Chapter Five

Expression studies on Xanthomonas adhesin-like protein (XadA) from *Xanthomonas oryzae* pv. *oryzae*
5.1 Abstract

Growth medium-dependent differential expression of the outer membrane-located XadA protein of Xoo has been reported, the protein expression being induced by a minimal medium but not by the peptone-sucrose (PS) medium used to culture Xoo. In the present study, the differential XadA expression was also found in a specialized minimal medium called XOM2 that is considered to mimic growth conditions inside the plant. A careful deletion of each media component of XOM2 revealed that XadA expression is glutamate dependent. The addition of glutamate to PS medium induces XadA expression within one hour. Surprisingly, xadA transcripts were found to be present under all nutritional conditions, indicating the involvement of a post-transcriptional control for XadA. Consistent with this possibility, functional deletions of the master transcriptional regulators of Xoo protein expression in planta were found to have no effect on glutamate-dependency of XadA expression. Primer extension studies show that the XadA 5’ UTR is 186 bp long and present under all growth conditions. Together, the results suggest that rapid expression of XadA is achieved by keeping the transcripts ready under all conditions but expressing XadA only after sensing the glutamate that is present in exudates from the hydathodal pores of the rice leaves.
5.2 Introduction

Dynamic environmental conditions like temperature, wind and rain regulate the conditions on the exposed aerial surfaces of the plant hosts. The key factor that attracts several microorganisms and promotes epiphytic growth on such hostile plant surfaces is the presence of organic nutrients that exude out from the plant sap (Andrews & Harris, 2000). Bacteria can sense these small molecules as signals to communicate with the plant hosts to regulate cellular responses of the whole pathosystem (Dunn & Handelsman, 2002). Foliar plant pathogens are more proficient in forming larger colonies on the leaf surfaces, even under environmental stress and tend to colonize openings such as stomata, hydathodes or wounds better than the non-pathogens (Wilson et al., 1999). Signals in the form of sugars, amino acids or some aromatic compounds have been found in the plant exudates and are considered as important chemoattractants for the bacteria to detect their hosts (reviewed in Andrews & Harris, 2000).

The internal milieu of the plant host is more conducive to microbial growth, being abundant in sugars, minerals and water (Marschner, 1995; Raven, 1984). *In planta* growth leads to the induction of bacterial genes that have virulence-related functions (Stachel et al., 1985; Osbourn et al., 1987; Beaulieu & van Gijsegem, 1990; Marco et al., 2003). In *Pseudomonas fluorescens*, some of these genes were shown to respond to small molecules like sugars, amino acids and organic acids that are presumably present in the wheat root exudates (van Overbeek & van Elsas, 1995).

Plant-inducible genes facilitate *in planta* growth of the pathogen through the expression of efficient mineral and sugar uptake systems (Expert et al., 1996; Blanvillain et al., 2007). The other essential functions associated with these plant-responsive genes are to encode toxins or hormones that interfere with the normal plant metabolism or development, in addition to proteins that can disrupt host-signalling pathways (van Gijsegem, 1997). The *hrp* (for hypersensitivity reaction and pathogenicity) gene cluster is a plant-inducible set of genes responsible for the causation of disease on host plants and eliciting a hypersensitive response (HR) on the non-host plants (reviewed in Lindgren, 1997). This cluster codes for genes involved in suppressing the innate immune responses that arise due to the detection of the pathogenic invasion by the host plant and promoting expression of host susceptibility factors (Mudgett & Staskawicz, 1998; Yang et al., 2006; reviewed in Jha & Sonti, 2009). In plant pathogens, the *hrp* genes encode a Type III secretion system (T3S) that forms a protein export
apparatus called the injectisome for delivery of T3S-secreted proteins directly into the plant cells (reviewed in Cornelius, 2006). Interestingly, expression of \( hrp \) genes is observed not only \textit{in planta} but also during growth in synthetic minimal media, where the level of expression depends on the nature of the carbon source provided (Arlat et al., 1992; Rahme et al., 1992; Wei et al., 1992). These media conditions are thought to mimic physiological conditions encountered by bacteria during \textit{in planta} growth.

A major mechanism of bacterial 'response regulation' is the two-component sensory transduction system (Stock et al., 2000). HrpG, is the two-component transcriptional activator responsible for the expression of \( hrp \) genes \textit{in planta} as well as in the synthetic plant mimic media (Brito et al., 1999). HrpX is an AraC-type sensor kinase that functions in response to activation by HrpG and in turn, activates the \( hrp \) effector operons (Wengelnik & Bonas, 1996). It is believed that HrpG can assimilate signals from various sensor genes that can detect plant milieu. One such sensor is PrhA that can sense physical contact with plant cells (Brito et al., 1999). Although no other sensors have been reported that may relay plant signals to HrpG, it is understood that there are contact-independent metabolic signal sensors functioning upstream of HrpG signalling pathway.

Xanthomonas adhesin-like factor A (XadA) is a surface-anchored protein, which belongs to the trimeric autotransporter adhesins (TAA) family, as inferred from the results of Chapter 4. An important observation regarding XadA expression in Xoo was that this protein could be detected only in a minimal medium and not in the peptone-sucrose (PS) medium routinely used to culture Xoo (Ray et al., 2001). This finding suggests that XadA is a differentially expressed protein responding to some nutrients in its growth medium. This result indicates a milieu-dependent variability at the level of XadA protein expression. In this chapter, a detailed analysis of the factors governing XadA expression is described. This study indicates that the expression of XadA is glutamate-dependent, post-transcriptionally regulated and might be essential for Xoo entry into the rice hydathodes.
5.3 Materials and Methods

5.3.1. Growth media and strains used in the study

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Table 5.1. List of strains & plasmids used plasmids used in the structural and functional analysis of XadA

*Escherichia coli* strains were grown in Luria-Bertani medium (Miller 1992) at 37°C and Xoo cultures were grown at 28°C in Peptone-Sucrose medium containing 10gm peptone and 10gm sucrose per litre (Ray et al. 2002). For induction of XadA, plant mimic medium XOM2 containing 1.8gm D (+) xylose (0.18%), 0.1gm L (-) Methionine (670 μM), 1.852gm Na-glutamate (10mM), 12mL of 20mM Fe-EDTA, 0.0068gm MnSO₄ (40 μM), 2.0gm KH₂PO₄ (14.7mM) and 1.0185gm MgCl₂ (5mM) were dissolved per litre. The pH was adjusted to 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 using 1N NaOH. 20mM Fe-EDTA solution was prepared by mixing 2.5gm FeSO₄.7H₂O and 3.36 gm Na-EDTA in 450ml water, boiling and cooling with constant stirring. Basal XOM2 medium (XB) was prepared by adding 1.8gm D (+) xylose (0.18%), 1.852gm Na-glutamate (10mM), and 2.0gm KH₂PO₄ (14.7mM) per litre and adjusting the pH to 5.5. The concentration of other amino acids used to replace Na-glutamate in the XB medium was also 10mM. Antibiotics rifampicin (50 μg ml⁻¹), kanamycin (15μg ml⁻¹) for Xoo and 50 μg ml⁻¹ for E. coli), cephalaxin (10 μg ml⁻¹) and cycloheximide (100 μg ml⁻¹) were used in this study (Sigma-Aldrich, USA). Cyclohexamide was used to minimize fungal contamination. The Xoo strains used in the study are listed in Table 5.1.
5.3.2. Cloning of XadA in various domain combinations into E. coli

The in silico analysis of XadA (Chapter 4) indicated that this protein has four distinct head-neck domains. With the aim of overexpression and crystallization, XadA was cloned in different combinations of the head-necks, apart from the full-length passenger domain of XadA. XadA was cloned with an N-terminal 6x-His-tag using pET28b expression vector under an isopropyl thiogalactoside (IPTG)-inducible T7 promoter (Novagen, USA). The various constructs of XadA used in this work are elaborated in Fig. 5.1.

The N-terminal 66-amino acid predicted signal peptide and the C-terminal membrane anchor domain were not included in any of the constructs. PCR primers for each construct were designed according to the xadA sequence of Xoo strain BXO43 (Genbank accession no. 288222) with NdeI and XbaI restriction endonuclease sites. The PCR products were obtained using regular PCR protocol using high fidelity Phusion polymerase (Finnzymes, USA), cloned into pET28b vector and transformed into BL21DE3 E. coli strain. DNA sequencing was performed to confirm the sequence of each construct.

5.3.3. Expression and purification of the XadA constructs in E. coli

A meticulous scheme was followed to overexpress the various XadA constructs, initially in small 10ml culture volumes and then, scaled up. Each clone was assessed for maximum expression under varying temperatures, time of induction and concentration of IPTG used. The different temperatures and time combinations used were 37°C for 4 hr, 30°C for 6 hr, 25°C for 10 hr, 18°C for 24 hr and 15°C for 30 hr while the IPTG concentrations were varied from the standard 1mM to 0.1mM. The induced cells were harvested, sonicated at 24W for 3 min and centrifuged. The supernatants were analysed on SDS-PAGE gels.

The constructs Xd12 and Xd4 were purified using native affinity purification protocol. Lysis buffer containing 100mM Tris-Cl pH 7.5, 300mM NaCl with 1mM PMSF was used to solubilise the induced cells harvested from one to eight litres of cultures and sonicated at 24W until complete lysis of the cells was visible (30-40 min). The lysate was centrifuged at 18,000rpm for 45 min, allowed to bind to the Ni-NTA\(^2+\) affinity column (Qiagen, USA) and washed thoroughly with wash buffer containing 100mM Tris-Cl pH 7.5, 300mM NaCl. Protein of interest was eluted using 50 mM, 100 mM and 250 mM imidazole (step elution) in 100mM Tris-Cl pH 7.5, 300mM NaCl. The protein peaks obtained during the
chromatography run were assessed using SDS-PAGE and fractions containing pure XadA proteins were pooled. The pooled fractions were concentrated using Amicon-Ultra 15 concentration units (Millipore, USA) and loaded on a Superdex-75 gel filtration column. In this step, the proteins were buffer-exchanged with 20mM Tris-Cl pH 7.5 and 20mM NaCl. The fractions containing pure protein were pooled and concentrated to obtain 1-3mg ml⁻¹ protein.

Figure 5.1. Schematic representation of the various constructs of XadA. A. The PFAM annotation of XadA representing the four 'head-neck' motifs is shown in cartoon form. The lines represent the various fragments of XadA that were cloned as 6x-His-tagged proteins. (Note: the various fragments are not drawn to scale). B. The region of XadA present in each XadA construct used in this study.
For XadA constructs that expressed largely in insoluble form, 6M Guanidine HCl was used to solubilize the inclusion bodies completely and the proteins were allowed to bind to the Ni-NTA$^{2+}$ column. The wash step was also a column refolding step with slow gradient between the buffer containing 8M Urea, 100mM Tris-Cl pH 7.5 and 300mM NaCl and another buffer containing 2M Urea, 100mM Tris-Cl pH 7.5, 300mM NaCl. The proteins were eluted very slowly with 250mM imidazole in the wash buffer containing 1M Urea, 100mM Tris-Cl pH 7.5, 300mM NaCl. Fractions containing pure proteins were pooled and subjected to slow dialysis at 4°C with buffer containing 1M urea and finally, 100mM Tris-Cl pH 7.5, 300mM NaCl and 1mM PMSF. Absence of precipitation at this step was considered as an indication of complete refolding. The proteins were treated like the native proteins after this step and subjected to gel filtration chromatography and SDS-PAGE analysis. The final concentrations of the proteins were 1-3mg ml$^{-1}$.

5.3.4 Assessing protease contamination in purified XadA proteins

Casein zymogram analysis was performed to rule out the possibility of protease contamination in the purified XadA preparations. For this analysis, the protein samples were mixed with sample buffer lacking reducing agents like DTT (4X sample buffer: 250mM Tris pH 6.8, 8% SDS, 40% glycerol, 0.4% bromophenol blue). α-Casein was added to a normal 12% SDS-PAGE mix at a concentration of 1mg ml$^{-1}$. After electrophoresis, gels were incubated for 30 minutes with 2.5% Triton X-100 and subsequently for 16hr at 37°C in 100 mM Tris-Cl, pH 7.4, containing 15 mM CaCl$$_2$$. Gels were stained with Coomassie Blue R-250 and destained with water.

5.3.5 Protocol used for attempting crystallization of XadA

XadA proteins Xd$_{12}$ and Xd$_4$ were found to be stable for longer periods than other XadA constructs. Purified Xd$_{12}$ and Xd$_4$ were screened against 198 crystallization conditions from Hampton Research Crystal Screen I and II, 96 conditions from Hampton Research Index Screen (Hampton research, USA) and 240 conditions from JBS screen (Jena Biosciences, USA). Each screen was monitored for at least three months for the presence of crystals.
5.3.6 Assessment of polydispersity in purified XadA proteins

The distribution of molecular mass in a protein sample is a good indicator of its quality, wherein the presence of molecules of various radii is a sign of polydispersity or multiple species in multiple conformations and molecular weights in the sample. Only single fractions collected from the protein peaks obtained in the final step of purification, i.e., gel filtration were used for this analysis in order to reduce the chances of introducing multiple conformers in the starting samples. Dyna Pro99 Dynamic Light Scattering (DLS) instrument (Wyatt Technology Corporation, USA) was used to record the intensity distribution plot, which is a measure of the molecular radius vs. Amplitude. Concentration of the proteins used was 0.2 mg ml\(^{-1}\). The samples were thoroughly centrifuged at 16,000 rpm and filtered using 0.2\(\mu\)m Anodisk filters (Millipore, USA). The protein samples were incubated for 5 min at room temperature before data acquisition over an acquired time of 10 min.

5.3.7 In silico analysis of disorder in XadA sequence

In order to assess whether XadA sequence was responsible for the disorder observed in these constructs, an in silico GlobPlot disorder analysis was performed (http://globplot.embl.de/). This software indicated that large stretches of XadA sequence are disordered and only very small globular regions were present. These globular regions are marked in Fig. 5.1 as ‘Glb’. The four globular stretches were cloned and overexpressed as 6x-his-tagged proteins and used for setting up crystallization screens as described above.

5.3.8 Secondary structure analysis of purified globular regions of XadA

The secondary structure elements of the Glb constructs were analysed using Far UV circular dichroism (CD) spectroscopy. These spectra were recorded on a Jasco J-715 spectrophotometer (Jasco Inc., USA) at room temperature. Concentration of the proteins used was 0.2 mg ml\(^{-1}\). 0.1cm path-length quartz cuvette was used to record the far UV spectra over 195-260 nm.
5.3.9. Polyclonal anti-XadA antibody generation

0.5mg of Xd12 and Xd4 were mixed thoroughly with incomplete Freund’s adjuvant and used for subdermal injection of two different healthy rabbits. Preimmune sera were collected before the injections. Booster doses of equal amounts of proteins were given to the animals 2 weeks later. After 3 weeks, a small amount of blood was withdrawn from both animals and test Western blotting was performed to assess the antibody efficiency as compared with the preimmune sera. 10ml of serum was finally collected from each animal to yield separate polyclonal antibodies against N- and C-terminal of XadA. The antibodies against Xd12 were effective at 1:3000 and Xd at 1:2000 dilution.

5.3.10. Xoo outer membrane protein preparations and Western blotting

Various Xoo strains were grown to saturation (24hr) in PS medium. The cultures were divided into 1ml aliquots and centrifuged at 8,000 rpm for 2min at 4°C. The supernatants were discarded; the pellets were washed with 1ml sterile water three times. The pellets thus obtained were resuspended in various growth media. For induction in the different minimal media, the pellets equivalent of 1ml Xoo cultures were resuspended in 1ml of the requisite medium and grown at 28°C for 36hr. Since Xoo cultures grow very fast in PS as compared to the minimal media, different strategies were followed to grow the cultures in PS. For short-term induction in PS medium (for assessing XadA expression within 1-12 hours), the pellets equivalent of 1ml Xoo cultures were resuspended in 1ml of the requisite medium and grown at 28°C. For long-term induction in PS medium (for assessing XadA expression 12-36 hr after growth in PS), the pellets equivalent of 1ml Xoo cultures were resuspended in 1ml of PS, of which a 10% inoculum was transferred to 1ml PS and grown at 28°C.

After induction, equal number of cells (normalised using O. D. units) were pelleted and resuspended in an outer-membrane protein extraction buffer containing125mM Tris-Cl pH 7.5 and 2% SDS. The samples were heated at 95°C for 3min and centrifuged at 13, 000 rpm for 5 min (method modified from Hussain et al., 2001). The supernatants were treated as outer-membrane protein preparation and used for Western blot analysis. The samples were loaded on 8 % SDS-PAGE gels and electro-transferred using ECL semi-dry transfer unit (Amersham Biosciences, USA) on a Hybond C-extra nitrocellulose membrane (GE, USA). The blots were blocked with 3% bovine serum albumin (BSA) made in TBST buffer (TBST: 50mM Tris pH
7.4, 150mM saline, 0.5% TWEEN-20), followed by treatment with primary anti-Xd12 antibody diluted in 1% blocking solution. After three rigorous washes with TBST for 15min each, the blots were treated with secondary goat anti-rabbit IgG alkaline-phosphatase conjugated antibody at a 1:10000 dilution (Sigma, USA), followed by three rigorous washes again. The blots were developed with a developing solution containing 0.05mg ml\(^{-1}\) nitroblue tetrazolium (NBT) and 0.05mg ml\(^{-1}\) bromo- chloro- indoyl phosphate (BCIP) dissolved in 100mM Tris pH 9.5, 100mM NaCl and 5mM MgCl\(_2\). After the appearance of the bands, the blots were washed with water, air-dried and photographed.

5.3.11. Enrichment and partial purification of XadA from Xoo cells

Cells were harvested from a 100ml PS-grown saturated 24hr culture of Xoo BXO43 and transferred to 250ml XOM2 medium. After 36hr of induction, the cells were harvested again and resuspended in 10ml of outer-membrane protein extraction buffer (125mM Tris-Cl pH 7.5, 2% SDS). The samples were heated at 95°C for 3min and centrifuged at 15,000rpm for 30min. The supernatants were treated as outer-membrane protein preparation and subjected to ammonium sulphate precipitation with 5%, 10%, 20%, 30% and 70% cuts. A western analysis was performed to trace XadA in the supernatants obtained after every step of ammonium sulphate precipitation and the fractions containing XadA were pooled. These fractions were dialysed for 36hr with several changes of 100mM Tris-Cl pH 7.5, 100mM NaCl to remove SDS, although it is known that complete removal of SDS from protein samples is very difficult.

5.3.12. RNA isolation and Real time PCR analysis of xadA transcripts

Xoo saturated (24hr) cultures grown in PS medium were pelleted and induced in different media conditions for 36hr. After induction, equal number of cells (normalised using O. D. Units) were subjected to total RNA isolation by using Trizol (Invitrogen, USA), following the manufacturer’s instructions. RNase-free DNase (Invitrogen, USA) treatment was given to the samples to ensure no genomic DNA contamination. The quality of the isolated RNA was assessed by 0.8% formamide-agarose gel electrophoresis and quantified with a Nanodrop spectrophotometer (NanoDrop technologies, USA).

2\(\mu\)g of freshly isolated RNA were used for first strand cDNA synthesis using Superscript II reverse transcriptase (Invitrogen, USA) and random hexamer primer. One \(\mu\)l of the 1:400 fold
diluted cDNAs were further used for real-time PCR (RT-PCR) analysis. RT-PCR enables both detection and quantification (as absolute number of copies) of a specific sequence in a cDNA sample using intercalating double stranded dyes such as SYBR green fluorescent dye. An increase in product is proportional to the increase in fluorescent intensity and the number of cycles required is measured, resulting in the quantification of the amount of cDNA. Triplicates of each cDNA sample were taken to set up the RT-PCR. 2.5 picomole of each of the primers and 2 μl of DNA sample were taken and volume of the reaction mix was made up to 17.5 μl with water followed by addition of equal amount of SYBR green master mix (Invitrogen, USA) to make up the final volume of 35 μl which would be sufficient for 3.5 PCR reactions. As a positive control, genomic DNA amount (Experiment positive control) was diluted to get a threshold cycle (C_T) value equivalent to the cDNA samples. RNA samples with no cDNA synthesis step were used as internal negative controls while 16s rRNA was used as an internal positive control. Average C_T value was calculated for triplicates of each sample. A difference greater than 0.3 in C_T value was rejected as an outlier. For each primer pair:

**Step1:** \( \text{AVERAGE } C_T (\text{cDNA}) - \text{AVERAGE } C_T (\text{RNA}) = \Delta C_T \)

**Step2:** \( \Delta C_T^{(\text{Experiment})} - \Delta C_T^{(16s \text{ rRNA})} = \Delta \Delta C_T \)

**Step3:** Relative amount of transcript in the experimental samples = \( 2^{-\Delta \Delta C_T} \)

The values obtained from the final step for each Xoo strain under different nutrient conditions were plotted.

**5.3.13. XadA primer extension and transcriptional start site mapping**

Primer extension was performed using a reverse primer annealing to the 5' end of the xadA transcript (annealing in the region from -1 to -22 of the translational start site). 5-10 μg of each RNA sample isolated as described previously was used as the template. The 5'end- \(^{32}\)P labelled primer was allowed to anneal to the transcript in the presence of 10 mM Tris pH 7.8, 1 mM EDTA, 250 mM KCl at 50°C, followed by incubation at 42°C for reverse transcription in the presence of 400 μM dNTPs and 5 units of AMV reverse transcriptase. At the end of 30 min, the extension products were processed and resolved on a 10% polyacrylamide-8 M urea denaturing gel in 1 X TBE. A parallel manual sequencing reaction was performed using
Perkin-Elmer sequencing kit with the previously described labelled primer and genomic DNA as the template. The gels were dried and subjected to phosphor imaging analysis. All bands were quantified using the Image Gauge program (Fuji, Japan).

5.3.14. Generation of mutations in \textit{hrpG}, \textit{hrpX}, and \textit{hfq} genes of \textit{Xoo}

Mutations in \textit{hrpG}, \textit{hrpX}, and \textit{hfq} genes of \textit{Xoo} strain BXO43 were generated by integration of recombinant pK18mob plasmid through homologous recombination (Fig. 5.2). An internal fragment of each gene to be mutated was polymerase chain reaction (PCR) amplified from BXO43 genomic DNA and cloned in pK18Mob plasmid. The plasmid DNA isolated from the positive clones was transformed by electroporation into BXO43 electrocompetent cells and the recombinants were isolated by selection for kanamycin resistance. Gene disruption was confirmed by PCR and sequencing of the PCR products. This process resulted in nonpolar mutations when the transcriptional orientation of the gene of interest and the promoter of \textit{lacZ} gene in pK18mob were in the same direction (Wei et al. 2007; Windgassen et al. 2000).
Figure 5.2. Schematic representation of the strategy used for generation of mutations in hprG, hprX and hfg genes of Xoo using pK18Mob vector.
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<th>Description</th>
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Table 5.2. List of primers used in the structural and functional analysis of XadA
5.4. Results and Discussion

5.4.1. XadA protein overexpressed in *E. coli* is intrinsically unstable

Various constructs of XadA (Fig. 5.1), containing different combinations of the four head­neck domains described in Chapter 4, were cloned as His-tagged proteins and overexpressed in *E. coli*. While constructs Xd$_{12}$ and Xd$_{4}$ yielded completely soluble proteins, the rest of the ‘Xd’ series of constructs showed insoluble proteins that could not be retrieved into soluble form under native conditions (without any denaturing agents). The level of expression of XadA in all the constructs was found to be low. In general, detergents such as Triton X-100, TWEEN-20 and Na-deoxycholate or polyols like glycerol and sorbitol can help in the solubilisation of inclusion bodies and bring more amount of protein into soluble form. However, the total XadA expression in every construct was very less and the effect of each additive on the solubility of XadA was only partial. Therefore, other than Xd$_{12}$ and Xd$_{4}$, affinity purification was performed under denaturing conditions for the rest of the constructs.

A common feature that was observed in all XadA constructs was the rapid degradation, despite the addition of protease inhibitors like PMSF and cocktails containing leupeptin, pepstatin and aprotinin even at low temperatures. The stability was found to be less than 24hr in most cases, with the appearance of protein ladder within a couple of hours after purification, resulting in complete degradation (Fig.5.3a). No protease contamination was found in any protein preparation, as indicated by casein zymograms (Fig.5.3b). Therefore, the instability of XadA proteins seems to be intrinsic.

Xd$_{12}$ and Xd$_{4}$ were found to be more stable than the rest of XadA proteins and were used for setting up crystallization screens, wherein no crystals were obtained. Dynamic light scattering analysis indicated the presence of multiple species of different oligomeric nature in the peaks showing pure and single protein bands immediately after purification (Fig.5.3c). For crystallization purpose, the protein of interest should have conformational purity and almost no polydispersity or in other words, the protein used for crystallization should be of a single molecular species with no oligomers and degradation products that can hinder crystallization. An *in silico* disorder analysis tool, GlobPlot, indicated the presence of very short globular stretches in XadA while suggesting frequently repeating regions of disorder. The globular stretches (Glb proteins), when cloned as His-tagged proteins, yielded highly stable and well-
expressing proteins (data not shown). However, these stable globular XadA proteins were very short in lengths and found to contain largely coiled coil secondary structures as predicted by the *in silico* analysis in Chapter 4 as well as from the CD spectrophotometric analysis (data not shown), rendering these proteins unfit for X-ray crystallography experiments.

Figure 5.3. Xoo XadA protein shows intrinsic instability when expressed in *E. coli*. (A) SDS-PAGE gel photograph showing the degradation of the 6x His-tagged 27 KDa Xd₄ construct of XadA after purification under native conditions. Lane 1: Xd₄ after NiNTA affinity purification; 2: Xd₄ after gel filtration using Superdex75; 3: The purified protein after concentration using a 5KDa-cutoff Amicon Ultra filter; 4: The purified concentrated protein after 24hrs incubation in 50mM Tris-Cl pH 7.5, 150mM NaCl, 1mM EDTA and 1mM PMSF; 5: The purified concentrated protein after 48hrs incubation in the buffer condition mentioned above. Note the appearance of lower bands (found to be degradation products by Western blot analysis using anti-XadA antibodies) and the decrease in the concentration of the 27KDa XadA band in lanes 4 & 5. (B) Casein zymogram of purified Xd₄ showing an absence of protease contamination (expected to be seen as zones of clearance in the position equivalent to the 27KDa Xd₄ band) in the protein preparation. Lane 1, 2 and 3 contain 50ng, 500ng and 750ng of a very freshly purified Xd₄ protein. (C) The intensity distribution plot obtained by dynamic light scattering analysis of a freshly purified Xd₄ protein. Note the presence of multiple peaks in the plot that represent the proteins of various radii even in the pure fractions of Xd₄ immediately after gel filtration (lane 2 Fig.5.2A), clearly indicating polydispersity.
5.4.2. XadA expression in Xoo is nutrient condition-dependent

It is reported that XadA expression is absent in peptone-sucrose (PS) rich medium that is regularly used for culturing Xoo and that it is induced in modified minimal medium (Ray et al., 2002). It was found that XadA expression is more consistent in a plant-mimic medium called XOM2 (Fig. 5.4a). This medium contains xylose as the sugar source and glutamate, methionine, Fe$^{3+}$, Mg$^{2+}$ and Mn$^{2+}$ as growth supplements. Tsuge et al. (2002) showed that this synthetic medium is capable of inducing the expression of hrp genes that are otherwise expressed only during growth inside the plant host. The secretion of the Hrp proteins in synthetic media by several plant pathogens is reportedly more at acidic pH values and reasoned to be so due to a closer approximation of the pH conditions inside plant apoplast (Furutani et al., 2003; Rossier et al., 1999; Huynh et al., 1989; Gopalan et al., 1996). Taking this into account, XadA expression was tested in XOM2 medium at different pH and was found to be induced at pH 5.0, 5.5, 6.0 and 6.5, with maxima at pH 5.5 (data not shown).

5.4.3. Glutamate regulates XadA expression

In order to find out whether the absence of XadA expression in rich medium is due to some inhibitory activity in PS or because of an inducer in XOM2 medium, the wild-type and xadA-strains were grown in a 1:1 mixture of PS and XOM2. Surprisingly, robust XadA expression was observed in the XadA$^+$ strains in this mixture, clearly suggesting that XadA expression is due to an induction effect in XOM2 while its absence in PS is mainly because of the lack of induction and not due to some inhibitory effect (Fig.5.4b).

Therefore, a search for the inducer in XOM2 medium was performed by reconstituting various XOM2 components and deleting each component one at a time. For this purpose, a basal XOM2 derivative (XB) containing 0.18% xylose and 14.7mM KH$_2$PO$_4$ (pH 5.5) was made. It was confirmed that XadA does not express in this medium. Xylose was replaced with equal amounts of glucose, sucrose and arabinose and XadA expression was not induced by any sugar in this basal medium (Fig. 5.5a).
Figure 5.4. Western blot analysis of XadA expression from outer-membrane protein pool of Xoo. (A) XadA is expressed in the plant-mimic medium XOM2 but not in the peptone-sucrose (PS) medium. Lanes WPS: Wild-type grown in PS; WXOM2: Wild-type in XOM2 pH 5.5; XPS: xadA- in PS; XXOM2. xadA- in XOM2 pH5.5; RPS: xadA+ revertant in PS; RXOM2. xadA+ Revertant in XOM2 pH5.5. (B) XadA expression is also observed in a mixture of PS and XOM2; Lanes WPS+XOM2: Wild-type in a 1:1 mixture of PS and XOM2 pH5.5; XPS+XOM2: xadA- in 1:1 mixture of PS and XOM2 pH5.5; RPS+XOM2: xadA+ revertant in a 1:1 mixture of PS and XOM2 pH5.5. The arrow indicates the expected 110KDa XadA band.

Each XOM2 component was added to XB pH 5.5 in different experiments and assessed for XadA expression by Western analysis. Strikingly, only when glutamate was added to XB pH 5.5, XadA expression could be regained (Fig. 5.5a). This glutamate containing XB medium was named XBGlu. Glutamate, along with other amino acids such as glutamine, asparagine and aspartate, is reported from the vasculature of rice and the guttation fluid of some grasses (Kamachi et al., 1991; Goatley and Lewis, 1966). Guttation fluid is the hydathodal exudate that appears as drops on leaf tips and apparently, serves as a chemoattractant for Xoo (Feng & Guo, 1975). XadA expression was also seen in XBGln medium containing glutamine instead of glutamate, albeit at a lower level, while similar replacement with aspartate and asparagine caused no induction (Fig. 5.5b).
Chapter Five

A Figure 5.5. XadA expression is glutamate dependent. Western blot analysis of the outer membrane protein preparation of Xoo using anti-XadA antibodies was performed. Xylose and KH$_2$PO$_4$ containing medium was treated as the basal medium XB. All the other media components were tested in XB background. (A) XadA expression of wild-type Xoo in various media components of XOM2. Lanes XBMet: XB + methionine; XBFe: XB + Fe-EDTA; XBGlu: XB + sodium glutamate; XBMn: XB + MnSO$_4$; XBMg: XB + MgCl$_2$; SB: Sucrose instead of Xylose in XB; GB: Glucose instead of Xylose in XB; AB: Arabinose instead of Xylose in XB. (B) XadA expression in BXO43 in the presence of nitrogenous amino acids. Lanes XBGlu: XB + sodium glutamate; XBGln: XB + Glutamine; XBAsp: XB + Aspartate; XBAsn: XB + asparagine; XBArg: XB + arginine; XBN+: XOM2B + NH$_4$Cl. The arrow indicates the expected 110KDa XadA band.

5.4.4. Glutamate induces a rapid and stable XadA expression

Addition of glutamate to PS medium at pH 5.5 also induced XadA expression (Fig. 5.6a). A time course of Xoo induction in XBGlu and XOM2 revealed that the XadA expression begins within 1hr (Fig. 5.6b). The expression increases up to 24hr, where it remains stable even up to 48-60hr after induction (data not shown). XadA protein was partially purified (section 5.3.11).
from a relatively large (250ml) culture of BX043 strain in XOM2 medium in order to assess the stability of XadA isolated from native source. XadA protein, thus isolated, was found to be relatively enriched several fold (Fig.5.7) and very stable at 4°C for several months (data not shown). The final XadA preparation might retain traces of SDS, even after thorough dialysis and the presence of detergent micelles might have a role in the long-term stability. However, it is important to note that with this method of purification, XadA can be relatively enriched in a stable form. This method, when scaled up, might be possibly used for obtaining full-length XadA protein for crystallization studies.

Figure 5.6. Characteristics of Glutamate-dependent XadA expression. Western blot analysis of the outer membrane protein preparation of Xoo using anti-XadA antibodies was performed. (A) Addition of glutamate into PS can induce XadA expression. Lanes WXBGlu: Wild-type Xoo grown in XB + Glu; 2. XXBGlu: xadA+ Xoo grown in XB + Glu; RXBGlu: xadA+ Revertant Xoo grown in XB + Glu; M: Marker; WPSSGlu: Wild-type Xoo grown in PS + Glu; 2. XPSGlu: xadA+ Xoo grown in PS + Glu; RPSSGlu: xadA+ Revertant Xoo grown in PS + Glu. (B) One hour of induction in the presence of glutamate is enough to induce XadA expression. All treatments in this figure are performed on wild-type Xoo cells grown in PS medium for 24 hours and induced for the requisite time-periods. Lanes PS1hr: Xoo induced in PS for 1hr; XOM21hr: Xoo induced in XOM2 for 1hr; XBGlulhr: Xoo induced in XBGlul for 1hr; M: marker; PS24hr: Xoo induced in PS for 24hr; XOM224hr: Xoo induced in XOM2 for 24hr; XBGlu24hr: Xoo induced in XBGlu for 24hr. The arrow indicates the expected 110KDa XadA band.
There is also a possibility of post-translational modification of XadA in Xoo in its native form, for example glycosylation, that might be absent when expressed in *E. coli*. Although the event of glycosylation is not very well studied in bacteria, certain Gram negative bacterial autotransporters are reported to be N-glycosylated (reviewed in Benz & Schmidt, 2002). Another plausible explanation for stability of XadA in Xoo could be that the lack of trimerization and/or proper folding in *E. coli* causes instability in the T1 loops that recur after every β-roll structure (Chapter 4; section 4.4.1), which might not be the case in XadA expressed in Xoo. The role of glutamate, if any, in conferring stability to XadA is also an interesting premise. This can be assessed by over-expressing XadA in *E. coli* in the presence of added glutamate.

![Figure 5.7. Relative enrichment of XadA obtained by the partial purification protocol.](image)

**Figure 5.7. Relative enrichment of XadA obtained by the partial purification protocol.** Western blot analysis of outer membrane protein preparations of wild type Xoo cells (WPS: PS grown Xoo or WXOM2: XOM2 grown Xoo cells). Step-wise ammonium sulphate precipitation was performed with one set of cultures (lanes marked ‘30% Amm. Sulphate’) while the control set was not precipitated (lanes marked ‘No precipitation’). The final volumes of the experiment and control preparations were equalized and finally equal volumes were loaded on the SDS-PAGE gel. The arrow indicates the expected 110KDa XadA band.

### 5.4.5. XadA expression is independent of the characterized response regulators of Xoo

The results clearly show that XadA is induced in the plant-mimic medium XOM2. Expression of bacterial genes in either plant-mimic medium or in planta is dependent on the HrpG and HrpX proteins (Mudgett & Staskawicz, 1998). *hrpG* and *hrpX* mutants of Xoo were generated in this study in order to determine if mutations in these genes would affect XadA
expression. Surprisingly, it was found that the expression of XadA in XOM2 is not affected by the either HrpG or HrpX mutants of Xoo (Fig. 5.8).

The role of another two-component transcriptional regulatory system, composed of the sensor protein ColS and the regulatory protein ColR, in XadA expression was assessed. colS and colR mutant strains (S. Sujatha, M.R. Vishnupiya, H. Patel, A. Pandey & R. Santi, unpublished results) too had no effect on XadA expression in XOM2 (Fig. 5.8).

![Figure 5.8. Western blot analysis of XadA expression in transcriptional regulator mutants of Xoo. HrpGPS: hrpG mutant grown in PS; HrpGXOM2: hrpG mutant grown in XOM2 pH5.5; HrpXPS: hrpX mutant grown in PS; HrpXXOM2: hrpX mutant grown in XOM2 pH5.5; ColSPS: colS mutant grown in PS; ColSXOM2: colS mutant grown in XOM2 pH5.5; ColRPS: colR mutant grown in PS; ColRXOM2: colR mutant grown in XOM2 pH5.5. The arrow indicates the expected 110KDa XadA band.]

5.4.6. Glutamate-dependent XadA expression is post-transcriptionally regulated

The presence of xadA transcripts in PS, XOM2 and XBGlu media was assessed by a PCR using whole cDNA samples as templates. Total RNA was isolated from Xoo cultures grown in different media and converted into cDNA using random hexamers for first strand synthesis. Strikingly, xadA transcripts are present under all media conditions (Fig. 5.9a). Real Time PCR
(RT-PCR) confirmed this result and indicated that PS-grown Xoo cells showed more transcripts of \textit{xadA} (Fig. 5.9b). The overall amount of \textit{xadA} RNA in comparison to the control 16s rRNA in all samples was found be very less, indicating a low expression of XadA in Xoo cells. Since \textit{xadA} strain BX0837 has a Tn5 insertion in the middle of the gene, it is known to express XadA protein in a truncated form (Ray et al., 2001). This is the reason for the presence of \textit{xadA} transcript in the BX0837 strain. Nevertheless, RT-PCR was performed with a different set of primers specific to the 3' end of \textit{xadA} and as expected, no transcript was seen in BXO837 samples (data not shown). The presence of \textit{xadA} transcript under all nutrient conditions and the induction of the protein expression in plant mimic-medium suggest a post-transcriptional regulation of XadA.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5_9}
\caption{	extbf{Figure 5.9.} \textit{xadA} transcripts are present in all media conditions. (A) PCR using cDNA templates prepared using total RNA from Xoo cells. RNA from WPS: Wild-type grown in PS; WXOM2: Wild-type in XOM2 pH 5.5; XPS: \textit{xadA} in PS; XXOM2. \textit{xadA} in XOM2 pH5.5 was isolated and converted into cDNA using first strand synthesis. PCR with corresponding RNA samples was performed to assess the genomic DNA contamination. PCR with wild-type genomic DNA and water were used as positive and negative controls, respectively. (B) Real Time PCR analysis of \textit{xadA} transcripts in various media conditions: WPS: Wild-type grown in PS; WXOM2: Wild-type in XOM2 pH 5.5; WBGlu: Wild-type grown in XB + Glutamate; XPS: \textit{xadA} mutant grown in PS; XXOM2. \textit{xadA} mutant in XOM2 pH5.5; XXBGlul: \textit{xadA} mutant grown in XB + Glutamate. Y-axis represents the relative enrichment of \textit{xadA} transcript in each media condition (X-axis) with respect to the 16s rRNA internal control.}
\end{figure}
5.4.7. The xadA transcript has a long 5' untranslated region

Another interesting feature that was observed in the xadA sequence was the lack of -10 and -35 promoter elements immediately upstream of the translational start site of xadA. Promoter prediction softwares (Neural Network Promoter Prediction software; BPROM software) predicted promoter elements atleast 150bp upstream of the translational start site (data not shown). However, the accuracy of these softwares is known to be low for bacterial sequences. Therefore, a primer extension analysis was performed using the total RNA isolated from wild-type Xoo cells grown in PS, XOM2 and in XB + glutamate medium. The primer extension product demonstrated that xadA has a long 5' untranslated region (UTR) and the transcriptional start site is -178bp upstream of the translational start site in Xoo cells grown in any of the three media (Fig. 5.10).

5.5. Inferences from the study

Pathogenic bacteria respond to the highly heterogeneous environment of their eukaryotic host by optimally regulating the expression of their genes. In order to understand the host-pathogen interactions better, it is crucial to identify the host cues that are used by the pathogen to regulate expression of virulence factors. Although some 'host-like' conditions can be mimicked in vitro (e.g., low iron, acidic pH, antimicrobial peptides and oxidative stress), the host environment remains too complex and dynamic to be accurately modelled in the laboratory.

In the case of rice-Xoo interactions, a reasonably good model of the rice xylem sap, wherein Xoo grows, has been constituted in the form of XOM2 medium in which, the expression of several plant-inducible promoters is observed. The expression of the trimeric autotransporter adhesin XadA is induced in this medium. Moreover, the presence of glutamate or glutamine only and no other amino acid including aspartate and asparagine could induce XadA expression. However, this induction was found to be independent of several known response regulators like HrpG and HrpX that transcriptionally regulate the expression of genes in response to either plant mimic media or growth in planta. Real time PCR analysis indicates that xadA transcripts are present in the PS medium and that expression of XadA protein in XOM2 appears to be regulated at the post-transcriptional level.
Figure 5.10. *xadA* transcript has a long 5'UTR. Primer extension analysis of *xadA* transcript, using a PCR primer that anneals to the *xadA* RNA around the translational start site, reveals that *xadA* has a long 5'UTR with the transcriptional start site at -178 with respect to the translational start site. All the experiments were performed with wild-type Xoo cells. RNA was isolated from WPS: Wild-type grown in PS; WXOM2: Wild-type in XOM2 pH 5.5; WXBGlu: Wild-type induced in XB + Glutamate and used for first strand synthesis using the α-P\(^32\) end-labelled primer. The lanes labelled as G, A, T and C represent manual sequencing using the corresponding labelled dNTPs.
A post-transcriptional control can be mediated at the translation step or after translation. In order to assess whether the long leader sequence of \( xadA \) has a role in the glutamate-dependent post-transcriptional regulation of XadA expression, a \( \beta \)-glucuronidase (GusA) reporter-tagged construct of \( xadA \) has been generated. This construct is a translational fusion of \( xadA \) with \( gusA \) \(~150\)bp downstream to the translational start site. GusA enzyme assay experiments with this Xoo construct are in progress. Very preliminary results with a \( xadA::gusA \) reporter suggest that XadA expression is controlled at least in part, at the translational level. A common mechanism of translational regulation of protein expression in bacteria is brought about either by a cis-acting RNA secondary structure or by non-coding small RNAs. Bacterial protein translation, in general, is coupled to the gene transcription process and small RNAs provide an alternative to this tight coupling by providing a means of stabilizing the mRNA for a later use in translation (Gottesman, 2005). These RNAs are typically 40-400bp in length, transcribed from the opposite strand from their targets and therefore, able to base pair extensively with their target mRNA (Majdalani et al., 2005). Apart from these cis-acting RNAs, several trans-acting small RNAs are also known to regulate bacterial gene expression, including genes involved in bacterial pathogenesis. Genome-wide searches for these small RNAs in \( E. \ coli \), and, more recently, in other bacteria as well have led to the identification of more than 200 predicted small RNAs and of these, the expression of 60 small RNAs has been confirmed by Northern blotting in \( E. \ coli \) K12 (Storz et al., 2004). In silico studies show the presence of small regulatory RNAs in the genomes of all the sequenced Xanthomonas strains (Luban & Kihara, 2007). In general, small RNAs are retained in their unstructured form to facilitate proper binding to their template by chaperones such as Hfq (host factor for the replication of the Q\( \beta \) phage RNA) and CsrA (carbon storage regulator) (Majdalani et al., 2005). Sequence homology-based identification of Hfq protein from Xanthomonas species is reported (Sauter et al., 2003). The expression of XadA was found to remain unaltered in an \( hfq \) mutant of Xoo under the various media conditions (data not shown). However, since there are non-coding RNAs in the Xoo transcriptome that function independent of Hfq, this mode of translational control of XadA expression cannot be ruled out.

The post-translational control of XadA expression is also plausible as XadA might be produced and rapidly degraded in PS medium while being stabilized in XOM2. It is interesting to note that XadA protein is intrinsically unstable in \( E. \ coli \). The degradation of
XadA, as soon as it is translated, in nutrient-rich condition might at least partially be responsible for the absence of XadA under these growth conditions. The possibility of a post-translational modification, such as glycosylation, in minimal plant-mimic conditions but not in PS or when overexpressed in *E. coli* needs to be explored. A search for putative N-glycosylation sites in XadA sequence yields at least seven sites (residues 63, 134, 140, 209, 556, 717 & 986) at very high confidence levels (data not shown) in the software NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/), which are located on the surface of XadA model presented in Chapter 4 (Fig. 4.7). In the absence of these post-translational modifications, XadA might be unstable. However, this mechanism of regulation of XadA expression might be less likely since the translation process is energetically demanding and it would be wasteful to synthesize a protein of 110KDa and then degrade it.

The data presented in the current study indicates the translation of XadA from a pre-existing pool of *xadA* transcript by Xoo upon sensing the presence of glutamate. It is interesting to note that there is a report stating that compounds in the rice hydathodal exudates act as chemoattractants for Xoo and that glutamate is the chemoattractant (Feng & Guo, 1975). It is also noteworthy that glutamate is one of the free amino acids found in hydathodal exudates from several grasses (Goatley & Lewis, 1966). It is interesting to note that XadA expression is also induced by glutamine, which is also present in the rice xylem sap (Oaks, 1992). The results suggest that rapid expression of XadA is achieved by keeping the transcripts ready under all conditions but expressing XadA only after sensing the glutamate that is exuded into the hydathodal pores on the rice leaf tips. In this manner, Xoo may be using glutamate as a cue that it is in the presence of rice hydathodes and that it is time to express XadA. Previous work in our lab has shown that XadA protein is involved in promoting plant colonization (Das et al., 2009).

The results discussed in this chapter also indicate that XadA is stably expressed in the glutamate-containing XOM2 medium. This feature might prove useful while designing a strategy for large-scale XadA purification. The problems encountered during the overexpression and purification of XadA from *E. coli* can be overcome by scaling up the XadA enrichment method described here.