outer membrane protein A precursor	*ompA*	6.84 / 38.26	6.35E-04	1.6
47	gi|635555568	outer membrane protein A	*ompA*	6.84 / 38.26	0.001	1.8
62	gi|635555568	outer membrane protein A	*ompA*	6.84 / 38.26	0.003	2.5
64	gi|635555568	outer membrane protein A	*ompA*	6.84 / 38.26	0.006	1.9
65	gi|635555568	outer membrane protein A	*ompA*	6.84 / 38.26	0.002	2.2
66	gi|635555568	outer membrane protein A	*ompA*	6.84 / 38.26	0.002	2.3
67	gi|635555568	outer membrane protein A	*ompA*	6.84 / 38.26	2.44E-01	2.1
68	gi|635555568	outer membrane protein A	*ompA*	6.84 / 38.26	0.013	1.8
69	gi|635555568	outer membrane protein A	*ompA*	6.84 / 38.26	0.006	1.9
70	gi|635555568	outer membrane protein A	*ompA*	6.84 / 38.26	0.005	1.7
71	gi|635555568	outer membrane protein A	*ompA*	6.84 / 38.26	0.006	1.7

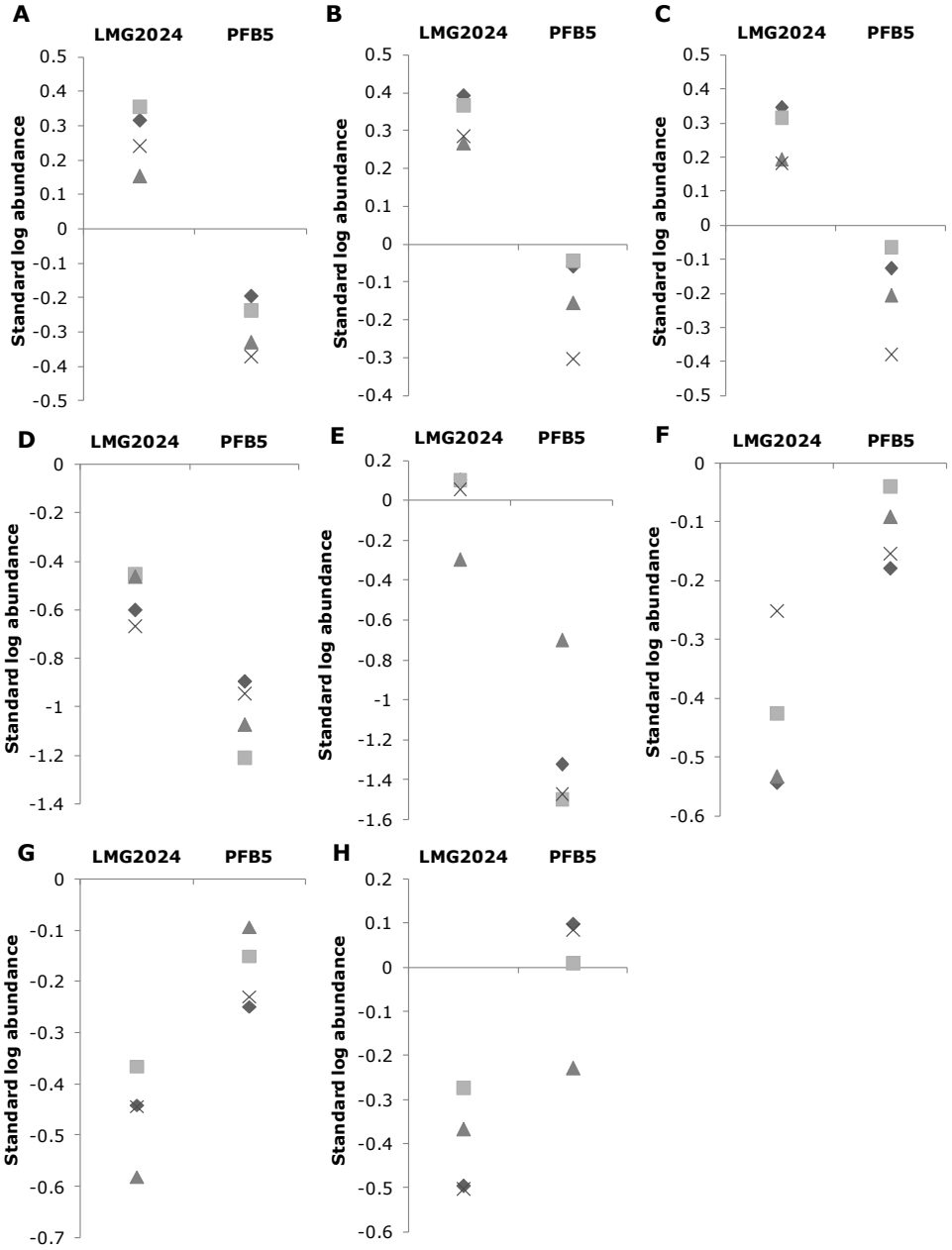


Figure 5.4: *A graphical view of the selected differential protein spots from two strains of E. amylovora, LMG2024 and PFB5 differing in virulence, grown in planta. Each symbol represents one of the four biological replicates. A. Spot 55 (OmpX), B. Spot 56 (OmpF), C. Spot 57 (OmpF), D. Spot 61 (FlgL), E. Spot 84 (IcsP/SopA), F. Spot 1 (YaeT), G. Spot 12 (TolC) and H. Spot 15 (TolC).*

5.5 Discussion

During this research, for the first time, OMPs of *E. amylvorora* were identified and characterized. Using an established extraction method (Hobb *et al.*, 2009) based on the use of N-lauroylsarcosine (Sarkosyl), an ionic detergent which provides a sarcosine-insoluble OM fraction. This extraction method was chosen following previous research that compared different extraction methods in another Gram-negative bacterium, *Campylobacter jejuni* (Hobb *et al.*, 2009). In total, nine methodologies were examined of which the OM fraction was analysed for purity and reproducibility by SDS-PAGE and immunoblotting using cellular markers for the cytoplasm, cytoplasmic membrane and OM (Hobb *et al.*, 2009).
In this report, a total 121 spots were identified by the SameSpots software 4.6 (Figure 5.1) of which 80% was identified. In total 30 unique proteins were found to be present in the outer membranes of *E. amylovora* (Table 5.2). The proteome profile which was used to identify these proteins included *in vitro* samples of 2 high virulent (PFB5 and BG16) and two low virulent (LMG2024 and PD437) WT strains of *E. amylovora*, and *in planta* samples of both PFB5 and LMG2024. This approach delivers a complete image of the outer membrane proteome with the added value of information about the OM proteome of this pathogen inside its host.

5.5.1 General proteome of the OM of E. amylovora

When comparing the predicted list of proteins (Table 5.1) with the identified proteins (Table 5.2) around 48% of the predicted proteins were found during identification. Absence of some of the other proteins not identified during the experiments may be explained by low abundance of these proteins in the samples. Although the used technique is very sensitive it can still miss low-abundant proteins. Furthermore, it has been known that large molecular mass proteins (> 80 kDa) can be missed (Molloy *et al.*, 2000), which can explain for example the absence of cellulose synthase operon protein C (146.81 kDa). Another reason may be the loss of these proteins during extraction, labeling or separation in first dimension. Moreover, 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.

5.5.2 In vitro comparison of the OM proteome of four wild type strains of E. amylovora

Next, a comparison was made between the outer membrane proteome of 4 WT strains of *E. amylovora*, with differential virulence, grown *in vitro*. No significant differences in normalised spot volumes were detected between the different strains except for four spots that appeared more abundant in LMG2024 in comparison with PFB5 (Figure 5.2). These spots answered to the previous set of requirements for the ANOVA p-value, power of the spots, fold change and q-value (Table 5.3). These proteins included two integral OMPs, OmpA and OmpF and also two proteins identified as structural components of bacterial flagella, FlgL and FliC. These results confirm the findings reported in Chapter 3. There we concluded that lower virulent strains contain more flagellin and motility associated proteins that can lead to an early activation of plant defense mechanisms. Moreover, 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 (Felix *et al.*, 1999; Jones & Dangl, 2006). This recognition may induce an immunity response in the host, called the PAMP-triggered immunity (PTI) which can block the pathogen (Jones & Dangl, 2006). Thereby these results further indicate the role of flagellin in the differential virulence between different strains of *E. amylovora*.

5.5.3 In planta comparison of the OM of two strains of E. amylovora

The results of a comparison between LMG2024 and PFB5, grown *in planta,* are shown in figure 5.2 and the identified proteins are listed in table 5.4. For the low virulent strain, LMG2024, 6 proteins were identified as being more abundant compared to the high virulent strain PFB5. These proteins included 3 integral OMPs, OmpA, OmpX, OmpF and an OmpF precursor, a structural component of the flagellum FlgL and the protease complex IcsP/SopA. As reported, IcsP/SopA is involved in proteolytic cleavage of IcsA in *Shigella flexneri* (Steinhauer *et al.*, 1999). Within its host cell cytoplasm, *S. flexneri* spreads by the directional assembly and accumulation of actin filaments at one pole of the bacterium, the pole were IcsA is located (Egile *et al.*, 1997). This protease belongs to the omptin family of enterobacterial surface proteases or adhesins, that share high sequence similarity and a conserved β-barrel structure in the OM. They are

multifunctional and they may exhibit differing virulence-associated functions (Kukkonen & Korhonen, 2004). However, a function for IcsP/SopA related to virulence has not yet been identified in *E. amylovora*.

In total 15 proteins were identified as being higher abundant in PFB5 in comparison with LMG2024. Eleven of these spots were identified as OmpA and one as an OmpA precursor. OmpA is one of the major outer membrane proteins of *E. coli* (10^5 copies per cell) (Molloy *et al.*, 2000) and it has a function in the structural integrity of the bacterial cell surface (Koebnik *et al.*, 2000). Presence of this large amount of OmpA in the higher virulent strain PFB5 in comparison with LMG2024 may explain the overall increased fitness of this high virulent strain (Chapter 4). Furthermore, 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 (Wu *et al.*, 2005). 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* (Fralick, 1996; Tikhonova & Zgurskaya, 2004). Moreover, it is suggested that TolC, together with AcrAB, mediates resistance against phytoalexins in *E. amylovora* (Al-Karablieh *et al.*, 2009). Phytoalexins are secondary metabolites, occurring in many chemical structures, that are synthesized *de novo* by the plant as response upon biotic and abiotic stress (Ahuja *et al.*, 2012; Vrancken *et al.*, 2013b).

5.5.4 Conclusion

During this research, a proteomics approach was used to determine the OMP composition of *E. amylovora*. OMPs were isolated using a reliable method based on the use of Sarkosyl which resulted in a pure OM fraction. Besides the Dps proteins (DNA protection during starvation), no cytoplasmic proteins were identified which emphasized the purity of the extraction method. To gain a general composition of the OMPs, samples of four *E. amylovora* strains grown *in vitro* and two strains grown *in planta* were combined. Moreover, this experimental set-up allowed us to identify differences between the used strains concerning their virulence. *In vitro* results confirmed previous findings of our research group (Chapter 3) 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. Previous results (Chapter 4) suggested that higher amylovoran synthesis plays an important role in protection of this strain against oxidative stress by capsule formation (Bugert & Geider, 1995) and biofilm formation (Flemming, 1993; Gilbert *et al.*, 1997). Both results indicate that PFB5 is better equipped to cope with plant defenses. Moreover, PFB5 may possess a better armed OM indicating a better survival strategy in comparison with the low virulent strain.

6

Gene expression profiling reveals major function for type III secreted proteins in virulence of *E. amylovora*

Research article – in preparation

6.1 Abstract

Erwinia amylovora, the causal agent of fire blight, uses a type III secretion system (T3SS) to translocate effector proteins into the cytoplasm of host cells to induce a hypersensitive response in non-hosts and promote infection in host plants. The T3SS is encoded by clustered *hrp* genes (hypersensitive response and pathogenicity). Until now, eleven proteins have been identified as being secreted by this pathway. In this study, primers were developed for the genes corresponding with eleven of these proteins excluding a DspA/E fragment. Gene expression profiling by means of RT-qPCR of these genes was performed on two highly virulent (PFB5 and BG16) and two hardly virulent (LMG2024 and PD347) strains of *E. amylovora* grown in an minimal *hrp*-inducing medium. Further, gene expression of the same set of genes was analyzed for the strains PFB5 and LMG2024 of *E. amylovora* which were grown inside apple rootstocks. All genes encoding for the effector proteins, with exception of *flgL3*, were up regulated in the highly virulent strains under *in vitro* conditions. Under *in planta* conditions, the same set of genes, now with the exception of *hrpA*, the gene encoding the Hrp pilin involved in secretion of the effector proteins, were also up-regulated.
Our results provide evidence that virulence in *E. amylovora* is closely related to production of effector proteins.

6.2 Introduction

Erwinia amylovora, a Gram-negative enterobacterium, is the causal agent of fire blight, a necrotic disease of rosaceous species such as apple and pear. Pathogenesis in *E. amylovora* is dependent on a functional type III secretion system (T3SS) (Oh & Beer, 2005) which is involved in the elicitation of a hypersensitive reaction (HR) in non-hosts (Baker *et al.*, 1993) and responsible for the oxidative burst during compatible interaction between pathogen and host (Venisse *et al.*, 2003). The T3SS forms a specialized syringe structure by which extracellular bacteria inject virulence proteins into the cytosol of its host (He *et al.*, 2004; Buttner & He, 2009). These virulence proteins are called type III effectors (T3Es) and are delivered into the host cytosol through a complex and ordered process (Buttner, 2012). The T3Es can suppress plant immunity or they can be recognized by the plant and hereby trigger an effector-triggered immunity (Jones & Dangl, 2006; Feng & Zhou, 2012). 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) (Oh & Beer, 2005; Mann *et al.*, 2012). The expression of the *hrp* genes in *E. amylovora* is dependent on environmental stimuli. They are expressed *in planta* under conditions of low nutrients and low pH and in a well defined culture medium thought to mimic the conditions of the plant's apoplast (Wei *et al.*, 1992b). Transcription of *hrp* genes is also regulated by HrpL, a sigma factor of the ECF (extra cytoplasmatic functions) subfamily. HrpL recognizes a conserved promoter motif, the *hrp* box (Wei & Beer, 1995). On the other hand, HrpS, a NtrC-family σ^{54} enhancer, is required for *hrpL* transcription in *E. amylovora* (Wei *et al.*, 2000). Furthermore, 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 (Ancona *et al.*, 2015).

A variety of genes have been identified encoding type III secretion proteins and structural components of the system. Nine *hrc* genes (HR and conserved) which are located together with the *hrp* genes on the PAI, are believed to be involved in the structure of the T3SS although the structure and morphology of the T3SS has not yet been identified for *E. amylovora* (Oh & Beer, 2005). In *E. amylovora*, eleven T3-secreted proteins have been reported to date (Nissinen *et al.*, 2007; Vrancken *et al.*, 2013b). Two harpins, HrpN and HrpW are secreted. These are glycine-rich, lack cysteine, are heat stable and are both involved in the induction of the HR in non host plants (Wei *et al.*, 1992a; Kim & Beer, 1998). Mutants in the *hrpN* gene showed to be non pathogenic (Wei *et al.*, 1992a; Barny, 1995), while HrpW on the other hand is not required for virulence (Kim & Beer, 1998). Furthermore, both HrpN and DspA/E are important factors in the elicitation of an oxidative burst in compatible host plants (Venisse *et al.*, 2003). DspA (Gaudriault *et al.*, 1997) and DspE (Bogdanove *et al.*, 1998), also known as DspA/E is a secreted effector homologous to the type III effector AvrE of *Pseudomonas syringae* (Gaudriault *et al.*, 1997). It is a large protein (198 kDa), required for pathogenicity in apple and pear (Gaudriault *et al.*, 1997; Bogdanove *et al.*, 1998). The N-terminus of DspA/E interacts with four similar putative leucine-rich repeat (LRR) receptor-like serine/threonine kinases (RLK) from apple (Meng *et al.*, 2006) and the C-terminus interacts with a pre-ferredoxin, an

electron carrier in photosystem I (Bonasera, 2006). Thereby DspA/E may inhibit signal transduction and photosynthesis in the host. DspB/F is a small protein suggested to function as a chaperone during the secretion of DspA/E (Gaudriault *et al.*, 2002). HrpA forms a Hrp pilin that extends outside the bacterial cell which may reach the host cell (Kim *et al.*, 1997; Jin *et al.*, 2001). Mutants of the *hrpA* gene are not able to cause HR in non host plants nor can they cause disease in hosts. HrpA is also important for the secretion of the effector proteins HrpW and DspA/E (Jin *et al.*, 2001). Eop1, the gene product of *orfB*, also calles EopB (*Erwinia* outer protein B) (Oh & Beer, 2005) is a member of the YopJ/AvrRxv/HopZ family of protease effectors which are homologues and conserved among plant and animal pathogens (Nissinen *et al.*, 2007). Eop2 and Eop3 were also secreted. Eop2 is homologues to the helper protein HopAK1 of *P. syringae* which induces a HR in tobacco (Alfano & Collmer, 2004; Nissinen *et al.*, 2007) and resembles HrpW (Nissinen *et al.*, 2007). However, the function in *E. amylovora* still remains unknown. The Eop3 effector is homologues to members of the HopX family which are common in *P. syringae* strains (Nissinen *et al.*, 2007). HrpJ, homologues to the protein YopN of *Yersinia spp*. (Bogdanove *et al.*, 1996) is required in pathogenesis and plays a major role in HR response in non hosts. Also a function for HrpJ in the accumulation of extracellular harpins was suggested (Nissinen *et al.*, 2007). Altough HrpK from *E. amylovora* is distantly related to the same protein of *P. syringae* (Alfano & Collmer, 2004), a *hrpK* mutant of *E. amylovora* was not restricted in virulence (Oh *et al.*, 2005) as was a *hrpK* mutant in *P. syringae* (Petnicki-Ocwieja *et al.*, 2005), so the function of this effector in *E. amylovora* has still to be determined. TraF is similar to proteins involved in plasmid transfer and pilus formation (Haase & Lanka, 1997; Nissinen *et al.*, 2007) and it also possesses a signal peptide for type II secretion (Nissinen *et al.*, 2007). Lastly, secretion of a homologue of the flagellar protein FlgE, FlgL, was observed (Nissinen *et al.*, 2007).

During this study, a comparison was made between different strains of *E. amylovora* regarding their secretome. Previous research has indicated eleven proteins as being secreted by the T3SS in *E. amylovora* (Nissinen *et al.*, 2007). Primers for the genes corresponding to these proteins, were designed and a gene expression profile by RT-qPCR was performed on four strains of *E. amylovora* with differential virulence, *in vitro*. For two strains these primers were

also tested on samples extracted from bacteria grown in an *in planta* model. These results confirm a role for several of these genes in virulence and in differences between those strains.

6.3 Materials and methods

6.3.1 Bacterial strains

For the experiments four wild type strains of *E. amylovora* were used including two low virulent strains, LMG2024 and PD437, both isolated from *Pyrus communis* and two high virulent strains, PFB5, isolated from *Prunus salicina* and BG16 (collection number SGB 225/12), isolated from *Malus sylvestris* (Chapter 3). Strains were stored at -80 °C in 10% glycerol and cultured on yeast peptone glucose agar (YPGA) plates at 24 °C.

6.3.2 Bacterial growth and isolation

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) (Huynh *et al.*, 1989) 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.

The extraction of viable bacterial cells from plant tissue was performed according to the procedure described in chapter 4. In short, apple rootstocks (Malling 9 clone T337, obtained from Carolus Trees (Sint-Truiden, Belgium)) were used as host. The two youngest leaves from the shoots were cut perpendicularly to the midvein using scissors dipped in a bacterial suspension of 1 x 10^8 CFU/ml. After approximately 10 days post inoculation (dpi) for PFB5 and 14 dpi for LMG2024, infection was systemic and sampling was performed. From every rootstock, the infected shoots were removed. After external sterilization of shoots and leaves by submerging them for 5 sec in 1% sodium hypochlorite, they were washed three times with sterile water. The shoots and leaves were cut in 0.5 cm pieces and extracted for 40 - 90 min in 250 ml of a sterile buffer (120 mM phosphate buffer, pH 8, 0.1% w/v sodium pyrophosphate, 0.1% v/v Tween-20, 25% w/v polyvinylpolypyrrolidone), and shaken (200 rpm, room temperature) in the presence of glass beads (0.1 mm – 1 mm). During this

stage bacteria were washed out the plant tissue into the buffer. After filtration through 2 Wattman filters (pore size 8 µm), the extract was centrifuged to pellet the bacteria. Hereafter the bacteria were washed three times with PBS. The samples were frozen at -80 °C until use.

6.3.3 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). After an incubation period of 5 min, bacteria were collected by centrifugation (5000 g, 10 min) and RNA was extracted using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. The RNA samples taken from the bacteria extracted from the plant tissue, were further purified and concentrated by a Na-Acetate and ethanol precipitation. DNA was removed using the TURBO DNA-free kit (Ambion) and final reverse transcription was carried out from 1 µg of DNase-treated total RNA using the PrimeScript RT Reagens Kit (Takara). The cDNA samples were ten-fold diluted using 1/10 diluted TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored at -20 °C until use. Primer3 (Whitehead Institue/MIT Center for Genome Research) was used to develop gene-specific primers based on proteins of interest (Table 6.1). Quantitative PCR (qPCR) was performed using Fast SYBR Green chemistry according to the manufacturer's instructions on an ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems, Belgium). Relative gene expression was calculated as $2^{-\Delta Cq}$ and was normalised with a normalization factor based on the expression of reference genes *rpoD*, *recA* and *rpoS*. Reference genes were tested using the GrayNorm algorithm and both normalised and non-normalised data were presented as an accuracy interval according to Remans *et al.* (2014). 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) (Table S.6.1).

Table 6.1: *Primers used for RT-qPCR.*

Protein	Gene	Gene ID	Locus tag	Left primer	Right primer	Amplicon size	Primer efficiency/ *in vitro* experiment%	Primer efficiency/*in planta* experiment%
DspA/E	*dspA/E*	8913119	EAMY_0557	CGCAGAATTGCTTAACGACA	AGCACTCCCTGATGTTGACC	96	92.88	83.65
HrpK	*hrpK*	8914195	EAMY_0519	AAGAAGGGGATGGGTATGCT	TTCAGGGCATCAAGGAACTC	98	105.51	92.55
Eop2	*eop2*	8911330	EAMY_0653	GCCTGACTGAGCATGTTGAA	CAACGGGACTTCGTTACAGG	100	95.51	87.12
HrpW	*hrpW*	8913502	EAMY_0556	CTGGCACCGTCTTCCAGTAT	TGTTTGATGGCAAAGGACAA	103	98.12	80.0
Eop1	*orfB*	8913496	EAMY_0554	TTCGGCCTGGATAAAGAGAA	ATTTGCACACAGGCACTGAC	98	99.91	93.66
HrpN	*hrpN*	8913118	EAMY_0552	ATCCTGAGGTGTTTGGCAAG	ATCTGGCTTGCTCAGTGCTT	98	108.28	114.0
HrpJ	*hrpJ*	8913100	EAMY_0535	CAGGGTTTGCATTTTCTGGT	GCAACATATTGAGCGCATTG	103	95.9	93.12
TraF	*traF*	8914448	EAMY_3322	CGTCAAGGTTAAAGCCGGTA	TAACTACAGCGCCTCGGTTT	89	95.28	92.70
Eop3	*eop3*	8911790	EAMY_2270	GGATTACCAGCGCTTCATTC	GTGGTGCTGGGGTCAGTATT	101	98.46	94.63
FlgL	*flgL3*	8914139	EAMY_2658	AGCTACCGTCTGGTGAATGG	GCAGCGGTCGCTATATTTTC	104	91.39	95.26
HrpA	*hrpA*	8913504	EAMY_0542	TGCTCAGGCGTCTAAAATGA	GCGTTGTGGCCAGAGTTTAT	96	128.09	97.56

Reference genes

	Gene	Gene ID	Locus tag	Left primer	Right primer	Amplicon size	Primer efficiency *in vitro%*	Primer efficiency *in planta%*
	rpoD	8914668	EAMY_RS19375	TCCGGTGCATATGATTGAGA	CGTTCGGCTAACTCTTCTGG	102	99.80	96.74
	recA	8914602	EAMY_RS21175	GCGACAAAATTGGTCAAGGT	CAGCATTGCGCGTAGTTTAG	98	98	94.47
	rpoS	8913432	EAMY_RS20985	GGATATTCTGGCCGATGAAA	TTGGCGTTGAGTTCAAACAG	102	101.9	93.8

6.4 Results

Based on the 11 type III secreted proteins of *E. amylovora* identified by Nissinen *et al.* (2007) of strain Ea 273, gene-specific primers were developed. To gain insights in the production of these proteins in strains of *E. amylovora*, exhibiting differences in virulence, gene-expression profiling was performed by RT-qPCR. First, four strains including two low virulent (LMG2024 and PD437) and two high virulent strains (BG16 and PFB5) were compared *in vitro*. Next, two strains exhibiting the most differences during the infection process, LMG2024 and PFB5 (Chapter 3 and Chapter 4), were chosen for an *in planta* comparison.

6.4.1 Gene expression of four strains grown in vitro

Four strains differing in virulence (Chapter 3 and Chapter 4), were used to compare gene expression profiles of the secretome. The bacteria were grown in an *hrp*-inducing minimal medium (HrpMM) (Huynh *et al.*, 1989). Eleven genes, corresponding to the secreted proteins found by Nissinen et al. (2007) (Table 6.1) (Nissinen *et al.*, 2007), were tested (Figure 6.1). Results were expressed, relative to the lowest virulent strain used, LMG2024. Relative gene expression of *hrpW* and *hrpK* (Figure 6.1A and C) showed a significant difference between PFB5 and the lower virulent strains, LMG2024 and PD437. Between both higher virulent strains, differences were not significant. When regarding expression of *dspA/E*, there was a significant difference between BG16 and the lower virulent strains and there was no significant difference between PBG16 and PFB5 (Figure 6.1B). The non-normalised values from PFB5 were significantly different from the lowest virulent strain LMG2024. The expression of *eop2* and *orfB* was respectively depicted in figure 1D and E. For both genes a significant difference was seen between low and high virulent strains. Normalised gene expression values for hrpN (Figure 6.1F) showed a significant difference between BG16 and the lower virulent strains. No significant difference could be observed between PFB5 and LMG2024 although non-normalised data did demonstrate a significant difference between both strains. RT-qPCR data showed a significant difference between low virulent strains and BG16 for the genes encoding HrpJ and HrpA (Figure 6.1G and K respectively). PFB5 forms an intermediate between the lower virulent strains and BG16. Relative gene expression of *traF* (Figure 6.1H) showed a significant difference between BG16 and the lower virulent strains.

Non-normalised data demonstrated significant differences between low and high virulent strains. The gene encoding Eop3 was up-regulated in a great extend in PFB5 in comparison with the other strains (Figure 6.1I). Significant differences were observed between PFB5 and the other three strains. For *flgL3* gene expression data showed a significant difference between LMG2024 and the other three strains (Figure 6.1J). This indicates a higher expression of the gene *flgL3* in the low virulent strain LMG2024.

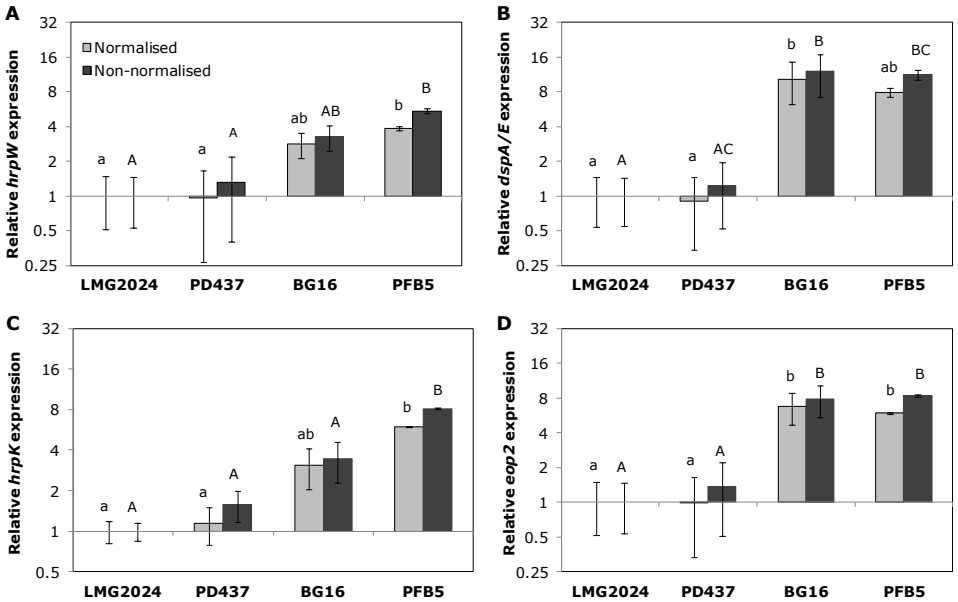


Figure 6.1: *Relative gene expression measured by RT-qPCR. Normalised data are represented in light gray and the non-normalised data in dark grey. Up- or down regulations are represented on a* log_2 *scale y axis relative to the least virulent strain. Columns represent data from four biological replicates ± standard errors. Letters indicate statistical differences (P < 0.05, one-way ANOVA after testing normality with Shapiro-Wilk test). Relative expression of A. hrpW, B. dspA/E, C. hrpK and D. eop2 is depicted.*

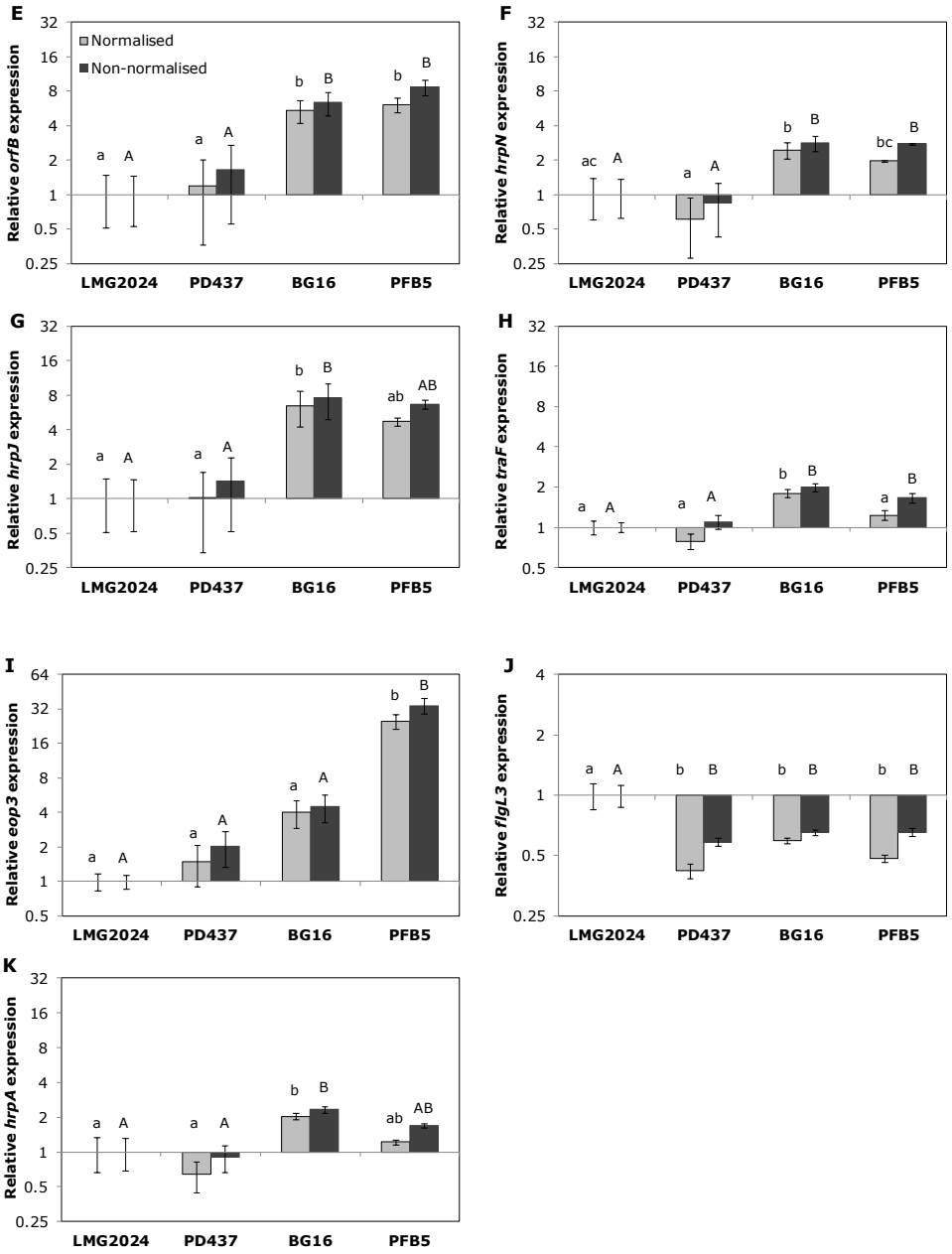


Figure 6.1 (continued): *Relative gene expression measured by RT-qPCR. Normalised data are represented in light gray and the non-normalised data in dark grey. Up- or down regulations are represented on a* log_2 *scale y axis relative to the least virulent strain. Columns represent data from four biological replicates ± standard errors. Letters indicate statistical differences (P < 0.05, one-way ANOVA after testing normality with Shapiro-Wilk test). Relative expression of E. orfB, F. hrpN, G. hrpJ, H. traF, I. eop3, J. flgL3 and K. hrpA is depicted.*

6.4.2 Gene expression of two strains grown in planta

Next, the same set of primers was used for the gene expression profiling of these genes for two strains grown *in planta*. As a host, shoots of apple rootstocks were used and the bacteria were isolated when infections were systemic, after which RNA was extracted and further processed. Results are depicted in figure 6.2. With exception of *hrpA*, all genes were up-regulated in the high virulent strain PFB5 in comparison with LMG2024. For both *traF* and *eop3*, significant differences were observed in both normalised and non-normalised data. For the expression of genes *hrpW*, *orfB*, *hrpJ* and *hrpN*, a significant difference between both strains was observed for the non-normalised data. A clear trend was visual for the normalised 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.

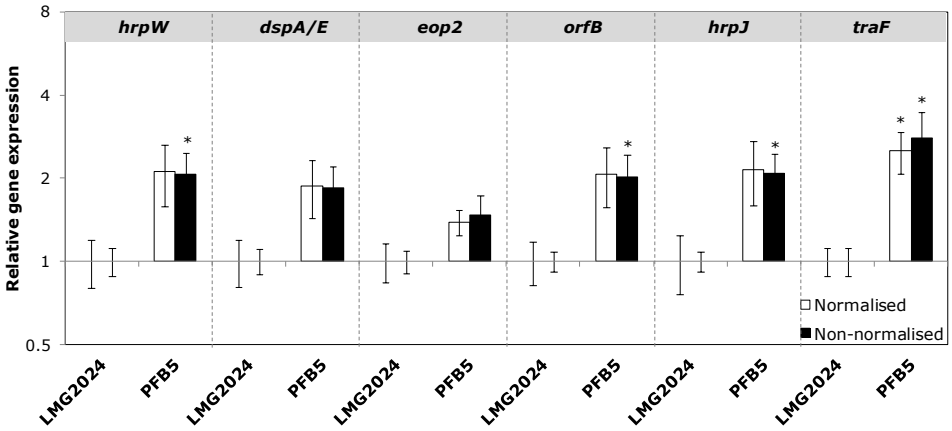


Figure 6.2: *Relative gene expression measured by RT-qPCR. Normalised data are represented in white and the non-normalised data in black. Up- or down regulations are represented on a log_2 scale y axis relative to the least virulent strain. Columns represent data from four biological replicates ± standard errors. Letters indicate statistical differences (P < 0.05, one-way ANOVA after testing normality with Shapiro-Wilk test). Relative expression of hrpW, dspA/E, eop2, orfB, hrpJ and traF is depicted.*

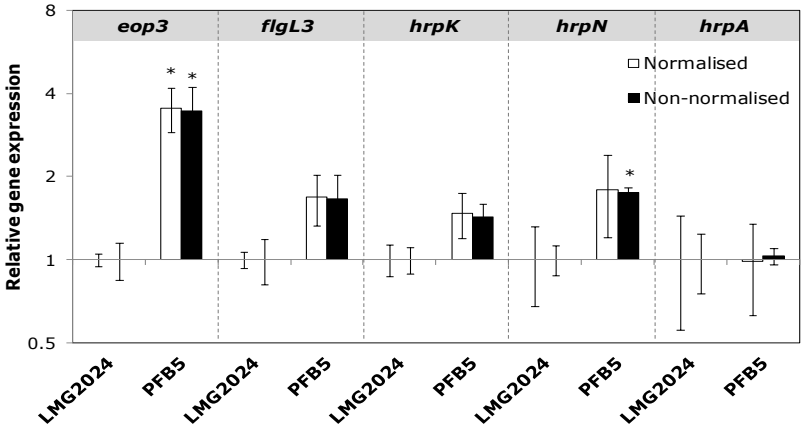


Figure 6.2 (continued): *Relative gene expression measured by RT-qPCR. Normalised data are represented in white and the non-normalised data in black. Up- or down regulations are represented on a* log_2 *scale y axis relative to the least virulent strain. Columns represent data from four biological replicates ± standard errors. Letters indicate statistical differences (P < 0.05, one-way ANOVA after testing normality with Shapiro-Wilk test). Relative expression of eop3, flgL3, hrpK, hrpN and hrpA is depicted.*

6.5 Discussion

The major type III secreted proteins have been identified for *E. amylovora* strain Ea 273 (Table 6.1) (Nissinen *et al.*, 2007). Gene-specific primers corresponding with these proteins were developed and gene expression was measured by RT-qPCR. Moreover, during this research the gene expression of the *hrp* genes in a culture medium resembling the plant's apoplast was measured and further there was also a comparison made with gene expression of these genes in bacteria grown inside a host plant. The gene expression profile for these genes was determined and compared for four strains with differential virulence grown in a HrpMM and for two strains grown in shoots of apple shoots. These results provide major insights in the different expression profiles between strains of *E. amylovora* exhibiting differential virulence towards the infection process.

When regarding the results of the *in vitro* comparison (Figure 6.1), all genes were up-regulated in the high virulent strains with exception of *flgL3* (Figure 6.1J). The gene *flgL3* encodes for a protein homologue to the flagellar protein FlgE (Nissinen *et al.*, 2007). This protein is the major component of the flagellar hook (Macnab, 1992; Berg, 2003) and important for motility. As previously reported (Chapter 3), LMG2024 is more motile in comparison with the other

strains in an *in vitro* environment. 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 is recognized by the plant (Gomez-Gomez & Boller, 2000; Boller & Felix, 2009; Sun *et al.*, 2013). 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 (Gomez-Gomez & Boller, 2000). Due to this higher abundance of PAMPs, this strain will be faster recognized by the plant.

In contradiction, *flgL3* was up-regulated in the higher virulent strain *in planta* (Figure 6.2). Following the previous hypothesis, the protein encoded by this gene can induce PTI but previous research (Chapter 3 and 4) suggested a higher amylovoran production for the high virulent strain PFB5 both *in vitro* as *in planta* which may prevent recognition of PAMPs by masking them. Further, amylovoran is important for biofilm formation, which encases the bacteria in an extracellular matrix that protects the bacteria (Koczan *et al.*, 2009).

Three type III secreted proteins have been shown to be important in pathogenicity of *E. amylovora*, DspA/E (Gaudriault *et al.*, 1997; Bogdanove *et al.*, 1998), HrpN (Wei *et al.*, 1992a; Barny, 1995) and HrpJ (Nissinen *et al.*, 2007). Gene expression profiling of the corresponding genes was depicted in figure 6.1 (B, F and G) and for all three genes, the higher virulent strains showed a higher expression. 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 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) (Meng *et al.*, 2006). Secondly, preferredoxin was found to interact with DspA/E. This protein is converted to ferredoxin which serves as an electron carrier in photosystem I (Bonasera, 2006). Next, DspA/E contributes to disease development by inhibition of the salicylic acid (SA) dependent innate immunity (DebRoy *et al.*, 2004). 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 (Dangl & Jones, 2001; Kunkel &

Brooks, 2002). Moreover, down-regulation of the jasmonic acid (JA) pathway by DspA/E has also proven important in the infection process of *E. amylovora* (Duge De Bernonville *et al.*, 2012).

Following these findings, we can suggest that the higher virulent strains, which showed a higher expression of the gene encoding DspA/E are able to interfere with the immune system of the plant, leading to successful infection. The lower virulent strains have a lower expression which can indicate a lower protein production of DspA/E and consequently a lower inhibition of plant immune responses. These results were reflected in the *in planta* comparison between LMG2024 and PFB5 (Figure 6.2) which leads to the same conclusion.

HrpN belongs to the class of harpins which are unlike the T3SEs, secreted and targeted to the intercellular spaces of plant tissues (Alfano & Collmer, 2004). Moreover, HrpN is only secreted and localized in the plants apoplast during infection (Perino *et al.*, 1999). 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* (Dong *et al.*, 2004). Furthermore, combined action of DspA/E and HrpN has been proven to induce an oxidative burst in host plants (Venisse *et al.*, 2003). Both genes are up-regulated in the higher virulent strains, both *in vitro* (Figure 6.1 B and F) and *in planta* (Figure 6.2). 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. Following this cell death, the pathogen obtains more nutrients to grow, divide and further colonize the host.

The expression of *eop3* was very high in PFB5 in comparison with the other strains, both *in vitro* (Figure 6.1I) and *in planta* (Figure 6.2). Eop3 is homologues to AvrPhE, a protein belonging to the HopX family of effector proteins (Mansfield *et al.*, 1994; Nissinen *et al.*, 2007). HopX has been shown to suppress programmed cell death by the inhibition of the pro-apoptotic protein Bax (Jamir *et al.*, 2004). This function could help PFB5 to secure nutrient supply by inhibiting programmed cell death. The gene encoding HrpA, the protein that forms the Hrp pilin, was upregulated in the higher virulent strains in vitro (Figure 6.1K), although, no difference was observed between LMG2024 and PFB5 *in planta* (Fig. 2).

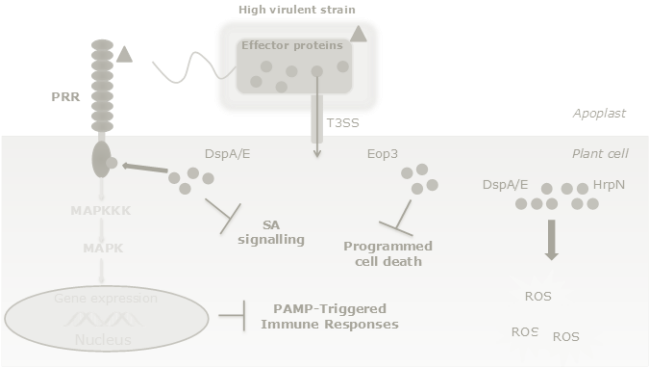
In conclusion, by gene expression profiling, we were able to identify important differences between strains of *E. amylovora* concerning the expression of genes encoding type III secreted proteins. Moreover, these differences could be linked to virulence since the strains used during this research exhibited differential virulence. An *in vitro* analysis shows that all genes corresponding to known type III secreted proteins of *E. amylovora* were up-regulated in higher virulent strains with exception of *flgL3*. This can lead to a higher recognition of the lower virulent strain by the host defense systems. Strains exhibiting high virulence potential showed higher expression of the gene encoding DspA/E. 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 (Wei *et al.*, 1992b), indicating the importance of this system to acquire nutrients.

6.6 Supplementary data

Table S. 6.1: *Quantitative PCR (qPCR) parameters according to the Minimum Information for publication of qPCR Experiments (MIQE) checklist derived from Bustin et al. (2010).*

Sample/Template	
Source	*Erwinia amylovora* cells extracted from plant tissue and *Erwinia amylovora* cells in liquid HrpMM medium
Method of preservation	After samples were taken, they were frozen in liquid N_2 and stored at -80°C
Storage time	Three weeks
Handling	Liquid supplemented with 2 volumes of RNAprotect Bacteria Reagent* (Qiagen, Venlo, The Netherlands).
Extraction method	RNeasy Mini Kit* (Qiagen, Venlo, The Netherlands)
RNA: DNA-free	TURBO DNA-free Kit* (Ambion, Lennik, Belgium)
Concentration	NanoDrop®: ND-1000 Spectrofotometer (Isogen Life Science, IJsselstein, the Netherlands)
RNA: integrity	Not tested; RNAprotect Bacteria Reagent* (Qiagen, Venlo,The Netherlands).
Assay optimisation/validation	
Accession number	See Table S.4.3
Amplicon details	Amplicon size see Table 3.2
Primer sequence	See Table 3.2
In silico	Primer-BLAST
Empirical	Primer concentration (300 nM), annealing temperature (60°C)
Priming conditions	Random hexamer priming
PCR efficiency	Dilution curves
Linear dynamic range	Samples are within the efficiency curve
RT/PCR	
Protocols	See Materials and Methods
Reagents	See Materials and Methods
NTC	C_q & melt curves
Data analysis	
Specialist software	7500 Fast System Sequence Detection Software, version 1.4 (Applied Biosystems, Lennik, Belgium, 2001-2006)
Statistical justification	4 biological replicates, one-way ANOVA
Normalisation	3 reference genes selected using GrayNorm

*All procedures were performed according to the manufacturer's protocol.



7

General discussion

7.1 General discussion

Fire blight, caused by the Gram-negative bacterium *Erwinia amylovora*, is a destructive disease which affects most members of the *Rosaceae* family of which apple (*Malus* spp.) and pear (*Pyrus* spp.) are economically the most important species. Other hosts include quince, blackberry, raspberry and many wild and cultivated ornamentals belonging to this family (Vanneste, 2000a). This devastating disease is spread by wind, insects, birds and human activity. Due to its destructive character and the lack of effective control mechanisms, *E. amylovora* is capable of dispersing rapidly both within susceptible plants and between trees in orchards which could result in great economic losses. Because of the expected rise in average global temperatures, the breeding of cultivars on susceptible rootstocks and the introduction of susceptible cultivars, fire blight will become an even greater threat for the fruit production in Europe in the near future (Deckers & Schoofs, 2008).

E. amylovora is considered to be a homogeneous species based on physiological, biochemical, phylogenetic and genetic analysis (Vanneste, 2000a; Smits *et al.*, 2010; Wang *et al.*, 2010; Zhao & Qi, 2011). However, differences in virulence have been observed in strains of *E. amylovora* isolated from nature (Cabrefiga & Montesinos, 2005; Lee *et al.*, 2010; Wang *et al.*, 2010), but the proteins underlying these differences in virulence have not been described.

Different virulence factors of *E. amylovora* have been identified including a functional type III secretion system (T3SS) to inject effector proteins into the cytosol of the host, exopolysaccharides (EPS) including amylovoran and levan, the sorbitol metabolism, the siderophore desferrioxamine, metalloproteases and two-component signal transduction systems (TCSTs) (see Chapter 1 for review).

To date, most research on the virulence of E. amylovora has been performed on a genomic level. On the contrary, proteome research of this pathogen is limited to the extracellular proteome of a raspberry and an apple isolate of *E. amylovora* grown in a *hrp*-inducing medium (Braun & Hildebrand, 2005), the identification of the type III secreted proteins (Nissinen *et al.*, 2007) and the lysine acetylome of *E. amylovora* (Wu *et al.*, 2013). The latter suggested that protein lysine acetylation or other post-translational modifications might be involved in the differential virulence between strains of *E. amylovora* (Wu *et al.*, 2013).

Within our research group, a proteomics analysis by two-dimensional differential gel electrophoresis (2D-DIGE) for *E. amylovora* has been optimized. The main objective of this study was to identify proteins important for the differential virulence in *E. amylovora*. To do so, strains differing in virulence were investigated both *in vitro* (Chapter 3) and *in planta* (Chapter 4). Moreover, a robust and reliable method was used to isolate the outer membrane and its proteome was also compared between these different strains *in vitro* and *in planta* (Chapter 5). Finally, gene expression by RT-qPCR was used to perform a comparative study of the type III secreted proteins in *E. amylovora* (Chapter 6) between the strains on a transcriptome level both *in vitro* and *in planta*.

During the different experiments four wild type strains were considered differing in virulence (Chapter 3). These include LMG2024, isolated from *Pyrus communis* (Belgian coordinated collections, Ghent university), strain PD437, also isolated from *Pyrus communis* (Plantenziektenkundige dienst, Wageningen, The Netherlands), strain PFB5, isolated from *Prunus salicina* (S.K. Mohan, Idaho, USA) and BG16 isolated from *Malus sylvestris* (Bulgaria) with collection number SGB 225/12. LMG2024 and PD437 are normal virulent strains while PFB5 and BG16 are highly pathogenic strains (Maes *et al.,* 2001; Chapter 3).

For all proteomics experiments both *in vitro* and *in planta*, LMG2024 and PFB5 were chosen for an in-depth comparison. This because of the overall difference in virulence that was observed during infections on apple rootstocks and the results of all proteomics data suggesting the most pronounced differences between those two strains. Further discussion will be limited to these two strains, LMG2024, a low virulent strain and PFB5, a high virulent strain.

For the comparison of both strains *in vitro* (Chapter 3), the bacteria were grown in a minimal medium containing sorbitol. Differences were observed in the overall metabolic processes (Figure 3.8) between both strains but the most interesting results were observed in motility and amylovoran synthesis. Proteomics results indicated a higher amount of both FliC, the main component of the flagellar filament, and the chemotaxis regulatory protein, CheY which can switch direction of movement (Berg, 2003). These results were confirmed by both a swarming assay and gene expression (Figures 3.6B and 3.7, respectively) suggesting a better motility and a higher flagella production for the low virulent strain. Flagellin contains a conserved domain that functions as a pathogen-

associated molecular pattern (PAMP), recognized by the pattern recognition receptor (PRR) flagellin-sensitive 2 (FLS2) receptor kinase (Gomez-Gomez & Boller, 2000; Boller & Felix, 2009; Sun *et al.*, 2013). Treatment of *Arabidopsis* with flagellin leads to the production of ROS, ethylene and the induction of the general defense of the plant (Gomez-Gomez & Boller, 2000). Furthermore, a PAMP-triggered immunity (PTI) may be induced (Jones & Dangl, 2006). In PFB5 on the other hand, a higher abundance of the protein GalF was observed. This protein is involved in the formation of UDP-glucose from α-D-glucose-1P which is essential for amylovoran production (Geider, 2000). To validate these results, an amylovoran assay and gene expression by RT-qPCR was performed (Figure 3.6A and 3.7, respectively), confirming the hypothesis that PFB5 produces higher amounts of amylovoran. The latter is an important virulence factor of *E. amylovora* and it was suggested that amylovoran is barely immunogenic, allowing the bacteria to elude host recognition and escape host defenses (Romeiro *et al.*, 1981; Geider, 2000). These results are depicted in figure 7.1. Due to its higher amount of flagellin and lower amount of amylovoran to mask this PAMP, LMG2024 is more likely to induce the PAMP-triggered immunity by a MAPK-pathway. Thereby LMG2024 will be faster recognized by the plant in comparison with PFB5, which was found to produce more amylovoran to mask the plant immune elicitors.

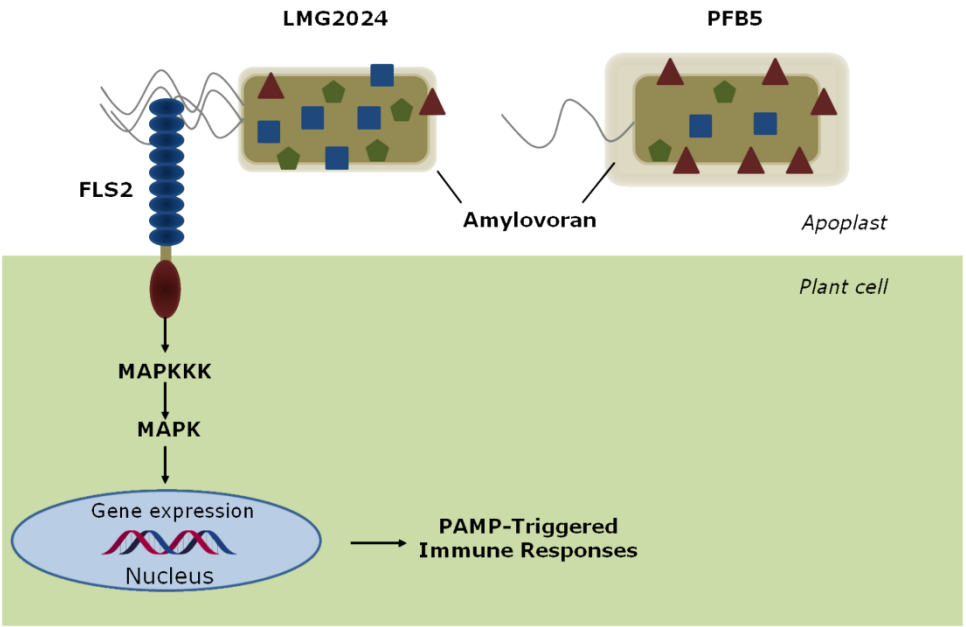


Figure 7.1: *Recognition of the pathogen-associated molecular pattern (PAMP) flagellin by the extracellular receptor-like kinase (RLK) FLS2. This triggers basal immunity which requires signaling through MAP kinase cascades and activation of gene expression. Morphological differences between both strains are also depicted. Other PAMPs are indicated in the cell including lipopolysaccharides (red triangles), elongation factors Tu (green pentagons) and cold shock proteins (blue squares). Figure modified from (Gomez-Gomez & Boller, 2000; Chisholm et al., 2006).*

To extrapolate these results to an *in planta* model, shoots of apple rootstocks were infected and when infection was systemic, viable bacterial cells were extracted from the host tissue. These samples were used as such for protein extraction without further interference of other media of growth conditions. These results give insights into the proteome of this pathogen inside its host (Chapter 4).

First, a fundamental difference was observed between LMG2024 and PFB5 concerning the proteins that were used in response to stress (Figure 4.4). Because *E. amylovora* is detected as an incompatible pathogen, infection leads to an oxidative burst by which the plant produces reactive oxygen species (ROS), thereby exposing the pathogen to high levels of oxidative stress when infecting its host (Venisse *et al.*, 2001; Venisse *et al.*, 2003). The low virulent

strain has a higher abundance of cold shock proteins, while PFB5 produces more heat shock proteins to deal with stress. Cold shock proteins can be recognized by the plant as PAMP inducing plant defenses. This may explain why PFB5 exhibits higher amounts of heat shock proteins.

When comparing the overall metabolic processes between both strains including carbohydrate, fatty acid and amino acid metabolism, it becomes clear that PFB5 is actively growing and dividing whilst LMG2024 seems in a stress condition and is impaired in many cellular activities. A detailed comparison of the carbohydrate metabolism between both strains showed that, all genes encoding depicted proteins (Table 4.5) with exception of *talA* and *ugd*, were up-regulated in PFB5 in comparison to LMG2024 (Figure 4.7). Moreover, the higher expression of the sorbitol dehydrogenase in the higher virulent strain indicates a better use of the present carbon source inside the plant and leads to a higher production of amylovoran. Indeed, two proteins, GalF and GalE were more abundant for PFB5 suggesting a higher amylovoran production since these proteins are necessary for the production of UDP-glucose and –galactose, two building blocks of amylovoran. Furthermore, amylovoran is involved in biofilm formation, a process that recently has been linked to pathogenesis in *E. amylovora* (Koczan *et al.*, 2009).

Different proteins involved in RNA processing were found to be more abundant for the higher virulent strain, PFB5, in comparison with LMG2024. These proteins included polyribonucleotide nucleotidyltransferase (Pnp) and enolase (Eno) which are both important components of the RNA degradosome in *E. coli* (Figure 4.5A). Following these results, all principal components of the RNA degradosome of *E. coli* were tested by gene expression including *rne*, *rhlB*, *eno*, *pnp*, *hfq* and *rho* (Figure 4.5C). All genes, besides *rne* and *rhlB* exhibited a higher expression in PFB5, suggesting a role for several components of this degradosome in virulence. In *Salmonella enterica*, Pnp functions as an important regulator of virulence (Clements *et al.*, 2002) and this protein has also been proposed to be important in the regulation of the production of outer membrane proteins by means of small RNAs (sRNAs) in *E. coli* (Andrade & Arraiano, 2008). The protein Hfq has already been described as a virulence regulator in *E. amylovora* (Zeng *et al.*, 2013). Moreover, genes regulated by Hfq can be classified under varying biological functions including host cell invasion, motility, central metabolism, LPS

biosynthesis, two-component regulatory systems and fatty acid metabolism (Chao & Vogel, 2010; Zeng *et al.*, 2013). In addition, two proteins involved in LPS-biosynthesis were more abundant for PFB5 which may imply protection against oxidative stress (Berry *et al.*, 2009).

These data confirm results from Chapter 3 indicating a lower concealment and a higher abundance of PAMPs in LMG2024 triggering the immune response of the host. Moreover, data from chapter 4 showed the presence of two extra PAMPs for LMG2024, namely cold shock proteins and the elongation factor Tu (EF-Tu). However, PFB5 exhibited a higher amount of LPS, another PAMP, but the higher amount of amylovoran may conceal these factors for RLKs of the plant. Moreover, both amylovoran and LPS have been identified as playing a role in protecting the cell against oxidative stress produced by the host.

The outer membrane (OM) and its proteins form the first interaction between *E. amylovora* and its environment. When entering a host plant, they are the first elements that interact with the host and are as first exposed to the defense mechanisms of the plant. In Chapter 5, the outer membrane from *E. amylovora* was extracted from the rest of the cell in order to investigate the proteome composition of this membrane and to identify differences between strains differing in virulence. In total 30 proteins were identified in the OM of *E. amylovora* (Table 5.2). *In vitro*, only four proteins were identified as being differently abundant between LMG2024 and PFB5 (Table 5.3) and all four appeared to be more present in the low virulent strain LMG2024. Conform with the *in vitro* experiment (Chapter 3), again flagellin (FliC) and another protein involved in flagellar structure, a flagellar hook associated protein (FlgL) were identified as being more abundant in the low virulent strain (Figure 5.3). These findings validate previous results already indicating a higher amount of flagellin for the low virulent strain.

The outer membrane proteome was also analyzed for LMG2024 and PFB5 when grown *in planta* (Table 5.4). Interestingly, again a structural component of the flagellum, FlgL, was more abundantly present in LMG2024 in comparison with PFB5. In PFB5 on the other hand, a higher abundance of the proteins YaeT and TolC was found. The first, YaeT, is an essential OMP involved in OM biosynthesis and is essential for viability (Voulhoux *et al.*, 2003; Bos *et al.*, 2007). This may indicate that PFB5 has a higher production of OMPs than LMG2024, emphasizing

the facts already mentioned in Chapter 4 that PFB5 may be dividing faster and growing better in comparison with LMG2024 that is trying to cope with the stress caused by the immunity defenses of the host. Secondly, TolC was more abundant for PFB5. A function for this protein in virulence and overall fitness has already been identified in *E. amylovora* (Al-Karablieh *et al.*, 2009). Moreover, TolC has been proven to interact with AcrAB leading to resistance against phytoalexins produced by the plant as a defense (Al-Karablieh *et al.*, 2009).

Previous results from Chapter 3 and 4 suggested a primary and fundamental difference in recognition of both strains by the host. This by the presence of PAMPs e.g. flagellin, cold shock proteins and the elongation factor Tu in the lower virulent strain, that are recognized by the host and initiate PAMP-triggered immunity (PTI) and by the amount amylovoran which may function in masking PAMPs in the higher virulent strain. PTI is the first response of the plant towards a recognized pathogen. A counteraction of the pathogen to this PTI is the injection of effector proteins into the cytosol of the host to interfere with this process, this is called effector-triggered susceptibility (ETS) (Jones & Dangl, 2006). In *E. amylovora*, eleven proteins are identified as being secreted by the T3SS (Nissinen *et al.*, 2007) and probably involved in this mechanism.

To identify the function of these effector proteins in the differential virulence between strains, gene expression was used to assess the differences between strains both *in vitro* and *in planta*. *In vitro* research showed a higher expression of all genes with exception of *flgE*, encoding the hook of the flagellum, in the high virulent strain (Figure 6.1). According to the results of the previous chapters 3, 4 and 5, there is a higher amount of flagella in the low virulent strain, LMG2024. All other genes were expressed more in the high virulent strain which may indicate a higher amount of effector proteins in the high virulent strain that may interfere with the PTI.

The same genes were measured for both strains grown *in planta* (Figure 6.2). In this case, all genes were expressed higher in PFB5, the higher virulent strain. Although not all functions of the effector proteins secreted by *E. amylovora* have been identified, some functions have been reported. Figure 7.2 summarizes known and predicted functions and represents possible interference of type III effector proteins of *E. amylovora* with the PTI in the host.

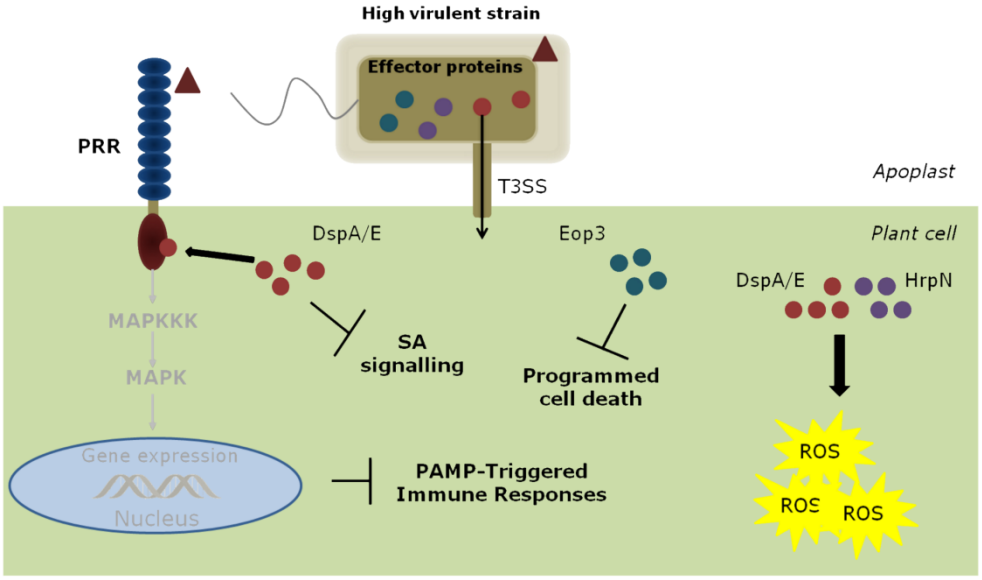


Figure 7.2: *Injection of type III secreted effector proteins into the host cytosol via the T3SS. Different function of these effectors in E. amylovora are depicted and there interference with plant immune responses is also shown. PAMP of the pathogen is recognized by a pattern recognition receptor (PRR). The bacterium injects effector proteins including DspA/E into the cytosol of the host which interfere with the cell signaling leading to plant defenses and PAMP-triggered immunity (PTI).*

DspA/E, a homolog of AvrE in *P. syringae*, contributes to disease development by several actions. First, it may interact with RLKs of the plant to interfere with PTI and induce effector-triggered immunity (ETI). Moreover, two groups of target proteins of DspA/E have been identified in apple, of which the first group includes four kinases named DIPM1-4 (DspE-interacting proteins from Malus 1 to 4) (Meng *et al.*, 2006). Secondly, preferredoxin was found to interact with DspA/E. This protein is converted to ferredoxin which serves as an electron carrier in photosystem I (Bonasera, 2006). Next, DspA/E, contributes to disease development by inhibition of the salicylic acid (SA) dependent innate immunity (DebRoy *et al.*, 2004). SA is a signal molecule of which levels are increased following pathogen infection. This leads to immune responses including systemic acquired resistance, basal resistance and even gene-for-gene resistance (Dangl & Jones, 2001; Kunkel & Brooks, 2002). Moreover, down-regulation of the jasmonic acid (JA) pathway by DspA/E has also proven important in the infection process of *E. amylovora* (Duge De Bernonville *et al.*, 2012).

Both HrpN and DspA are important factors in the elicitation of an oxidative burst in compatible host plants (Venisse *et al.*, 2003). Furthermore, *E. amylovora* needs this oxidative burst for a successful infection (Venisse *et al.*, 2001). It was suggested that this ROS production is needed to invade plant cells since *E. amylovora* itself does not produce pectolytic or cellulolytic enzymes (Seemuller & Beer, 1976) nor phytotoxic metabolites (Eastgate, 2000) to invade its host cells and acquire nutrients. Eop3 is also secreted, this protein is homologues to AvrPhE, belonging to the HopX family (Mansfield *et al.*, 1994; Nissinen *et al.*, 2007). HopX has been shown to suppress programmed cell death by the inhibition of the pro-apoptotic protein Bax (Jamir *et al.*, 2004).
Since all these genes were expressed more, we may hypothesize that the higher virulent strain, which secretes more of these effectors, has a higher ability to suppress the innate immune system of the plant.

An overview of all important proteins and genes is listed in table 7.1.

Table 7.1: *Summary table with the most important proteins or genes identified to be more abundant in one of the two strains during the conducted research.*

	In vitro		**In planta**	
	LMG2024	**PFB5**	**LMG2024**	**PFB5**
Complete proteome	➢ ↑ Flagellin (FliC) ➢ ↑Chemotaxis regulatory protein (CheY)	➢ ↑Amylovoran (GalF)	➢ ↑Cold shock proteins ➢ ↑Elongation factor (EF-Tu)	➢ ↑Heat shock proteins ➢ ↑Carbohydrate metabolism ➢ ↑SrlD ➢ ↑Amylovoran ➢ ↑Biofilm ➢ ↑LPS ➢ ↑RNA processing ➢ ↑Fatty acid metabolism
Proteome outer membrane	➢ ↑Flagellin (FliC) ➢ ↑FlgL	➢ No proteins identified	➢ ↑FlgL3 ➢ ↑IcsP/SopA	➢ ↑YaeT ➢ ↑TolC
Secretome[a]	➢ ↑*flgE*	➢ ↑All other genes	➢ No genes	➢ ↑All genes

[a]No proteomics results available for this experiment, results from gene expression by RT-qPCR were used

7.2 Future perspectives

During this research, the main focus was to shed more light upon the proteome of *E. amylovora*. As a result, different aspects of the proteome of *E. amylovora* were described during this dissertation. A comparative proteome study was performed on strains differing in virulence, grown *in vitro* and *in planta*. Moreover, we were able to isolate the proteins of the outer membrane of this pathogen and again we carried out a comparative analysis of the outer membrane proteome of two strains grown *in vitro* and *in planta*. Thereby we performed an open screening by which we identified proteins important in virulence and important for the pathogen to survive inside the host.

A next step in this investigation would be to make the comparison for both strains grown *in vitro* versus *in planta*. Thereby we may identify the proteins accounting for survival inside the host. Also a proteome analysis of the proteins secreted by these strains could provide more insights in the secretion behavior and the difference between different strains. Subsequently, investigation of the targets of these effectors could provide more information on the infection process. Further, would it be interesting to identify the proteins secreted by other secretion systems including the type II and type VI secretion systems. Proteins secreted by these secretion systems may also be important in virulence and may also contribute to differences between strains.

During this dissertation artificial infections were performed and a method was optimized to extract viable bacterial cells from the plant tissue. This technique could be used to extract bacterial cells from infected plant material sampled from naturally occurred infections. Further, bacteria from ooze droplets may provide more information on the natural infection behavior of *E. amylovora*.

During this work different proteins were identified that can be used for the development of more specific treatments. The higher amounts of amylovoran and higher concentration of lipopolysaccharides in the higher virulent strain, for example, could be exploited to interfere with infection. The recognition of bacterial surface polysaccharides by lectines of the innate immune system has been described in human medicine (Sahly *et al.*, 2008). Moreover, a mannose receptor of the macrophages is able to recognize bacterial capsular polysaccharides and lipopolysaccharides (Zamze *et al.*, 2002). These findings could be extrapolated to a plant-pathogen interaction. In order to fight fire blight

infections, it would be interesting to find a way to remove the capsular amylovoran layer, thereby exposing the pathogen to the plant's defense mechanisms.

The use of antimicrobial peptides (AMPs) could also be employed to develop a new treatment against this disease. These peptides are produced by living organisms but the design of new molecules has been accomplished (Montesinos & Bardaji, 2008). AMPs are oligopeptides with a varying numbers of amino acids, ranging from 5 to over a hundred (Bahar & Ren, 2013). Promising results already have been published for four structurally different groups of de-novo synthesized peptides that are highly active against several plant pathogens including *Pseudomonas syringae*, *Pectobacterium carotovorum* and *Xanthomonas vesicatoria* (Zeitler *et al.,* 2013). In time it might be possible to find or even develop AMPs to interfere with specific proteins involved in pathogenicity of *E. amylovora*.

Another interesting path for developing a control method against fire blight, is that of the Alphabodies. These protein scaffolds are an alternative to antibodies and can be fashioned for targeting protein-protein interfaces (Desmet *et al.*, 2014). Our results indicated the importance of several proteins for virulence and infection that could be used to develop a specific alphabody protein for this target, thereby undermining the virulent ability of the pathogen. Interesting targets include proteins involved in amylovoran synthesis or the effector protein DspA/E.

Finally, the evolvement of proteomic techniques implicates future perspectives for this investigation. Until recently it was only possible to investigate the proteome for one single parameter, mainly protein abundance. But new mass spectrometry-based proteomic approaches aim to combine several parameters, thereby providing multidimensional biological information about the proteome. For example, investigation of protein degradation synthesis and turnover can be combined with the examination of other protein characteristics including protein isoforms or variants (Larance & Lamond, 2015). These advantages could lead to a better image of the proteome of *E. amylovora*, highlighting other properties of the proteins involved in virulence or the infection process that not yet have been studied.

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Scientific contributions

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International journals

- **Kucharíková S., Tournu H., Holtappels M., Van Dijck P., and Lagrou K.** (2010) In vivo Efficacy of anidulafungin against Candida albicans mature biofilms in a novel rat model ofcatheter-associated candidiasis. *Antimicrobial Agents and Chemotherapy* **54**(10): 4474-4475
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- **Vrancken K., Holtappels M., Schoofs H., Deckers T. and Valcke R.** (2013) Pathogenicity and infection strategies of the fire blight pathogen Erwinia amylovora in Rosaceae: State of the art. *Microbiology-Sgm* **159:** 823-832
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- **Holtappels M. and Valcke R.** (2014): Proteome investigation of the plant pathogen *Erwinia amylovora. Acta Horticulturae* (ISHS) **1056**:187-190
- **Holtappels M., Vrancken K., Schoofs H., Deckers T., Noben J.P. and Valcke R.** (2014) The plant pathogen *Erwinia amylovora*: a proteome investigation. Accepted at *Acta Horticulturae* – Proceedings of the 29th International Horticultural Congress

Presentations

- **2014:** IHS 2014 Brisbane, Brisbane, Australia

  Title: The plant pathogen *Erwinia amylovora*: a proteome investigation.
- **2013:** XIIIth ISHS International Fire Blight Workshop, Zurich, Switzerland

  Title: Proteome investigation of the plant pathogen *Erwinia amylovora.*

Posters

- **2014:** XVI International Congress on Molecular Plant-Microbe Interactions, Rhodos, Greece

  Title: In vitro comparison of two wild type strains of *Erwinia amylovora* on a proteomic level
- **2012:** Two-day symposium of the Belgian Proteomics Association (BePAc), Belgium, Ghent

  Title: Proteome investigation of the plant pathogen *Erwinia amylovora*

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