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

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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, XpsD Δ 414-759, 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 campestris* pv. *campestris* (Dums *et al.*, 1991; Hu *et al.*, 1992a), *out* 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 *exe* 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* (Wei *et al.*,

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 *Shigella 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 PulD 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 demonstrated to be defective in the secretion of extracellular enzymes, which include polygalacturonate lyase, α -amylase, protease and endoglucanase (Hu *et al.*, 1992a). 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; Russel, 1994). Among them, subcellular location of *K. oxytoca* PulD and the filamentous phage protein pIV, which is required for phage morphogenesis, have been carefully studied. PulD 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 (Brisette & Russel, 1990) or fractionated with Triton X-100 (Russel & Kaźmierczak, 1993). Recent studies by Kaźmierczak *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*-

*Bst*EII 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 *Bam*HI fragment of the *xpsD* gene at the *Bam*HI 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 *Eco*RI fragment cloned in pTZ18U. The plasmid pCT5 was constructed by ligating the *Eco*RI 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'-CGCCAGGGTTTCCAG-TCACGAC-3') and 'b' (5'-AGTGGTGGCGATGACGTCG-GCCTGTGCGCT-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'-GACGTCATCGCCACCACTCCGCCACCGGA-3') and 'd' (5'-TCAGCGGCACCACGCGCACCTCGA-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 *Hind*III and *Sac*II 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 μ g ml⁻¹; ampicillin, 50 μ g ml⁻¹; rifampicin, 100 μ g ml⁻¹; and kanamycin, 50 μ g ml⁻¹.

Site-directed mutagenesis. The site-directed *in vitro* mutagenesis with the Altered Sites System purchased from Promega was used in creating *Xba*I 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'-TCGCGGCGCACGTCT-AGAGCGGAGTGGTGG-3' for the upstream *Xba*I site and 5'-GTAACGCTACCGTTCTGGTCTAGACTAGCGG-3' for the downstream *Xba*I site. A single primer with the following sequence was used in the construction of the mutant

*xpsD*4414-759 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 lysozyme 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. Sera 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.

[³H]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 (10%, 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 Enlightning 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 lysozyme 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% (v/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 followed 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 & Kaźmierczak (1993).

Immunofluorescence. The procedures of Koehler *et al.* (1992) were followed with slight modifications. *X. campestris* pv. *campestris* cells grown in L-broth to OD₆₀₀ 0.6 were washed with PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄ l⁻¹, pH 7.4) plus 10 mM MgCl₂ 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× magnification.

Complementation. The chimeric *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 0.2% (w/v) starch for α -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(pKC118) as well, albeit in slightly different proportions (Fig. 1a, lane 3). In contrast, only the 48 kDa protein was perceived 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 pKC118 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(pKC118) was approximately ten times that detected in XC1701.

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 XC1708(pMH7). (b) Autoradiogram of [³H]palmitate-labelled *X. campestris* pv. *campestris*. *X. campestris* pv. *campestris* XC1708(pCPP30) (lane 1) and XC1708(pKC118) (lane 2), grown in XOL + 0.5% maltose, were labelled with [³H]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 *.

[³H]Palmitate labelling of XpsD

When *X. campestris* pv. *campestris* XC1708(pKC118) was labelled with [³H]palmitate, followed by immunoprecipitating 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-fractionated 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 immunologically 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. 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 LacZ-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* DH5 α (pMH9), XpsD was degraded completely by treating with $200 \mu\text{g trypsin ml}^{-1}$ or $1 \text{ mg proteinase K ml}^{-1}$

Fig. 3. Sucrose gradient sedimentation analysis of the total membrane preparation of *X. campestris* pv. *campestris* XC1701. The total membrane fraction, prepared from cells disrupted with a French press, was analysed on a 25–61% (w/w) sucrose gradient. Concentrated samples from every other fraction were separated in an SDS-polyacrylamide gel. Top panel: gels stained with Coomassie brilliant blue; bottom panel: immunoblots detected with antibody against the LacZ-XpsD fusion protein. In the bottom panel, only the portion where XpsD migrated is depicted. The top of the gradient is on the left. Buoyant densities are shown for the fractions not labelled with fraction numbers.

Fig. 4. (a) Protease sensitivity of XpsD in intact *E. coli*. *E. coli* DH5 α (pMH9), labelled with [³⁵S]methionine at 37 °C for 30 min, was treated with various concentrations of proteinase K or trypsin on ice for 1 h. Protease-treated samples precipitated with antibody against LacZ-XpsD and antibody against *E. coli* OmpA were separated in an SDS-polyacrylamide (10%) gel. (b) Trypsin sensitivity of XpsD in *X. campestris* pv. *campestris*. *X. campestris* pv. *campestris* XC1708(pKC118), labelled with [³⁵S]methionine at 28 °C for 30 min, was resuspended in 10 mM Tris/HCl, pH 7.8, with (EDTA-treated cells) or without (intact cells) 5 mM EDTA. Digestions were conducted on ice for 1 h. Trypsin-treated samples, with (top panels) or without (bottom panels) precipitation with antibody against LacZ-XpsD, were separated in an SDS-polyacrylamide (10%) gel. The top panels show autoradiograms of immunoprecipitated samples and the bottom panels are Coomassie-brilliant-blue-stained gels of samples not precipitated with antibody.

(Fig. 4a). In order to substantiate the intactness of the protease-treated cells, we included anti-OmpA antibody in the immunoprecipitation reactions. *E. coli* OmpA is completely resistant to proteases in intact cells. On the other hand, in the cells permeabilized with EDTA, a trypsin-resistant fragment of 24 kDa was produced (Tomassen & Lugtenberg, 1984). In the experiments shown in Fig. 4(a), OmpA was not degraded to any significant extent at all concentrations of either protease, neither was the 24 kDa protein fragment detected in any trypsin-treated sample.

When labelled *X. campestris* pv. *campestris* XC1708(pKC118) was treated with trypsin, it was observed that the XpsD was not as sensitive as that in *E. coli*.

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, α -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 α -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 α -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

Fig. 5. Comparison of sensitivity of non-fatty-acylated XpsD with wild-type XpsD against trypsin or proteinase K digestion. XC1708(pKC107) and XC1708(pCT5), labelled with [35 S]methionine for 30 min, were treated with trypsin or proteinase K (PK) at various concentrations (in mg ml $^{-1}$), with or without EDTA, as depicted above each lane, followed by immunoprecipitation with anti-LacZ-XpsD antibody, SDS-PAGE and fluorography. –, Digestion without EDTA; +, digestion with the addition of 5 mM EDTA. Numbers marked on the left depict molecular mass standards in kDa.

amy gene of *X. campestris* pv. *campestris* (Hu *et al.*, 1992b). 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 [35 S]methionine-labelled intact XC1708(pKC107) or XC1708(pCT5) with trypsin or proteinase 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 proteinase K ml $^{-1}$. Similar trypsin or proteinase 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 XpsD Δ 414-759 and XpsD Δ 28-427

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 XpsD Δ 28-427 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, pIV Δ 15-242, has been observed previously by Russel & Kaźmierczak (1993).

Subcellular location of the C-terminal truncated protein XpsD Δ 414-759

Fractionation via spheroplast formation revealed that the C-terminal truncated protein XpsD Δ 414-759 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

Fig. 6. Protease digestion of XpsD Δ 414-759. XC1708(pMH7), labelled with [35 S]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.

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 XpsD Δ 414-759 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 XpsD Δ 414-759 is exposed to the cell surface.

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

The extracellular protein secretion property of the trans-conjugants 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 XpsD Δ 414-759 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 (Brissette & 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 *phoA* (Russel & Kaźmierczak, 1993). Unlike pIV, a C-terminal truncated XpsD protein, XpsD Δ 414-759, 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 XpsD Δ 414-759 was synthesized at a slightly higher level than the plasmid-encoded wild-type XpsD. The membranous XpsD Δ 414-759 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. campestris* pv. *campestris*

(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). Although 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 (Köplin *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 IS1 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 & Schoulaker-Schwarz, 1994). Examination of the *xpsD* gene nucleotide sequence revealed a stretch of six adenines at nucleotides 1644-1649, counted from the adenine 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 (C AAG 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 proteolytic 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.

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