Insertion mutagenesis of XpsD, an outer-membrane protein involved in extracellular protein secretion in *Xanthomonas campestris* pv. *campestris*

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XpsD is an outer-membrane protein required for extracellular protein secretion in *Xanthomonas campestris* pv. *campestris*. Cross-linking and gel-filtration chromatography analyses have suggested that it forms a multimer. To determine its structure–function relationship, linker-insertion mutants were constructed in an *xpsD* gene carried on a plasmid. To assay for secretion function, each mutant gene was introduced into an *xpsD*::Tn5 mutant strain (XC1708) and assayed for α -amylase secretion on starch plates. To test whether the mutant genes exerted a dominant-negative effect, each was introduced into the parental strain XC1701 and examined for secretion interference. Nine functional, one semi-functional and eleven non-functional mutants were obtained. All the non-functional mutants, except two for which the mutant proteins were undetectable on immunoblots, showed interference of normal secretion. The insertion sites in the different mutant proteins are randomly distributed throughout the entire sequence of the XpsD protein. All the permissive insertion sites are located where β -turn or coiled secondary structure is predicted. Over half of the non-permissive sites are located within predicted helical or β -sheet regions. By pretreating total membranes of XC1701 in SDS at 50 °C, an immunoreactive band with high molecular mass (HMM) could be detected that remained in the stacking gel during SDS-PAGE. The semi-functional and all functional mutant proteins formed HMM complexes that were as SDS-resistant as those of the wild-type, whereas all except three of the non-functional mutant proteins formed HMM structures that were less resistant to SDS than the wild-type. By analysing the appearance of SDS-resistant HMM complexes, we were able to detect conformational alterations in XpsD that are too subtle to be detected by other assays.

Keywords: random linker insertion, outer membrane protein, type II secretion pathway, negative dominance, SDS-resistant high-molecular-mass protein

INTRODUCTION

In Gram-negative bacteria, macromolecules, which include secreted enzymes and toxins, and cell-surface structures such as pili or filamentous phage particles,

have to traverse both the inner membrane (IM) and the outer membrane (OM) before reaching the cell surface. The type II secretion pathway is a two-step process (Salmond & Reeves, 1993) in which proteins with an N-terminal signal peptide are first exported across the IM via the Sec-dependent pathway and processed. In the second step, the processed proteins are secreted across the OM via a secretion machinery composed of 12–14 proteins (Lory, 1992; Pugsley, 1993). XpsD is an OM protein required for a type II secretion pathway in *Xanthomonas campestris* pv. *campestris* (Hu *et al.*,

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Abbreviations: DSM, disulfide-bonded multimers; HMM, high molecular mass; IAA, iodoacetamide; IM, inner membrane; β -ME, β -mercaptoethanol; OM, outer membrane.

1992a; 1995). Extracellular enzymes secreted via this pathway include polygalacturonate lyase, endoglucanase, α -amylase and protease (Daniels *et al.*, 1984; Gough *et al.*, 1988; Hu *et al.*, 1992b; Tang *et al.*, 1987). Besides eight proteins involved in the type II pathway, XpsD is also related to a component of the N-terminal-signal-peptide-independent type III secretion pathway (Lory, 1992; Pugsley, 1993; Salmond & Reeves, 1993), the pIV protein of filamentous phages (required for phage assembly and export) (Hill & Petersen, 1982; Luiten *et al.*, 1985; Russel, 1994a), the PilQ proteins of *Pseudomonas aeruginosa* (Martin *et al.*, 1993) and of *Neisseria gonorrhoeae* (Drake & Koomey, 1995) and the BfpB protein of enteropathogenic *Escherichia coli* (required for formation of type IV pili; Ramer *et al.*, 1996). All these proteins share amino acid sequence similarity to various degrees with the XpsD protein, particularly within the C-terminal 200 amino acid residues.

Like the BfpB protein of *E. coli* (Ramer *et al.*, 1996), XpsD is a lipoprotein, fatty-acylated at its mature N-terminal cysteine residue, while its signal peptide is cleaved by signal peptidase II (Hu *et al.*, 1995). However, lipid modification of the XpsD protein is not essential for normal function, since replacement of the N-terminal signal peptide with one that was cleavable by leader peptidase I did not affect its function (Hu *et al.*, 1995).

The XpsD protein, consisting of 759 amino acid residues, is predicted to be rich in β -sheets with the exception of a region from residue 340 to 460. Unlike that of the pIV protein, the N-terminal half of the XpsD protein is localized in the outer membrane. A C-terminally truncated XpsDA414–759 protein appeared in the OM fraction when analysed on a sucrose gradient and was detectable on the cell surface when intact cells were treated with an antibody against the XpsD protein (Hu *et al.*, 1995).

By performing gel-filtration chromatography, we previously demonstrated that the XpsD protein forms a multimer (Chen *et al.*, 1996). Its homologues, the pIV protein of filamentous phage (Kazmierczak *et al.*, 1994) and the *N. gonorrhoeae* PilQ (Omc) protein (Newhall *et al.*, 1980), were also shown to form homo-multimers of 10–12 subunits. It has been suggested that these OM proteins, in multimeric form, may serve as secretion channels for the translocation of extracellular enzymes and release of assembling phage or pili. Our previous analysis of truncated mutant XpsD proteins indicated that the C-terminal half is required, and sufficient, for multimer formation (Chen *et al.*, 1996). An N-terminally deleted mutant protein, XpsDA29–428, was capable of forming multimers indistinguishable from the wild-type XpsD multimer when analysed by gel-filtration chromatography. By contrast, the C-terminally deleted mutant protein, XpsDA414–759, could not form such multimers. Although not necessary for multimer formation, the N-terminal half is clearly indispensable for normal function of the XpsD protein; in the C-terminal half of the XpsD protein, at least one loop region is also

functionally important. Mutants carrying both XpsD(A553L/I554D) and XpsDA545–553 proteins were defective in secretion, while remaining capable of forming multimers similar to the wild-type (Chen *et al.*, 1996).

To further our understanding of the XpsD protein structure, in this study we have analysed 21 semi-randomly inserted linker-insertion mutant XpsD proteins. In the N-terminal half of XpsD, we found that the positions of functional insertions are interspersed with those of non-functional insertions. Three mutants with insertions in the C-terminal half of the XpsD protein showed secretion defects as well as structural alterations. Interspersed among them are functional insertions.

METHODS

Bacterial strains, plasmids and media. The parental strain *X. campestris* pv. *campestris* XC1701 is a spontaneous rifampicin-resistant mutant from a natural isolate, XC17 (Hu *et al.*, 1992a). XC1708 (*xpsD*::Tn5) is a Tn5-derived mutant of XC1701 (Hu *et al.*, 1992a). *E. coli* DH5 α [F^- ϕ 80d *lacZ* Δ M15 *deoR* *recA1* *endA1* Δ (*lacZYA-argF*)U169 *hsdR17*($r_k^-m_k^+$) *supE44* *thi-1* *gyrA96* *relA1*] was used in all cloning work in this study. The broad-host-range plasmid pCPP30 (Tc^r) was a kind gift from D. Bauer (Cornell University, Ithaca, NY, USA). To increase the likelihood of obtaining insertions in the *xpsD* gene, we constructed a smaller plasmid (pTKC118). The 2.6 kb *HindIII*–*EcoRI* fragment of pKC118 (Hu *et al.*, 1992a), which contained the entire coding sequence of the *xpsD* gene, was introduced into pTZ18U (Ap^r) (Mead *et al.*, 1986). The resulting plasmid, pTKC118, is 5.46 kb, while pKC118 is 10.1 kb. L broth and L agar were prepared according to Miller (1972). Starch plates for assaying α -amylase secretion were prepared by including 0.2% soluble starch in XOL minimal medium (Hu *et al.*, 1992b). Antibiotics were added where appropriate at the following concentrations: 15 μ g tetracycline ml⁻¹; 50 μ g ampicillin ml⁻¹; 100 μ g rifampicin ml⁻¹; and 50 μ g kanamycin ml⁻¹.

Semi-random linker-insertion mutagenesis of the *xpsD* gene. pTKC118 was partially digested with *HaeIII*, *RsaI* or *AluI*, or completely digested with *SmaI*. DNA fragments were recovered from an agarose gel, ligated with a self-annealed 12-mer oligonucleotide, 5'-CCCAGCTCGGG-3', and used to transform *E. coli* DH5 α . Self-annealing of the 12-mer oligonucleotide was performed by incubating it in TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA) at a final concentration of 1 μ g ml⁻¹ at 80 °C for 1 min, then transferring it to a beaker of water at 65 °C for slow cooling to room temperature (Lathe *et al.*, 1984). The self-annealed oligonucleotide was then treated with T4 polynucleotide kinase prior to ligating into the digested plasmid. To enrich for mutants, we isolated plasmid DNA from the pooled transformants and treated it with *SacI*. The linearized plasmid DNA was then recovered from an agarose gel, self-ligated and used to transform *E. coli* DH5 α .

DNA sequencing. Target DNA was amplified from the mutant *xpsD* gene by PCR. After PCR, excess primers and nucleotides were removed by treating the PCR product with 10 units exonuclease I (USB/Amersham Life Science) and 2 units shrimp alkaline phosphatase (USB/Amersham Life Science) in a final volume of 7 μ l at 37 °C for 15 min. After the enzymes were inactivated by heating at 80 °C for 15 min, the DNA was

ready for direct sequencing. The dideoxy sequencing method (Sanger *et al.*, 1977) was followed.

Electroporation. Mutant *xpsD* genes with confirmed insertion sites by DNA sequencing were subcloned downstream of the *lac* promoter in the broad-host-range plasmid pCPP30. Then the recombinant plasmid was electroporated into *X. campestris* pv. *campestris* XC1701 or XC1708. The electroporation conditions described by Chen *et al.* (1996) were followed.

Plate assay for α -amylase secretion. Cells grown on L agar were toothpicked onto XOL agar plus 0.2% starch and incubated at 28 °C overnight. Appearance of a clear zone surrounding the colony indicated secretion of α -amylase.

Preparation of total membranes. Total membranes of *X. campestris* pv. *campestris* were prepared as the pellet from a low-speed centrifugation of the cell lysates obtained after lysozyme/EDTA/sucrose treatment followed by freeze-thawing and DNase I digestion (Brissette & Russel, 1990). Briefly, 1 ml cells grown in L broth to OD₆₀₀ 1.3 were harvested and washed with water once before resuspending in 25 μ l buffer containing 200 mM Tris/HCl (pH 8.0), 2 mM EDTA. After addition of 30 μ l 40% sucrose and 5 μ l lysozyme (5 mg ml⁻¹), the whole mixture was incubated at room temperature for 10 min followed by two cycles of freeze-thawing. To this cell lysate, 400 μ l distilled water, 12 μ l 1 M MgCl₂ and 2 μ l DNase I (1 mg ml⁻¹) were added, followed by incubation at room temperature for 20 min. The DNase I treated cell lysate was centrifuged at 12000 r.p.m. for 10 min and the pellet was saved as the total membrane fraction. In some experiments, iodoacetamide (IAA) was added to a final concentration of 50 mM immediately before breaking the cells by freeze-thawing.

SDS-PAGE and immunodetection. Total membranes were resuspended in sample buffer [125 mM Tris/HCl, pH 6.8; 4% (w/v) SDS; 5% (w/v) β -mercaptoethanol (β -ME); 0.05% bromophenol blue; 20% (w/v) glycerol] before boiling for 5 min or treating for 15 min at 50 °C or on ice. After sample treatment, proteins were separated in an SDS-polyacrylamide gel (10%, w/v, acrylamide) followed by electroblotting onto a nitrocellulose membrane and immunodetection. The detailed procedures were as described previously (Hu *et al.*, 1992a). For detecting HMM-XpsD (high-molecular-mass XpsD), 3.75% acrylamide was used for preparing the stacking gel. In all other cases, 5% acrylamide was used.

RESULTS

Isolation of linker-insertion mutants of the *xpsD* gene

To study the structure–function relationship of the OM protein XpsD, we constructed a series of mutant *xpsD* genes containing insertions of a 12 bp linker. The linker was designed, in addition to introducing a unique *SacI* restriction site, to create in-frame insertions of four amino acid residues in all three reading frames. Furthermore, a proline and a glycine residue, both of which are prone to make turns in the polypeptide chain, appear in two reading frames (Table 1). Screening of the plasmid DNA from a total of 252 transformants revealed that 117 became cleavable with *SacI*. The 12 bp linker in more than half of these *SacI*-cleavable plasmids was located within the cloned *xpsD* fragment. The insertion sites were located primarily by restriction enzyme digestions and confirmed by DNA sequencing. A total of

21 different insertion mutations (Table 1), which included one at the unique *SmaI* site, were obtained. Ten of the insertions were at *HaeIII* sites, four at *AluI* sites and six at *RsaI* sites. Within the *xpsD* coding sequence, there are 19 *HaeIII*, 8 *AluI* and 8 *RsaI* sites, so at least half of the target insertions were achieved.

Nearly half of the mutant XpsD proteins are functional

Depending on the reading frame, the inserted 12 bp linker encodes either PELG, PSSG or XRARX. Two types of XRARX insertions were generated in this study; all insertions at alanine residues are replaced with ARARA, and all at tyrosine residues with SRARD (Table 1). Each mutated gene, cloned on the broad-host-range plasmid pCPP30, was electroporated into the *xpsD*::Tn5 mutant strain XC1708 and examined for α -amylase secretion on starch plates. More than half of each type of insertion, other than SRARD, produced an XpsD protein positive for α -amylase secretion (Table 1). Active secretion, as opposed to passive leakage by cell lysis, of α -amylase by the functional mutants was confirmed by Coomassie brilliant blue staining and immunoblot analysis of the SDS-polyacrylamide gel on which the extracellular fraction was analysed. Based on the secretion properties of the XC1708 strain containing each *xpsD* mutant gene, we categorized the insertion sites into the functional and the non-functional. The former are probably located in regions of the XpsD protein that are less constrained than the latter. In other words, functional insertions did not significantly affect the proper function of the XpsD protein. One mutant, with the insertion at Pro-123, had an intermediate secretion phenotype and was classified as semi-functional. The insertion sites of H76 (Ala-182) and of H54 (Ala-186) are only four residues apart, defining a permissive region flanked by Ala-182 and Ala-186. Moreover, DNA sequencing revealed that we had accidentally introduced a 12 bp *HaeIII* fragment (5'-CCTCGCCATCGG-3') into the H76 mutant, upstream of the linker, generating a mutant XpsD protein with amino acid residues SPSARARA inserted downstream of Ala-182. This additional insertion between Ala-182 and Ala-186 did not affect the normal function of the protein, indicating that this might be a region favourable for introducing extra amino acid residues.

All but two non-functional mutant XpsD proteins are detectable on immunoblots

To determine whether stable proteins were produced by all the mutated *xpsD* genes, we performed immunoblot analysis on total cell extracts. Full-length XpsD proteins were clearly detected in all insertion mutants except two, one at Ala-169 and the other at Glu-334 (data not shown). These results indicated that most of the non-functional mutants remained capable of producing structurally stable XpsD proteins. Since the insertions are located in the mature portion of the protein, it was not anticipated that cellular processes such as transcription, translation, or translocation (signal sequence

Table 1. Properties of the linker-insertion mutant XpsD proteins

Clone	Insertion site	Replacing amino acids	Secretion in*		Immunoblot	HMM†
			XC1708	XC1701		
H64	Ala-89	ARARA	3.0±0.0	3.1±0.3	+	+
H2	Gly-162	GPSSG	3.0±0.2	3.1±0.3	+	+
H76	Ala-182	ASPSARARA	3.0±0.2	3.0±0.1	+	+
H54	Ala-186	ARARA	2.8±0.1	3.2±0.1	+	+
H62	Ala-380	ARARA	2.7±0.2	2.8±0.1	+	+
A80	Gln-423	QPELG	2.8±0.2	2.9±0.1	+	+
R25	Ser-427	SPELG	2.9±0.3	3.2±0.4	+	+
H6	Gly-518	GPSSG	3.2±0.1	3.2±0.2	+	+
A3	Ser-604	SPSSG	2.7±0.2	2.9±0.2	+	+
TS2	Pro-123	PPSSG	1.8±0.2	2.7±0.1	+	+
H74	Ala-169	ARARA	1.0±0.0	3.2±0.4	—	—
A43	Glu-334	EPELG	1.1±0.2	2.9±0.1	—	—
H26	Gly-116	GPSSG	1.3±0.3	2.1±0.2	+	+
H32	Ala-132	ARARA	1.2±0.2	2.1±0.3	+	+
R31	Val-149	VPSSG	1.2±0.2	1.8±0.1	+	+
R19	Tyr-497	SRARD	1.2±0.1	1.9±0.2	+	—/+
R38	Ser-690	SPELG	1.4±0.2	1.8±0.2	+	—/+
R37	Tyr-199	SRARD	1.5±0.2	1.6±0.2	+	—
R33	Tyr-212	SRARD	1.1±0.2	1.8±0.2	+	—
H58	Ala-344	ARARA	1.4±0.3	2.6±0.1	+	—
A25	Ala-645	ARARA	1.3±0.1	1.8±0.2	+	—

* Numbers represent the ratio calculated from dividing the diameter of the clear zone by the colony diameter (mean of five repeats $\pm$ SD). XC1701 without plasmid resulted in a ratio of 3.0 ± 0.2 and XC1708 without plasmid gave rise to a ratio of 1.1 ± 0.2 . Both controls were calculated from the results of 15 experiments.

† + HMM-positive, — HMM-negative under all conditions tested, —/+ HMM-negative at 50 °C but HMM-positive on ice.

processing) would be affected. Neither could plasmid loss be the cause of the absence of the XpsD protein in the strains containing insertions at Ala-169 or at Glu-334, since we could detect the recombinant plasmid DNA. Perhaps the insertions at these two sites rendered the XpsD protein unstable, due to significant changes in its secondary structure. Such changes may have either blocked the formation of proper tertiary or quaternary structures or caused incorrect localization and/or assembly of the mutant XpsD protein in the outer membrane, which in turn might provoke protein degradation.

A dominant-negative effect is exerted by non-functional mutant XpsD proteins

To determine if the non-functional mutants are dominant-negative to the wild-type *xpsD* gene, we introduced each mutant *xpsD* gene into XC1701 and assayed for α -amylase secretion of the transformants on starch plates. All non-functional insertions, except the two located at Ala-169 and Glu-334, caused secretion interference (Table 1). Insertions at Ala-169 and Glu-334 are unlikely to interfere with secretion in XC1701, since their products are unstable. The mild defect in the mutant XpsD with an insertion at Pro-123 did not cause

significant interference. The interference observed for the Ala-344 insertion appeared much less severe than those of the other mutants (Table 1). This could be due to a low steady-state level of the protein. One likely explanation for the dominance of the mutant proteins is the formation of mixed complexes composed of wild-type and the non-functional mutant XpsD proteins (Chen *et al.*, 1996). Alternatively, the mutant proteins might interfere with the assembly of the wild-type multimeric complex. The dominance might also arise from competition between mutant and wild-type proteins for binding to other components of the secretion apparatus or for binding to the secreted proteins. This might be the case for the mutant with the insertion at Val-149, since quantitative analysis revealed that the steady-state level of the mutant protein was significantly lower than that of the chromosomally encoded wild-type protein (data not shown).

Wild-type HMM-XpsD is SDS resistant at 50 °C

An HMM form appearing as immunoreactive material in the stacking gel during SDS-PAGE has been reported for three XpsD homologues, the PilQ protein of *N. gonorrhoeae*, the PulD protein of *Klebsiella oxytoca* and the pIV protein of *E. coli* filamentous phage (Drake &

Fig. 1. Immunoblot detection of HMM-XpsD in XC1701 pretreated with sample buffer containing 4% or 0.5% SDS (100 and 50 indicate pretreatment temperatures in °C).

Koomey, 1995; Hardie *et al.*, 1996; Linderroth *et al.*, 1996). Moreover, Linderroth *et al.* (1996) demonstrated that HMM-pIV represents the pIV multimer. In 4% SDS, the HMM-pIV was detectable when samples were pretreated at temperatures below 65 °C. It was stable to boiling when glycerol (or urea) and β -ME were simultaneously present in the sample buffer. Since we have shown previously that the XpsD protein forms multimers (Chen *et al.*, 1996), we wondered whether the XpsD multimer could be detected as an HMM form. HMM-XpsD was indeed observed when samples were pretreated at 50 °C or on ice in sample buffer (4% SDS with β -ME), but not when boiled (Fig. 1). Reduction of

the SDS concentration from 4% to 0.5% did not make the HMM-XpsD more resistant to boiling (Fig. 1). Unlike HMM-pIV, HMM-XpsD remained sensitive to boiling, even when β -ME and glycerol (or urea, data not shown) were both present in the sample buffer. The monomeric XpsD was detectable under all conditions tested. Therefore, HMM-XpsD is more prone to SDS dissociation than HMM-PulD (Hardie *et al.*, 1996) and HMM-PilQ (Drake & Koomey, 1995); the latter two remained stable to pretreatment in sample buffer at 100 °C.

SDS-resistant HMM-XpsD is present in all functional and some non-functional mutants

We examined the mutant XpsD proteins by pretreating total membranes in sample buffer at 50 °C for 15 min, and found that the HMM form appeared in all nine functional, the semi-functional and three of the 11 non-functional mutants (Gly-116, Ala-132 and Val-149) (Fig. 2). Almost all HMM-XpsDs were detected as a sharp band close to the well. Only the HMM form of the Val-149 XpsD mutant protein repeatedly appeared as a smear (Fig. 2). It also featured a low-molecular-mass protein band (Fig. 2), which could be a degradation product. These two phenomena may be related.

The absence of the HMM form in two of the mutant XpsD proteins (Ala-169 and Glu-334) is probably due to their low steady-state levels. On the other hand, six of the non-functional mutant XpsD proteins (Tyr-199, Tyr-212, Ala-344, Tyr-497, Ala-645 and Ser-690) were produced at steady-state levels comparable with the wild-type; they also showed a dominant-negative effect, suggesting that these mutant XpsD proteins might be able to form mixed complexes with the wild-type protein. It is possible that 50 °C is too high a temperature for these mutant XpsD proteins to remain associated as

Fig. 2. Immunoblot detection of HMM-XpsD from the linker-insertion mutant XpsD proteins listed in Table 1 (100 and 50 indicate pretreatment temperatures in °C).

Fig. 3. Immunoblot detection of HMM-XpsD from mutant XpsD proteins with insertions at Tyr-199, Tyr-212, Ala-344, Tyr-497, Ala-645 and Ser-690, pretreated in standard sample buffer at 50 °C (50) or on ice (ice).

Fig. 4. Immunoblot detection of DSM when pretreated in sample buffer without β -ME. Linker-insertion mutant XpsD proteins with insertions at Tyr-497 or Ala-645 were pretreated on ice in sample buffer containing 4% SDS without β -ME. IAA was added before cell lysis (+) or not added at all (-).

HMM forms. By incubating total membranes for 15 min on ice, instead of at 50 °C, we were able to detect HMM forms in two of them; one with the insertion at Tyr-497 and the other at Ser-690 (Fig. 3). The former appeared slightly fainter and less sharp than the latter. Apparently, the HMM forms of these two mutant XpsD proteins were not as stable as that of the wild-type; however, they were more stable than those of the other four mutant XpsD proteins. To examine if milder conditions than treatment in sample buffer are necessary for detecting HMM forms in the other four mutants, we lowered the SDS concentration in the sample buffer from 4% to 0.5%, and/or eliminated β -ME. All treatments were performed on ice. None of these milder treatments resulted in the appearance of HMM forms (data not shown). Instead, new, slowly migrating protein bands appeared in the running gel in samples prepared in the absence of β -ME from the mutant with the insertion at Ala-645 (Fig. 4); similar species were occasionally detected in the mutant with the insertion at Tyr-497. Their apparent molecular masses were lower than the HMM form, but higher than the monomeric form of the XpsD protein. These species were not detected when the wild-type or the other mutant XpsD proteins were treated in the same way (Fig. 1 and data not shown). There are three cysteine residues in XpsD.

Besides the N-terminal cysteine of the mature protein, the other two are residues 647 and 660, both of which are located in the vicinity of Ala-645. To test if these slowly migrating bands represent disulfide-bonded multimers (DSM), we examined the effect of IAA on their appearance. IAA was added before cell lysis. These slowly migrating bands were no longer detectable in the Ala-645 mutant when IAA was included (Fig. 4).

To determine if the absence of the HMM form was a consequence of the inability of the mutant XpsD proteins to form multimeric complexes, we examined Triton X-100 membrane extracts prepared from the Tyr-199, Tyr-212, Ala-344 and Ala-645 mutants by gel-filtration chromatography as described previously (Chen *et al.*, 1996). The mutant proteins appeared in the same fractions as the wild-type protein (data not shown), indicating that the mutant proteins do form multimeric complexes.

DISCUSSION

The entire amino acid sequence of the XpsD protein could be divided into an N-domain and a C-domain separated by a central glycine- and serine-rich region, which is predicted to favour β -turn structure (residues 351–450) (Fig. 5). The GS-rich region is present in only one XpsD homologue, the OutD protein of *Erwinia chrysanthemi* (Lindeberg & Collmer, 1992), suggesting that the GS-rich region in the XpsD protein may not be essential for normal function. In agreement with this prediction, all three mutant proteins with extra amino acid residues inserted within the GS-rich region (Ala-380, Gln-423 and Ser-427) are functional. A putative linker region of 104 residues has been proposed in the middle of the XpsD protein from sequence alignments analysed by Genin & Boucher (1994).

We observed correlations between HMM production and multimer formation for two previously constructed deletion-mutant XpsD proteins. The XpsD Δ 29–428 protein, which formed multimers appearing in the near-void volume upon gel-filtration chromatography (Chen *et al.*, 1996), produced an HMM form that migrated slightly faster on SDS-polyacrylamide gels than the wild-type HMM form, due to its smaller molecular size (data not shown). By contrast, the XpsD Δ 553–759 protein formed multimers with fewer subunits than the wild-

Fig. 5. The positions of linker insertions in the XpsD protein. The top line indicates the scale in amino acids. In the lower section, locations of functional (filled arrows), semi-functional (downward-pointing open arrowheads) and non-functional (upward-pointing open arrowheads) insertions are indicated. Segments predicted (Chou & Fasman, 1978) to form α -helices of ten residues or more are represented as hatched rectangles and probable β -sheets of eight residues or more as open ovals. Predicted β -turns or coils are left unmarked.

type multimers (Chen *et al.*, 1996), and no longer produced HMM-XpsD (data not shown). These observations suggested that HMM-XpsD may represent the XpsD multimers that remain associated in the presence of SDS, similar to the pIV-HMM (Linderth *et al.*, 1996).

Of the five mutant proteins with insertions in the C-domain, only the Tyr-497 insertion site is located within a likely membrane-spanning segment, where a typical amphipathic β -strand is predicted. The corresponding protein was detectable at steady-state levels comparable with the wild-type protein and exerted secretion interference. When analysed on a gel-filtration column, it appeared to form multimers as well as the wild-type protein (data not shown). If this β -strand is indeed part of a β -barrel, apparently its disruption did not affect multimer assembly. However, the stability of the multimers to SDS treatment was reduced by the replacement of Tyr-497 with SRARD.

The other four insertions within the C-domain are located outside long stretches of predicted α -helix or β -strand (Fig. 5). Only two of these mutant proteins (Gly-518 and Ser-604) are functional. When we aligned the sequence of the XpsD protein with the PulD protein of *K. oxytoca*, we found that Gly 518 was located within a region defined as module V in the PulD protein, which was shown to be dispensable (Daefer *et al.*, 1997). Insertions at Ala-645 and Ser-690 rendered the mutant XpsD proteins non-functional. Apparently these two sites are not as permissive as Gly-518 and Ser-604. Insertions at either site caused XpsD multimers to become more prone to SDS dissociation than the wild-type. Upon gel-filtration chromatography, both mutant proteins appeared in the near-void volume, as did the wild-type XpsD (data not shown). These results suggest that the integrity of the region around Ala-645–Ser-690 may be essential for keeping the protein in the correct functional conformation. Moreover, conformational alterations caused by amino acid insertion at these two sites, while not detectable by gel-filtration chromatography, could be distinguished clearly by looking for SDS-resistant HMM. Ser-690 is located five residues downstream of a highly conserved glycine residue, which was demonstrated to be irreplaceable with any other amino acid residues in the filamentous phage pIV protein (Russel, 1994b).

DSMs appeared when the mutant XpsD protein with the insertion at Ala-645 was pretreated with SDS in the absence of β -ME, but not when IAA was included during the cell lysis. These results suggested that the insertion of RARD downstream of Ala-645 may have exposed the cysteine residues at position 647 and/or 660 for disulfide bond formation between nearby XpsD monomers. On the other hand, the absence of DSM forms of wild-type XpsD protein suggests that the two cysteines are normally either buried or too far apart for DSM formation. Apparently, such a conformation is essential for normal function of the XpsD protein. Linderth *et al.* (1996) isolated a stabilizing pIV mutant as a general suppressor of several defective pIV gene mutations. This mutation (causing a single amino acid replacement, S318I) alone has no discernible phenotype in plaque formation, but gives rise to extremely heat-stable and SDS-resistant HMM. Amino acid sequence comparison revealed that Ser-318 of the pIV protein is in alignment with Thr-648 of the XpsD protein. It is interesting to note that Thr-648 is in close proximity to Ala-645, where an insertion produced an HMM-destabilizing effect in XpsD.

Five non-functional mutant proteins with insertions in the N-domain are produced at similar steady-state levels as the wild-type protein. They could be distinguished based on SDS-resistant HMM complex formation. Three mutant proteins, with insertions at Gly-116, Ala-132 and Val-149 respectively, formed SDS-resistant HMM complexes as efficiently as the wild-type protein. Gly-116 is located in a region with predicted β -turn structure, Ala-132 is located in a predicted β -strand and Val-149 is located in a putative α -helix (Fig. 5). These mutations apparently affected the protein function without making HMM-XpsD more prone to SDS dissociation, suggesting that mutations at these three sites have minimal effects on the overall conformation of the protein. Recent studies suggested that the N-terminal domains of filamentous phage pIV protein (Daefer *et al.*, 1997) and the OutD protein of *E. chrysanthemi* (Shevchik *et al.*, 1997) carried a specificity determinant of the secretion machinery. It would be interesting to examine if any of the three insertion sites located within the N-domain of the XpsD protein might be involved in the determination of secretion specificity.

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