XpsG, the major pseudopilin in *Xanthomonas campestris* pv. campestris, forms a pilus-like structure between cytoplasmic and outer membranes

Nien-Tai HU*, Wei-Ming LEU†, Meng-Shiunn LEE†, Avon CHEN‡, Shu-Chung CHEN‡, Yu-Ling SONG‡ and Ling-Yun CHEN‡[1]

*Institute of Biochemistry, National Chung Hsing University, Taichung 402, Taiwan, †Institute of Biotechnology, National Chung Hsing University, Taichung 402, Taiwan, and ‡Institute of Biochemistry, Chung Shan Medical University, Taichung 402, Taiwan

GspG, -H, -I, -J and -K proteins are members of the pseudopilin family. They are the components required for the type II secretion pathway, which translocates proteins across the outer membrane of Gram-negative bacteria to the extracellular milieu. They were predicted to form a pilus-like structure, and this has been shown for PulG of *Klebsiella oxytoca* by using electron microscopy. In the present study, we performed biochemical analyses of the XpsG protein of *Xanthomonas campestris* pv. campestris and observed that it is a pillar-like structure spanning the cytoplasmic and outer membranes. Subcellular fractionation revealed a soluble form (SF) of XpsG, in addition to the membrane form. Chromatographic analysis of SF XpsG in the absence of a detergent indicated that it is part of a large complex (> 440 kDa). *In vitro* studies indicated that XpsG is prone to aggregate in the absence of a detergent. We isolated and characterized a non-functional mutant defective in forming the large complex. It did not interfere with the function of wild-type XpsG and was not detectable in the SF. Moreover, unlike wild-type XpsG, which was distributed in both the cytoplasmic and outer membranes, it appeared only in the cytoplasmic membrane. When wild-type XpsG was co-expressed with His_6-tagged XpsH but not with untagged XpsH, SF XpsG bound to nickel and co-eluted with XpsH. This result suggests the presence of other pseudopilin components in the XpsG-containing large-sized molecules.

Key words: chromatography, Gram-negative bacteria, pillar-like structure, type II secretion.

INTRODUCTION

In the type II secretion apparatus involved in the transport of extracellular proteins across the outer membrane in Gram-negative bacteria, five type IV pilin-like proteins (GspG, -H, -I, -J and -K) have been identified. They were designated as pseudopilins because of their sequence similarity at the N-terminus with type IV prepilin [1]. The sequence G↓(F/M)XXXE followed by a hydrophobic region of 15–20 amino acid residues is conserved in all pseudopilins. Moreover, the cleavage between G↓F by the type IV prepilin leader peptidase is required for their normal functioning [2–4].

Type IV pilin forms a helical polymer (the type IV pilus), 6 nm in diameter and several micrometres in length, on the surface of a variety of bacterial species. Crystallographic analysis of *Neisseria gonorrhoeae* pilin revealed an elongated ladle-shaped structure [5]. The N-terminal segment of a long α-helical spine forms the ladle handle. A computational model of a right-handed fibre with five subunits per turn was proposed. The fibre is formed with a core of packed hydrophobic α-helices surrounded by a tubular β-wrap. In the detergent-solubilized state, a dumb-bell-shaped dimer is thermodynamically favoured for minimal exposure of its N-terminal hydrophobic α-helix.

The similarity between pseudopilin and pilin at their N-termini led to the suggestion that pseudopilin forms a pilus-like structure [6,7]. The pilus-like structure was proposed to connect the cytoplasmic membrane with the outer surface of the cell, either to conduct proteins through the outer membrane [8] or to act as a piston to push secreted proteins into the secretion pore located in the outer membrane [9]. The major pilin-like protein of *Klebsiella oxytoca*, PulG, was shown to form multimers of 2–5 subunits on formaldehyde cross-linking [10]. In addition to homodimer formation, XcpT (GspG of *Pseudomonas aeruginosa*) was also shown to form heterodimers with other pseudopilins by cross-linking and affinity chromatography [11]. Despite these observations, demonstration of a pilus-like structure composed of pseudopilin has been unsuccessful until recently. Using electron microscopy, Sauvonnet et al. [12] observed pilus-like bundles with diameters of 15–25 nm on the surface of *Escherichia coli* that overexpressed the *pul* operon of *K. oxytoca*. The presence of PulG in the pilus-like structure was confirmed with immuno-gold labelling. However, this surface-exposed pilus-like structure could not be detected when the Pul components were expressed at low levels. Furthermore, the characteristics of the pilus-like structure inside the cells remain unclear.

XpsG, 143 amino acids in length, is the major pilin-like protein in *Xanthomonas campestris* pv. campestris. We observed that the XpsG of the wild-type strain existed in two forms, a major membrane-associated form (MF) and a minor soluble form (SF). By size-exclusion chromatography, the XpsG in SF was found to be very large in the absence of a detergent. To exclude the possibility that these complexes may have formed as unordered structures during *in vitro* manipulation, we looked for a mutant of *xpsG* that had lost its function without affecting the stability of the protein. Such a mutant was obtained and designated as XpsG-F120. It was undetectable both in the outer membrane and in the SF. We hypothesize that, as a result of cell passage through the French press, the pilus-like structure connecting the two membranes in intact cells was broken and released as SF in the wild-type strain.

EXPERIMENTAL

Preparation of antibodies raised against XpsG and XpsH proteins

A 375 bp DNA fragment encoding amino acid residues 20–143 of the XpsG protein and a 267 bp fragment encoding amino acid

Abbreviations used: DOC, deoxycholate; MF, membrane form; NTA, nitrilotriacetic acid; SF, soluble form.

[1] To whom correspondence should be addressed (e-mail chenly@csmu.edu.tw).

residues 82–169 of the XpsH protein were produced by PCR amplification. The following primers were used in PCR: PG-forward: 5′-CACTTGGGATCCTTGCTGGAAATCATC-ATCGT-3′; PG-reverse: 5′-CACTTGGTCGACTTACTGGT-ACTTGATGTCCGA-3′; PHC-forward: 5′-CACTTGGGAT-CCCAGCGCTTTCTGATCGACCCGCAG-3′; PHC-reverse: 5′-CACTTGGTCGACTGGCGCTGGCGTCCGCAG-3′. The PCR product was digested with *Bam*HI and *Sal*I, and subcloned into pET32a (Novagen) in-frame with the upstream *trxA* gene. The recombinant plasmid was examined with restriction enzyme digestion and confirmed by DNA sequencing data. The plasmid encoding the TrxA-XpsGHis$_6$ or TrxA-XpsHHis$_6$ protein was transformed into competent cells of *E. coli* BL21(DE3). Overproduction of the fusion protein was accomplished by induction with 2 mM isopropyl β-D-thiogalactoside in Luria broth. The fusion protein was purified after specific binding with nickel-nitrilotriacetic acid (Ni-NTA) under native conditions, in accordance with the manufacturer's instructions (Qiagen). New Zealand White rabbits were immunized with the Ni-NTA-purified TrxA-XpsGHis$_6$ or TrxA-XpsHHis$_6$ protein. A primary injection of 500 μg of the protein with complete Freund's adjuvant was given to the rabbits, followed by several injections of 250 μg of the protein with incomplete adjuvant on alternate weeks.

Construction of Δ*xpsG* strain XC1713

Two *Nco*I restriction sites, one at the 36th and the other at the 131st codon of the *xpsG* gene, were generated after site-directed mutagenesis for the construction of an in-frame deleted *xpsG* (Δ*xpsG*) gene. Site-directed mutagenesis was conducted in accordance with the manufacturer's instructions by using the GenEditor™ *in vitro* site-directed mutagenesis system (Promega). Single-stranded DNA prepared from the plasmid pMS1, in which a 3.7 kb *Eco*RI fragment containing complete *xpsG*, *-H*, and *-I* genes subcloned in the phagemid pBluescript II SK(−) (Stratagene), was used as the template. Primers with the following sequences were used: mG-1: 5′-GTGCTCACCCATGGTCG-GCAG-3′; mG-2: 5′-GATGGCCGCCCATGGCGGCAGCA-G-3′. Deletion of the *xpsG* gene was conducted by digesting the mutated plasmid with *Nco*I followed by self-ligation.

The Δ*xpsG* gene was introduced into the parental strain XC1701 genome to replace the wild-type *xpsG* gene by using a two-step selection procedure described by Kamoun et al. [13]. Briefly, the Δ*xpsG* gene with its flanking sequences, subcloned in a suicidal plasmid that carries *kan* and *sacB* genes, was introduced into XC1701 via electroporation [14]. Colonies that grew on Luria–Bertani agar plates containing kanamycin (50 μg/ml) were selected and examined for sucrose sensitivity. Subsequently, the kanamycin-resistant, sucrose-sensitive bacteria were grown in Luria broth for 4 h or overnight at 28 °C, before spreading (with or without dilution) on Luria–Bertani agar plates containing 5% (w/v) sucrose. Colonies appearing on this plate were then examined for kanamycin sensitivity and α-amylase secretion on XOL agar plates [15] supplemented with 2% (w/v) starch.

Four sucrose-resistant, kanamycin-sensitive and non-secretive colonies were picked and examined by PCR analysis for the presence of the Δ*xpsG* gene and absence of the wild-type *xpsG* gene in the genomic DNA. We designated the correct mutant strain as XC1713. When we introduced a plasmid pFG that only contained the wild-type *xpsG* gene into XC1713, we observed the recovery of secretion on the starch plate. Immunoblot analysis of the XpsM, XpsN and XpsD proteins, encoded by sequences downstream to the *xpsG* gene, was also performed to confirm that the Δ*xpsG* gene of XC1713 did not affect its downstream gene expression.

Construction of Δ*xpsH* strain XC1717

An identical strategy and template were used for constructing the Δ*xpsG* strain XC1713. The following primers were used for generating upstream and downstream *Nco*I sites: mH-1: 5′-GCAGCGACCCATGGCGCGTTGCGCGTCTGCC-3′ and mH-2: 5′-GCGCCAGGACCATGGGCGCCATCCAGTT-3′ respectively. As a result, amino acid residues 2–123 of XpsH were deleted.

Construction of His$_6$-tagged *xpsG* and *xpsH* genes

An in-frame *Xho*I site was introduced immediately upstream of the stop codon of each gene by PCR amplification of coding sequences with a common 5′-primer in combination with two different 3′-primers designed to be annealed specifically with each gene. The plasmids pFG and pFH served as a template in the PCR. Each plasmid contains the entire coding sequence of the *xpsG* or *xpsH* gene in a broad host-range vector pCPP30 downstream of the *lac* promoter. The 5′-primer PSB anneals with the *lac* promoter region, 5′-CCCAATACGCAAACCGCCTCT-3′. The 3′-primers are as follows: XpsG-3His$_6$: 5′-CACTT-GCTCGAGCTGGTACTTGATGTCCGA-3′ and XpsH-3His$_6$: 5′-CACTTGCTCGAGTGGCGCTGGCGTCCGCAG-3′. The PCR product was digested with *Hin*dIII and *Xho*I followed by ligation with a broad host-range vector, in which (CAC)$_6$-TGA had been introduced in-frame downstream of the *Xho*I site. The resulting plasmids were designated as pFG-His and pFH-His.

Construction of pAC2 that encodes the mutant XpsG-F120

A unique *Sal*I site was introduced in the *xpsG* gene cloned in pBluescript II SK(−) via site-directed mutagenesis using the GenEditor™ system, resulting in the plasmid pAC1. In this process, the mutagenesis primer PGSalI 5′-GGCCAGGCCGTC-GACCTGATC-3′ was used. Digestion of the plasmid pAC1 with *Sal*I, followed by the insertion of a double-stranded adaptor, generated the plasmid pAC2. The double-stranded adaptor was obtained by the annealing of the Sal-His(+) primer 5′-TCGACC-ACCATCATCATCATG-3′ and the Sal-His(−) primer 5′-TCG-ACATGATGATGATGGTGG-3′. The plasmid pAC2 encodes the mutated XpsG-F120 protein with the insertion of seven amino acid residues DH$_5$V downstream of Phe^{120} in the XpsG protein.

Membrane and soluble extracts of the XpsG protein

X. campestris pv. campestris XC1701, grown in Luria broth to a stationary phase, was washed with water to remove exopolysaccharide and resuspended in buffer A [20 mM Tris/HCl (pH 8.0), 1 mM PMSF, 0.5 mM dithiothreitol, 0.2 M NaCl]. Cells were disrupted by passing twice through a French press at 96600 kPa. Cell lysate was ultracentrifuged at 60000 rev./min (Beckman rotor Ti 90) for 1 h and the supernatant collected was designated as SF. Membrane vesicles collected as a pellet by ultracentrifugation were solubilized in buffer B [50 mM Tris/HCl (pH 8.0)] with 2% (v/v) Triton X-100 or 0.5% deoxycholate (DOC). MF of Triton X-100 or DOC extracts was collected in the supernatant by ultracentrifugation at 30000 rev./min (Hitachi rotor P50S2) for 1 h.

Anion-exchange FPLC

The procedures of Chen et al. [14] were followed. Briefly, MF of Triton X-100 extract or the SF prepared from the parental-strain XC1701 was passed into the FPLC High-Q column (1 ml size; Bio-Rad Laboratories) pre-equilibrated with buffer C [20 mM Tris/HCl (pH 8.0), 1 % (v/v) Triton X-100]. Protein fractions were eluted with buffer C containing a 0–1 M NaCl linear gradient. Triton X-100 was omitted from buffer C in all processes for the analysis of SF. The collected fractions (1 ml each) were analysed by immunoblotting with antibody raised against XpsG.

Gel-filtration FPLC

The MF or SF of cell extracts was chromatographed on an FPLC Superdex HR200 (Pharmacia Biotech) HR column (size; 25 ml) that was pre-equilibrated with buffer D [20 mM Tris/HCl (pH 8.0), 0.2 M NaCl] containing 1 % (v/v) Triton X-100 or 0.5 % DOC. Triton X-100 or DOC was not present in the analysis of SF. The gel-filtration Superdex HR200 column was calibrated with the following molecular-mass standards: thyroglobulin (669 kDa, 16.1 min), apoferritin (443 kDa, 21.1 min), β-amylase (200 kDa, 23.5 min), BSA (66 kDa, 26.3 min) and carbonic anhydrase (29 kDa, 31.8 min).

Ni-NTA affinity chromatography

SF or MF of the cell extract was mixed with 1 ml of Ni-NTA resin (Qiagen), which had been pre-equilibrated with a 15 × volume of buffer D at 4 °C overnight. The resin mixture was then loaded on to a 10 cm × 1 cm (diameter) column (Bio-Rad Laboratories) with a flow rate of 0.5 ml/min. After sequential washings with 20 × volume of equilibration buffer containing 25 mM imidazole, the bounded protein was eluted with the same buffer containing 250 mM imidazole and collected at 1 ml/fraction. Proteins collected in each fraction were precipitated with trichloroacetic acid at a final concentration of 10 % (w/v) and analysed by SDS/PAGE and immunoblotting.

Sucrose-gradient sedimentation analysis

The procedure of Lee et al. [16] was followed.

RESULTS

Detection of a SF of XpsG

To characterize the pilin-like protein, XpsG, we raised the antibody against a thioredoxin–XpsG fusion protein produced in *E. coli*. After immunoblot analysis, we detected a distinct band in the parental strain of *X. campestris* pv. campestris XC1701 that was absent from the Δ*xpsG* strain XC1713 (Figure 1A). The mobility of this protein by SDS/PAGE (16 kDa) agrees with the predicted molecular mass of XpsG (15.15 kDa), indicating that it is XpsG. To determine the subcellular localization of XpsG, cells were broken in a French press and separated into two fractions by ultracentrifugation. Although the α-subunit of RNA polymerase was detected only in the supernatant, the outer membrane protein XpsD appeared only in the pellet. By contrast, XpsG was detected in both fractions (Figure 1B). XpsG detected in the supernatant was one-fourth of that in the pellet, the low concentration suggesting that it was not due to the presence of un-precipitated membrane vesicles. We designated the XpsG in the supernatant as SF and that in the pellet as MF. But theoretically, the SF includes both cytoplasmic and periplasmic fractions, and the membrane fraction consists of outer membrane, cytoplasmic membrane and protein aggregates.

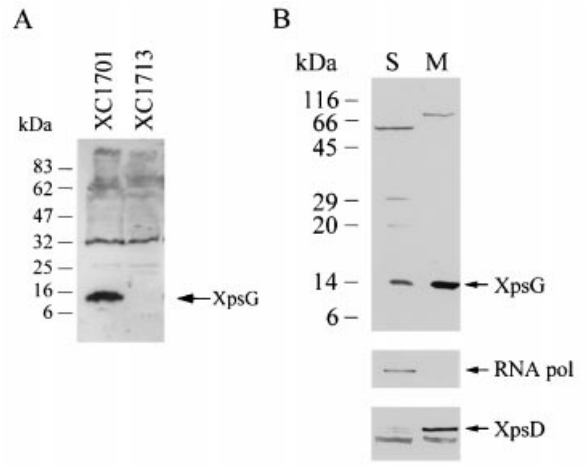

Figure 1 Detection of XpsG in SF

(**A**) Antibody raised against the TrxA–XpsGHis$_6$ fusion protein specifically recognized a protein with apparent molecular mass of 16 kDa in the parental strain XC1701, but not in the Δ*xpsG* mutant strain XC1713. (**B**) French press-broken cells of XC1701 were divided into supernatant (S) and pellet after ultracentrifugation at 60 000 rev./min for 1 h. The pellet resuspended in Triton X-100 was further centrifuged at 30 000 rev./min for 1 h. The supernatant collected from the second ultracentrifugation was saved as membrane (M) fraction. RNA pol, α-subunit of RNA polymerase; XpsD, the pore-forming outer membrane protein required for type II secretion [22].

Chromatographic analysis of MF XpsG and SF XpsG

To determine if the XpsG protein forms pilus-like structures inside the cell, we attempted its purification by conducting ion-exchange and gel-filtration chromatography. After ion-exchange chromatography, the Triton X-100-extracted MF XpsG fractionated at 7–11 min (Figure 2A, upper panel). Further analysis of the Superdex HR200 size-exclusion column equilibrated with 1 % (v/v) Triton X-100 revealed that the MF XpsG fractionated with a major peak at 22–26 min ranging in molecular mass from 66 to 443 kDa (Figure 2B, upper panel), which appears to be too small for a pilus-like structure. In addition, when the column was equilibrated with 0.5 % DOC, the MF XpsG appeared at 29–32 min with a molecular mass of approx. 32 kDa (Figure 2B, lower panel), twice the size of XpsG, suggesting that multimeric XpsG is likely to be disrupted by detergent treatment. Therefore it was not feasible to purify an intact pilus-like structure from the membrane fraction. On the other hand, the SF XpsG behaved differently. In the absence of a detergent, it fractionated at 11–13 min after ion-exchange chromatography (Figure 2A, lower panel). In the analysis of the size-exclusion column equilibrated with buffer in the absence of detergent, the SF XpsG fractionated in or near the void volume at 16–20 min (Figure 2C), implying a size of > 443 kDa. Assuming that XpsG forms a pillar-like structure in the periplasm, with one end buried in the cytoplasmic membrane and the other in the outer membrane, it would not be surprising if it was easily sheared during cell disruption, thereby releasing it from its membrane anchorage. We postulate this to be the source of SF XpsG.

Effect of detergent on SF XpsG

Size-exclusion chromatography of SF XpsG in the presence of Triton X-100 or DOC resulted in the same elution profiles as those of MF XpsG (results not shown), implying that both SF and MF XpsG probably shared similar characteristics and a similar overall structure. When the DOC concentrations in the

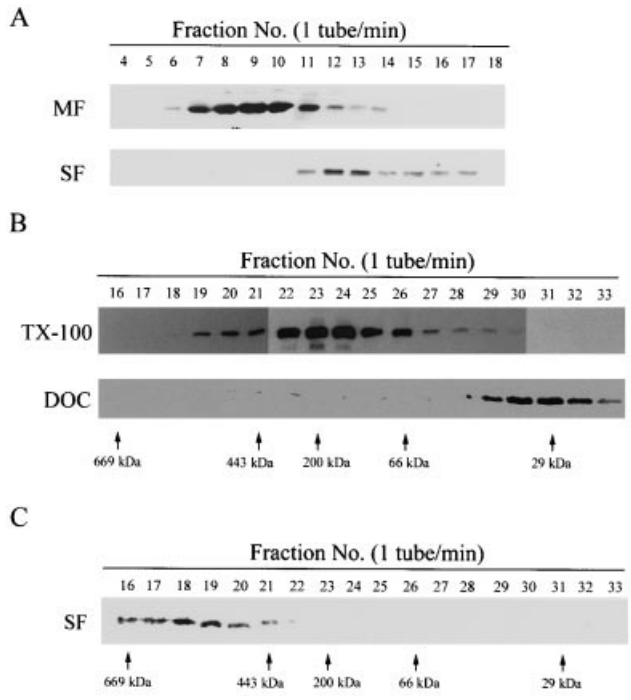

Figure 2 Chromatographic analyses of SF XpsG and MF XpsG and immunoblotting

(**A**) Anion-exchange chromatography analyses of MF XpsG of XC1701 in the presence of 1% (v/v) Triton X-100 and SF XpsG in the absence of Triton X-100. (**B**) Gel-filtration chromatography analysis of MF XpsG of XC1701 on a Superdex HR200 column equilibrated and eluted with buffer D containing 1% (v/v) Triton X-100 (upper panel) or 0.5% DOC (lower panel). (**C**) Gel-filtration chromatography of SF XpsG of XC1701 on a Superdex HR200 column that was equilibrated only with buffer D.

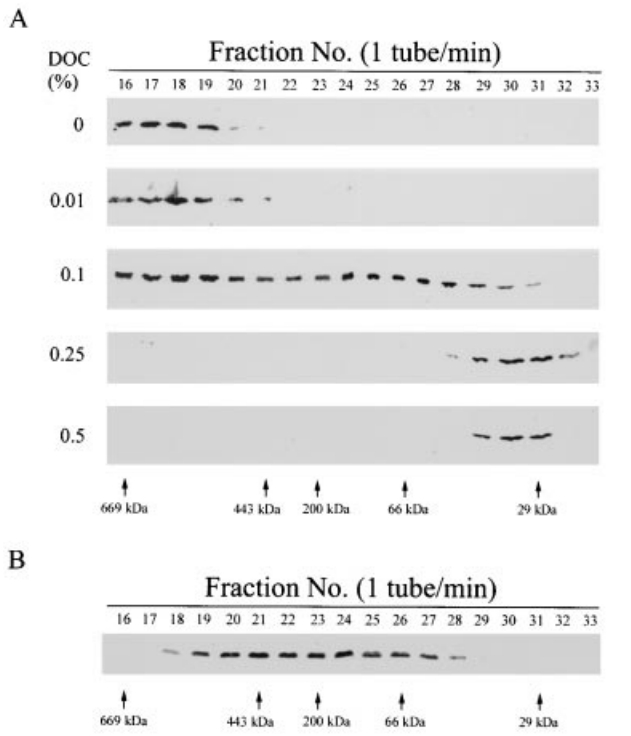

Figure 3 Effect of DOC on SF XpsG

(**A**) Gel-filtration chromatography of SF XpsG of XC1701 in the presence of various concentrations of DOC. (**B**) Second gel-filtration chromatography in the absence of DOC for pooled fractions 29–31 collected by chromatography in the presence of 0.5% DOC.

equilibration buffer of size-exclusion columns were varied, SF XpsG eluted either near the void volume (i.e. > 443 kDa) or as dimer-sized molecules (Figure 3A). A wide distribution of XpsG (between 16 and 31 min) was clearly detected at 0.1% DOC. Apparently, the size of SF XpsG varies significantly with the concentrations of DOC. In an attempt to reassemble the large XpsG complex from dimeric XpsG, we collected the 0.5% DOC-dissociated XpsG (Figure 3A, bottom panel) and applied it to a second size-exclusion column in the absence of DOC (Figure 3B). This XpsG neither ran as a dimer, nor resumed its original large size. Instead, XpsG appeared at 19–27 min, suggesting that some unknown factor required for reassembly may have been removed. We conclude that size determination of the XpsG multimeric structure in the presence of detergent is not reliable. Moreover, the results imply that the XpsG is prone to assemble or aggregate in the absence of a detergent. The large SF XpsG complex, instead of representing the pilus-like structure, probably occurred as an artifact from an *in vitro* process. To discover the biological relevance of the complex, we searched for an *xpsG* mutant that had lost its biological function and the ability to form the complex.

Analysis of XpsG-F120 mutant protein

We obtained one mutant with an insertion of seven amino acids (DH_5V) downstream of residue Phe^{120} (encoded by the plasmid pF120; Figure 4A). Overexpression of the mutant protein XpsG-F120 did not exert a negative-dominant effect in the parental strain XC1701, nor did it complement the Δ*xpsG* strain XC1713 (Figure 4B). After SDS/PAGE, the migration of XpsG-F120 was slower when compared with wild-type XpsG, reflecting the increase in its molecular mass (16.05 kDa; Figure 4C). In addition to the XpsG-F120 band, a band migrating faster than wild-type XpsG was detected in the membrane fraction whenever the XpsG-F120 was expressed. It is probably a degradation product of XpsG-F120. XpsG-F120 expressed in the presence or absence of wild-type XpsG was detectable only in the membrane fraction. By contrast, co-expressed wild-type XpsG remained in two forms. Interestingly, the XpsG-F120, although expressed substantially, did not join with the wild-type XpsG protein in the formation of large-sized molecules in SF (Figure 4C).

To examine if the MF XpsG from wild-type and mutant strains possess the same characteristics, we analysed the distribution of XpsG protein between two membrane bilayers. Sucrose-gradient sedimentation analysis revealed that the wild-type XpsG co-fractionated with the cytoplasmic-membrane marker succinate dehydrogenase (results not shown) in fractions 11–17 as well as with the outer-membrane marker OprF (results not shown) in fractions 22–28 (Figure 5, upper panel). This observation is consistent with the hypothesis that the assembled XpsG forms pillar-like structures across the periplasm and attaches *in vivo* with both the outer and cytoplasmic membranes. By contrast, the mutant XpsG-F120 only appeared in the cytoplasmic membrane fractions (Figure 5, lower panel). The absence of mutant protein in both soluble and outer membrane fractions suggested that the XpsG-F120 had lost its ability to form the pillar-like structure and therefore accumulated only in cytoplasmic membrane.

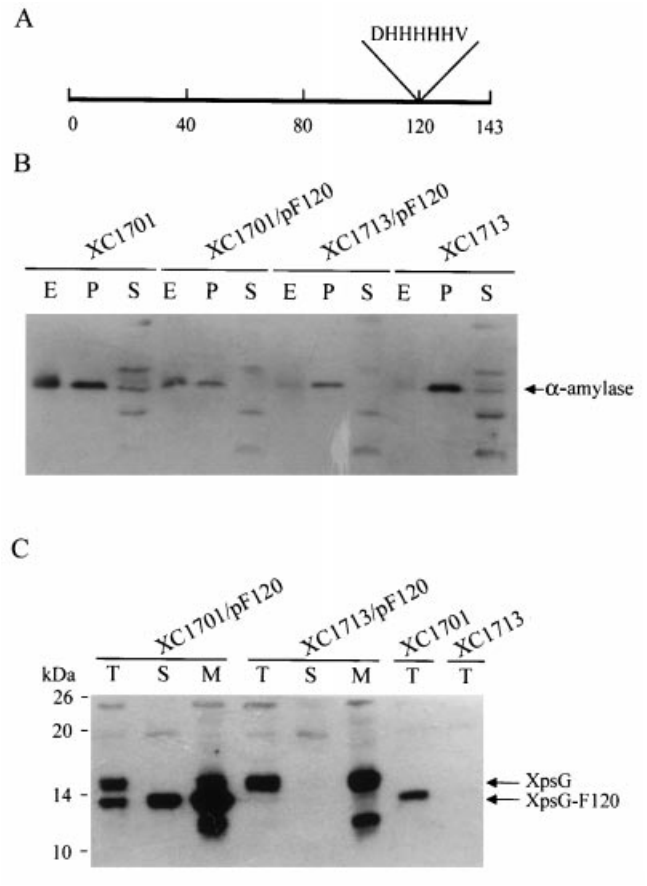

Figure 4 Analysis of mutant protein XpsG-F120

(**A**) Diagrammatic representation of XpsG-F120 mutant with seven amino acid residues DH_5V insertion downstream of Phe^{120}. (**B**) Subcellular distribution of the secreted protein α-amylase. The procedures of Lee et al. [16] were followed. E, extracellular fraction; P, periplasmic fraction; S, spheroplast fraction. (**C**) Subcellular localization of XpsG, as described in the Experimental section. T, total lysate; S, soluble fraction; M, membrane fraction.

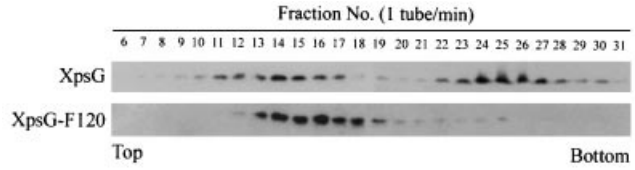

Figure 5 Sucrose-gradient sedimentation analysis of wild-type XpsG and mutant XpsG-F120

The procedures of Lee et al. [16] were followed. The cytoplasmic-membrane marker succinate dehydrogenase activity is shown in fractions 13–19 and the outer-membrane marker OprF in fractions 24–28.

Chromatographic analysis of extracellular XpsG

After suspending cells harvested from plates, Sauvonnet et al. [12] observed the detachment of PulG-containing pilus-like structures from the surface of cells that overexpress the Pul secretion components. This result raised the question whether any large-sized molecules could be detected in the extracellular fraction. We observed that detection of XpsG in the extracellular fraction was possible only when XpsG was produced in significantly higher level than that in the wild-type strain (Figure 6A). Secretion remains normal in this complemented strain XC1713/pFG (results not shown). Further analysis by size-exclusion chromatography in the absence of detergent indicated that the extracel-

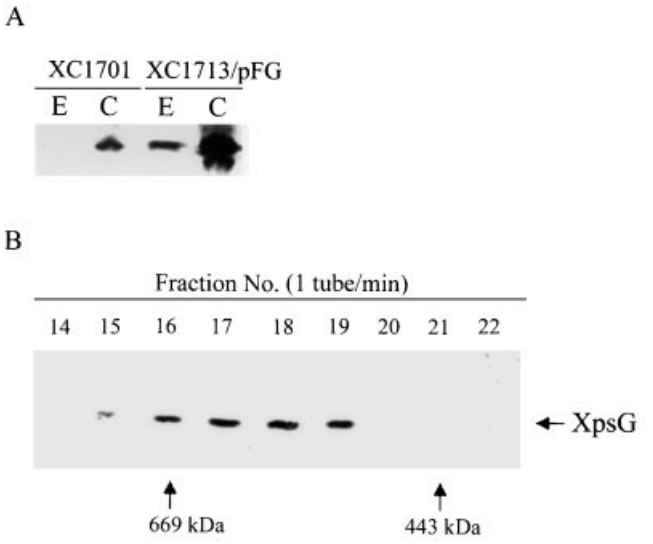

Figure 6 Gel-filtration chromatography of extracellular XpsG

(**A**) An equivalent amount of extracellular fraction (E) and cellular fraction (C) from wild-type strain XC1701 or from Δ*xpsG* strain XC1713, complemented with wild-type *xpsG* gene encoded on plasmid pFG, was separated by SDS/PAGE and immunoblotting with antibody raised against XpsG. (**B**) Gel-filtration chromatography and immunoblotting analysis of the extracellular XpsG from XC1713/pFG in the absence of a detergent, under the same conditions as that for SF XpsG in Figure 2(C).

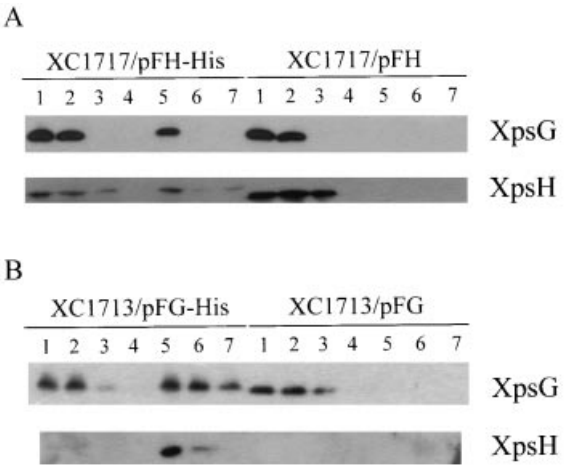

Figure 7 Ni-NTA affinity chromatography

(**A**) Δ*xpsH* mutant XC1717, containing plasmid pFH-His that overexpresses $XpsHHis_6$ or plasmid pFH that overexpresses XpsH, was analysed by Ni-NTA affinity chromatography. (**B**) Δ*xpsG* mutant XC1713, containing plasmid pFG-His that overexpresses $XpsGHis_6$ or plasmid pFG that overexpresses XpsG was analysed by Ni-NTA affinity chromatography. Lanes 1, soluble fraction; lanes 2, column flow-through; lanes 3–4, fractions washed with 25 mM imidazole; lanes 5–7, fractions eluted with 250 mM imidazole. Upper and lower panels, immunoblots detected by an antibody raised against XpsG and XpsH respectively.

lular XpsG of XC1713/pFG has an elution profile similar to that of the SF XpsG from XC1701 (Figures 6B and 2C).

Co-fractionation of XpsG with one other pseudopilin component after nickel-affinity chromatography

Heterodimers of XcpT with XcpU, XcpV and XcpW, (the pseudopilins of *P. aeruginosa*), have been described by cross-linking studies [11]. To investigate the presence of other pseudopilins in the large XpsG-containing complex, we made use of the nickel-binding property of His_6-tagged XpsH protein and analysed the co-expressed wild-type XpsG protein by affinity-column chromatography and immunoblot analysis of XpsG. His_6-tagged XpsH protein probably complemented the *xpsH* chromosomal mutant strain XC1717 (N.-T. Hu, W.-M. Leu, M.-S. Lee, A. Chen, S.-C. Chen, Y.-L. Song and L.-Y. Chen, unpublished work) and its elution profile was confirmed by an antibody raised against XpsH (Figure 7A, lower panel). XpsG was revealed to be co-eluting with His_6-tagged XpsH (encoded by pFH-His) but not with wild-type XpsH (encoded by pFH; Figure 7A, upper panel). Similarly, the XpsH co-fractionated only with the His_6-tagged XpsG (Figure 7B). Inability to detect XpsH in the crude extract may be due to the low expression of the chromosomal gene. These results demonstrated that SF XpsG contains at least one other pseudopilin component, although it may not be present in equal amounts.

DISCUSSION

In the present study, we found an SF of XpsG in *X. campestris* pv. campestris separable from the MF. The XpsG in SF is unlikely to represent the MF that is left unprecipitated after ultracentrifugation for two reasons. The marker protein for the outer membrane (XpsD) was detected only in the membrane fraction. Moreover, the wild-type XpsG was detectable in both soluble and membrane fractions, whereas the co-expressed mutant XpsG-F102 was detected only in the membrane fraction. The latter observation also excludes the possibility that the SF XpsG may represent an unordered structure created *in vitro*. If indeed an unordered structure had formed *in vitro*, we may detect both mutant and wild-type XpsG in the SF when they are co-expressed. However, co-appearance of mutant and wild-type XpsG in the SF was not observed.

Both cytoplasmic and periplasmic fractions are contained in the SF after ultracentrifugation of French press-broken cells. Since the SF XpsG was detectable in the wild-type strain, aggregation of XpsG in the cytoplasm was probably not due to accumulation of any overproduced or unbalanced Xps components in the cytoplasm. Thus the SF XpsG probably originates from the periplasm. However, unlike other periplasmic proteins, XpsG was not released on spheroplasting (results not shown), suggesting that the SF XpsG is probably not present as independent components in the periplasm. A vigorous extraneous force such as that generated after cell passage into the French press may be required to release XpsG in the soluble, i.e. periplasmic, fraction.

A pilus-like structure made of pseudopilins has long been sought in the type II secretion apparatus. By the biochemical approach, we have shown here for the first time a type of the XpsG that forms a large complex with other pseudopilins. Further analysis of a defective mutant XpsG-F120, which no longer produced these complexes, prompted us to propose a model for the pilus-like structure in the type II secretion apparatus (Figure 8). The pilus-like structure, assembled from the major pilin-like protein XpsG, probably existed *in vivo* as a pillar-like

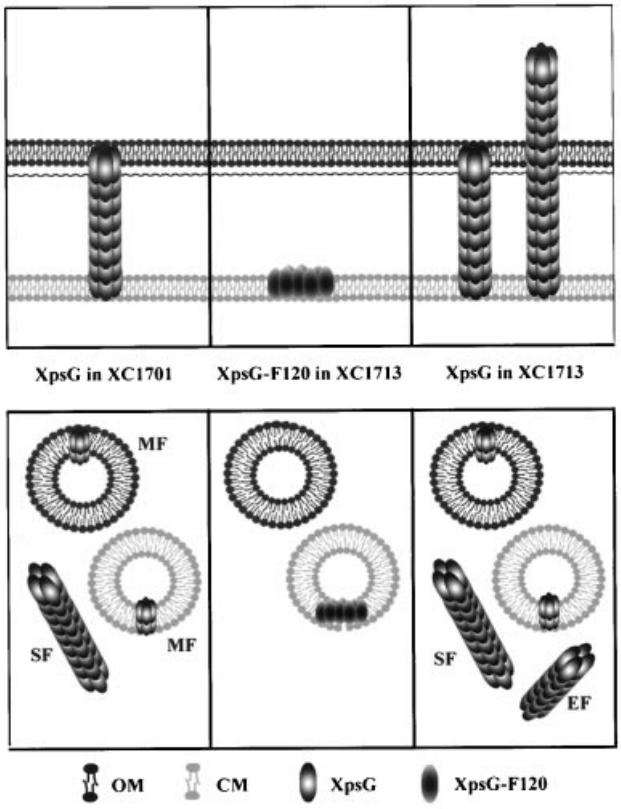

Figure 8 Schematic diagram of the pilus-like structure *in vivo* (upper panel) and after cell breakage (lower panel)

Left panels, chromosomal gene-encoded wild-type XpsG in wild-type strain XC1701; central panels, mutant XpsG-F120 in Δ*xpsG* strain XC1713; right panels, plasmid pFG-encoded wild type XpsG in Δ*xpsG* strain XC1713. (**A**) Pilus-like structure *in vivo*, across the cytoplasmic membrane (CM) and outer membrane (OM). PG, peptidoglycan. (**B**) Distribution of pilus-like structure after cell breakage. EF, extracellular form.

structure across the periplasm, with both ends tightly bound to the membranes (Figure 8, upper left panel). The N-terminal end might be anchored in the cytoplasmic membrane directly or indirectly by a platform, as proposed for the cytoplasmic membrane protein PilC of *P. aeruginosa* Tfp [17] or PilR of the R64 thin pilus [18]. The C-terminal end could either be anchored in the outer membrane or be fixed within the peptidoglycan network that lies beneath the outer membrane. How exactly the pillar-like structure is bound to either membrane is not known. However, the connections might be quite strong, thus leading to breakage at the junctions when vigorous extraneous force is applied to the cells (Figure 8, lower left panel).

The mutant XpsG-F120, by contrast, is defective in such a way that it can no longer assemble into the pillar-like structure proposed to span the two membranes (Figure 8, upper central panel). Probably, this explains why we were not able to detect the mutant XpsG-F120 in the SF or in the outer membrane. The observation that the mutant XpsG-F120 did not exhibit a dominant negative effect agrees with the hypothesis that it is defective in its ability to assemble into a pilus-like structure. The susceptibility of the mutant XpsG-F120 to degradation indicates its conformational difference from the wild-type protein, which could account for its inability to assemble into a pilus-like structure. A model proposed for pilus assembly suggests that the

C-terminal domain of pilin plays a critical role [19]. This may explain why insertion of seven amino acids downstream of Phe^{120}, located at the C-terminal domain, affects only its pilus-forming ability but not its N-terminal processing or cytoplasmic-membrane anchorage ability.

At normal expression levels, the pseudopilins form a pilus-like structure, probably not detected on the cell surface [20] or in the extracellular fraction. Overexpression of the PulG, along with other Pul secretion components [20] at high levels made their detection on cell surface and in extracellular fraction possible. Our study showed that overexpression of the XpsG alone was sufficient for its detection in the extracellular fraction. XpsG and the cellular SF XpsG have similar sizes after gel-filtration chromatography in the absence of a detergent. We propose that the cell surface-exposed molecules probably are pilus bundles continuous with the pillar-like structure existing in the periplasm (Figure 8, upper right panel). Appropriately assembled secretin is required for cell surface exposure of pseudopili [20], suggesting that the pillar-like structure probably extends to the cell surface through the secretion pore. We also performed immunoblot analysis for extracellular XpsG in the *xpsD* mutant. A lower signal than that in XC1713/pFG was detected, indicating less-effective extrusion of the pillar-like structure to the cell surface in the absence of functional secretin. We do not know exactly how in the *xpsD* mutant a small amount of XpsG reached the extracellular milieu. Possibly, a second secretin, like that in *P. aeruginosa* [21], is present in *X. campestris* pv. campestris.

We thank Marjorie Russel for reading the manuscript and providing helpful suggestions and Dr M.-T. Yang (Institute of Molecular Biology at National Chung Hsing University) for providing the antibody raised against an α-subunit of RNA polymerase of *X. campestris* pv. campestris. This work was supported by grants to L.-Y.C. from National Science Council, Taiwan (NSC-88-2311-B-040-001-B31, NSC-89-2311-B-040-001-B31 and NSC-89-2311-B-040-007-B32).

REFERENCES

1 Pugsley, A. P. (1993) The complete general secretory pathway in Gram-negative bacteria. Microbiol. Rev. **57**, 50–108
2 Strom, M. S., Nunn, D. and Lory, S. (1991) Multiple roles of the pilus biogenesis protein pilD: involvement of pilD in excretion of enzymes from *Pseudomonas aeruginosa*. J. Bacteriol. **173**, 1175–1180
3 Nunn, D. N. and Lory, S. (1992) Components of the protein-excretion apparatus of *Pseudomonas aeruginosa* are processed by the type IV prepilin peptidase. Proc. Natl. Acad. Sci. U.S.A. **89**, 47–51
4 Pugsley, A. P. and Dupuy, B. (1992) An enzyme with type IV prepilin peptidase activity is required to process components of the general extracellular protein secretion pathway of *Klebsiella oxytoca*. Mol. Microbiol. **6**, 751–760
5 Parge, H. E., Forest, K. T., Hickey, M. J., Christensen, D. A., Getzoff, E. D. and Tainer, J. A. (1995) Structure of the fibre-forming protein pilin at 2.6 Å resolution. Nature (London) **378**, 32–38
6 Pugsley, A. P. and Possot, O. (1993) The general secretory pathway of *Klebsiella oxytoca*: no evidence for relocalization or assembly of pilin-like PulG protein into a multiprotein complex. Mol. Microbiol. **10**, 665–674
7 Hobbs, M. and Mattick, J. S. (1993) Common components in the assembly of type 4 fimbriae, DNA transfer systems, filamentous phage and protein-secretion apparatus: a general system for the formation of surface-associated protein complexes. Mol. Microbiol. **10**, 233–243
8 Russel, M. (1998) Macromolecular assembly and secretion across the bacterial cell envelope: type II protein secretion systems. J. Mol. Biol. **279**, 485–499
9 Filloux, A., Michel, G. and Bally, M. (1998) GSP-dependent protein secretion in Gram-negative bacteria: the Xcp system of *Pseudomonas aeruginosa*. FEMS Microbiol. Rev. **22**, 177–198
10 Pugsley, A. P. (1996) Multimers of the precursor of a type IV pilin-like component of the general secretory pathway are unrelated to pili. Mol. Microbiol. **20**, 1235–1245
11 Lu, H. M., Motley, S. T. and Lory, S. (1997) Interactions of the components of the general secretion pathway: role of *Pseudomonas aeruginosa* type IV pilin subunits in complex formation and extracellular protein secretion. Mol. Microbiol. **25**, 247–259
12 Sauvonnet, N., Vignon, G., Pugsley, A. P. and Gounon, P. (2000) Pilus formation and protein secretion by the same machinery in *Escherichia coli*. EMBO J. **19**, 2221–2228
13 Kamoun, S., Tola, E., Kamdar, H. and Kado, C. I. (1992) Rapid generation of directed and unmarked deletions in *Xanthomonas*. Mol. Microbiol. **6**, 809–816
14 Chen, L. Y., Chen, D. Y., Miaw, J. and Hu, N. T. (1996) XpsD, an outer membrane protein required for protein secretion by *Xanthomonas campestris* pv. campestris, forms a multimer. J. Biol. Chem. **271**, 2703–2708
15 Hu, N. T., Hung, M. N., Chiou, S. J., Tang, F., Chiang, D. C., Huang, H. Y. and Wu, C. Y. (1992) Cloning and characterization of a gene required for the secretion of extracellular enzymes across the outer membrane by *Xanthomonas campestris* pv. campestris. J. Bacteriol. **174**, 2679–2687
16 Lee, H. M., Wang, K. C., Liu, Y. L., Yew, H. Y., Chen, L. Y., Leu, W. M., Chen, D. C. and Hu, N. T. (2000) Association of the cytoplasmic membrane protein XpsN with the outer membrane protein XpsD in the type II protein secretion apparatus of *Xanthomonas campestris* pv. campestris. J. Bacteriol. **182**, 1549–1557
17 Nunn, D. (1999) Bacterial type II protein export and pilus biogenesis: more than just homologies? Trends Cell Biol. **9**, 402–408
18 Sakai, D. and Komano, T. (2002) Genes required for plasmid R64 thin-pilus biogenesis: identification and localization of products of the *pilK*, *pilM*, *pilO*, *pilP*, *pilR*, and *pilT* genes. J. Bacteriol. **184**, 444–451
19 Keizer, D. W., Slupsky, C. M., Kalisiak, M., Campbell, A. P., Crump, M. P., Sastry, P. A., Hazes, B., Irvin, R. T. and Sykes, B. D. (2001) Structure of a pilin monomer from *Pseudomonas aeruginosa*. Implications for the assembly of pili. J. Biol. Chem. **276**, 24186–24193
20 Sauvonnet, N., Gounon, P. and Pugsley, A. P. (2000) PpdD type IV pilin of *Escherichia coli* K-12 can be assembled into pili in *Pseudomonas aeruginosa*. J. Bacteriol. **182**, 848–854
21 Martinez, A., Ostrovsky, P. and Nunn, D. N. (1998) Identification of an additional member of the secretin superfamily of proteins in *Pseudomonas aeruginosa* that is able to function in type II protein secretion. Mol. Microbiol. **28**, 1235–1246

Received 1 February 2002/3 April 2002; accepted 4 April 2002
Published as BJ Immediate Publication 4 April 2002, DOI 10.1042/BJ20020194