The *Pseudomonas syringae*-derived HrpA pilins – molecular characterization and biotechnological application of the transcripts

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ACADEMIC DISSERTATION

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Summary

Many Gram-negative bacteria pathogenic to plants and animals possess type III secretion systems that are used to cause disease. Effector proteins are injected into host cells using the type III secretion machineries. Despite vigorous studies, the nature of the secretion signal for type III secreted proteins still remains elusive. Both mRNA and proteinaceous signals have been proposed. Findings on coupling of translation to secretion by the type III secretion systems are also still contradictory.

This study dealt with the secretion signal of HrpA from *Pseudomonas syringae* pathovar *tomato*. HrpA is the major component of the type III secretion system-associated Hrp pilus and a substrate for the type III secretion systems. The secretion signal was shown to reside in the first 15 codons or amino acids, a location typical for type III secretion signals. Translation of HrpA in the absence of a functional type III secretion system was established, but it does not exclude the possibility of coupling of translation to secretion when the secretion apparatus is present.

The *hrpA* transcripts from various unrelated plant pathogenic bacteria were shown to be extremely stable. The biological relevance of this observation is unknown, but possible explanations include the high prevalence of HrpA protein, an mRNA secretion signal or timing of secretion. The *hrpA* mRNAs are stable over a wide range of temperatures, in the absence of translating ribosomes and in the heterologous host *Escherichia coli*. The untranslated regions (UTRs) of *hrpA* transcripts from at least 20 pathovars of *Pseudomonas syringae* are highly homologous, whilst their coding regions exhibit low similarity. The stable nature of *hrpA* messenger RNAs is likely to be due to the folding of their 5' and 3' UTRs. *In silico* the UTRs seem to form stem-loop structures, the hairpin structures in the 3' UTRs being rich in guanidine and cytosine residues.

The stable nature of the *hrpA* transcript directed the studies to the stabilization of heterologous transcripts and to the use of stable messenger RNAs in recombinant protein production. Fragments of the *hrpA* transcript can be used to confer stability on heterologous transcripts from several sources of bacterial and eukaryotic origin, and to elevate the levels of production of the corresponding recombinant proteins several folds. *hrpA* transcript stabilizing elements can be used for improving the yields of recombinant proteins also in *Escherichia coli*, one of the commonly used hosts in industrial protein production.

Original publications

This thesis is based on the following articles and manuscript that in the text are referred to by their Roman numerals.

I Hienonen E, Roine E, Romantschuk M, Taira S.
mRNA stability and the secretion signal of HrpA, a pilin secreted by the type III system in Pseudomonas syringae. Mol. Genet. Genomics. 2002 266:973-8.

II Hienonen E, Rantakari A, Romantschuk M, Taira S.
The bacterial type III secretion system-associated pilin HrpA has an unusually long mRNA half-life. FEBS Lett. 2004 571:217-20.

III Hienonen E, Romantschuk M, Fenel F, Taira S.
Transcript stabilization by mRNA sequences from *hrpA* of *Pseudomonas syringae*. Manuscript (submitted to J. Biotechnol.).

Abbreviations

ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
CBD	chaperone binding domain
C-terminus	carboxy-terminus
EPEC	enteropathogenic *Escherichia coli*
G/C	guanidine/cytosine
HR	hypersensitive reaction
Hrc	hypersensitive reaction and pathogenesis conserved
Hrp	hypersensitive reaction and pathogenesis
MLD	membrane localization domain
mRNA	messenger ribonucleic acid
NPT	neomycin phosphotransferase
N-terminus	amino-terminus
ORF	open reading frame
PAPI	Poly(A) polymerase
PNPase	polynucleotide phosphorylase
PPK	polyphosphate kinase
PR	pathogenesis related
pv.	pathovar
R	resistance gene or protein
RBS	ribosome binding site
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
rt-PCR	reverse transcriptase polymerase chain reaction
SD	Shine-Dalgarno
spp.	subspecies
Tir	translocated intimin receptor

Abbreviations continued

tRNA	transfer ribonucleic acid
TTSS	type III secretion system
UTR	untranslated region
Yop	*Yersinia* outer protein
Ysc	*Yersinia* secretion

1 Introduction

1.1 SECRETION SYSTEMS OF GRAM-NEGATIVE BACTERIA

Bacteria secrete various kinds of proteins into their extracellular environment. The secreted proteins are needed outside the cell for nutritional, defense or other purposes such as communication with other organisms. Gram-negative bacteria use several different pathways to secrete proteins outside the bacterial cell (reviewed by Stathopoulos *et al.* 2000, Pallen *et al.* 2003, Henderson *et al.* 2004). Unlike Gram-positive bacteria that only have one cell membrane and a thick cell wall consisting of peptidoglycan, Gram-negative bacteria have two membranes interspaced with a thin peptidoglycan layer in the periplasmic space. The secretion systems of Gram-negative bacteria are categorized into type I to V secretion systems in a somewhat arbitrary manner and the classification system is constantly changing with the discovery of novel variants of secretion systems. The so called type III and IV secretion systems differ from other secretion pathways by their ability to translocate their substrates directly into eukaryotic cells.

Some secreted proteins cross both bacterial membranes in a single step as in the type I and III secretion systems whereas others have a periplasmic intermediate. Those with a two-step secretion process must first cross the inner membrane using for example the general secretion pathway. The proteins are selected for secretion through this system by their well characterized N-terminal (amino-terminal), cleavable secretion signals with a short, positively charged N-terminus, a central hydrophobic region and a more polar C-terminal (carboxy-terminal) region (Paetzel *et al.* 1998). In the periplasmic space the proteins partially fold and they are directed to different secretion systems by their remaining secretion signals. The signals may lie in the primary polypeptide sequence of the secreted proteins or be conformational ones as is suspected to

be the case for the so called type II secretion signals (reviewed by Sandkvist 2001). Signals might even appear in the transcript instead of the polypeptide sequence as suggested for the type III secretion systems (Anderson and Schneewind 1997, Anderson *et al.* 1999, Mudgett *et al.* 2000).

1.2 TYPE III SECRETION SYSTEMS

Type III secretion systems (TTSSs) can be divided into two categories, the flagellar systems and the virulence or non-flagellar systems, the latter including symbiosis-associated systems. The similarities and differencies between the two categories are schematically represented in Figure 1.

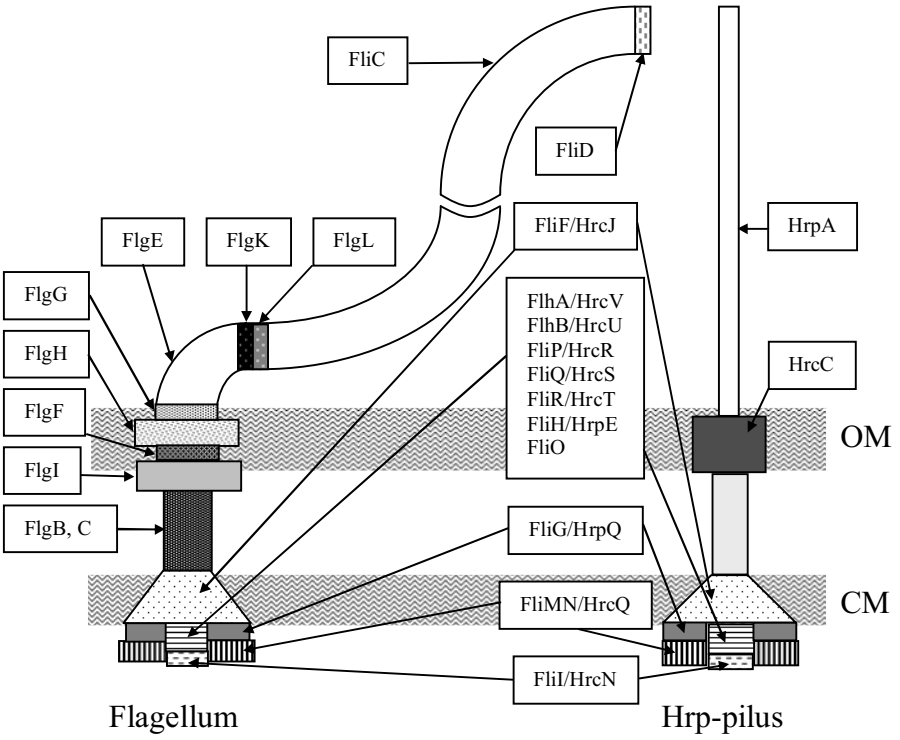

Figure 1. Schematic drawing of type III secretion systems. Hrp/Hrc denotes proteins of the virulence TTSS of *Pseudomonas syringae* and Flg/Flh/Fli flagellar proteins. Homologous proteins are separated by a slash and indicated with identical patterns. OM= outer membrane, CM= cytoplasmic membrane. Modified from the Masters thesis of Chun-Mei Li (2001) with permission.

1.2.1 Evolution and distribution

The flagellar and the non-flagellar secretion systems share a common ancestor (Foultier *et al.* 2002, Gophna *et al.* 2003). The divergence between the systems may have occurred hundreds of millions of years ago. The order of appearance of the virulent and the flagellar secretion systems is still unclear. The flagellar secretion system is readily considered the more ancient one on the basis that the eukaryotic hosts have evolved later, whereas the ability to move by the use of flagella would be an older feature. Gophna and co-workers (2003) did not find support for the claim by Macnab (1999) and Galán and Collmer (1999) that the non-flagellar TTSS would have evolved from the flagellar secretion system. They found the levels of diversity to be similar in the non-flagellar TTSS and the flagellar systems, thus the systems have a similar degree of antiquity. In addition, they noted the peculiarity of why a simpler non-flagellar TTSS would have evolved from the more complex flagellar system.

The TTSSs have spread among bacteria by horizontal gene transfer and are found in distantly related bacterial species (Foultier *et al.* 2002). TTSSs are often encoded by genes on pathogenicity islands flanked by mobile elements or on plasmids and they do not follow the G/C (guanidine/cytosine) content of their hosts. Some species of bacteria have more than one TTSS. These systems are a result of successive horizontal gene transfers, not of intragenomic gene duplications (Troisfontaines and Cornelis 2005). After their acquisition, the genes encoding the TTSSs have not undergone major reorganisation (Foultier *et al*. 2002).

Non-flagellar TTSSs are found in many Gram-negative bacteria pathogenic to plants, animals, including man and insects, and even amoebas, and in symbionts. The bacteria include for example species of *Aeromonas*, *Bordetella*, *Burkholderia*, *Chlamydia*, *Chromobacterium*, *Citrobacterium*, *Desulfovibrio*, *Edwardsiella*, *Erwinia*, *Escherichia*, *Pantoea*, *Photorhabdus*, *Pseudomonas*, *Ralstonia*, *Rhizobium*, *Salmonella*, *Shigella*, *Sodalis*, *Vibrio*, *Xanthomonas* and

Yersinia (reviewed by Galán and Collmer 1999, Pallen *et al.* 2005, Troisfontaines and Cornelis 2005).

Type III secretion genes occur also in the symbiotic bacterium, *Rhizobium* (Freiberg *et al.* 1997, Viprey *et al.* 1998). Viprey and others have shown (1998) that the non-flagellar TTSS in *Rhizobium* is expressed later than the Nod genes. TTSSs are probably needed for nodule initiation, but may not be vital for nitrogen fixation. Viprey and others (1998) have also demonstrated that type III secreted proteins affect the formation of nodules and act as host specificity determinants. Some plant-*Rhizobium* interactions seem to benefit from the proteins, whilst others exhibit the exact opposite phenotype.

The flagellar secretion systems have also been reported to function in the secretion of virulence proteins in many bacteria (Young *et al.* 1999, Ghelardi *et al.* 2002, Konkel *et al.* 2004). The functional conservation of the TTSS apparatuses has been experimentally demonstrated by secretion of proteins originating from one bacterial species by the secretion machinery of another species. Examples range from the secretion of a virulence protein from *Shigella flexneri* by *Yersinia pseudotuberculosis*, secretion and translocation of an effector protein of *Y. pseudotuberculosis* by *S. typhimurium* (Rosqvist *et al.* 1995), to the secretion of effectors from *Pseudomonas syringae* (Ham *et al.* 1998) and *Y. enterocolitica* (Anderson *et al.* 1999) by the TTSS of *Erwinia chrysanthemi* expressed in *E. coli*, and to the secretion of YlpA, a flagellar TTSS secreted virulence factor of *Y. enterocolitica* through the two virulence TTSSs of *Yersinia* and the flagellar secretion system (Young and Young 2002, Warren and Young 2005).

1.2.2 Effects caused by the TTSSs of plant pathogenic bacteria

TTSSs are used by pathogenic bacteria to secrete proteins outside the bacterial cell (e.g. harpins) and to translocate proteins directly into the host cells

(effector proteins, formerly known as avirulence proteins in plant pathogens). In plant pathogenic bacteria the TTSSs are known as Hrp systems because of their effects on the plant i.e. HR (hypersensitive reaction) and pathogenesis. HR is a defensive plant reaction, a localized cell death that restricts the spread of the pathogen. Plant resistance gene (*R*) products can recognise virulence factors either directly or indirectly by their actions (the guard hypothesis). According to the guard hypothesis, the guardian *R* gene products monitor their guardees that may also be the targets of the (a)virulence proteins (reviewed by Van Der Biezen and Jones 1998, Dangl and Jones 2001). After recognition of the pathogen by the *R* gene products, several responses will follow. Reactive oxygen intermediates (ROIs) are produced, HR is induced, systematic defence signalling will follow and induce pathogenesis related (PR) genes in distant parts of the plant and make the plant resistant to a wide variety of pathogens. In experimental infections HR is used as a fast and easy assay for the presence of bacteria with an active TTSS. Visible cell collapse typically appears within 24 hours of infection (Roine *et al.* 1997a). The pathogenic functions of effectors are discussed in section 1.2.5.

1.2.3 The structures of the non-flagellar type III secretion apparatuses

TTSSs share homologous proteins that for example in the animal pathogenic bacterium *Yersinia* are called Ysc for *Yersinia* secretion and in plant pathogenic bacteria Hrc for hrp conserved. The conserved proteins form the core of the secretion machinery. Ten of the 11 conserved genes encoding the TTSSs are also conserved in the flagellar type III system, the exception being HrcC/YscC (reviewed by Hueck 1998, Cornelis and Van Gijsegem 2000, Büttner and Bonas 2002). The secretion is mediated by the secretion/translocation apparatuses that comprise of two rings in the inner and outer membranes, of long appendages called needles in animal pathogens or pili in the case of the

plant pathogenic bacteria and of a translocon that forms a pore in the host cell membrane. The secretion apparatus is built of conserved proteins such as the outer membrane protein HrcC, the inner membrane/membrane-spanning protein HrcJ, the inner membrane proteins HrcR, HrcS, HrcT, HrcU and HrcV, the cytoplasmic proteins HrpQ, HrcQ, HrpE and the ATPase (adenosine triphosphatase) HrcN that provides energy for the secretion process in plant pathogenic bacteria (see figure 1 for locations of the conserved proteins). Some components of the TTSS are secreted through the general secretion system (Sukhan *et al.* 2001, Kimbrough and Miller 2002, Gauthier *et al.* 2003). These include the inner membrane and membrane-spanning components and at least partly the outer membrane proteins of the secretion apparatus. The needle or pilus proteins as well as components of the translocon are secreted by the TTSS.

Needles of animal pathogenic bacteria are approximately 40-80 nm in length and their external diameter is 6 to 13 nm, and internal diameter in the range of 2 nm (reviewed by Ghosh 2004). At the tips of the needles are structures needed for the assembly of the translocation pore. These structures comprise of LcrV, YopB and YopD in the case of *Yersinia* (reviewed by Cornelis 1998, Mueller *et al.* 2005) and of EspA in *E. coli* (Ghosh 2004). The EspA filaments have an outer diameter of about 12 nm and an inner diameter of 2.5 nm and are in average 40 to 140 nm in length.

TTSS-associated pili of plant pathogenic bacteria have an external diameter of ca. 8 nm, and can be several micrometers in length (reviewed by Ghosh 2004). J. Lee and colleagues (2001) demonstrated that the harpin HrpZ from *P. syringae* binds lipid bilayers. They also demonstrated that HrpZ can form ion-conducting pores. They hypothesised that HrpZ may either facilitate nutrient release or the translocation of effector proteins into eukaryotic cells, thus being functionally equal to the translocation structures of the animal pathogenic bacteria. Harpin proteins are further discussed in section 1.2.5.

Proteins secreted by the TTSSs are thought to be transported through the needles or pili (Jin and He 2001, Li *et al.* 2002). Proteins must be in an at least partly unfolded state to fit in the narrow type III secretion channels. Thus large or extensively folded proteins do not make good substrates for the TTSS (Akeda and Galán 2005). Proteins destined for secretion must either fold slowly or be unfolded before secretion. The TTSS-associated ATPase InvC of *Salmonella* has been shown to unfold SptP and to release the effector protein from its chaperone SicP (Akeda and Galán 2005). The ATPase YscN of *Yersinia* may also work in the unfolding of type III secreted proteins (Wilharm *et al.* 2004).

The ingeniousness of the TTSSs is represented by enteropathogenic *Escherichia coli* (EPEC) that inserts a receptor for the bacterium itself into the membrane of the host cell (Kenny *et al.* 1997, reviewed by Nougayrède *et al.* 2003). The translocated intimin receptor (Tir) binds intimin, an outer membrane protein of EPEC. Both proteins are encoded by genes in the locus of enterocyte effacement (LEE) of EPEC along with the TTSS. Kenny and others (1997) have shown that the delivery of Tir into mammalian cells is facilitated by the TTSS. Michgehl and co-workers (2006) have proven that Tir can also be inserted into host cell membrane independently of the TTSS. After tyrosine phosphorylation Tir binds intimin, which leads to cytoskeletal nucleation and pseudopod formation (Rosenshine *et al.* 1996) that are morphological changes in the cell structure indicative of bacterial infection and of active TTS.

1.2.3.1 HrpA pilin of P. syringae

HrpA is the major structural protein of the Hrp pilus in *P. syringae* (Roine *et al.* 1997a, Hu *et al.* 2001). HrpA is capable of reassembling into pili *in vitro* (Roine *et al.* 1997b) and is essential for virulence and triggering of HR in plants as a result of the delivery of effectors into plant cells (Roine *et al.* 1997a). The

Hrp pilus has been shown to cross the plant cell wall (Brown *et al.* 2001). The pili have been shown to both grow and secrete proteins from their tip (Jin and He 2001, Li *et al.* 2002).

The pilus and needle subunit proteins are generally small (ca. 60-120 amino acids) and form mainly α-helical structures (Koebnik 2001, Weber *et al.* 2005, Zhang *et al.* 2006). The C-terminus of HrpA is needed for the assembly of the pili and for pathogenicity (Taira *et al.* 1999), whereas the N-terminus is not needed for filament formation (Roine *et al.* 1997b, Taira *et al.* 1999). Similar architecture exists in the *Xanthomonas campestris* major pilus protein called HrpE (Weber and Koebnik 2005). The primary sequences of HrpA proteins, however, are not very similar. The proteins from *P. syringae* pathovar (pv.) *tomato* DC3000 and *P. syringae* pv. *syringae* 61, for example, are only 27% identical and 43% similar (Deng *et al.* 1998).

1.2.4 Regulation of non-flagellar type III secretion

The TTSSs may be activated upon contact with the host cell or in the apoplast of plants and thereafter deliver proteins from the bacterial cytoplasm into the eukaryotic cells. Environmental signals, such as host cell contact, pH, temperature, carbon source and host cell-derived molecules regulate the transcription, translation and secretion of TTS-associated proteins in an elaborate manner. The TTSSs can also be induced experimentally. In plant pathogenic bacteria minimal medium with a low pH and fructose as the preferred carbon source is used for the expression and secretion studies of the Hrp-regulon (Huynh *et al.* 1989). Examples of regulatory cascades in TTS gene expression have been found in several bacterial species, but the complete networks remain to be solved.

The *hrp* clusters of plant pathogenic bacteria have been classified into two groups that differ in gene organization and sequence as well as in their *hrp* gene

expression (reviewed by Alfano and Collmer 1996). Group I includes bacteria such as *E. amylovora* and *P. syringae* and group II *Ralstonia solanacearum* and *X. campestris*. The regulatory cascades of *hrp* gene expression in *P. syringae* are summarized in Figure 2.

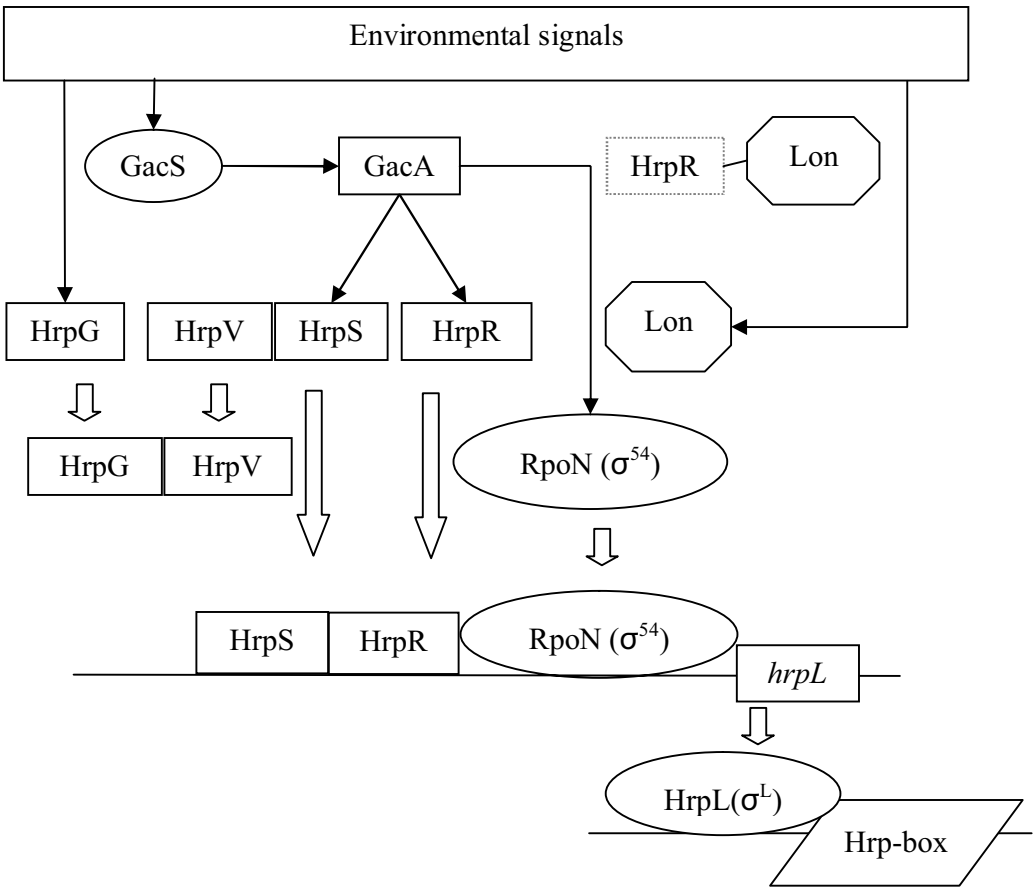

Figure 2. Regulation of expression of TTS-associated genes in *P. syringae*.

The genes of TTS-associated proteins of group I bacteria are characterized by Hrp-boxes. In *Pseudomonas syringae*, the Hrp-box motifs are recognized by the alternative sigma factor HrpL (Xiao and Hutcheson 1994). The alternative sigma factor RpoN or σ^{54} regulates the expression of *hrpL* (Hendrickson *et al.* 2000). HrpR/S complex is needed for the expression of *hrpL* as well (Xiao *et al.*

1994). HrpV acts as a negative regulator of *hrp* gene expression (Preston *et al.* 1998). HrpG forms a complex with HrpV, which leads to the dissociation of HrpV from HrpS and the activation of *hrp* gene expression (Wei *et al.* 2005). In conditions mimicking the *in planta* environment, the HrpR/S complex is active due to the lack of Lon-mediated degradation of HrpR (Bretz *et al.* 2002). The expression of *hrpR* and *hrpS* as well as *rpoN* is controlled by GacA (Chatterjee *et al.* 2003). GacA is a response regulator of a two-component system, GacS being the sensor kinase. According to Chatterjee and colleagues (2003) GacA regulates several genes in control of various systems in *P. syringae* pv. *tomato*. Wei and colleagues (2000) have suggested that HrpA might also be involved in the regulation of expression of TTSS. HrpA would act upstream of *hrpRS* in regulating the expression of the operon.

Group II bacteria use AraC family transcriptional activators for induction of *hrp* gene expression (reviewed by Alfano and Collmer 1996). A non-diffusible molecule in the plant cell wall is recognised by PrhA and leads to induction of *hrp* genes (Aldon *et al.* 2000).

At the temperature of +37°C an increase in the amount of extracellular amino acids (glutamate, glutamine, aspartate or asparagine), in serum proteins, such as albumin, or a decrease in calcium concentration triggers type III secretion in animal pathogenic bacteria (Lee VT *et al.* 2001). The temperature regulation of TTS in *Shigella*, enteroinvasive *E. coli* (Falconi *et al.* 1998) and *Y. enterocolitica* (Rohde *et al.* 1999) involves conformational changes in the virulence plasmid that encodes the genes of the TTSS.

In *Yersinia* three classes of genes controlling the expression and/or secretion of *Yersinia* outer proteins (Yops) have been identified (reviewed by Anderson *et al.* 2002). Mutations in class I genes, such as *yopN* result in the loss of calcium regulation of synthesis and secretion, class II genes (*yopD* and *lcrH*) in loss of calcium response of synthesis, and class III genes that are components of the secretion apparatus, in loss of synthesis under low-calcium conditions.

Yersinia employs repressors and post-transcriptional control to regulate its virulence gene activation. The complexity of regulation is exemplified by the involvement of a number of proteins including YscM1, YscM2, SycH, YopD and LcrH (Pettersson *et al.* 1996, Cambronne *et al.* 2000, Cambronne and Schneewind 2002, Cambronne *et al.* 2004). Host cell contact leads to the TTSS-dependent secretion of LcrQ/YscM, a negative regulator of Yop expression (Pettersson *et al.* 1996, Cambronne *et al.* 2000). According to Cambronne and colleagues (2004), secretion of the regulator *per se* is not required for the relief of repression of Yops, but the binding of SycH chaperone to regulators of expression is (Cambronne *et al.* 2000, Cambronne *et al.* 2004). The regulation of Yops in *Yersinia* may involve degradation of *yop* messenger ribonucleic acids (mRNAs) in the absence of secretion (Anderson *et al.* 2002). Anderson and others (2002) suggest that the binding of YopD and LcrH to *yop* mRNA may prevent the initiation of translation of the transcript. It remains to be seen whether there is a more common connection between the stability of TTSS-associated transcripts and type III secretion and if this connection involves an mRNA secretion signal.

In *P. aeruginosa* secretion is coupled to transcription by a cascade of ExsE, ExsC, ExsD and ExsA (Rietsch *et al.* 2005, Urbanowski *et al.* 2005). ExsE is secreted from the cells as the secretion apparatus is opened and the secretion is aided by ExsC, a chaperone and an anti-anti-activator. After the secretion of ExsE, ExsC is free to bind ExsD, an anti-activator. The binding of ExsC to ExsD releases ExsA, a DNA-binding protein, to activate the transcription of the type III secreted proteins.

1.2.5 Type III secreted effectors and harpins

The components of the TTSS are conserved among bacteria, but the effector proteins are not (Galán and Collmer 1999). Type III effectors of plant

pathogenic bacteria can determine the host range of the pathogen (Alfano and Collmer 2004). Type III secreted effector proteins in plant pathogens were first named avirulence proteins since they were discovered for their ability to elicit plant defences, thus avirulence. Later it was noted that the proteins can act as pathogenicity factors in some plants, whilst other plants have matching *R* genes that recognize the effector proteins and launch defences. The effects caused on plants by the TTSS-dependent proteins are discussed from the view of the plant in section 1.2.2.

Type III secreted proteins help the pathogen to grow in host, to defeat its defences and to cause disease symptoms (Alfano and Collmer 2004). Type III secreted effectors have more sequence homology to eukaryotic than to bacterial proteins (Cornelis 2002) and may have been acquired from eukaryotes (Galán 2001, Cornelis 2002, Troisfontaines and Cornelis 2005).

Little has been known about the cellular functions of the type III effectors in plant pathogenic bacteria, but more information is been discovered at an increasing rate. The effector proteins often act on the plasma membrane or the nucleus of plant cells (Alfano and Colmer 2004). The effector proteins are believed to stimulate or interfere with host cellular processes. They may affect the host cell morphology or metabolisms and favour the pathogen by suppressing the defences of the plant. The effectors may suppress programmed cell death, jasmonic acid and salicylic acid signalling, the expression of defence genes and cell wall-based defences, such as cell wall thickening papillae (Hauck *et al.* 2003, Abramovitch and Martin 2004, Mudgett 2005, Li *et al.* 2005).

P. syringae pv. *tomato* DC3000, for example, has more than 30 effector proteins (Guttman *et al.* 2002, Buell *et al.* 2003). Effector proteins of *P. syringae* pv. *tomato* for example suppress the salicylic acid dependent callose deposition (HopPtoM and AvrE) (DebRoy *et al.* 2004), target host immunity associated proteins to the host proteasome/ubiquitination system to suppress the extracellular cell wall-associated defenses (HopM1) (Nomura *et al.* 2006),

inhibit programmed cell death by mimicking host ubiquitin ligases (AvrPtoB) (Janjusevic *et al.* 2006) and suppress early-defense signalling (AvrPto and AvrPtoB) (He *et al.* 2006).

The genes induced by the TTSSs in plants are also associated with defence, such as the salicylic acid dependent pathway (Hauck *et al.* 2003). These genes are probably not induced to high enough levels to be effective against infection in susceptible plants.

Effects similar to those executed by the effectors of plant pathogens occur with the animal-targeting effectors: the inflammatory response is downregulated, phagocytosis is inhibited, apoptosis is induced in macrophages and lymphocyte activation is impaired (reviewed by Cornelis 2002).

Known and predicted functions of effector proteins include phosphatases, kinases, ADP-ribosyltransferases, adenylate cyclases, proteases, phosphodiesterases, syringolide synthases and transcription factors (reviewed by Cornelis and Van Gijsegem 2000, Innes 2003, Grant *et al.* 2006). According to Guttman and colleagues (2002) effectors have a high overall serine and asparagine content, and low leucine, isoleucine and valine content. They also have a low aspartate and lysine content in their N-termini. These observations are in line with those of Petnicki-Ocwieja and others (2002) who analysed by computer the N-termini of type III secreted proteins from *P. syringae* (see also section 1.2.7 on type III secretion signals). According to Guttman and colleagues (2002) the N-termini of type III secreted effectors resemble chloroplast and mitochondrial targeting sequences. Many *P. syringae* effectors are believed to localize in the chloroplasts, and some animal pathogens target their effectors to mitochondria. The significance of this finding, whether it speaks for the common evolutionary origin for the secretion and targeting mechanisms or for analogous functional requirements or for something else, remains elusive (Guttman *et al.* 2002).

Some effectors are modified inside host cells by host enzymes. Modifications may be needed for the virulence and avirulence functions of the

effectors. Effectors may have evolved to mimic host cell proteins that are modified. AvrPto of *P. syringae* is both myristoylated (Shan *et al.* 2000, Anderson *et al.* 2006) and phosphorylated (Anderson *et al.* 2006) in tobacco and tomato leaves, and these modifications affect the outcome of the interaction between plants and bacteria.

Harpins are glycine-rich proteins that lack cystein residues, are heat-stable and can elicit HR when injected into non-host plants (He *et al.* 1993, Alfano and Collmer 1996). They may facilitate the delivery of effector proteins into the plant cell cytoplasm (J. Lee *et al.* 2001) as noted in section 1.2.3 on the structures of the non-flagellar type III secretion apparatuses.

1.2.6 Type III secretion signals

Despite extensive research, the signal for type III secretion is still enigmatic. In the non-flagellar secretion systems, the signals found so far lie in the region covering the first 7 to 28 codons (for examples see Sory *et al.* 1995, Anderson and Schneewind 1999, Mudgett *et al.* 2000, Rüssmann *et al.* 2002, Ramamurthi and Schneewind 2005). Both N-terminal amino acid signals, perhaps amphipathic ones (Lloyd *et al.* 2001), and 5' terminal mRNA signals (Anderson and Schneewind 1997, Anderson *et al.* 1999, Mudgett *et al.* 2000) have been proposed.

Aldridge and Hughes (2001) have proposed several models for the secretion of TTSS substrates. The secretion signal could be an mRNA signal, leading to co-translational secretion, an N-terminal signal, or assisting secretion chaperones could be used. Their models are mostly based on flagellar TTSS, but may be applicable to the non-flagellar TTSS as well. They propose that the secretion of late-substrates, such as effectors for the non-flagellar TTSS, could be hindered by ribosomes translating the structural components of the machinery in a co-translational manner, until the secretion machinery is ready

(the closed gate model). In this model secretion could still be assisted by chaperones associated with the growing polypeptide near the secretion machinery. After completion of the TTS machinery, the ribosome gate would open and allow the secretion of the effector proteins. In the open gate model, on completion of the TTS machinery, the secretion chaperones sense the secretion substrates flowing to the cytoplasm from the full secretion channel, thus inducing the shutdown of secretion or a switch between substrates.

Evidence for the mRNA secretion signal has come mostly from experiments illustrating that point mutations or some frame-shift mutations that completely alter the amino acid sequence of the secretion signals of YopE, YopN or YopQ of *Y. enterocolitica* do not prevent secretion of reporter proteins (Anderson and Schneewind 1997, Anderson and Schneewind 1999). Goss and colleagues (2004) demonstrate that some synonymous mutations in YopN abolish the secretion of hybrid proteins. Ramamurthi and Schneewind (2005) could show that a single synonymous mutation almost completely abolished the secretion of a reporter protein fused to the minimal secretion signal of YopE from *Y. enterocolitica*, whereas changing the reading frame of the secretion signal being comprised of the first 15 codons (minimal secretion signal and the suppressor region) did not abolish reporter protein secretion. Synonymous mutations were also made to the secretion signal of YopQ by Ramamurthi and Schneewind (2002). Some of the mutations abolished secretion of the reporter protein, whilst others did not. Frameshifts in the secretion signal of InvJ in *Salmonella* did not abolish secretion (Rüssmann *et al.* 2002). However, synonymous mutations changing the mRNA sequence of *invJ* did not hinder secretion either.

Lee and Schneewind (2002) opted for the mRNA signal based on the notion that the type III pathway of *Y. enterocolitica* cannot be occluded by folded proteins with TTS signals. They reasoned that the completed polypeptides rejected by the TTSS cannot re-enter the pathway because they no longer are attached to mRNA signals. The rejection of folded polypeptides from TTSS was also demonstrated by Sorg and colleagues (2005). They also proved that

some impassable substrates can inhibit the expression of other substrates of TTSSs.

The N-terminal secretion signal is supported by Warren and Young (2005) who made a frameshift mutation in the secretion signal of YlpA of *Y. enterocolitica*. The frameshift resulted in poor secretion of YlpA. Lloyd and colleagues (2001) made frame-shift mutations to the first 11 codons of *yopE* of *Y. pseudotubercolis*. Their mutations that changed the amino acid sequence of the protein drastically reduced the secretion of YopE in a chaperone-deficient *yerA*- background, whereas mutations altering the mRNA sequence while leaving the amino acid sequence intact allowed secretion. Ramamurthi and Schneewind (2003a) noted that the construct made by Lloyd and others (2001) mutated codons 12 and 13 that are a part of the suppressor region sensitive to mutations.

Some of the differences in the results of several groups may be attributed to the discrepancy in the behaviour of different TTS substrates, whilst others may be the result of the use of differing constructs. Ramamurthi and Schneewind (2002, 2003a, 2003b, 2005) studied the effect of the length of the secretion signal to tolerance of frame-shift mutations. Their observations illustrate that even though the minimal secretion signal of YopQ of *Y. enterocolitica* is in the first 10 codons, codons 11-15 help in tolerance for mutations. They call this tolerance region the suppressor region, and show it to be sensitive to mutagenesis (2003a). The same was proven to be true for YopE (2005). The first 7 codons are sufficient for secretion, but codons 8-15 can suppress mutations in the minimal secretion signal.

Rüssmann and colleagues (2002) hypothesised that instead of an mRNA secretion signal, secretion of TTSS-dependent proteins could be accomplished by the use of polypeptide sequences that do not acquire structures rapidly. Lloyd and colleagues (2001) noted the amphipathic nature of the N-termini of Yops, and created a functional synthetic, amphipathic serine/isoleucine secretion signal for YopE. Petnicki-Ocwieja and others (2002) analysed by

computer the N-termini of type III secreted proteins from *P. syringae*. They defined the following rules: the first five amino acids include solvent exposed, equivalent amino acids, no acidic amino acids (aspartate or glutamate) reside in the first 12 amino acids and the first 50 amino acids are in general rich in polar amino acids (especially serine and glutamine) and amphipathic.

Some TTS substrates only seem to have one secretion signal. These proteins include parts of the secretion machinery (Anderson and Schneewind 1999). Other secreted proteins that are often translocated into host cells have a second secretion signal located further downstream of the first signal (Sory *et al.* 1995, Schesser *et al.* 1996, Cheng *et al.* 1997, Mudgett *et al.* 2000, Chiu and Syu 2005). The second signal comprises of a binding site for small, cytoplasmic proteins known as TTS chaperones (Anderson and Schneewind 1999). The TTS chaperones are described in more detail in the following section (1.2.7). The N-terminal secretion signal has been referred to as the primary secretion signal and the chaperone binding domain (CBD) as the translocation domain. Some experiments argue against the CBD acting as a translocation signal. A *Y. enterocolitica* strain lacking most effectors delivered YopE with its CBD deleted into eukaryotic cells (Boyd *et al.* 2000). No secretion or translocation could be detected without the primary 5' secretion signal. Conflicting evidence has been published by Cheng and colleagues (1997), who have demonstrated that a fusion protein of NPT with YopE in *Y. enterocolitica* was secreted without the N-terminal secretion signal.

Co-translational secretion has been proposed for some of the TTS substrates of both the flagellar and non-flagellar types (Karlinsey *et al.* 2000, Anderson and Schneewind 1999). As an extreme example, Anderson and Schneewind (1999) showed that YopQ is only translated when a functional TTSS is present. The results of Anderson and Schneewind (1999) showing that YopQ was only present in the culture medium and not found in the cytosol of *Y. enterocolitica* are in conflict with the results of Trček and others (2002) who could detect YopQ in the cytosol of *Y. enterocolitica* and show post-translational secretion.

Coupling of translation to secretion might be mediated by type III chaperones (Karlinsey *et al.* 2000). In contrast, some TTSS-dependent proteins have been shown to be secreted from a pre-made pool. For example, translation of YopE is not coupled to its secretion in *Y. pseudotuberculosis* (Lloyd *et al.* 2001). Post-translational secretion in this case is dependent on chaperones. The same seems true for YopE of *Y. enterocolitica* (Cheng *et al.* 1997).

No conclusive proof on the secretion signal for TTS has been provided that would account for the secretion of all the known type III secreted proteins. Both the nucleotide and polypeptide sequences of TTS-dependent proteins vary greatly. Sequence or secondary structure data unambiguously proving the existence of a general mRNA signal is lacking. No unequivocal evidence exist that would establish how an amphipathic, unstructured polypeptide signal that seems to exist only in a portion of TTS proteins could exclude the secretion of all non-TTS proteins, whilst promoting the secretion of all TTS proteins either.

The secretion signal for the flagellar TTSS also needs further investigations. Flagellar export chaperones interact with the C-termini of their substrates, unlike their virulence system counterparts (Evdokimov *et al.* 2003). FliS, the chaperone of the flagellar filament protein FliC seems to prevent the premature polymerization of FliC in the cytosol. According to Evdokimov and others (2003) the FliS-related flagellar type III chaperones share no common evolutionary ancestry with the non-flagellar type III secretion chaperones. Majander and co-workers (2005) used the 173 bp untranslated region upstream of the *fliC* gene of *E. coli* to secrete heterologous proteins through a modified flagellar TTSS. They were also able to secrete heterologous proteins fused to FliC without its 5' UTR. They concluded that either the 5' UTR of *fliC* or other regions, perhaps with the help of chaperones are needed for the secretion through the flagellar TTSS. In *S. typhimurium* the TTS signal of the flagellin is in amino acids 26 to 47 that are sufficient for the export of polypeptides fused to them (Végh *et al.* 2006). These residues are among the most conserved of the

disordered N-terminal region of flagellins. They are hypothesised to form amphipathic helical structures.

1.2.7 TTS chaperones

TTS chaperones are small (ca. 15 kDa) proteins that have an acidic pI and an amphipathic alpha-helix in their C-termini (Alfano and Collmer 2004). They often act as dimers and are encoded adjacent to their cognate effector proteins (reviewed by Feldman and Cornelis 2003). Chaperones are likely to have evolved from common ancestral proteins (Birtalan *et al.* 2002).

TTS chaperones have been mostly studied in animal pathogenic species but exist also in plant pathogens. Many roles have been assigned to type III chaperones (reviewed by Feldman and Cornelis 2003, Ghosh 2004). They may act as anti-aggregation and -folding factors. In complex with effector proteins type III chaperones may, at least in some cases, form three-dimensional signals recognized by the TTSS (Birtalan *et al.* 2002). A hierarchy of secretion could be introduced by chaperones to the effectors (Boyd *et al.* 2000, Birtalan *et al.* 2002). Protein fusions containing only the N-terminal secretion signal are unable to compete with effector proteins harboring both the N-terminal secretion signal and the chaperone binding site (Boyd *et al.* 2000). Hierarchy is not conferred on the stage of transcription, at least in the case of *P. syringae* pv. *phaseolicola* (Thwaites *et al.* 2004).

TTS chaperones have been shown to act only on the domains they bind to (Birtalan *et al.* 2002). Results of Birtalan and co-workers (2002) on *Y. pseudotuberculosis* SycE-YopE complex and of Luo and colleagues (2001) on *Salmonella* SigD-SigE and *E. coli* Tir-CesT complexes exhibit that chaperones do not promote global unfolding of these effectors. According to the results of Birtalan and colleagues (2002) chaperones do not protect effectors against proteolysis. On the contrary, Losada and Hutcheson (2005) have demonstrated

that chaperones of *P. syringae* do protect their cognate effectors against Lon-mediated degradation. The chaperones may keep their cognate effectors in an unfolded or non-globular state that is competent for secretion through the TTSS (Stebbins and Galán 2001).

Boyd and co-workers (2000) have studied the CBD of YopE of *Y. enterocolitica*. They showed that the removal of YopE residues binding to SycE downstream of the minimal CBD (amino acids 15 to 50) leads to the mutant protein being secreted by *Y. enterocolitica* independently of SycE. They concluded that amino acids 50 to 77 inhibit secretion of YopE in the absence of its chaperone. Ehrbar and colleagues (2006) also hypothesise that an inhibitory factor would bind the CBD of newly synthesised proteins and prohibit transport via the TTSSs. The binding of the cognate chaperone would release this inhibitory factor. The work of Letzelter and colleagues (2006) on the effector protein YopE of *Y. enterocolitica* has verified that a deletion of the CBD (residues 20 to 77) of YopE does abolish the need for its cognate chaperone for secretion and translocation. They have, however, concluded that the CBD creates the need for the chaperone by reducing the solubility of the effector protein.The CBDs may act as the membrane localization domains (MLD) of effectors and the chaperones can prevent their insolubility in the bacterial cytoplasm (Letzelter *et al.* 2006). SycO of *Y. enterocolitica* binds the MLD of YopO and SycE covers the MLD of YopE. Letzelter and colleagues (2006) hypothesize that the primary function of type III chaperones could be to cover the MLDs of membrane-associated effector proteins inside the bacteria and that the function of targeting the proteins to the secretion machinery would have evolved later.

The role of the CBD in secretion pathway specificity of type III secreted proteins has been studied by Lee and Galán (2004) and Ehrbar and colleagues (2006). They both share the view of the importance of the CBD, but the details vary. Lee and Galán (2004) believe that the chaperones confer secretion-pathway specificity, whereas according to Ehrbar and others (2006) the CBD

prevents secretion in the absence of the chaperone. Birtalan and colleagues (2002) believe that the CBDs may not act as inhibitors of secretion in the absence of their cognate chaperones, but the inhibition may be a by-product of being aggregation-prone regions. The results of Letzelter and colleagues (2006) on effectors of *Y. enterocolitica* show that the chaperones may indeed mask the aggregation-prone MLDs. Lee and Galán (2004) demonstrated that SopE was only secreted through the flagellar TTSS in the absence of the CBD. This was interpreted as a sign of an ancestral flagellar secretion signal, actions of which can be masked by the CBD and its chaperone. Changing the CBD of SopE from *Salmonella* led to its secretion via the flagellar TTSS as well as the SPI-1 TTSS in the experiments performed by Ehrbar and colleagues (2006). Without its cognate chaperone wild type SopE is not secreted at all, whilst the chaperone binding site mutant is secreted by both TTSSs (Ehrbar *et al.* 2006).

Type III chaperones may regulate the expression of some TTS-associated genes. An interaction between the SicA TTS chaperone and the transcriptional activator InvF was shown by Darwin and Miller (2001). They demonstrated that both proteins are needed for the activation of some TTS promoters of proteins needed for invasion in *S. typhimurium*. They also suggest a model in which SicA could dock the transcription and translation machineries near the TTSS and thus couple translation to secretion.

1.3 mRNA DEGRADATION

Typical mRNA half-lives in *E. coli* range from 3 to 8 min. (Bernstein *et al.* 2002). In *Bacillus subtilis*, the half-lives of 80 % of mRNAs are less than 7 min. (Hambraeus *et al.* 2003). According to Bernstein and colleagues (2002) factors such as UTR length, G/C content or codon composition, predicted secondary structure stabilities, degree of single-strandedness, or the frequency of RNase E cleavage sites could not be used to predict the stability of transcripts. The

transcripts of genes with similar functions were, however, found to have similar stabilities. No correlation was found between the stable mRNAs in *E. coli* versus their counterparts in *B. subtilis* (Hambraeus *et al.* 2003). Similar to the results of Bernstein and colleagues (2002), Hambraeus and colleagues (2003) found no structures predominant in the 5' UTRs of stable or unstable *Bacillus* transcripts. The stability of the interaction between the ribosome binding site (RBS) and the ribosome was not different between the stable and unstable transcripts either. Similar results have also been obtained from a eukaryote, *Saccharomyces cerevisiae* (Wang *et al.* 2002). Transcripts encoding subunits of multicomponent, stoichiometric complexes had similar decay rates. Transcript half-lives did not correlate with ribosome density, or with ORF (open reading frame) size or codon bias.

Three main theories exist on the functional degradation of mRNAs. They have been tested experimentally and using mechanistic modelling (Carrier and Kealing 1997b, c). Carrier and Keasling (1997b) used a model that takes into account the binding of RNA polymerase at the promoter, transcription elongation and termination, ribosome binding at the RBS, translation elongation and termination, as well as protein degradation. According to the so-called 5' binding theory, the ribonuclease (Rnase) will bind to the free 5' end of the transcript and move along the mRNA behind the ribosomes until it reaches the cleavage site. This theory failed to predict the effects of ribosome loading and translational rate on transcript stability. The ribosome binding theory assumes that the RNase can bind to any site in the transcript that is not covered by a ribosome. This theory was not able to predict the 5' to 3' direction of degradation of mRNAs. Carrier and Keasling (1997b) found the hybrid 5' binding/ribosome protection theory of mRNA degradation to be superior to the other two theories. In the hybrid theory the nuclease binds to the 5' end of the transcript and loops to a cleavage site. The RNase will cleave at the site if a protecting ribosome is not present.

1.3.1 RNases

E. coli has at least five endoribonucleases: RNase III, RNase E, RNase G and RNase I/M (reviewed by Kushner 2002). There may be some functional overlap between RNases E and G. RNase I/M is found in the periplasm. Exonucleases include RNase II, RNase R, RNase BN, RNase PH, RNase D, RNase T and polynucleotide phosphorylase (PNPase) (reviewed by Kushner 2002). PNPase and RNase II as well as PNPase and RNase R exhibit some functional redundancy. In addition, there is an oligoribonuclease that degrades the short 4- to 7-mers created by PNPase and RNase II. Other proteins involved in mRNA decay include RNA helicases, poly(A) binding proteins and auxiliary proteins, such as Hfq (reviewed by Kushner 2004).

The site of the first endonucleolytic cleavage of a transcript is not random (Belasco *et al.* 1986). mRNA degradation is initiated by a cleavage by RNase E (Carrier and Keasling 1997b). The structure of the catalytic domain of RNase E has been solved and it resembles partly a deoxyribonuclease (DNase) (Callaghan *et al.* 2005). It has a 5' sensing site in addition to the catalytic cleavage site. The binding of the 5' end of the mRNA to the enzyme changes its conformation, and induces the cleavage reaction (Callaghan *et al.* 2005).

RNase III cleaves double-stranded ribonucleic acid (RNA) molecules either with a single-stranded or a double-stranded break (Ehretsmann *et al.* 1992, Grunberg-Manago 1999). In bacteria, mRNA degradation is 5' to 3' directional (von Gabain *et al.* 1983, Selinger *et al.* 2003). Exonucleases RNase II and PNPase degrade mRNA in a 3' to 5' direction. Degradation by RNase II ends in 5' monophosphates and by PNPase in nucleoside diphosphates (Ehretsmann *et al.* 1992, Grunberg-Manago 1999). PNPase can also synthetise polynucleotide tails to the ends of mRNAs (Mohanty *et al.* 2004). Relatively strong secondary structures can act as barriers to exonucleases but can be overcome by oligoadenylation (Coburn and Mackie 1996). Poly-adenylation may help in the degradation of mRNAs that have Rho-independent termination creating stem-

loop structures that inhibit RNase II and PNPase (Kushner 2002, 2004). Poly(A) polymerase (PAPI) adds about 10 to 60 nucleotides to the 3' end of the mRNA and provides single-stranded tails for PNPase (O'Hara *et al.* 1995, Grunberg-Manago 1999). Efficient polyadenylation by PAPI of mRNAs with Rho-independent transcription terminators requires Hfq, an RNA-binding protein (Mohanty *et al.* 2004). The partial elimination of polyadenylation stabilizes some mRNAs and alters their degradation patterns (O'Hara *et al.* 1995).

1.3.1.1 The degradosome

The degradosome has endo- (RNase E) and exonuclease (PNPase) as well as helicase activities (RhlB helicase) (reviewed by Grunberg-Manago 1999, Rauhut and Klug 1999). The activity of the helicase is ATP (adenosine triphospate)-dependent (Py *et al.* 1996). The role of enolase that is a glycolytic enzyme in the degradosome is not clear yet, but it might be structural not functional (Py *et al.* 1996, Grunberg-Manago 1999). Other proteins found to be associated with the degradosome include DnaK, a chaperone, and polyphosphate kinase (PPK) that removes inhibitory polyphosphate and nucleotide diphosphates (NDPs) and regenerates ATP (Blum *et al.* 1997). Blum and others (1997) have also shown that PPK binds RNA. GroEL, a chaperone and a member of the heat shock protein family, may also be associated with the degradosome, although this association is still elusive (Sohlberg *et al.* 1993).

The degradosome is formed on an RNase E scaffold and it interacts with PAPI. Removal of the C-terminus of RNase E does not impair cell growth, but does affect mRNA degradation and degradosome foundation (Lopez *et al.* 1999). Lopez and others (1999) speculate that RNase E and PNPase act for the most part independently and that mRNA degradation does not need to proceed fast. The advantages of having a ribonuclease complex might include the

elimination of the need for a free PNPase molecule to find the newly formed 3' end of the mRNA (Grunberg-Manago 1999).

1.3.2 Protection against RNases

1.3.2.1 Ribosomes affect mRNA stability

Experimental data exist on the protecting/stabilizing effect of ribosomes on mRNAs. Ribosomes have been proven to interfere with RNase E nuclease activity (Braun *et al.* 1998, Vytvytska *et al.* 2000). However, it has been noted that not all untranslated regions are unstable (von Gabain *et al.* 1983).

Puromycin strips transcripts of ribosomes (Odom *et al.* 1990), whereas aminoglycosides, such as kanamycin (Hirokawa *et al.* 2002) and chloramphenicol (Pato *et al.* 1973) are inhibitory to the release of mRNA from ribosomes. According to Pato and co-workers (1973) 80-90 % of mRNAs are stabilized by chloramphenicol, and puromycin destabilizes the transcripts to half their original stability. They also demonstrate that the portions of mRNA chains that are synthesised after the addition of chloramphenicol degrade faster than the portions that are already protected by ribosomes.

The stabilizing effect of some antibiotics can be due to the titration of RNases by the increase of ribosomal ribonucleic acid (rRNA) synthesis following the translational block (Lopez *et al.* 1998). *lacZ* mRNA lacking a RBS, for example, was stabilized by all tested translation inhibitors. The changes of these antibiotics on the stability of the translated mRNAs may be due to additive or antagonistic effects of ribosome stalling or stripping, respectively, and titration of RNases (Lopez *et al.* 1998). The trans-effects (titration) seem to overcome the cis effects (absence of stabilizing ribosomes) in long-term. It is also possible that the degradosome is inhibited by translation inhibitors. One of its components may be unstable, thus requiring ongoing

synthesis and replacement or the antibiotics may change the structure of the complex to inhibit its actions or hamper its access to mRNAs (Lopez *et al.* 1998).

1.3.2.2 Secondary structures of transcripts

Some structures, notably hairpins, in the UTRs of transcripts can stabilize mRNAs. 5' hairpins are believed to protect the mRNAs against RNases, especially RNase E that requires a free 5' end for beginning the degradation of the transcript. The 5' stabilizing hairpins prevent the binding and thus action of RNase E. Hairpins and other paired regions that can protect transcripts against RNases are conserved through evolution (James *et al.* 1989, Chen *et al.* 1991). Thus a hypothetical secondary structure that can only be found in the transcript from one species is probably not existent *in vivo* (Chen *et al.* 1991). The sequences *per se* may not be conserved, but changes in one side of the paired region result in complementary changes in the binding bases. As an example, the 5' UTRs of *ompA* transcripts from several species are sequencially divergent, but they fold in a similar fashion into two imperfect stem-loops (Chen *et al.* 1991).

Heterologous transcripts can be stabilized by the use of naturally existing or man-made elements. Often hairpin structures or stem-loops are used in the ends of the transcript. Typically, 3- to 5-fold elevations in half-lives are seen with the use of stabilizing elements (Wong and Chang 1986, Chen *et al.* 1991, Carrier and Keasling 1997a, Carrier *et al.* 1998). The 5' stabilizing region of *ompA* and the 3' region of *B. thuringiensis cry* gene, for example, elevated the half-lives of a fragment of the *bla* transcript from approximately three to 15 min (Belasco *et al.* 1986, Chen *et al.* 1991) and of the penicillinase gene from *B. licheniformis* from two to six min. (Wong and Chang 1986), respectively. According to the experiments of Belasco and co-workers (1986), the stabilizing

effect of *ompA* was only seen when using translational fusion constructs. Inserting a stop codon between *ompA* and the gene coding for the recombinant partner destabilized the transcript.

Although 5' hairpins can stabilize mRNAs there is no uniform correlation between secondary structure folding energy and mRNA half-lives (Carrier and Keasling 1999). RNase III is known to degrade double-stranded regions, and is probably responsible for the poor stabilizing effect of some hairpin structures. Carrier and Keasling (1999) have also confirmed that unpaired bases in the 5' end of the transcript destabilize it, even if a hairpin structure is present.

Also 3' elements can be used to stabilize heterologous mRNAs. The *cry* terminator fragment containing an inverted repeat forming a hypothetical stem-loop structure was used to stabilize transcripts fused to it both in *E. coli* and in *B. subtilis* (Wong and Chang 1986). Engineered 3' hairpins have also been demonstrated to stabilize transcripts (Smolke *et al.* 2000, Smolke and Keasling 2002).

Although RNA secondary structures in the 3' regions of transcripts are sufficient to protect the mRNAs against PNPase and RNase II *in vitro*, additional factors may be needed for the stabilizing effect they confer *in vivo* (McLaren *et al.* 1991). In the experiments of McLaren and colleagues (1991), the exoribonuclease stalling effect of different stem-loop structures was only a few minutes *in vitro*.

In some cases, also the sequence of the loop in a hairpin structure has been proven to be of importance (Tuerk *et al.* 1988). The sequence UUCG stabilized hairpin structures. Hairpin loop sequences may be recognized by some proteins.

Sometimes the elements used do not form stable secondary structures themselves, but need trans-acting elements for the creation of the stabilizing structures (Agaisse and Lereclus 1996). The STAB-SD elements that are Shine-Dalgarno (SD) sequences found in the 5' UTRs of many Gram-positive bacteria can confer stability to sequences downstream of them (Agaisse and Lereclus 1996). These SD sequences do not act in translation initiation. No secondary

structures form in these regions according to computer analysis, but the 3' end of the 16S rRNA binds to these sequences. This interaction is likely to block access of RNases to these sequences.

1.3.3 Transcript stability as a mechanism to control gene expression

Transcript stability is also dependent upon growth conditions, oxygen availability and temperature changes, and the efficiency of translation (reviewed by Grunberg-Manago 1999). Ribosomes and polymerases may protect transcripts against RNases. Transcript stability is also influenced by growth rate and the occurrence of rare codons. The removal of rare codons can have either a positive or a negative effect on mRNA stability. It may uncover regions containing cleavage sites, or help the ribosomes cover the mRNA faster, thus masking it from RNases (Carrier and Keasling 1997c).

The labile nature of most mRNAs reflects the fact that transcript instability is an effective way to adapt to rapid changes in the environment (reviewed by Ehretsmann *et al.* 1992). In the case of polycistronic mRNAs the expression of proteins can be controlled on the level of stability of the different mRNA segments.

Transcript stability can be a mechanism for the cells to control the expression of a gene. By alternating the degradation speed of a transcript, the cell can respond to specific conditions. In some cases the half-life of a transcript varies according to the growth-rate of the cells (Nilsson *et al.* 1984). The least stable of the *ompA* mRNA fragments has a half-life of 15 min. at a doubling rate of one per 40 min., but only 4 min. when the cells are dividing once in 200 min. This ensures that equal amounts of protein are present in the cells at all growth rates. The same kind of reduction in half-life was seen with the *cat* transcripts, from 2 min. to 0.4 min (Nilsson *et al.* 1984). The stability of the *ompA* transcript is controlled by a complicated system, parts of which still

remain unsolved. The dependence of the half-life of the *ompA* transcript on the growth-rate of the cell is mediated by host factor I, Hfq (Vytvytska *et al.* 1998). Hfq has been shown to bind small RNAs around its central pore (Schumacher *et al.* 2002). Hfq can unwind secondary structures of RNAs hence destabilizing surrounding RNA structures and permitting new RNA-RNA interactions (Schumacher *et al.* 2002). Hfq binds to the 5' UTR of *ompA*. According to Vytvytska and colleagues (1998), the amount of Hfq is dependent on growth-rate, being the highest in slowly growing cells. The binding-site of Hfq coincides with one of the RNase E cleavage sites in the 5' UTR of *ompA* transcript (Moll *et al.* 2003). Still, binding of Hfq destabilizes *ompA* mRNA since it hinders 30S ribosomal subunits from binding to the 5' UTR of *ompA* and stabilizing it (Vytvytska *et al.* 2000, Moll *et al.* 2003). The growth-rate dependent regulation of Hfq may be further mediated by some small RNA or component (Rasmussen *et al.* 2005).

A small regulatory RNA also binds to the translational initiation region of *ompA* mRNA (Rasmussen *et al.* 2005, Udekwu *et al.* 2005). According to Rasmussen and colleagues (2005) the growth-phase regulation of *ompA* mRNA is different from the growth-rate regulation. They state that the antisense regulator RNA MicA (SraD) accumulates in the stationary phase and is a growth-phase dependent regulator. Hfq facilitates the binding of MicA to *ompA* mRNA (Rasmussen *et al.* 2005, Udekwu *et al.* 2005) and strains lacking Hfq have less stable MicA RNA (Rasmussen *et al.* 2005). MicA interferes with ribosome binding (Udekwu *et al.* 2005). MicA has been found in several bacteria, and the differences in their sequences are often located in single-stranded regions or have compensatory changes in the stem-regions (Udekwu *et al.* 2005). The regions complementary to *ompA* are highly conserved.

1.4 PROTEIN PRODUCTION

Industrially important proteins are produced in large quantities in microorganisms. Problems of protein production *in vivo* include plasmid loss, especially with high copy number plasmids and toxic or growth rate reducing proteins (reviewed by Baneyx 1999). Figure 3 illustrates the critical points in the design of recombinant protein production from vector design to production and purification of the heterologous protein product.

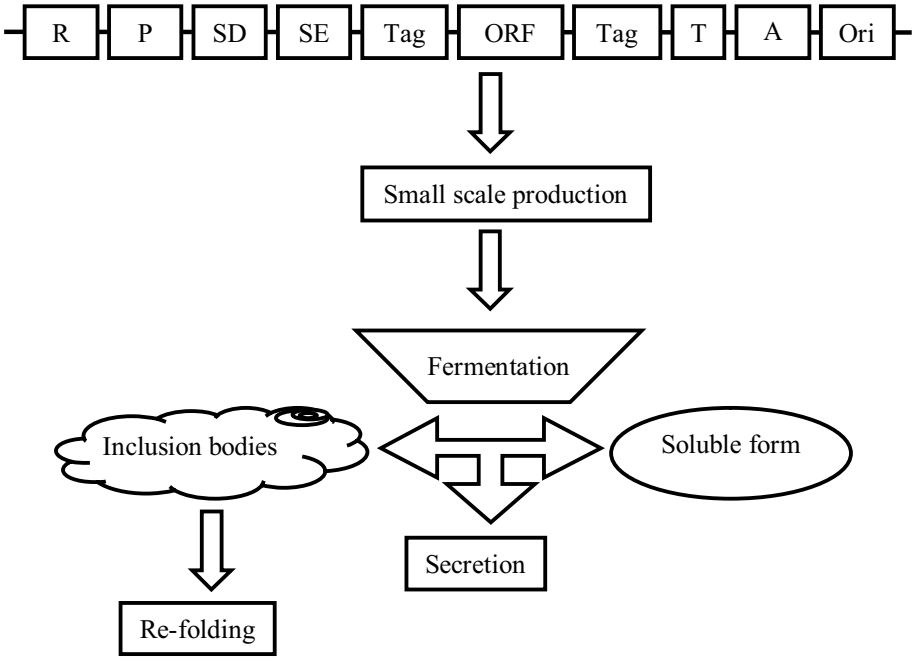

Figure 3. Steps to be considered in recombinant protein production: vector design, scale-up and purification in an active form. R= regulator element, P= promoter, SD= Shine-Dalgarno sequence, SE= stabilizing element, Tag= polypeptide motif that may help in the purification or improve the solubility of the recombinant protein (optional), ORF= open reading frame (protein to be produced), T= transcriptional terminator/stabilizing element, A= antibiotic resistance marker and Ori= origin of replication.

1.4.1 Transcript stabilizing elements in recombinant protein production

Transcript stability can play a role in recombinant protein production if the amount of mRNA is the limiting factor. The use of transcript stabilizing elements may lead to corresponding elevations in both mRNA stability and protein synthesis (Wong and Chang 1986, Carrier and Keasling 1997a, Carrier *et al.* 1998). The strains with plasmids coding for stabilized transcripts produced in these experiments 2- to 5.3-fold higher amounts of recombinant proteins than the control strains. The effect of the stabilizing elements on recombinant protein yields was greater with low inducer concentrations (Smolke *et al.* 2000). mRNA stabilizing elements burden the cells metabolism, since precursors or machinery may become limiting in the synthesis of cellular components (Carrier *et al.* 1998). mRNA stabilizing elements for recombinant protein production are on their best in low copy plasmids, since the stability plays an important part in the amount of protein produced in a wide range of inducer concentrations, whereas other factors become limiting at high induction concentrations with high copy number plasmids (Carrier *et al.* 1998). Also, the amounts of protein produced from a low copy plasmid were greater than those from a high-copy plasmid at relatively low induction conditions. Thus low copy plasmids with mRNA stabilizing elements could be very useful in continuous cultures (Carrier *et al.* 1998).

1.4.2 *In vitro* translation systems

The advantages of *in vitro* translation compared to the *in vivo* protein production include the ability to add unnatural amino acids and produce toxic, poorly expressed and unstable polypeptides (reviewed by Spirin 2004). These systems consist of cell-free extracts of *E. coli*, wheat germ cells or rabbit reticulocytes, or of pure bacterial translation system components with some

necessary additives, such as ions. The proteins produced in cell-free *in vitro* translation systems need less purification steps than proteins produced using whole cells since fewer contaminating proteins are present in the *in vitro* translation systems. The continuous-action cell-free translation systems can function for weeks with the addition of consumable substrates and mRNA and the removal of reaction products. Folding modulators and the removal of reducing activity of the cell extracts are needed with certain proteins to catalyse the production of disulfide bonds in the polypeptide chain and otherwise hinder incorrect folding of the polypeptide (Spirin 2004, Baneyx and Mujacic 2004).

1.4.3 Host organism for the production of foreign proteins

The choice of the host organism depends on the protein to be produced. Some eukaryotic proteins may not be efficiently modified in prokaryotes. Commonly used production hosts include *E. coli*, lactic acid bacteria, *Bacillus*, molds, yeasts, insect cells, mammalian and plant cell cultures and transgenic animals and plants (reviewed by Jana and Deb 2005, Hunt 2005). Expression strains deficient in the production of certain proteases (Park *et al.* 1999) or with a C-terminal deletion of RNase E (Lopez *et al.* 1999) may be especially helpful in the production of highly degradable recombinant proteins or those with labile mRNAs (reviewed by Sørensen and Mortensen 2005), respectively. *E. coli* strains have also been created for the production of membrane proteins and inclusion body prone proteins (reviewed by Sørensen and Mortensen 2005).

1.4.4 Vector design for the production of recombinant proteins

The amount of the recombinant protein needed together with the possible negative effects of overproduction determine the vector to be used. The gene coding for the desired protein product can be inserted into the chromosome or

in plasmids. Copy number of the plasmid, i.e. origin of replication, and the choice of promoter as well as the antibiotic selection marker affect the expression of the recombinant protein (Jana and Deb 2005).

High copy number often equals high productivity, but also high metabolic burden on the cells. Low copy number plasmids are more stable and less of a burden to the cell. The production of recombinant proteins requires energy and may be stressful for the production host (reviewed by Sørensen and Mortensen 2005). Due to the stress, components of the protein production machinery may be down-regulated and proteolysis may increase. Some expression vectors have so called dual regulation, i.e. both the promoter and the copy number of the plasmid are under the control of the same inducer (reviewed by Jana and Deb 2005).

Promoters and upstream elements must be chosen on the basis of the application used. In most cases the ideal promoter is highly controlled but efficient, can be induced to varying degrees, is easily transferable between strains to be tested, and easily inducible with an inexpensive inducer or by thermal induction (reviewed by Jana and Deb 2005). Promoter leakage can be a problem especially when protein production is wanted only at a specific stage, such as after the induction of another protein or after a sufficient biomass has been formed. This is the case when a chaperone or a component of an export pathway is needed before the production of the actual end product, when a multicomponent protein is being produced or in the case of a metabolic pathway.

High accumulation of the transcript and efficient translation are key elements in the production of large quantities of proteins. In high amounts, mRNA may cause ribosome destruction and cell death (Baneyx 1999, Hunt 2005). An optimal RBS (Ma *et al.* 2002) and codon usage resembling that of the production host, or the addition of genes coding for minor transfer ribonucleic acids (tRNAs) (Brinkmann *et al.* 1989), are essential for high-level translation (reviewed by Jana and Deb 2005). Translation can be hindered by unusual

codons usage, especially in the case of eukaryotic proteins produced in prokaryotes. The effect of overproducing genes encoding minor tRNAs may be more complex than enhanced translation. Brinkmann and colleagues (1989) noted that $tRNA^{Arg}$ had an effect on cell viability and plasmid stability as well as on yields of recombinant proteins abundant in rare arginine codons.

Co-expression of all components of multi-component protein complexes in a single cell can be achieved in various ways: several plasmids each carrying one gene coding for one component of the complex, one plasmid with multiple promoters each promoting the expression of one polypeptide or polycistronic plasmids. The advantage of polycistronic plasmids is the high number of components that can be expressed simultaneously (Tan 2001). The number of different plasmids that can be maintained in a cell is limited due to incompability issues.

1.4.5 Folding of recombinant proteins

Incorrectly folded proteins form inclusion bodies or they are degraded (reviewed by Baneyx and Mujacic 2004). Using low growth temperature reduces both proteolysis (Baneyx *et al.* 1991) and protein misfolding by reducing the strength of hydrophobic interactions (reviewed by Baneyex and Mujacic 2004).

Misfolding increases when folding modulators are titrated due to the vast over-production of the heterologous protein. This especially happens with the use of strong promoters and high inducer concentrations. Chaperones can sometimes disintegrate aggregates and refold misfolded proteins. Simultaneous induction of chaperones may help to hinder wrong folding of recombinant proteins (Jana and Deb 2005, Tolia and Joshua-Tor 2006). Simultaneous expression of partners of a multi-component protein complex may facilitate their recovery in active form (Li *et al.* 1997). Tags, such as thioredoxin, maltose

binding protein (MBP) and N-utilizing substance A (NusA) may also be used to enhance the solubility of proteins fused to them (Hammarström *et al.* 2002, reviewed by Hunt 2005, Sørensen and Mortensen 2005).

Occasionally, inclusion bodies can be useful, such as in the case of toxic or unstable proteins. In inclusion bodies they will not cause toxic effects or be degraded, and can sometimes be refolded easily after purification (reviewed by Tsumoto *et al.* 2003, Baneyex and Mujacic 2004). Tsumoto and colleagues (2003) have reviewed the steps needed for the refolding of proteins from inclusion bodies. Proteins are first solubilised by adding detergents, urea or guanidine HCl, and then refolded by gradually decreasing the concentration of the solubilising agent. This decrease can be achieved by dialysis, gel filtration, solid resin phase refolding or dilution. Small molecule additives such as urea or guanidine HCl along with co-solutes may be used to facilitate the correct folding of recombinant proteins. Co-solutes include certain amino acids, sugars and salts and act as aggregation suppressors that reduce side chain interactions or folding enhancers that enhance protein-protein interactions.

Some proteins naturally contain disulphide bonds that are normally not produced in the cytosol of bacteria. In the periplasm disulfide bonds may form (Schlapschy *et al.* 2006, reviewed by Baneyx and Mujacic 2004). Disulphide bond formation may also be achieved in the cytoplasm by the use of certain commercially available mutant host strains (reviewed by Sørensen and Mortensen 2005). Over-expression of enzymes needed for the formation of disulphide and other native bonds may enhance the production of native-state proteins in the periplasm (Schlapschy *et al.* 2006).

1.4.6 Purification of recombinant proteins

Protein purification may be facilitated by the use of tags (reviewed by Hunt 2005). These include glutathione S-transferase (GST), polyhistidine (His6),

maltose binding protein, and many more. Purification may be further facilitated by the secretion of the heterologous protein product to the periplasm of bacteria or outside the cells. Advantages of periplasmic localization or secretion in addition to disulphide bond formation in the periplasm include easier purification since fewer proteins are present in the periplasm and the cell culture medium than inside cell cytoplasm, decreased proteolysis with fewer proteases present, and correctly processed termini of the polypeptide (Talmadge and Gilbert 1982, reviewed by Mergulhão *et al.* 2005, Sørensen and Mortensen 2005). Care must be taken on the translational level of recombinant proteins, since the amount most suitable for secretion may be narrow and must be determined experimentally (Simmons and Yansura 1996).

Periplasmic proteolysis may be further diminished by the use of strains with mutations in the genes coding for proteases, by mildly acidic growth medium and by the use of zinc that inhibits protease activity (Baneyx *et al.* 1991). Known protease recognition and degradation sites may also be mutated in recombinant proteins (Bielli *et al.* 2001). Holin and lysozyme have been employed for release of proteins from *E. coli* after the production of the recombinant protein (Morita *et al.* 2001). In addition to periplasmic leakage that may be enhanced by mechanical, chemical or enzymatic treatments or by the use of leaky strains, type I and II secretion systems are most commonly used for recombinant protein secretion (reviewed by Mergulhão *et al.* 2005). The use of the flagellar TTSS of *E. coli* for the purpose of producing heterologous proteins in the supernatant was recently described (Majander *et al.* 2005). A mutant strain lacking the *fliC* and *fliD* genes coding for the flagellar filament protein and the capping protein, respectively, was created for this purpose. Up to 15 mg of protein was secreted per litre of culture medium, which corresponded to over 50 % of the total secreted protein.

Bacillus species (reviewed in Simonen and Palva 1993) and fungi (reviewed in Conesa *et al.* 2001) naturally secrete high amounts of proteins. This phenomenon has been used for the production and purification of recombinant

proteins. Fungi, including yeasts, are able to perform many of the post-translational modifications needed for the proper function of most recombinant proteins of eukaryotic origin (Cereghino and Cregg 2000, Conesa *et al.* 2001). Problems may arise from the differencies in modification patterns of the production host and the organism from which the heterologous gene originated (Cereghino and Cregg 2000). In addition, some fungal and *Bacillus* strains have been ascertained the generally recognized as safe (GRAS) status and are thus a tempting choice for the production of proteins for human use.

2 Aims of the study

The TTSSs have been widely studied, but basic knowledge of some crucial aspects of the system is still lacking. The hypothesis of a novel kind of a secretion signal, an mRNA signal, led to research on the mRNA of *hrpA*, a type III secreted protein and a component of the TTSS. The aims of this study were to map the regions needed for the secretion of HrpA of *P. syringae* and factors affecting the accumulation of the *hrpA* transcript. The discovery of the extremely long half-life of *hrpA* transcripts directed the studies from a major virulence system of a multitude of pathogenic bacteria, the TTSS, to a more applied direction i.e. to the studies of mRNA stabilizing elements of *hrpA*. The aims of this biotechnological part of the project were to narrow down the regions needed for the stabilizing effect of *hrpA*, to demonstrate the scope of the application with diverse transcripts and finally, to demonstrate the usability of the stabilizing system in recombinant protein production.

3 Materials and methods

The materials, methods, strains and plasmids used in this thesis are described in more detail in the articles and manuscript and are listed in Tables 1 and 2.

Table 1. Methods used in this study

Method	Article
Primer extension	I*
HR assay	I
Immunoblotting	I, III
Mfold analysis of RNA secondary structures	II, III
RNA isolation, Northern blots, probes	I, II**, III
Plasmid constructs	I, II, III
Induction of protein production	I, II, III

*Performed by E. Roine.

**For *E. carotovora* subspecies *carotovora* performed by A. Rantakari.

Table 2. Strains and plasmids used in this study

Strain	Source	Article
E. coli DH5alpha	Bethesda Research Laboratories	I, II, III
E. coli MC1061	LGC Promochem	III
*Pst** DC3000	D. Cuppels, London, Ont., Canada	I, II, III
Pst DC3000 *hrpA*-	Roine *et al*. 1997a	I, II
Pst DC3000 Rif^{S} **	D. Cuppels, London, Ont., Canada	II, III
*Ecc**** SSC1	Saarilahti *et al*. 1986	II
pBBR1MCS	Kovach *et al.* 1994	I
pDN18	Nunn *et al.* 1990	II
pTCKJ02	Carrier *et al.* 1998	III

**Pseudomonas syringae* pv. *tomato*

**Rifampicin sensitive

****Erwinia carotovora* subspecies *carotovora*

4 Results and discussion

4.1 The secretion signal of *hrpA*

The secretion signals of the TTSS-dependent proteins have been studied extensively, but the data is somewhat contradictory (see section 1.2.6 on the secretion signals for TTS). The transcription start site of the *hrpA* transcript was located to a site 42 nucleotides upstream of the translation start codon using primer extension analysis. Promoter and protein fusion constructs revealed that the secretion signal of HrpA of *P. syringae* pv. *tomato* resides in the first 15 codons of *hrpA* mRNA or amino acids of HrpA protein (I). The location of the signal is typical for type III secreted proteins. The nature of the secretion signal, whether it resides in the mRNA or in the amino acid sequence, remains elusive.

4.2 Translation of HrpA is not dependent on secretion

Some TTSS-associated proteins are secreted in a co-translational way with no intracellular pool detected (Anderson and Schneewind 1999). HrpA protein can be expressed without a functional secretion system (I). HrpA transcribed from a *lacZ* promoter was translated in *P. syringae* under Hrp-non-inducing conditions. Thus translation and secretion are not coupled for HrpA, even though the secretion compatibility of pre-made HrpA has not been established.

4.3 Regions important for the accumulation of *hrpA* transcript

Whilst studying the secretion signal of *hrpA* from *P. syringae*, the importance of specific regions for transcript accumulation became apparent. The first 15 codons needed for the secretion of HrpA, as well as the 5' UTR of the transcript, are important for the accumulation of the *hrpA* mRNA (I).

Additional regions involved in the accumulation of the transcript reside in the 3' UTR of *hrpA* (II). Replacing the 5' UTR of *hrpA* with that of *lacZ* or insertions made in the 3' UTR substantially reduced the levels of *hrpA* transcripts. Computer analysis revealed that the 3' UTR of *hrpA* mRNA forms a hypothetical GC-rich stem-loop structure. The insertions in the 3' UTR that reduced the *hrpA* mRNA levels were predicted to change the hairpin structure in this region (II).

The regions in the 5' and the 3' UTRs of *hrpA* transcripts of *P. syringae* pathovars form, according to computer analysis, extremely similar stem-loop structures (III). The sequences of the elements forming these structures are almost identical even though the regions between these conserved sequences, including the coding regions, do not exhibit high homology. This suggests that the stem-loops are functionally important (James *et al.* 1989, Chen *et al.* 1991). The GC-rich hairpins in the 3' UTRs are likely to serve as barriers for the attacks of RNases and might also serve as transcriptional terminators. The role of the stem-loop structures formed in the 5' UTRs of *hrpA* transcripts, if other than stabilization of the transcripts, remains elusive.

4.4 Half-lives of *hrpA* transcripts

In *P. syringae* pv. *tomato hrpA* is transcribed as two mRNAs; *hrpA* that is 0.4 kb and *hrpAZ* that is 1.7 kb. The *hrpA* transcript is extremely stable in different species as analysed by Northern blots (II). Its half-life was measured as 20-40 min. in *P. syringae* pv. *tomato*. The transcript degraded slowly in the first time points after inhibiting *de novo* RNA synthesis with rifampicin, and faster after 40 min. when the *hrpAZ* signal had disappeared. The half-life of the *P. syringae* pv. *phaseolicola* race 4 *hrpA* transcript was measured in *P. syringae* pv. *tomato* DC3000, since a rifampicin sensitive strain was unavailable, and was calculated as 34 min. The half-life of *hrpA* of *E. carotovora* subspecies

(spp.) *carotovora* (recently renamed as *Pectobacterium carotovorum*) was calculated to be 47 min.

According to reverse transcriptase polymerase chain reaction (rt-PCR) measurements (Thwaites *et al.* 2004) the half-life of *hrpA* from *P. syringae* pv. *phaseolicola* race 7 was 8 min. Results obtained by Northern blot analysis of *hrpA* transcripts and contradictory to those achieved by rt-PCR and demonstrate that the *hrpA* mRNA is stable in *P. syringae* pv. *phaseolicola* race 7 (Hienonen E, unpublished results). The differences between the results (Thwaites *et al.* 2004, II and Hienonen E, unpublished data) cannot be attributed to the strains used, but might be explained by the use of different methods. Northern blots measure all the different folding forms of the mRNA without any preference, whereas rt-PCR employed by Thwaites and colleagues (2004) might give a more biased view if one form of the transcript is more readily amplified by the polymerase than others.

Ribosomes may protect transcripts from degradation by RNases (Braun *et al.* 1998, Vytvytska *et al.* 2000). The high stability of *hrpA* mRNA is, however, not dependent on translation or on the presence of ribosomes, as the transcript is equally stable after the addition of antibiotics that release the ribosomes from the mRNA (III). The stability of the *hrpA* transcript is not dependent on additional species-specific factors either, since the transcript is stable also in *E. coli* (II). The stability of the *hrpA* transcript does not control the temperature dependence of the TTSS induction (van Dijk *et al.* 1999) as the transcript is stable under temperatures ranging from 18 to 37 °C (II).

4.5 Stabilization of heterologous transcripts by *hrpA*

The *hrpA* transcript can be used to stabilize heterologous transcripts originating from Gram-negative and Gram-positive bacteria and from eukaryotic species (III). These heterologous transcripts include *neo*, *xln2*, *gfp*

and *fbp54*. Their half-lives were elevated from a few minutes to the range of 17 min in *E. coli* and 25 min in *P. syringae*. Both translational fusion and out-of-frame constructs were stabilized to the same extent. Some transcript stabilizing elements only work as translational fusions (Belasco *et al.* 1986), and if non-fusion recombinant protein is required, a cleavage site must be inserted between the coding regions of the two proteins. The *hrpA* transcript from *P. syringae* pv. *phaseolicola* has the same stabilizing effect on heterologous transcripts as that of *P. syringae* pv. *tomato*.

The regions in the 5' and 3' elements of the *hrpA* mRNA needed for the accumulation of the transcript (I and II) are also necessary and sufficient for the stabilization of heterologous transcripts as shown by deletion analysis (III). The 3' UTR of *hrpA* has a major effect on transcript stability, whereas more minor influence on stability was assigned to the 5' regions. This is in accordance with the result that the transcript *hrpAZ* is less stable than *hrpA*, having a half-life of 11 min. (II), which is still above the average 3-8 min. of bacterial transcripts. The sequences of the transcripts *hrpA* and *hrpAZ* are identical in the 5' region, but differ in the 3' regions.

4.6 Recombinant protein yields can be improved using the transcript stabilizing elements from *hrpA*

The stabilizing effect of *hrpA* elements on heterologous transcripts was also shown to have an application in the production of heterologous proteins (III). Transcript stabilizing elements of *hrpA* fused to the gene encoding neomycin phosphotransferase (NPT) resulted in higher yields of NPT as compared with the control lacking transcript stabilizing elements. The strain of *E. coli* with *hrpA* mRNA stabilizing elements produced up to 5.5 times more NPT than the control as analysed by Coomassie blue staining of poly acryl amide gels.

Further optimization, such as a less leaky promoter, would be needed for more extensive and quantitative studies.

5 Conclusions

This study addressed questions relating to the type III secretion system-associated proteins of plant pathogenic bacteria. In addition to the information on the major virulence system of various Gram-negative pathogens, new knowledge was generated on the production of recombinant proteins in bacteria.

As was in the beginning of this project, it still remains unclear whether the secretion signal for type III secretion is an amino acid signal or an mRNA signal. The search for the secretion signal of the type III secretion-dependent HrpA protein from the plant pathogenic bacterium *P. syringae* did reveal that the signal resides in the first few codons of the mRNA or amino acids of the protein, as is typical for type III secreted proteins. The fact that HrpA can be translated independently of a functional secretion machinery was not unexpected since coupling of translation to secretion has been shown for only a few type III secreted proteins (Karlinsey *et al.* 2000, Anderson and Schneewind 1999). HrpA may not be among the co-translationally secreted type III proteins since no chaperone has been found for HrpA thus far and type III chaperones may contribute to the secretion of co-translationally secreted proteins (Darwin and Miller 2001). The secretion competence of pre-made HrpA still remains to be shown.

The studies on the secretion signal of HrpA that potentially is RNA in nature led to the discovery of the extreme stability of the *hrpA* transcript. The half-life of the transcript is roughly five- to tenfold that of typical bacterial mRNAs (Bernstein *et al.* 2002). Conservation of the sequence of the untranslated regions of otherwise dissimilar *hrpA*s from several *P. syringae* pathovars, the hypothetical stem-loop structures forming in those regions and the stable nature

of the transcript also in *E. carotovora* indicate a biological relevance of the long half-life. The conservation of the presumably stabilizing secondary structures and the relevance of the stability of the transcripts in nature are intriguing issues and require further investigation. The relative amounts of TTS-dependent proteins may be at least in part controlled by the stabilities of their transcripts, HrpA being one of the most abundant TTSS-associated proteins (Roine *et al.* 1997a). Additional research is needed to establish whether stability of mRNAs and conservation of secondary structures are features commonly shared between *hrpA* and analogous transcripts. No published data exists on the stabilities of transcripts coding for pilus or needle components from species other than *P. syringae* and *E. carotovora*.

Stability is an endogenous feature of the *hrpA* mRNA since the transcript is stable also in *E. coli*. The conserved untranslated regions of the transcript are likely to protect the *hrpA* mRNA against the attacks of RNases. Secondary structures may cover the RNase cleavage sites or hinder the attachment and cleavage by RNases that require free ends or cleave only single-stranded RNA. Data from this study on the start site of transcription of *hrpA* and the mapping of the regions involved in transcript accumulation and stability were used to investigate the stabilizing effect of the 5' and 3' regions of *hrpA* mRNA on heterologous transcripts. The half-lives of transcripts originating from diverse organisms were elevated substantially when fused with stabilizing elements of *hrpA* from pathovars of *P. syringae*. The transcript stabilizing system based on the *hrpA* mRNA is at least as potent as those previously described in the literature and summarised in the introduction section. Compared with other stabilizing mRNA structures used for elongating the half-lives of heterologous transcripts, the half-lives achieved with this system are equal or even longer.

The stabilizing effect of *hrpA* mRNA on heterologous transcripts was proven to be of use in the production of recombinant proteins in bacteria, including the widely-used industrial production host *E. coli*. Recombinant protein yields were elevated manifold with the addition of the transcript stabilizing elements despite

the teething problems associated with this novel production system. Compared with the previously published results that are reviewed in the introduction section on the use of transcript stabilizing elements in the production of recombinant proteins in bacteria, the findings using this system are among the best published. Prospective development in the form of better control of induction and more defined growth conditions will presumably further improve the yields of recombinant proteins using this transcript stabilization system based on the *hrpA* transcripts from pathovars of *P. syringae*.

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