XpsD, an Outer Membrane Protein Required for Protein Secretion by Xanthomonas campestris pv. campestris, Forms a Multimer*

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XpsD is an outer membrane lipoprotein, required for the secretion of extracellular enzymes by Xanthomonas campestris pv. campestris. Our previous studies indicated that when the xpsD gene was interrupted by transposon Tn5, extracellular enzymes were accumulated in the periplasm (Hu, N.-T., Hung, M.-N., Chiou, S.-J., Tang, F.-., Chiang, D.-C., Huang, H.-Y. and Wu, C.-Y. (1992) J. Bacteriol. 174, 2679–2687). In this study, we constructed a series of substitutions and deletion mutant xpsD genes to investigate the roles of NH₂- and COOH-terminal halves of XpsD in protein secretory function. Among these secretion defective xpsD mutations, one group (encoded by pCD105, pYL4, pKdA6, and pKD2) caused secretion interference when co-expressed with wild type xpsD, but the other (encoded by pMH7, pKdPs, and pKDT) did not. Cross-linking studies and gel filtration chromatography analysis indicated that the wild type XpsD protein forms a multimer in its native state. Similar gel filtration analysis of xpsD mutants revealed positive correlations between multimer formation and secretion interfering properties exerted by the mutant XpsD proteins in the parental strain XC1701. Those mutant XpsD proteins (encoded by pCD105, pYL4, pKdA6, and pKD2) that caused secretion interference formed multimers that are similar to the wild type XpsD multimers and those (encoded by pMH7, pKdPs, and pKDT) that did not formed smaller ones. Furthermore, gel filtration and anion exchange chromatography analyses indicated that the wild type XpsD protein co-fractionated with XpsD(Δ29–428) or XpsD(Δ448–650) protein but not with XpsD(Δ74–303) or XpsD(Δ553–759) protein. We propose that the mutant XpsD(Δ29–428) protein caused secretion interference primarily by forming mixed non-functional multimers with the wild type XpsD protein in XC1701(pCD105), whereas the mutant XpsD(Δ74–303) did so by competing for unknown factor(s) in XC1701(pYL4).

XpsD is an outer membrane (OM) protein of Xanthomonas campestris pv. campestris required for the secretion of extracellular proteins with a cleavable NH₂-terminal signal peptide (1–3). It is likely to be involved in the second step of a two-step secretory pathway, the type II pathway (4–6). Mutations in the xpsD gene caused the accumulation of extracellular enzymes in the periplasm (1). These enzymes are probably exported from cytoplasm in the first step via a Sec-like pathway (7). Homologues of XpsD are widespread in Gram-negative bacteria. Among them, the PulD protein of Klebsiella oxytoca (8), the OutD protein of Erwinia chrysanthemi (9, 10), the OutD protein of Erwinia carotovora (11), the XcpD protein of Pseudomonas aeruginosa (12), and the ExeD protein of Aeromonas hydrophila (13) have been shown to be involved in the same type of secretion pathway. In addition, between 11 to 13 more genes in each of these bacteria are required for the second step of secretion (5, 6). Some other XpsD homologues, however, are involved in a different type of secretory pathway, the type III pathway, for proteins without a cleavable NH₂-terminal signal peptide. These XpsD homologues, which include the YscC protein of Yersinia enterococlitica (14), the HrpH protein of Pseudomonas syringae (15), the MxiD protein of Shigella flexneri (16), and the InvG protein of Salmonella typhimurium (17), work with a different set of accessory proteins (6). There are other proteins which share significant sequence homology with XpsD, such as the plV proteins of the filamentous phages f1, 12-2, and Ike of Escherichia coli (18–20), the plV protein of the filamentous phage pf3 of P. aeruginosa (21), the pIIQ protein of P. aeruginosa (22), and the OrfE protein of Haemophilus influenzae (23). These proteins are required, respectively, for filamentous phage release (24, 25), pilus assembly (26), and genetic transformation (23, 26). Alignment of all these protein sequences revealed the strongest homology at their COOH termini for approximately 200 amino acid residues (22, 27).

Recent studies by Kaczmerczk et al. (28) indicated that plV, which is required for filamentous phage release, appeared to form a stable complex composed of 10–12 monomers. Moreover, the OutD protein of E. chrysanthemi, an XpsD homologue required for type II secretion, was co-precipitated with plV by antibody against plV (28), suggesting a complex formation between the two proteins. OutD is also highly homologous to plV at the COOH-terminal end, implicating the involvement of this region in the mixed complex formation (28).

Secondary structure prediction indicated that XpsD is rich in β-structures. At least 14 amphipathic β-strands were predicted based upon Jähnig’s algorithm (29). Near the middle of XpsD, there is a region of more than 100 amino acids that is longest compared with other XpsD homologues. It is rich in Gly and Ser, which is observed in only one other XpsD homologue, i.e. the OutD protein of E. carotovora (11). This long loop divides the entire XpsD into two domains: the upstream NH₂-terminal domain terminates at the 330th amino acid residue and the downstream COOH-terminal domain begins at the 440th residue.
In this report, we show that XpsD also forms multimeric complex as pV does. In order to find out what roles do NH₂ and COOH-terminal domains play in the complex formation, we constructed a series of deleted xpsD mutants, examined each for secretion interference in presence of wild type xpsD gene and conducted gel filtration and anion exchange chromatography analyses on them.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmid Constructs—All the X. campestris pv. campestris strains used in this study were from our previous work (1). The parental strain XC1701 is a spontaneous rifampicin-resistant mutant from a natural isolate XC17. Both XC1708 (xpsD::Tn5) and XC17433 (ΔxpsD,F,G,H,I,J,K,L,M,N,D) are Tn5-derived mutants of XC1701. The detailed structures of other xpsD mutations are depicted in Fig. 1. All of them were cloned in a broad host range plasmid pCPP30 (IncP replicon, tet'), which was kindly provided by D. Bauer of Cornell University. The xpsD-phaA fusion gene on pKD20 plasmid was constructed by inserting the signal peptide-less strain BMH71-18 (E. coli) into the XbaI site of pCD2. Escherichia coli strain BMH71-18 (stupri lac-proAB muts::Tn10 (F'proAB lacIq23A1M5)) used in site-directed mutagenesis was purchased from Promega Corp. All plasmids were maintained in E. coli DH5α [F'pSC101::λZaM15 dea recA1 endA1 (laczYA-argF)U169 hsdR17 (rK-,mK+) F'proAB lacIq23A1M5] or M109 (supE44 thiI lacPROAB recA1 endA1 hsdR17 gyrA96 relA1 F'traD36 proAB Iq23A1M5)] (31).

Site-directed Mutagenesis—Site-directed in vitro mutagenesis with the Altered Sites System purchased from Promega Corp. was used to generate new XbaI sites in the xpsD gene. The xpsDΔ29–428 and xpsDΔ29–428 + deletions were constructed in our previous work (3). For the construction of the xpsDΔ545–553 mutation, the following two primers were used: 5′-TTTCCCCAAGAAATTCTAGACGAGCGCCTTTGGCG-3′ for the upstream XbaI site and 5′-CTACGGCCTATACTTAGAGCTGCTACTTTTTGCC-3′ for the downstream XbaI site. Electroporation—X. campestris pv. campestris strains grown to an OD₆₀₀ of 0.6–0.8 were washed in 0.3–0.5 ml of sterile water to remove exopolysaccharide. One and one-half ml of the washed cells were resuspended in 100–200 μl of sterile water, into which 100 ng of plasmid DNA (in 5–10 μl) was added. Electroporation was performed three times with the Gene Pulse/Pulse Controller (Bio-Rad), at 200 ohms, 25 microfarads, and 2.5 kV in a cuvette with a 0.4-cm gap. The shocked cells were diluted in 1 ml of L broth and incubated shaken for another 10 min at 28°C. The treated cultures were collected from Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis samples and analyzed by immunoblotting. Gel Filtration Chromatography—Fractions containing the XpsD protein eluted from DE52 column were collected and precipitated by the addition of solid ammonium sulfate to a final concentration of 70%. The pellet collected from centrifugation was dissolved in a small volume of Buffer B (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.2 mM NaCl, 1% deoxycholic acid) and chromatographed on FPLC Superdex HR-200 (Pharmacia Biotech Inc., 25-ml size) which was pre-equilibrated with Buffer B. Fractions were collected and analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting. The column was calibrated with the following molecular mass standards: thyroglobulin (669 kDa), apolipoprotein (443 kDa), β-amylase (200 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa).

RESULTS

Mutant xpsD Genes—In order to study the XpsD protein structure, we constructed a series of xpsD deletion mutations (Fig. 1). We introduced five different XbaI sites into xpsD gene in various combinations. Some are silent, such as the xpsD genes encoded on pKU2 and pCD103 plasmids; the other generates missense mutations, such as the xpsD gene encoded on pKD2 plasmid. In-frame internal deletion mutants were created with XbaI and BclI. The mutant XpsD proteins can be classified as: (i) truncated proteins XpsD(Δ29–428) (pCD105), XpsD(Δ74–303) (pYL4), XpsD(Δ141–759) (pMH7), XpsD(Δ448–650) (pIKPs), XpsD(Δ545–553) (pKDa6), and XpsD(Δ553–759) (pKDT) and (ii) missense proteins XpsD(Δ544–754) (pIKU2), XpsD(Δ553LΔ554D) (pIKD2), and XpsD(Δ298–7428N429D) (pKDC03). All constructions were confirmed with restriction digestion and DNA sequencing results.

Complementation Test of the xpsD Mutations—Each mutant xpsD gene was inserted downstream to the lac promoter on pCPP30 plasmid and introduced into XC1708 (xpsD::Tn5) via electroporation. The transformants selected on tetracycline plates were examined for α-amylase and protease secretion (data not shown). Moreover, the fate of α-amylase in all these transformants was investigated by immunoblot analysis of the extracellular, periplasmic, cytoplasmic, and membranous fractions with antibody against α-amylase. Results from both experiments indicated that only XC1708(pKU2) and XC1708(Δ29–428) secreted α-amylase extracellularly as well as the parental strain XC1701 and XC1708(pKCl18) (Figs. 1 and 2). All of the other XC1708 transformants and XC1708 itself accumulated α-amylase in the periplasm.

Stability of Mutant xpsD Gene Products—In order to determine that the negative results of the complementation tests were not due to protein instability, we performed immunoblot analysis with antibody against XpsD on total cell extracts of XC1708 (data not shown) and on partially purified proteins made in XC17433 (eluted from DE52 chromatography). XC17433 is a mutant strain which lacks the entire xps gene cluster on its chromosome. It is apparent that all mutant xpsD gene products examined, except XpsD(Δ29–428) (data not shown), were detected, as well as the wild type XpsD synthesized from pKCl18 (Fig. 3). However, the major protein bands observed for XpsD(Δ141–759) (with estimated molecular mass of 43 kDa) and XpsD(Δ553–759) (with estimated molecular mass

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mass of 58 kDa) migrated faster than expected and appeared broader than the other XpsD proteins. Both observations indicated that these two proteins are probably less stable than the others. Only small amounts of degradation products were noted for XpsD(D74–303) and XpsD(A553L/I554D) (Fig. 3). The weak intensity of the protein band observed for XpsD(D545–553) was probably due to low sample amount, not representative of protein instability. This interpretation is supported by the absence of degradation products from XpsD(D545–553) analyzed on gel filtration chromatography (Fig. 5).

Interference of the Parental Strain Protein Secretion by the Presence of Mutated xpsD Gene—When these mutated xpsD genes were introduced into the parental strain XC1701 and tested for extracellular protein secretion, it was observed that some mutant genes exerted negative dominance over the chromosomally located xpsD1 gene (Fig. 1). They include those encoded by pCD105, pYL4, pKdA6, and pKD2. On the other hand, no apparent secretion interference was observed with three other nonfunctional mutant XpsD proteins encoded by pMH7, pKdPs, and pKD7.

In Vivo and In Vitro Cross-linking of Wild Type XpsD—The negative dominance of some xpsD mutations suggested possible involvement of protein-protein interactions between wild type and mutant XpsD proteins. We performed protein cross-linking on the cell membrane prepared from XC1701 cells (in vitro) as well as on growing cultures of XC17433(pKC118) (in vivo). XpsD could be cross-linked in both cases by glutaraldehyde as detected on immunoblots (Fig. 4, arrow). While the former result suggested that XpsD is likely to be part of a multimeric complex, the latter further suggested that XpsD could be cross-linked in absence of all the other Xps proteins encoded by the xps gene cluster.

XpsD Protein Complex Formation Analyzed on Gel Filtration Chromatography—In order to confirm the multimer formation of XpsD, we introduced each mutant, as well as the wild type xpsD gene, into XC17433. Thus we could perform gel filtration chromatography of the detergent-extracted XpsD in absence of

Fig. 1. Summary of the xpsD mutations and their secretion and multimer formation properties. The structural gene of xpsD1 on pKC118 plasmid was shown on top with its derivatives shown below. Truncated regions were left blank. The numbers in parentheses next to the restriction sites used indicate the nucleotide numbers. The open box represents the phoA gene without its NH2-terminal signal peptide sequence. The designations listed under the column "XpsD" show the deleted residues ( ), missense substitutions, and fusion. "+" indicates positive secretion, multimer formation, or positive interaction with wild type XpsD. "-" indicates secretion negative, no multimer formation, or no interaction with wild type XpsD. "ND" indicates intermediate level of secretion with clear zones of diameters between those of XC1701 and XC1708. ND, not determined.

Fig. 2. Subcellular fractionation of α-amylase in XC1708 transformants. Except for positive (XC1701) and negative (XC1708) controls, each transformant is indicated by the plasmid contained within XC1708. The fractions are designated as follows: C, cellular; P, periplasmic; M, membranous; E, extracellular.

Fig. 3. Immunoblot of DE52-purified XpsD. DE52-purified XpsD was analyzed on SDS-polyacrylamide gel followed by immunoblotting. Samples were loaded as follows: lane 1, XC17433(pKC118); lane 2, XC17433(pYL4); lane 3, XC17433(pMH7); lane 4, XC17433(pKdPs); lane 5, XC17433(pKdA6); lane 6, XC17433(pKD2); lane 7, XC17433(pKD2).
the other Xps proteins that are also required for protein secretion. The membrane fraction prepared from French press disrupted cells was extracted with Triton X-100. We noticed that presence of 10 mM EDTA during Triton X-100 extraction was vital in solubilizing the wild type XpsD protein from XC17433(pKC118) (data not shown). This observation agreed with the OM localization of XpsD (3). The Triton X-100-EDTA extract was subsequently chromatographed on a DE52 column and eluted with 0.2M NaCl. The DE52 eluate concentrated via 70% ammonium sulfate precipitation was then chromatographed on an FPLC gel filtration column in presence of 1% sodium deoxycholate. Immunodetection of fractionated samples indicated that the wild type XpsD protein encoded by pKC118 appeared in near void volume (retention time of 15–16 min) (Fig. 5A). Likewise, the mutants XpsD(D74–303), XpsD(D545–553), XpsD(D29–428), and XpsD(A553L/I554D) were eluted near the void volume (Fig. 5A and B). Some of the former three proteins also exhibited longer retention time. In contrast, XpsD(D414–759), XpsD(D448–650), and XpsD(D553–759) were eluted at retention times of 23–25, 19–21, and 23–25 min, respectively (Fig. 5A). These results indicated that wild

Fig. 4. Glutaraldehyde cross-linking of XpsD in vitro and in vivo. Glutaraldehyde (GA) (final concentrations indicated above each lane) was added to a Triton X-100 membrane extract of XC1701 (A); B, growing XC17433(pKC118) cells. XpsD was detected by immunoblotting. The arrows indicate cross-linked XpsD.

Fig. 5. FPLC gel filtration chromatography analysis of XpsD and its mutant XpsD proteins. Triton X-100 membrane extract was passed through a DE52 anion exchanger. Samples were eluted with 0.2 M NaCl and precipitated with 70% ammonium sulfate. After centrifugation, precipitates were solubilized in a small volume of Buffer B and chromatographed on an FPLC gel filtration column (Superdex HR-200, 25 ml), pre-equilibrated with the same buffer. Proteins in the eluted fractions were separated on SDS-polyacrylamide gel, followed by immunoblot using antibody against XpsD (A) or XpsD(D29–428) (B). Each mutant XpsD protein was produced in XC17433(xpsE,F,G,H,I,-
J,K,L,M,N,D) harboring the respective plasmid.

Fig. 6. Analysis of the interaction between XpsD and XpsD(D553–759) or XpsD(D448–650) by FPLC gel filtration chromatography. Samples were prepared from XC1701(pKDT) (A) and XC1701(pkDts) (B), following the same procedures as described in the legend to Fig. 7.

Fig. 7. Analysis of the interaction between XpsD protein and XpsD(D29–428) or XpsD(D74–303) protein by FPLC anion exchange chromatography. Triton X-100 extracts of the membranes prepared from French press disrupted cells were passed through an FPLC anion exchange column (Mono Q, HR 5/5, 1 ml), eluted with a 0–1.0M NaCl linear gradient. Fractions 8–18 were analyzed separately on SDS-polyacrylamide gel, followed by immunoblot analysis with antibody against XpsD. Samples were prepared from XC17433(pKC118), XC17433(pCD105), XC17433(pYL4) (A); XC1701(pCD105) (B); and XC1701(pFL4) (C).
type XpsD protein may form high molecular weight complexes that are stable to deoxycholate. Mutant XpsD(D29–428), XpsD(D448–650), and XpsD(D553–759) proteins also formed similar complexes but probably with weaker associations. On the other hand, mutant XpsD(D414–759), XpsD(D448–650), and XpsD(D553–759) proteins could no longer form such complexes.

Next, we wanted to find out if those mutant XpsD proteins that did not exhibit negative dominance could not form multimeric complexes with the wild type XpsD protein. As expected, the XpsD(D553–759) protein appeared between 21 and 24 min on FPLC Superdex HR-200 column when it was co-expressed in XC1701(pKDT) culture with the wild type XpsD protein, which appeared in the near void volume (15–16 min) (Fig. 6A). This result indicated that the XpsD(D553–759) protein probably did not form a complex with the wild type XpsD protein, consistent with our model of negative dominance based on protein-protein interaction (Fig. 8B). However, the XpsD(D448–650) protein synthesized in XC1701(pKdPs) co-migrated with the wild type XpsD protein (Fig. 6B). This result suggested that the XpsD(D448–650) protein formed a complex, either with the wild type XpsD protein, or with other protein (including itself). The former possibility contradicts with the observation that the XpsD(D448–650) protein did not interfere with secretion in XC1701, unless we assume that the heteromultimer of wild type XpsD and XpsD(D448–650) is functionally equivalent to the wild type XpsD homomultimers for some unknown factor(s) (Fig. 8C).

**DISCUSSION**

All the xpsD truncated mutants inspected in this study were defective in the secretion of α-amylase and protease, causing accumulation of α-amylase in the periplasm. These XpsD proteins can be divided into two groups: Group I, including XpsD(D29–428) (pCD105), XpsD(D448–650) (pKL4), and XpsD(D553–553) (pKdA6), displayed negative dominance over the wild type XpsD protein, and Group II, including XpsD(D414–759) (pMH7), XpsD(D448–650) (pKdPs), and XpsD(D553–759) (pKDT), did not. In this study we found good correlation between negative dominance and the formation of XpsD multimers: all Group I proteins formed multimers that appeared in the near void volume, whereas all Group II proteins did not. This indicates that Group I proteins exerted

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**Fig. 8.** Diagrammatic presentations for the interference of normal protein secretion by mutant XpsD proteins. Co-expressed with the wild type XpsD protein encoded by the chromosomal DNA of XC1701 are mutant XpsD proteins: A, XpsD(D29–428); B, XpsD(D553–759); C, XpsD(D74–303); and D, XpsD(D448–650).
negative dominance by interacting with the wild type XpsD protein. This was supported by our observations that the XpsD(29–428) protein co-fractionated with the wild type XpsD protein, whereas the XpsD(553–759) protein did not.

The amino acid sequences common in the Group I proteins are residues 1–28, 429–544, and 554–759. At the NH2 terminus, a conserved lipoprotein signal peptidase cleavage site (---LLAG--C--) (33) is located between residues 21 and 22. Fatty acylation of XpsD has been demonstrated with [3H]palmitate labeling of wild type XpsD (3). This suggested that the NH2-terminal amino acid residues 1–28 either absent entirely in the XpsD(29–428), XpsD(545–553), and XpsD(A553L/I554D) proteins formed multimers by themselves, or affect protein secretion in Xc1701. We propose that these two regions (429–544 and 554–759) are required for multimer formation. Although the mutant XpsD(29–428), XpsD(74–303), XpsD(129–465) and XpsD(553L/554D) proteins formed multimers by themselves, all of them are not functional in protein secretion. We suggest that the regions covered within residues 74–303 and the amino acid residues 553A, 554I, albeit unrelated with multimer formation, is important for protein secretion.

Due to the small size of deoxycholate micelles (28, 34), we carried out gel filtration chromatography in the presence of deoxycholate. Wild type XpsD eluted in the near void volume with an estimated molecular mass of approximately 1,000 kDa, suggesting that it forms stable multimeric complexes. At this stage we do not know whether XpsD forms homomultimers or heteromultimers. A homomultimer of XpsD of this size would contain at least 12 mature XpsD polypeptides (77 kDa each). This is comparable with the filamentous phase pV multimers, estimated to be 10–12-mers (28).

A gated channel formed by pV was proposed for releasing of assembled filamentous phase particles (25). The COOH-terminal 200 amino acid sequence of XpsD are highly homologous to pV and other analogous proteins (22, 27). Moreover, XpsD co-expressed with pV was precipitated with antibody against pV, which does not cross-react with XpsD(29). When we introduced pV gene into Xc1701, we clearly observed secretation interference. Both results suggested complex formation between XpsD and pV.

REFERENCES

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