Subcellular location of XpsD, a protein required for extracellular protein secretion by Xanthomonas campestris pv. campestris

Nien-tai Hu,1 Ming-Ni Hung,1 Chao-Tsai Liao1,2 and Ming-Huei Lin1,3

Author for correspondence: Nien-tai Hu. Tel: +886 4 2874754. Fax: +886 4 2861905. e-mail: 711nchu@vax9k.nchu.edu.tw

The last ORF of an xps gene cluster, designated xpsD, is required for the secretion of extracellular enzymes across the outer membrane in Xanthomonas campestris pv. campestris. It could encode a protein of 759 amino acid residues. A consensus N-terminal lipoprotein signal peptide was revealed from its deduced amino acid sequence. A [3H]palmitate labelling experiment indicated that XpsD was fatty-acylated. Differential extraction with Triton X-100 disclosed that XpsD was fractionated with the outer membrane. Sucrose gradient sedimentation analysis of total membranes also indicated that XpsD was mainly located in the outer membrane. At least part of XpsD is exposed to the cell surface as suggested by trypsin experiment results. Intact cells pretreated with antibody against XpsD could indirectly be labelled with fluorescent agent. When the N-terminal lipoprotein signal peptide was replaced with a nonlipoprotein signal peptide cleavable by signal peptidase I, non-fatty-acylated XpsD was synthesized. Its subcellular location was indistinguishable from that of the fatty-acylated XpsD. Complementation of an xpsD::Tn5 mutant of X. campestris pv. campestris indicated that this non-fatty-acylated XpsD remains functional in extracellular protein secretion. A stable, C-terminal truncated protein, XpsDΔ414-759, was synthesized from a mutated xpsD gene. Although it stayed associated with the outer membrane and exposed to the cell surface, it no longer could complement the xpsD::Tn5 mutant of X. campestris pv. campestris.

Keywords: Xanthomonas campestris pv. campestris, general secretion pathway, outer-membrane protein, lipoprotein, main terminal branch

INTRODUCTION

Extracellular proteins synthesized in Gram-negative bacteria have to cross two membrane barriers before being secreted. In some cases, independent of an N-terminal signal peptide, the secretion is accomplished in one step, for instance, the secretion of α-haemolysin by Escherichia coli (Holland et al., 1990), proteases by Erwinia chrysanthemi (Létoffé et al., 1990) and alkaline protease by Pseudomonas aeruginosa (Guzzo et al., 1991). In other cases, proteins are secreted via a general secretory pathway in two steps: they reach the periplasm via the general export pathway before they are translocated across the outer membrane (Wandersman, 1992; Pugsley, 1993). An N-terminal signal peptide is processed when the preprotein is being exported across the inner membrane. Fourteen proteins encoded by the pulC–O and pulS genes of Klebsiella oxytoca are required for the secretion of pullulanase across the outer membrane (reviewed by Pugsley et al., 1990). Homologues of pul genes required for the secretion of various extracellular enzymes have since been found to be widespread among different plant and animal pathogens. These homologues include xps genes of Xanthomonas campestrispv. campestris (Dums et al., 1991; Hu et al., 1992a), xux genes of E. chrysanthemi (Condemine et al., 1992; He et al., 1991; Lindeberg & Collmer, 1992) and Erwinia carotovora (Murata et al., 1990; Reeves et al., 1993), xcp genes of P. aeruginosa (Filloux et al., 1990; Bally et al., 1991, 1992; Akrim et al., 1993) and exx genes of Aeromonas hydrophila (Jiang & Howard, 1992; Howard et al., 1993). A third secretory pathway (Salmond & Reeves, 1993; Van Gijsegem et al., 1993) was observed in the secretion of Yop proteins by Yersinia enterocolitica (Michiels et al., 1991), in harpin secretion by Erwinia amylovora (Weil et al.,...
N.-T. HU and OTHERS

1992) and by Pseudomonas syringae (He et al., 1993), in PopA secretion by Pseudomonas solanacearum (Arlat et al., 1994) and in secretion of Ipa proteins by Shibella flexneri (Allaoui et al., 1992, 1993). This type of pathway is similar to the one-step pathway in its independence of an N-terminal signal peptide. On the other hand, one of the determinants required for this pathway (YscC, HrpH, HrpA and MxiD) is analogous to one component of the two-step pathway, the PullD protein of K. oxytoca (d’Enfert et al., 1989) and its homologues.

X. campestris pv. campestris causes black rot in the crucifers. Nonpathogenic Tn5 mutants have been isolated and a-amylase, protease and endoglucanase (Hu et al., 1992). Cloning and sequencing of the genes complementing the secretion-defective mutants revealed an xps gene cluster (Dums et al., 1991; Hu et al., 1992a). The last ORF, designated xpsD, of the gene cluster could encode a protein composed of 759 amino acid residues. Examination of its amino acid sequence disclosed near its N-terminus a stretch of hydrophobic amino acid residues followed by a conserved lipoprotein signal peptide cleavage site, -LAG C-, that would be cleaved by leader peptidase II and modified with lipid at C (cysteine), the first amino acid residue of the mature protein (Hayashi & Wu, 1990). More than ten bacterial or phage proteins which are required for various biological functions share significant sequence homology with the mature XpsD (Pugsley, 1993; Russell, 1994). Among them, subcellular location of K. oxytoca PullD and the filamentous phage protein pIV, which is required for phage morphogenesis, have been carefully studied. PullD analysed on sucrose gradients was mainly detected in the outer membrane (d’Enfert et al., 1989). On the other hand, filamentous phage pIV was clearly observed in both inner and outer membranes when analysed on sucrose gradients (Brissette & Russell, 1990) or fractionated with Triton X-100 (Russell & Kázmiereczak, 1993). Recent studies by Kázmiereczak et al. (1994) further suggested that pIV may form a multimer. Secondary structure prediction indicated that XpsD is rich in amphipathic β-sheets, which were demonstrated to be involved in outer-membrane integration of porins (Weiss et al., 1991; Cowan et al., 1992).

In this study, we determined the subcellular location of XpsD in its original host X. campestris pv. campestris and demonstrated that its fatty acylation is not required for its correct subcellular location or its role in extracellular protein secretion. In contrast, a truncated protein, XpsDA414-759, was no longer functional in protein secretion, despite its association with the outer membrane.

METHODS

Bacterial strains, plasmids and media. Xanthomonas campestris pv. campestris xpsD::Tn5 mutant XC1708 was constructed previously in this laboratory (Hu et al., 1992a). The broad-host-range vector pCPP30 was kindly provided by David Bauer (Department of Plant Pathology, Cornell University, Ithaca, NY, USA). The plasmid pKC107 contains a 6-4 kb EcoRI–BglII fragment from X. campestris pv. campestris, which includes the wild-type xpsD gene, in pCPP30. The same fragment cloned in a phagemid pSelect-1 was designated pKC101. The plasmids pMH9 and pKC118 each contain the complete xpsD gene alone downstream of the lac promoter on vectors pTZ18U (Mead et al., 1986) and pCPP30, respectively.

The plasmid pCT1 was constructed by cloning a BamHI fragment of the xpsD gene at the BamHI site of pTZ19U (Mead et al., 1986) downstream of the lac promoter. The plasmid pMH7 was acquired by cloning a C-terminal truncated xpsD gene (xpsDA414-759) in pCPP30. The plasmid pCD105 was obtained by cloning the truncated xpsDA28-427 gene in pCPP30. The plasmid p18UA5 contains the complete amy gene on a 1-8 kb EcoRI fragment cloned in pTZ18U. The plasmid pCT5 was constructed by ligating the EcoRI fragment of pCT3, which contains a chimeric amy–xpsD gene, with pCPP30. The SOE (gene splicing by overlap extension) strategy of Horton et al. (1989) was followed in the construction of pCT3.

Four oligomers designated ’a’, ’b’, ’c’ and ’d’ were synthesized for conducting polymerase chain reactions (PCR). For the synthesis of an ‘amy’ fragment, p18UA5 was included as the template, and oligomers ’a’ (5’-CGGCAAGGTTTTCGGCTACGAC-3’) and ’b’ (5’-AGTGTTGCGATGACGTCG-GCCTGTCGCC-3’) were used as primers. This ‘amy’ fragment contains the nucleotide sequence encoding the signal peptide plus the first three amino acid residues of the mature protein of α-amylase, and the 5’ flanking sequence which includes the promoter region of the amy gene (Hu et al., 1992b). In a separate tube, pKC101 was included as template and oligomers ’c’ (5’-GACGTCATCGCCAACACACTCGCCAC-GGA-3’) and ’d’ (5’-TCACGGAGTGCACGCCAAGCCTCG-3’) were used as primers for the synthesis of an ‘xpsD’ fragment which contains the nucleotide sequence encoding the N-terminus of the processed XpsD protein minus its first amino acid residue, cysteine. Primers ’b’ and ’c’ were so designed that 18 nucleotides on the 5’ end of each are complementary to each other. The ‘amy’ and the ‘xpsD’ fragments, recovered from the agarose gel slices using Geneclean, were mixed and underwent six cycles of PCR (94 °C for 1 min 30 s, 37 °C for 2 min and 58 °C for 3 min) before primers ’a’ and ’d’ were added for an additional 30 cycles of PCR (94 °C for 1 min, 60 °C for 2 min and 72 °C for 1 min for all cycles except the last one, which was 72 °C for 2 min). An ‘amy–xpsD’ fragment of 1 kb was generated from this PCR. It was recovered from the agarose gel, digested with HindIII and SalII and ligated with pKC101 digested with the same enzymes.

M9 medium, M9 salts, L-broth and L-agar were prepared according to Miller (1972). The XOL medium for growing X. campestris pv. campestris was prepared as reported before (Hu et al., 1992a). Antibiotics were added where appropriate at the following concentrations: tetracycline, 15 μg ml⁻¹; ampicillin, 50 μg ml⁻¹; rifampicin, 100 μg ml⁻¹; and kanamycin, 50 μg ml⁻¹.

Site-directed mutagenesis. The site-directed in vitro mutagenesis with the Altered Sites System purchased from Promega was used in creating XbaI sites in the xpsD gene for the construction of xpsDA28-427 and xpsDA414-759 genes. Single-stranded template was generated by superinfecting an E. coli strain containing the complete xpsD gene cloned in the phagemid pSelect-1 with a helper phage, M13KO7 (Vieira & Messing, 1987). Two primers were used in the construction of the mutant xpsDA28-427 gene: 5’-TCGGCGGGCGACATCT-AAGGGGGAGTGTGGG-3’ for the upstream XbaI site and 5’-GTAACGTACCGTCTGTCGAGCTAGACGCGG-3’ for the downstream XbaI site. A single primer with the following sequence was used in the construction of the mutant

1396
xpsDA414-759 gene: 5'-TGCCGCTACTTCCGTCTAGAAG-CTCCCTGACG-3'.

Production of antibody against LacZ-XpsD fusion protein. E. coli JM101(pCT1) grown in L-broth containing 50 μg ampicillin ml⁻¹ to OD₆₀₀ 0-4 was induced with 0-3 mM IPTG for 2 h. Total cell lysates were prepared by treating with 1 mg lysosome ml⁻¹ in 20 mM Tris/HCl, pH 7-0, 1 mM EDTA on ice for 30 min, followed by sonication, and electrophoresed in an SDS-polyacrylamide gel containing 8% (w/v) acrylamide: N,N',-methylene bisacrylamide (29:1, w/w). The major band containing the overproduced LacZ-XpsD fusion protein was collected for immunizing the rabbit. One hundred and fifty micrograms of the fusion protein was injected each time. Serum was prepared following the procedures of Harlow & Lane (1988) were stored at -70°C and tested subsequently for antibody in immunoblot analysis.

Western blot and immunodetection. Proteins separated in SDS-polyacrylamide gels were electroblotted onto nitrocellulose membranes with transfer buffer (192 mM glycine, 25 mM Tris/HCl, pH 8-3, 20%, v/v, methanol). The blot, after having been treated with blocking buffer (5%, w/v, nonfat milk powder in 150 mM NaCl, 1 mM EDTA, 50 mM Tris/HCl, pH 7-4, 0-05%, v/v, Tween 20), was reacted overnight at room temperature with the antiserum raised in rabbit against the LacZ-XpsD fusion protein (1:1000 dilution) and detected with the second antibody against rabbit IgG conjugated to peroxidase.

³[H]Palmitate labelling. X. campestris pv. campestris, grown in XOL +0.5% maltose to OD₆₀₀ 0-8, was labelled with 200 μCi (7400 kBq) [9,10-³H]palmitic acid ml⁻¹ (55 Ci mmol⁻¹; 2035 GBq mmol⁻¹) for 2 h at 28°C. Labelling was stopped with an equal volume of cold 10% (w/v) trichloroacetic acid (TCA). After having been left on ice for 10 min, the mixture was centrifuged (7000 g) at 4°C for 1 min. The precipitate was then washed twice with cold acetone, dried and boiled in lysis buffer (10 mM Tris/HCl, pH 7-5, 1 mM EDTA, 1%, w/v, SDS) for 10 min. After centrifugation at 7000 g for 10 min, the supernatant was transferred to a new tube and saved as labelled cell lysate at -20°C.

Immunoprecipitation. Labelled cell lysates were reacted with antiserum against the LacZ-XpsD fusion protein (at 1:600 dilution) alone, or plus the antiserum against E. coli OmpA (at 1:8000 dilution), in Triton buffer (2%, v/v, Triton X-100, 50 mM Tris/HCl, pH 7-5, 150 mM NaCl, 0.1 mM EDTA) at 4°C overnight. Seventy-five microlitres of protein A-Sepharose CL-4B (1000 g) (w/v, in Triton buffer, was then added and incubated for another hour at 4°C. After centrifugation at 7000 g for 1 min, the pellet was washed twice with Triton buffer and once with 10 mM Tris/HCl, pH 8-0. The washed pellet was boiled in electrophoresis sample buffer for 5 min. After centrifugation at 7000 g for 10 min, the supernatant was ready for SDS-PAGE. When the electrophoresis was finished, the gel was soaked in Enlightening autoradiography enhancer (Du Pont NEN Research Products) at room temperature with shaking for 30 min, dried and exposed to X-ray film at -70°C.

Proteinase K or trypsin treatment of intact cells. Early exponential phase cultures (OD₆₀₀ = 0-6) were used for proteinase K or trypsin experiments. E. coli was pregrown in M9 minimal salts with 0-4% glucose and 0-5% Casamino acids. Before labelling the cells were washed with M9 salts and incubated at 37°C for 1 h in M9 medium (without Casamino acids) supplemented with 0-3 mM IPTG. For every 1 ml culture, 20 μCi [³⁵S]methionine (740 kBq) (>1000 Ci mmol⁻¹; 37000 GBq mmol⁻¹) were added. After incubation at 37°C for 30 min, the labelling reaction was stopped by chilling in ice. X. campestris pv. campestris was pregrown and labelled in similar conditions, except that M9 salts were replaced with XOL salts and all incubations were performed at 28°C. The labelled cells were then centrifuged at 7000 g for 5 min, resuspended in 10 mM Tris/HCl, pH 7-8, and 10 mM MgCl₂, and treated with various concentrations of proteinase K or trypsin on ice for 1 h. Ice-cold 10% TCA and protease inhibitor (final concentration of 5 mM PMSF in the case of proteinase K; final concentration of 25 μg soybean trypsin inhibitor ml⁻¹ in the case of trypsin) were added to stop the reaction. After centrifugation at 7000 g for 1 min, the TCA precipitates were treated the same way as in the palmitate labelling experiment to obtain the labelled cell lysates, which were immunoprecipitated and electrophoresed as before.

Subcellular fractionation. The periplasmic fraction was collected as the supernatant after centrifugation of spheroplasts, prepared by treating H₂O-washed cells with 1 mg lysosome ml⁻¹ in 20% (w/v) sucrose, 30 mM Tris/HCl, pH 8-0, 1 mM EDTA on ice for 1 h, at 12000 r.p.m. for 30 min. Prior to sonication, spheroplasts were washed once with the same buffer without lysozyme and resuspended in 10 mM Tris/HCl, pH 8-0. The pellet collected after centrifugation of sonicated spheroplasts at 12000 r.p.m. for 30 min was resuspended in lysis buffer and saved as the membrane fraction, and the supernatant saved as the cytoplasmic fraction. Both periplasmic and cytoplasmic fractions were precipitated with cold 10% TCA and washed twice with 95% (w/v) ethanol before being resuspended in lysis buffer. Proteins solubilized by boiling each fraction in lysis buffer for 3 min followed by centrifugation at 12000 r.p.m. for 10 min were separated in SDS-polyacrylamide gels.

Cell disruption and membrane fractionation on a sucrose gradient. X. campestris pv. campestris cells, grown in L-broth to late exponential phase and washed twice with distilled water, were resuspended in 10 mM HEPES, pH 7-5 (approximately 1 ml for 100-150 mg wet wt cells), plus DNase (50 μg ml⁻¹) and RNase (50 μg ml⁻¹), and disrupted by passing through a French pressure cell at 18000-20000 p.s.i. (124.2-138 MPa) twice. Unbroken cells were removed by centrifugation at 3500 g for 10 min. Prior to centrifugation, 1 mM MgCl₂ was added. The total membrane preparation was pelleted by centrifugation at 342000 g for 60 min, washed once with 10 mM HEPES, pH 7-5, and resuspended in the same buffer. The concentrated membrane preparation was then loaded on a step sucrose gradient (25-61% w/w) and centrifuged at 131000 g for 20 h at 4°C. The density of each fraction, collected at 1 ml per fraction, was calculated from the sucrose concentration determined with a refractometer. Samples in alternate fractions were precipitated with cold 5% TCA, washed twice with ethanol and analysed in SDS-polyacrylamide gels used by Western blotting and immunodetection.

Differential fractionation of membranes with Triton X-100. The procedure of Russel & Kazmierczak (1993) was modified as follows. The total membrane fraction, prepared as described above, except with a pressure of 5000 p.s.i. (34-5 MPa) was resuspended with 1% Triton X-100, 10 mM MgCl₂, 50 mM Tris/HCl, pH 8, and incubated at room temperature for 30 min followed by centrifugation at 156000 g for 30 min. The supernatant was precipitated with chloroform/methanol (1:2-4, v/v), resuspended in 4% SDS and saved as the inner-membrane fraction. The pellet was extracted again by resuspending with 1% Triton X-100, 10 mM EDTA, 50 mM Tris/HCl, pH 8, and incubating at room temperature for 30 min followed by centrifugation at 12000 g for 1 h. The supernatant from the second extraction was concentrated as before and saved as the...
outer-membrane fraction. The soluble and nonextractable fractions were prepared following the procedure of Russel & Kazmierczak (1993).

**Immunofluorescence.** The procedures of Koechler et al. (1992) were followed with slight modifications. *X. campestris pv. campestris* cells grown in L-broth to OD$_{600}$ 0.6 were washed with PBS (8 g NaCl, 0.2 g KCl, 1-44 g Na$_2$HPO$_4$, 0.24 g KH$_2$PO$_4$ l$^{-1}$, pH 7.4) plus 10 mM MgCl$_2$ three times before suspending in the same buffer. Ten microlitres of washed cells was dropped onto glass slides, air-dried, overlaid with 1% (w/v) bovine serum albumin (BSA) and incubated at room temperature for 1 h. The slides were subsequently incubated at room temperature with antibody against the LacZ-XpsD fusion protein (1:100 dilution in PBS + BSA), followed by incubation with goat anti-rabbit IgG antibody conjugated with fluorescein isothiocyanate (FITC) (1:50 dilution in PBS + BSA). Before and after each incubation the slides were washed with water a few times and air-dried. Five microlitres 50% (v/v) glycerol was dropped on the slide before it was covered with a cover slip and examined under a fluorescence microscope at 1000 times magnification.

**Complementation.** The chimeric amy-amy::xpsD gene was tested for its ability to recover the function of a defective xpsD gene by first introducing it into XC1708 via triparental conjugation. The conjugation procedure of Hu et al. (1992a) was followed. The transconjugants were then examined on XOL medium plus 2% (w/v) starch for a-amylase secretion and on XOL medium plus 0.1% (w/v) skimmed milk for protease secretion. The parental strain XC1701 was included as a positive control, and XC1708 was included as a negative control.

**RESULTS**

**Antibody raised against the LacZ-XpsD fusion protein**

A LacZ-XpsD fusion protein with an apparent molecular mass of 68 kDa could be produced from *E. coli* JM101(pCT1). This fusion protein contains the amino acid residues 37-588 of XpsD. Under inducing conditions, the fusion protein represents at least 30-40% of total protein (not shown). Antibody raised against this fusion protein could detect in the parental strain of *X. campestris pv. campestris* XC1701 a major protein with an apparent molecular mass of 77 kDa and a minor protein of 48 kDa (Fig. 1a, lane 1). Both were observed in XC1708(pCK118) as well, albeit in slightly different proportions (Fig. 1a, lane 3). In contrast, only the 48 kDa protein was detected in the xpsD::Tn5 mutant XC1708 (Fig. 1a, lane 2). The molecular mass of the 77 kDa protein agrees with what was predicted from the amino acid sequence deduced from the nucleotide sequence of the xpsD gene. However, XpsD made in *X. campestris pv. campestris* migrated slightly slower than that from *E. coli* (not shown), the cause of which is not clear. Expression of the xpsD gene encoded in pCK118 is under the control of a lac promoter. However, the expression level of XpsD in *X. campestris pv. campestris* was not noticeably increased by including the inducer IPTG. Consequently, IPTG was excluded in the following experiments with *X. campestris pv. campestris*. The amount of XpsD detected in XC1708(pCK118) was approximately ten times that detected in XC1701.

**[H]Palmitate labelling of XpsD**

When *X. campestris pv. campestris* XC1708(pCK118) was labelled with [H]palmitate, followed by immunoprecipitation with antibody against the LacZ-XpsD fusion protein, two protein bands were revealed on the autoradiogram, one with apparent molecular mass of 77 kDa and the other of approximately 48 kDa (Fig. 1b, lane 2). Neither was detected in XC1708 containing the cloning vector pCPP30 (Fig. 1b, lane 1). The 48 kDa protein will be discussed later.

**Triton X-100 fractionation of wild-type XpsD**

Differential extraction of membrane vesicles with Triton X-100 revealed that XpsD synthesized from XC1701 was fractionated with outer-membrane vesicles (Fig. 2). Co-fractionation with it was a 44 kDa protein detectable with antibody against the *E. coli* outer-membrane protein OmpA. The major outer-membrane protein OprF of *P. aeruginosa* is also immunochemically related to OmpA (Woodruff & Hancock, 1989; Hancock et al., 1990; Ullstrom et al., 1991). The immunologic relatedness among the three proteins suggested that the 44 kDa protein may be an analogue of OmpA and OprF. We designated this 44 kDa protein OprF. A major portion of OprF was repeatedly observed in the nonextractable fraction. We do not have a good explanation for this observation. We noticed that a minute amount of OprF was also extracted with inner-membrane vesicles. On the other hand, a major protein of 50 kDa revealed on Coomassie-brilliant-blue-stained gels appeared solely in the inner-membrane fraction (Fig. 2, indicated by an arrowhead). The majority of XpsD synthesized from

---

**Fig. 1.** (a) Detection of XpsD protein in a whole cell extract of *X. campestris pv. campestris* using antibody against the LacZ-XpsD fusion protein. Samples were loaded in the following order. Lanes: 1, XC1701; 2, XC1708; 3, XC1708(pKC118); 4, XC1708(pMH7). One-tenth as many cells were loaded for XC1708(pKC118) and XCl708(pMH7). (b) Autoradiogram of [H]palmitate-labelled *X. campestris pv. campestris* XCl708(pCPP30) (lane 1) and XC1708(pKC118) (lane 2), grown in XOL+0.5% maltose, were labelled with [H]palmitate for 2 h at 28°C. Labelled cell lysates were precipitated with antibody against LacZ-XpsD fusion protein, followed by electrophoresis in an SDS-polyacrylamide gel (10%) and fluorography. The 48 kDa protein is designated by *.

---

N.-T. HU and OTHERS
Subcellular location of XpsD from *X. campestris*

Fig. 2. Triton X-100 fractionation of *X. campestris* pv. *campestris* XC1701, XC1708(pKC118) and XC1708(pCT5). S, soluble proteins (periplasmic plus cytoplasmic); IM, inner-membrane proteins; OM, outer-membrane proteins; NE, nonextractable proteins. Each fraction was prepared as described in Methods followed by separation in SDS-PAGE. The gel was subsequently stained with Coomassie brilliant blue or transferred to nitrocellulose paper followed by reacting with antibody against Lad-XpsD fusion protein (labelled XpsD) or antibody against *E. coli* OmpA (labelled OprF) and detection with second antibody conjugated to peroxidase. The arrow points to the 50 kDa protein detected in the inner membrane. Only portions of immunoblots are shown.

XC1708(pKC118) was detected in outer-membrane and nonextractable fractions in almost equal amount (Fig. 2). The minor band migrating faster than the major XpsD observed in XC1708(pKC118) is probably a degradation product.

Sucrose gradient sedimentation analysis of wild-type XpsD

When total membrane vesicles of the parental strain XC1701 were analysed on sucrose gradients, XpsD was detected in fractions with buoyant densities ranging from 1.145 g cm\(^{-3}\) to 1.286 g cm\(^{-3}\) (Fig. 3). Likewise, the 44 kDa OprF protein appeared in the same fractions (Fig. 3). On the other hand, protein profiles of the fractions with buoyant densities lower than 1.145 g cm\(^{-3}\) were distinctly different from those with higher densities. Noticeably, a major protein with an apparent molecular mass of 50 kDa was predominantly present at a density of 1.129 g cm\(^{-3}\), where inner-membrane vesicles were expected to appear. Absence of XpsD in fractions 4–10, where inner-membrane vesicles were expected, indicated that it is not likely to be associated with the inner membrane. Sucrose gradient sedimentation analysis of XC1708(pKC118) resulted in an almost identical distribution of XpsD protein (data not shown), suggesting that overexpression of XpsD did not alter the subcellular location of XpsD to any significant extent that could be detected on sucrose gradients.

Protease sensitivity of wild-type XpsD in intact cells

We examined XpsD protease sensitivity by treating labelled cells with trypsin or proteinase K. In *E. coli* DH5α(pMH9), XpsD was degraded completely by treating with 200 μg trypsin ml\(^{-1}\) or 1 mg proteinase K ml\(^{-1}\)
Degradation of XpsD was not observed until the concentration of trypsin was raised to 1 mg ml$^{-1}$ (Fig. 4b, top, left panel). A dominant fragment of approximately 60 kDa and several minor fragments of smaller sizes were generated (Fig. 4b, top, left panel). In order to ascertain that the degradation of XpsD caused by treating with 1 mg trypsin ml$^{-1}$ was not due to cell leakage caused by the high concentration of trypsin, we performed the same control experiments as in the case of E. coli by following the degradation of the 44 kDa protein with anti-OmpA antibody. For unknown reasons, the 44 kDa outer-membrane protein in X. campestris pv. campestris could not be precipitated with the antibody against E. coli OmpA. To circumvent this problem, we divided all trypsin-treated samples into two. The major portion of each sample was immunoprecipitated with anti-LacZ-XpsD antibody as before. The remaining portion was separated in an SDS-polyacrylamide gel without immunoprecipitation and stained with Coomassie brilliant blue. As shown in Fig. 4(b) (bottom, left panel), both the number and densities of the stained proteins in the sample treated with 1 mg trypsin ml$^{-1}$ were almost identical to those treated with trypsin at lower concentrations. In contrast, in the cells pretreated with 5 mM EDTA, stained protein bands apparently diminished in number and density in all trypsin-treated samples (Fig. 4b, bottom, right panel). At the same time, the trypsin-resistant XpsD fragments were completely degraded (Fig. 4b, top, right panel). These results suggested that, either in E. coli or in X. campestris pv. campestris, some protease susceptible sites of XpsD are accessible from the exterior of cells.

**Immunofluorescence labelling of wild-type XpsD in intact cells**

Antibody labelling experiments were conducted to further examine the likelihood that XpsD is exposed to the cell surface in X. campestris pv. campestris. XC1708(pKC118), fluoresced, while XC1708 did not. Fluorescence, albeit weak, was also observed in the parental strain XC1701 (not shown).

Intactness of the air-dried cells was examined as follows. Among other proteins, a-amylase is secreted by the parental strain XC1701, whereas it accumulates in the periplasm in the $xpsD::Tn5$ mutant strain XC1708 (Hu et al., 1992a). When the antibody against a-amylase was included in the immunofluorescence experiments, XC1701 or XC1708(pKC118) showed fluorescence. In contrast, no fluorescence was observed in XC1708, in which synthesis of a-amylase was demonstrated by a Western blot of the total cell extract (not shown). These results indicated that periplasmic proteins are not detectable under our experimental conditions.

**Replacement of the XpsD lipoprotein signal peptide with a nonlipoprotein signal peptide**

In order to study the significance of the lipid moiety of XpsD in its subcellular location, we replaced the sequence encoding the signal peptide of XpsD with that from an...
The newly introduced signal peptide was designed to allow the protein product to be translocated across the inner membrane without lipid modification. The absence of lipid modification was substantiated by palmitate labelling experiments. When the parental XpsD was labelled with \(^{3}H\)palmitate, the variant XpsD was not labelled (not shown). Its export to the outer membrane was examined as follows.

**Subcellular location of non-fatty-acylated XpsD**

The non-fatty-acylated XpsD produced from XC1708(pCT5) was examined for its subcellular location via fractionation on a sucrose gradient, differential extraction with Triton X-100 and its protease sensitivity in intact cells. Results from all three experiments indicated that the non-fatty-acylated XpsD appeared to be exported to the outer membrane as well as the parental XpsD. The distribution of non-fatty-acylated XpsD on a sucrose gradient is almost identical to that of the parental XpsD (not shown), disclosing that it is mainly located in the outer membrane. Its fractionation with Triton X-100 also revealed that it is extracted with the outer-membrane vesicles (Fig. 3). Treatment of \(^{3}S\)methionine-labelled intact XC1708(pKC107) or XC1708(pCT5) with trypsin or protease K revealed no major difference in protease sensitivity between the fatty-acylated XpsD and the non-fatty-acylated XpsD (Fig. 5). In this particular experiment, the XpsD protein, with or without lipid modification, was degraded completely with 0.5 mg trypsin ml\(^{-1}\) and almost completely with 1 mg protease K ml\(^{-1}\). Similar trypsin or protease K degradation products were observed in both strains. Minor differences between two trypsin-treated (0.5 mg ml\(^{-1}\)) samples could be attributed to slight variation in sample sizes. When 5 mM EDTA was included in trypsin treatment, almost all degradation products disappeared in both strains. All these results suggested that lack of fatty-acylation did not provoke any detectable conformational change in XpsD.

**Construction of the mutant xpsD genes that produce truncated XpsD proteins XpsDA414-759 and XpsDA28-427**

Alignment of XpsD with its homologues revealed a GS (glycine and serine)-rich region that was only observed in XpsD and the OutD protein of *E. chrysanthemi* (Condemine *et al.*, 1992). This GS-rich region in XpsD is located between amino acid residues 347 and 427. This region was chosen arbitrarily for dividing XpsD into halves, an N-terminal half and a C-terminal half. In order to produce a C-terminal truncated protein, we introduced a premature termination codon in the cloned xpsD gene, via site-directed mutagenesis, at amino acid residue 414 of the XpsD protein. When pMH7 was introduced into the xpsD::Tn5 mutant XC1708, a 45 kDa protein cross-reactive with the anti-LacZ-XpsD antibody was produced (Fig. 1a, lane 4).

An internal deletion was created to produce an N-terminal truncated protein, the deletion being from amino acid residue 28 to 427, so that the truncated protein could be exported via its N-terminal signal peptide sequence. Unfortunately, no cross-reactive material with the expected molecular mass (38 kDa) could be detected with antibody against the LacZ-XpsD fusion protein on a Western blot of the total cell extract of XC1708(pCD105). The possibility that a premature termination codon might have been generated in the mutated region as a result of mutagenesis or cloning has been ruled out from DNA sequencing data. Short labelling followed by immune precipitation was performed to examine protein stability. No conclusion could be reached. Besides protein instability, lack of cross-reactivity between the anti-LacZ-XpsD antibody and the truncated protein XpsDA28-427 could also account for the absence of its detection. Amino acid sequence downstream of residue 588 of XpsD was excluded from the LacZ-XpsD fusion protein, which was used to raise antibody against XpsD. Lack of cross-reactivity between antibody against the complete filamentous phage protein pIV and a truncated protein, pIVA15-242, has been observed previously by Russel & Kazmierczak (1993).

**Subcellular location of the C-terminal truncated protein XpsD A414-759**

Fractionation via spheroplast formation revealed that the C-terminal truncated protein XpsDA414-759 was present in both periplasmic and membrane fractions in *X. campestris* pv. *campestris* (not shown). Sucrose gradient sedimentation analysis indicated that the truncated protein present in membranes appeared in similar fractions as the complete XpsD, cofractionating with the outer-membrane protein OprF (not shown).

When XC1708(pMH7) was treated with as high as 2 mg trypsin ml\(^{-1}\), approximately 20% of the truncated XpsD...
trypsin at various concentrations on ice for 1 h, without (a) or with (b) prior treatment with 5 mM EDTA. All samples were precipitated with antibody against LacZ-XpsD, followed by SDS-PAGE and fluorography. Only a portion of the fluorogram is shown.

![Fig. 6. Protease digestion of XpsDA414-759. XC1708(pMH7), labelled with [35S]methionine for 30 min, was treated with trypsin at various concentrations on ice for 1 h, without (a) or with (b) prior treatment with 5 mM EDTA. All samples were precipitated with antibody against LacZ-XpsD, followed by SDS-PAGE and fluorography. Only a portion of the fluorogram is shown.](image)

remained undegraded (Fig. 6a). Complete digestion of the truncated XpsD was observed only when the cells were pretreated with EDTA (Fig. 6b), which presumably permeabilized the outer membrane. These results agreed with the observation that some of the truncated protein XpsDA414-759 synthesized from pMH7 was detected in the periplasmic fraction.

The fluorescence observed in anti-LacZ-XpsD antibody-labelled XC1708(pMH7) was almost indistinguishable from that in XC1708(pKC118), which contained the complete, wild-type XpsD, suggesting that some portion of the truncated XpsDA414-759 is exposed to the cell surface.

**Functional assay of non-fatty-acylated XpsD and truncated XpsD proteins**

The extracellular protein secretion property of the transconjugants XC1708(pCT5) and XC1708(pMH7) was tested on XOL plates containing starch or milk. Clear zones appearing around colonies after overnight incubation at 28 °C indicated secretion of α-amylase and protease, respectively. Diameters of the clear zones generated from XC1708(pCT5) were of the same size as those from the parental strain XC1701 as well as from XC1708(pKC118) (not shown). On the contrary, XC1708(pMH7) was defective in secretion (not shown). Results from fluorescence experiments also suggested that the truncated XpsDA414-759 was not able to complement the secretion defect in XC1708. In contrast to XC1708(pKC118), no fluorescence was detected in XC1708(pMH7) from indirect labelling with antibody against α-amylase.

**DISCUSSION**

Two aspects of the subcellular location of XpsD were pursued in this study. The first was focused on the subcellular location of wild-type XpsD. We demonstrated in this study that XpsD is an outer-membrane protein with part of its sequence exposed to the cell surface. The second aspect concerned the requirement of the lipid moiety of XpsD and its N-terminal or C-terminal halves for localization of XpsD in the outer membrane. It was shown that fatty acylation is not required for XpsD to be located in the outer membrane. On the other hand, the N-terminal half of XpsD polypeptide is sufficient, by itself, for outer-membrane localization. Because the C-terminal half of XpsD cannot be detected with the antibody raised against the LacZ–XpsD fusion protein, its subcellular location was not examined. However, it is homologous to the C-terminal two-thirds of the filamentous phage protein pIV, which was shown to be located in the outer membrane by Russel & Kaźmierczak (1993).

A 16 kDa, N-terminal fragment of the filamentous phage protein pIV remained undigested after trypsin digestion of f1-infected E. coli. This fragment was demonstrated to be intrinsically resistant to trypsin and localized in the periplasm (Brisette & Russel, 1990), suggesting that the N-terminal one-third of pIV may be located in the periplasm. This was confirmed by gene fusion studies of the pIV gene with phaA (Russel & Kaźmierczak, 1993). Unlike pIV, a C-terminal truncated XpsD protein, XpsDA414-759, was located in the membrane as well as in the periplasmic fraction. Its periplasmic location could be attributed to its high level of expression. We noticed that XpsDA414-759 was synthesized at a slightly higher level than the plasmid-encoded wild-type XpsD. The membrane XpsDA414-759 cofractionated with the outer-membrane protein OprF on a sucrose gradient and was susceptible in intact cells to protease digestion and labelling with antibody against the LacZ–XpsD fusion protein. Such difference in subcellular location between the two respective N-terminal portions of XpsD and pIV may have significance in their different biological roles, the former being required for extracellular protein secretion and the latter for phage assembly and release (Russel & Kaźmierczak, 1993). Each N-terminal portion may be involved in interaction with different proteins, pI in the case of pIV (Russel, 1993). It would be interesting to find out if XpsD interacts with any other protein(s) encoded in the xps gene cluster.

Despite its wide distribution on sucrose gradients, XpsD apparently cofractionated with the outer-membrane protein OprF. The peak of the distribution appeared between fractions 20–30, which was not so apparent in the particular experiment shown in Fig. 3. This was because, despite the inclusion of extra amounts of SDS, samples in fractions 20–24 were not solubilized as completely as in other fractions as evidenced from the Coomassie-blue-stained gel. XpsD detected in fractions 12–18 could represent that present in the light tail end of outer-membrane distribution, or the XpsD located in the membrane vesicles composed of attachment sites between inner and outer membranes (Ishidate et al., 1986). The observation that almost no XpsD made in XC1701 was extracted with Triton X-100 in the inner-membrane fraction seemed to agree with the former interpretation.

The lipoprotein signal peptide of the xpsD gene product was replaced with a nonlipoprotein signal peptide, plus the first three amino acid residues of the mature protein, derived from the amy gene of X. campesiris pv. campesiris.
(Hu et al., 1992b). Such a construction was designed to avoid aberrant signal peptide processing or inefficient export. Both were observed in the case of the Klebsiella lipoprotein pullulanase when cysteine, the modified amino acid of lipoprotein, alone was substituted (Murooka & Ikeda, 1989; Kornacker et al., 1991). Sucrose gradient analysis and protease studies indicated that the subcellular location of the variant XpsD was not significantly different from the parental XpsD, suggesting that the replacement with an authentic nonlipoprotein signal peptide has allowed efficient translocation of XpsD across the inner membrane. Plate assays for the secretion of α-amylase or protease indicated that fatty acylation of XpsD is probably not essential for its role in protein secretion. Among other homologues, XpsD is the only known lipoprotein. However, a small outer-membrane protein, PulS, required for pullulanase secretion by K. pneumoniae, is also a lipoprotein (d'Enfert & Pugsley, 1989). Its homologue OutS required for secretion of pectate lyase by E. chrysanthemi appeared to be a lipoprotein as well (Condemine et al., 1992). However, we have not tested the ability of XpsD in substituting its homologues for protein secretion or vice versa, experiments conducted by He et al. (1991) suggested that sec-dependent secretion systems from different Gram-negative bacteria may not be interchangeable. Before further experiments are conducted, we cannot rule out the possibility that the lipid moiety of XpsD might be significant in determining the selectivity of secreted proteins or other untested properties, such as efficiency, of the secretion machinery.

The difference in XpsD protease sensitivity between X. campestris pv. campestris and E. coli could be attributed to: (i) difference in cell surface, (ii) different XpsD conformational structures adopted in different species, (iii) presence or absence of other Xps proteins, or (iv) any combinations of the above three. In contrast to the lipopolysaccharides (LPS) of enteric bacteria, no heptose was found in the LPS of X. campestris pv. campestris (Volk, 1966). The ladder-like separation pattern of Salmonella typhimurium LPS in SDS-polyacrylamide gels was not observed with X. campestris pv. campestris LPS. Instead, three faint bands appeared at the high molecular mass region (Köpplin et al., 1993). These observations suggested the presence of distinct features in X. campestris pv. campestris LPS, which may influence the accessibility of XpsD to proteases.

Comparison of the trypsin digestion products of XpsD revealed differences between E. coli and X. campestris pv. campestris. A major band with a molecular mass of approximately 60 kDa and two groups of minor bands with smaller molecular masses, one around 45 kDa and the other around 31 kDa, appeared in X. campestris pv. campestris treated with 1 mg trypsin ml⁻¹ (Fig. 4b, top, left panel) or 0-5 mg trypsin ml⁻¹ (Fig. 5). Such bands were not observed in trypsin-treated E. coli that expressed XpsD (Fig. 4a). These differences are probably not related to the other Xps proteins. Almost identical protease digestion products were observed in XC1708(pKC118) and in XC17433(pKC118) (not shown). The latter contained no Xps proteins other than XpsD. Presumably, different conformations of the XpsD external loop structures in different host cells could account for the observed differences.

Pullulanase produced by K. oxytoca was detected on the cell surface by immunodetection (d’Enfert et al., 1987). In E. coli expressing pullulanase, it was detected on the cell surface as well only when the genes required for its secretion were present. These results suggested that the attachment of pullulanase on the cell surface may be an intermediary step in pullulanase secretion. However, when the lipoprotein signal peptide of pullulanase was replaced with the nonlipoprotein signal peptide of MalE, it was shown that, in contrast to fatty-acylated pullulanase, non-fatty-acylated pullulanase was secreted directly into the milieu without prior attachment to the cell surface (Poquet et al., 1993). Further studies are required to assess the significance of detecting secreted, nonlipoprotein α-amylase on the cell surface of X. campestris pv. campestris.

We considered the absence of surface-exposed α-amylase in the secretion-defective mutant strain XC1708 as an indication of intactness of cells in immunofluorescence experiments. The validity of such control experiments is probably not influenced by the lack of knowledge about the significance of surface-exposed α-amylase in secretion-competent strains.

A protein with an apparent molecular mass of 48 kDa repeatedly appeared along with the 77 kDa XpsD, either on a Western blot of X. campestris pv. campestris cell extracts or on an autoradiogram of immunoprecipitated, [³H]palmitate-labelled cells. It was not detected in the strain XC17433, where the entire xps gene cluster was deleted (not shown). It was discernible in the xpsD::Tn5 mutant XC1708, where Tn5 was inserted close to C-terminal end of XpsD (Fig. 1a, lane 2). Replacement of the XpsD lipoprotein signal peptide with a nonlipoprotein signal peptide did not abolish the appearance of the 48 kDa protein, which, however, was no longer labelled with [³H]palmitate. These observations suggested that this protein probably contains the N-terminal end of XpsD and its production was not influenced by the absence of lipid modification at the N-terminus or Tn5 insertion near the C-terminal end. It has been demonstrated that the dnaX gene of E. coli encodes two DNA polymerase III holoenzyme subunits, τ (71 kDa) and γ (47 kDa), that share an identical N-terminal sequence. They are produced from the same ORF as a result of a −1 translational frameshifting event (Blinkowa & Walker, 1990; Tsuchihashi & Kornberg, 1990). A stretch of six adenines and a downstream hairpin structure were noted to flank the termination codon of the γ subunit (Tsuchihashi & Kornberg, 1990). Similar structures were associated with expression of the genes of the bacterial insertion sequence IS7 and the trpR gene of E. coli (Escoubas et al., 1991; Benhar et al., 1992). The latter was later demonstrated to be caused by a translational bypassing event and unrelated with the hexa-adenine-stretch (Benhar & Engelberg-Kulka, 1993; Engelberg-Kulka & Schouler-Schwarz, 1994). Examination of the xpsD gene nucleotide sequence revealed a stretch of six adenines at nucleotides 1644–1649, counted from the adenosine of the initiation codon. A termination codon in the −1 or +1 reading
frame appears within 30 nucleotides downstream of the hexa-adenine-stretch. However, translational termination in either case would produce a protein with a molecular mass of 55 kDa. Besides, no reduction in the production of the 48 kDa protein was observed from two mutated xpsD genes, one with two-base substitutions (CAAG AA) interrupting the hexa-adenine-stretch and the other with an inframe deletion removing the hexa-adenine-stretch plus some flanking sequences (data not shown). It is unlikely that the appearance of the 48 kDa protein is due to a frameshifting event occurring downstream of the hexa-adenine-stretch in the xpsD gene. Other possible causes that might have prompted the production of the 48 kDa protein, such as translational bypassing or protenolytic cleavage, have not been ruled out. Determination of the origin of the 48 kDa protein may provide clues as to whether it plays any role in extracellular protein secretion.

ACKNOWLEDGEMENTS

N. T. Hu would like to dedicate this work to the late Philip J. Bassford, Jr. The authors would also like to thank M. Russel for reading the manuscript, U. Henning for kindly sending antibody against E. coli OmpA, P. Ray and E. A. Parrish for kind help in raising the antibody against a-amylase, Y.-N. Chang for suggesting the SOE method, D. Bauer for providing plasmid pCPP39, K.-C. Wang for constructing plasmids pKC101, pKC107 and pKC118, D.-Y. Chen for pCD105 and the 48 kDa protein studies and C.-H. Lin for determining the partial DNA sequence of pCD105. This work was supported by a grant from National Science Council of Republic of China: NSC82-0211-B005-037.

REFERENCES


Received 31 October 1994; revised 20 February 1995; accepted 22 February 1995.