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Roles of the minor pseudopilins, XpsH, XpsI and XpsJ, in the formation of XpsG-containing pseudopilus in *Xanthomonas campestris* pv. campestris

Wei-Wen Kuo, Hung-Wei Kuo, Chun-Chia Cheng, Hsiang-Ling Lai & Ling-Yun Chen*
Institute of Biochemistry, Chung Shan Medical University, Taichung 402, Taiwan, Republic of China

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Summary

Due to their similarity to type IV pilus (Tfp) subunits, the pseudopilins, XpsG, -H, -I, -J and -K, have been predicted to form a pilus-like structure in the type II secretion (T2S) pathway. While overexpression of GspG can result in the formation of bundle structures, the functions of other pseudopilin are not known yet. In this study, we investigate the mutual interaction among the pseudopilins and characterize the specialized minor pseudopilin, XpsJ. By using gel filtration and Ni-NTA affinity chromatography, a linearly ordered interactive relationship is revealed among the four pseudopilins, XpsG–XpsI–XpsH–XpsJ. Notably, unlike the mutant XpsJ194 staying in the inner membrane, wild type XpsJ stayed in the outer membrane and blocked the extension of overexpressed XpsG to outside of the cell. By analogy with the Type I pilus structures, we hypothesize that the XpsH and XpsI might act as an adaptor to connect XpsJ with the major pseudopilin XpsG, and XpsJ might act as a tip to restrict the out-growth of XpsG in the pilus-like structure of the T2S pathway.

Introduction

Protein secretion is required for bacterial pathogenicity in delivering hydrolytic enzymes and toxins for host infection. In addition to the inner membrane (IM), Gram-negative bacteria face a challenge to permit proteins for crossing the outer membrane via specific terminal branches. In type II secretion (T2S) pathways, mature folded proteins are translocated into the extracellular milieu by a secretion apparatus composed of at least 12 gene products [1]. Among them, GspG, -H, -I, -J and -K proteins, were designated as pseudopilins due to their homology to type IV prepilin in the sequence G↓(F/M)XXXE followed by 15–20 hydrophobic amino acids at the N-terminus [2–4]. These pseudopilins and type IV pilins share not only the same N-terminal hydrolysis and methylation processes by prepilin peptidase [3], but also a similar hydrophobic N-terminal α-helix. In type IV pilus (Tfp), these structures enable the oligomerization of the pilin monomers [5, 6]. Furthermore, the findings of the N-terminal α-helix and four anti-parallel β-strands by X-ray crystallography in PulG lacking Tfp homologous region are similar to the structure of Tfp [7]. These common features suggest that they are both evolutionarily related and these pseudopilins may form a pilus-like structure to participate in protein secretion by linking the inner and outer membranes [8] or form a scaffold for the assembly of other components in the machinery [9].

The formation of a pilus-like pseudopilin structure has been documented in several studies. The relative abundance of GpsG compared to other pseudopilins indicates that it may be the major component [3]. The multimerization of PulG was shown through chemically cross-linking [10].

*To whom correspondence should be addressed. Fax +886-4-2324-8195; E-mail: chenly@csmu.edu.tw

In *Pseudomonas aeruginosa*, possessing both type II secretion and type IV piliation pathways, heterodimer formation of XcpT (GspG) with other pseudopilins was identified by cross-linking and affinity chromatography [11]. The first demonstration of pilus-like structure assembly by pseudopilins was revealed by Sauvonnet and colleagues who overexpressed the pullulanase T2S system of *Klebsiella oxytoca* in *E. coli* and observed that PulG was assembled into pilus-like bundles examined by immuno-gold labeling [12]. The failure of the defective expression of PulJ and PulK to affect the formation of surface exposed pilus structure composed of PulG suggests that they both are not required for piliation. On the other hand, the single short fibers formed in PulI-lacking bacteria suggest that PulI must perform an essential role in pilus formation. However, expression of *pul* genes from a chromosomal level did not result in observable surface-extension bundle [12]. Therefore, more experiments are needed to establish the roles of pseudopilins in pilus formation.

Recently, a closely related work [13] further investigated the relevance of this structure with the secretion and adhesion functions by overexpressing $XcpT_G$ found to assemble into a pilus-like structure, called a type II pseudopilus in *P. aeruginosa*. Particularly, this appendage was identified to increase the bacterial adherence capacity and reduce the bacterial exoprotein releasing ability [13]. Our previous study indicates the formation of a pilus-like structure spanning between the cytoplasmic and outer membrane in *Xanthomonas campestris* pv. campestris [14]. Remarkably, we also observed the interaction between XpsG and XpsH by Ni-NTA affinity chromatography. Although the biogenesis of secretion pilus is thought to be in a similar manner to the Tfp, however, in addition to the pilin subunit PilA found to be involved in the assembly of Tfp, the role of other minor pilins have not been established. Fortunately, studies of type I pili which is one of the most investigated pilus family, provide some clues to predict the minor pseudopilin functions in the T2S apparatus.

In this study, the roles of the minor pseudopilins in T2S pathways were investigated. Using gel filtration and Ni-NTA affinity chromatography, we observed that XpsG, -H, -I and -J can form a multiprotein complex, and XpsG and XpsJ may stay both sides of the complex with a linearly ordered interactive sequence, XpsG–XpsI–XpsH–XpsJ. It was also revealed that XpsJ, opposite to the inner membrane location of mutant XpsJ-194, stays in the outer membrane and blocked the extracellular distribution of overexpressed XpsG. We hypothesize that the minor pseudopilin XpsH and XpsI may act as adaptors and XpsJ as an adhesive tip within the pilus-like structure in theT2S pathway.

Materials and methods

Bacterial strains and plasmids

Xanthomonas campestris pv. campestris XC1701 was isolated as a spontaneously derived rifampicin-resistant mutant of the wild isolate XC17 [15]. All other mutants were derivatives of XC1701. The mutants of *X. campestris* pv. campestris used in this study were produced using procedures described previously [14]. XC1713, XC1717, XC1716 and XC1718 were mutated in the *xpsG, xpsH, xpsI and xpsJ*, respectively. Identical strategy and template were used for constructing Δ*xpsI* strain XC1716 and Δ*xpsJ* strain XC1718 [14]. Primers used for XC1716 and XC1718 generating upstream and downstream *Nco*I sites are mI-1: 5′-GACGCCAGCGCCATGGAAGCATCAGCGC-3′, mI-2: 5′-CCGCGTCGCAATGCCCATGGCGCCGGTAGAC-3′ and mJ-1: 5′-GCGGCGAAGCCATGGCGCAACGCAGCG-3′, mJ-2: 5′-GTGGCGGTGGAGCCATGGCGCAATGAGG-3′, respectively. As a result, amino acid residues 2–93 of XpsI and 44–209 of XpsJ were deleted.

The procedures used to produce plasmids encoding XpsG, XpsH, XpsI and XpsJ were also described previously [14]. *xpsI* and *xpsJ* were cloned into a vector pCPP30 represented as pFI and pFJ, respectively. Methods similar to those described in previous report [14] were used for constructing His_6-tagged *xpsG, xpsH, xpsI* and *xpsJ* genes. To construct His_6-tagged *xpsI and xpsJ*, the plasmid pFI and pFJ served as a template in the PCR. The 5′-primer PSB anneals with the *lac*promoter region, 5′-CCCAATACGCAAACCGCCTCT-3′. The 3′-primers are XpsI-3His: 5′-CACTTGCTCGAGCGCGGGGTTGCCCTGTGC-3′ and XpsJ-3His: 5′-CACTTGCTCGAGTTGCGCCACGGCTCCACC-3′. The PCR product was digested with *Hin*dIII and *Xho*I followed by ligation with a broad host range vector pSYP9, in which $(CAC)_6$-TGA

have been introduced in-frame downstream of a *Xho*I site. The resulting plasmids are designated as pFI-His and pFJ-His.

Construction of deletion mutants of xpsJ gene

The same procedures were used for constructing His_6-tagged *xpsG, xpsH, xpsI* and *xpsJ* genes [14]. The 3′-primers are J199his: 5′-CACTTGCTCGAGCGCCACCACCACCGGCGG-3′, J198his: 5′-CACTTGCTCGAGCACCACCACCGGCGGCCA-3′, J197his: 5′-CACTTGCTCGAGCACCACCGGCGGCCAGGC-3′, J196his: 5′-CACTTGCTCGAGCACCGGCGGCCAGGCCGA-3′, J194his: 5′-CACTTGCTCGAGCGGCGGCCAGGCCGAGCC-3′, J193his: 5′-CACTTGCTCGAGCCAGGCCGAGCCGCCGCT-3′ and J180his: 5′-CACTTGCTCGAGCGGCCAGGCCGAGCCGCC-3′. The PCR product was digested with *Hin*dIII and *Xho*I followed by ligation with vector pSYP9.

α-amylase activity assay

α-amylase activity was determined by measuring the reducing sugars released from soluble starch as modified by Somogyi [16]. *X. campestris* pv. campestris grown in XOL medium to exponential phase [attenuance (D_{600}) of approx. 1.0] were centrifuged, the supernatant was defined as enzyme solution. The reaction mixture containing 0.1 ml of enzyme solution, 0.1 ml of 1.5% soluble starch solution pH 7.0 incubated at 37 °C for 10 min. After this time the reaction was stopped by addition of 0.8 ml of stop solution (0.5 M acetic acid : 0.5 M HCl = 5:1). After 2 min, remove 0.1 ml of the reaction mixture from the test tube. Add this aliquot to iodine solution (4% KI 1.25% iodine), then add water to 1 ml. The amounts of reducing sugars released were determined by OD_{600} absorbance.

Preparation of antibodies against XpsI and XpsJ proteins

A 351-bp and 516-bp DNA fragments encoding amino acid residues 29–138 and 26–209 of the XpsI and XpsJ proteins were produced from PCR amplification. Primers used in PCR are PI-forward: 5′-CACTTGGGATCCTCCTTGTCCGGTGCGGCG-3′, PI-reverse: 5′-CACTTGAAGCTTCGCGGGGTTGCCCTGTGC-3′, PJ-forward: 5′-CACTTGGGATCCCTGGCCTTTGCCACGTTG-3′, PJ-reverse: 5′-CACTTGAAGCTTTCATTGCGCCACGGCTCC-3′. The PCR product was digested with *Bam*HI and *Hin*dIII, and subcloned into pET32a (Novagen) in-frame with the upstream *trxA* gene. The recombinant plasmid was examined with restriction enzyme digestion and confirmed with DNA sequencing data. The plasmid encoding the TrxA–XpsIHis$_6$ or T rxA–XpsJHis$_6$ protein was transformed into competent cells of *Escherichia coli* BL21(DE3). Overproduction of the fusion protein was accomplished by induction with 2 mM isopropyl β-D-thiogalactoside in Luria–Bertani broth. The fusion protein was purified upon specific binding with nickel nitrilotriacetic acid (Ni-NTA) under native condition, in accordance with the manufacturer's instructions (Qiagen). New Zealand White rabbits were immunized with the Ni-NTA purified TrxA–XpsIHis$_6$ or TrxA–XpsJHis$_6$. 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.

Isolation of Triton X-100 extract of membrane proteins

X. campestris pv. campestris grown in Luria–Bertani broth to exponential phase [attenuance (D_{600}) of approx. 1.0] were harvested, and the cells were broken by passing through a French press three times at 124,200 kPa (18,000 lbf/in^2). Unbroken cells were removed by centrifugation at 4300 × *g* for 10 min. Membrane vesicles, precipitated upon ultracentrifugation at 23,000 × *g* for 45 min, were extracted by incubating with buffer A [20 mM Tris–HCl, pH 8.0, 1 mM PMSF, 0.5 mM DTT, 0.2 M NaCl] at 4 °C for 30–60 min with gentle shaking. The Triton X-100 extract of membrane proteins was collected as the supernatant after ultracentrifugation at 23,000 × *g* for 30 min.

Size-exclusion FPLC

The procedures of Chen et al. [17] were followed. Briefly, Triton X-100 extracts of membrane proteins was chromatographed on a FPLC Superdex 200 HR column (Pharmacia Biotech Inc., 25-ml size) that was pre-equilibrated with buffer B

(20 mM Tris–HCl, pH 8.0, 0.2 M NaCl, 1% Triton X-100).

Nickel affinity chromatography

Triton X-100 extracts of membrane proteins were loaded on to a 1 ml Ni-NTA column (Qiagen), which had been pre-equilibrated with buffer B. The extracts were circulated several times at a low flow rate (0.33 ml/min). After the binding stage was completed, the column was washed with 20 vol. of wash buffer (buffer B plus 20 mM imidazole). Fractions (1 ml aliquots) eluted with elution buffer (buffer B plus 250 mM imidazole) were collected. Proteins collected in each fraction were precipitated with TCA at a final concentration of 10% (w/v) and analyzed by SDS-PAGE followed by immunoblotting.

Sucrose gradient sedimentation analysis

Procedures of Lee et al. [18] were followed.

Results

Specific recognition of XpsI and XpsJ with antisera raised against the truncated proteins purified from E. coli

To determine the XpsG, -H, -I and –J functions in *Xanthomonas campestris* pv. campestris, Δ*xpsG* mutant strain XC1713, Δ*xpsH* mutant strain XC1717, Δ*xpsI* mutant strain XC1716 and Δ*xpsJ* mutant strain XC1718 were prepared. The requirement of XpsG, -H, -I and -J for the α-amylase secretion was identified in complementation tests (data not shown). The antibody identifications of XpsG and XpsH were described in the previous study (Figure 1a, b) [14]. Here, antibodies were raised against XpsI and -J proteins produced from overexpressed plasmid pCPP30 in the strains XC1716 and XC1718, respectively. After immunoblot analysis, a distinct band observed in the strain of XC1716 harboring plasmid pFI or pFI-His (Figure 1c). The band recognized as the fusion protein XpsI-His_6 appeared with an upper shift from XpsI, suggesting the identification of XpsI protein. Similar results were also observed in experiments using Δ*xpsJ* strain XC1718 harboring plasmid pFJ or pFJ-His (Figure 1d). Unlike XpsG, XpsH, -I and -J were not detectable in the parental strain XC1701 (Figure 1). Thus XpsG seems to be a major pseudopilin while XpsH, -I and -J are minor pseudopilins.

Association of XpsH and XpsI with each other, as well as with the major pseudopilin XpsG and a third minor pseudopilin XpsJ

Heterodimers between the major pseudopilin $XcpT_G$ and the minor pseudopilin $XcpU_H$, $XcpV_I$ or $XcpW_J$ of *P. aeruginosa* have been demonstrated by crosslinking studies [11]. In addition, the interaction between the major pseudopilin XpsG and the minor pseudopilin XpsH was identified in our previous study by affinity chromatography [14]. Here, we examined the interactions among XpsG, -H, -I and -J using the same procedures. After Triton X-100 extraction, membrane proteins of the complemented strain

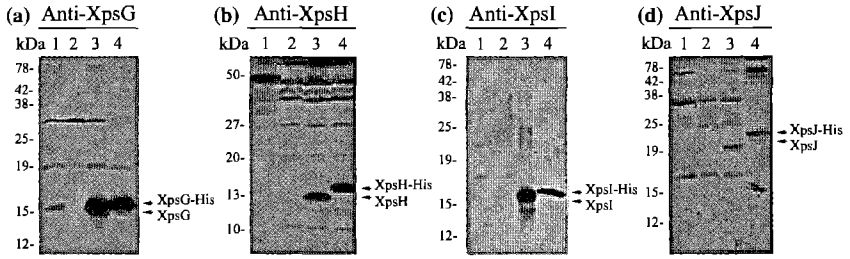

Figure 1. Identification of XpsG, -H, -I and -J antibodies in *Xanthomonas campestris* pv. campestris. Cells were cultured and grown to an OD_{600} of 1.0 at 30 °C. 200 μl volume was taken and centrifuged for 5 min followed by resuspending the pellet with 20 μl sample buffer. The cell lysate was further partial purified by Ni-NTA affinity chromatography in lane 3 and 4 of panel (c) and (d). Proteins were detected by SDS/PAGE and immunoblotting analysis. In each panel, lane 1 is wild type strain XC1701. Lane 2 in (a) is XC1713 (Δ*xpsG*); (b) is XC1717 (Δ*xpsH*); (c) is XC1716 (Δ*xpsI*); (d) is XC1718 (Δ*xpsJ*). Lane 3 in (a) is XC1713 harboring plasmid pFG (XC1713/pFG); (b) is XC1717/pFH; (c) is XpsI protein; (d) is XpsJ protein. Lane 4 in (a) is XC1713/pFG-His; (b) is XC1717/pFH-His; (c) is XpsI-His; (d) is XpsJ-His.

XC1717/pFH or XC1717/pFH-His was applied to the Ni-NTA affinity column and the eluted fractions were analyzed by immunoblotting with antisera against XpsG, -H, -I and -J. Despite their difference in signal intensity, both of the minor pseudopilin XpsI and XpsJ, as well as the major pseudopilin XpsG, co-eluted with the His_6-tagged XpsH (encoded by pFH-His), but not with the XpsH without His_6-tag (encoded by pFH, Figure 2). These observations imply the existence of a multiprotein complex comprising XpsG, -H, -I and -J.

Association between XpsH and XpsI not requiring XpsG, nor XpsJ

The association between XpsH and XpsI was clearly observed in previous experiment. They co-eluted with each other reciprocally, suggesting the likelihood of a direct association. To examine the accuracy of such a prediction, we introduced the plasmids encoding the XpsH, with or without His_6-tag, into XC1713 (Δ*xpsG*) and XC1718 (Δ*xpsJ*), and analyzed the elution profiles from the Ni-NTA affinity column. Immoblotting with antisera against XpsH and XpsI revealed that both stayed together (Figure 3), agreeing with the proposition that the association between XpsH and XpsI is independent of XpsG and XpsJ. We also examined the influence of *xpsG* knockout mutation on the association of XpsJ with the His_6-tagged XpsH, and the influence of *xpsJ* mutation on co-elution of XpsG with the His_6-tagged XpsH. The results revealed that neither mutation affect the association of the other one with XpsH. These results indicated that XpsG and XpsJ is likely to exist at the two ends of the complex composed of XpsG, -H, -I and -J.

Requirement of XpsI for the association between XpsH and XpsG, not for the association between XpsH and XpsJ

As observed in previous experiment, XpsG and XpsJ are independent of each other in their association with XpsH. Since XpsH associates with XpsI directly, it prompted us to ask if XpsG, or XpsJ, also associates directly with XpsH. To solve the problem, we made use of the Δ*xpsI* mutant XC1716, into which we introduced the plasmids encoding XpsH, with or without the His_6-tag. We then examined if XpsG or XpsJ co-eluted with the His_6-tagged XpsH in the absence of XpsI. As observed in Figure 4, only XpsJ, but not XpsG, co-eluted with the His_6-tagged XpsH, suggesting that XpsI is required for the association between XpsH and XpsG, but not for the association between XpsH and XpsJ. Taken together with the observation that the association between XpsH and XpsJ is independent of XpsG (Figure 3a), the results implicated a direct association between XpsH and XpsJ. However, the

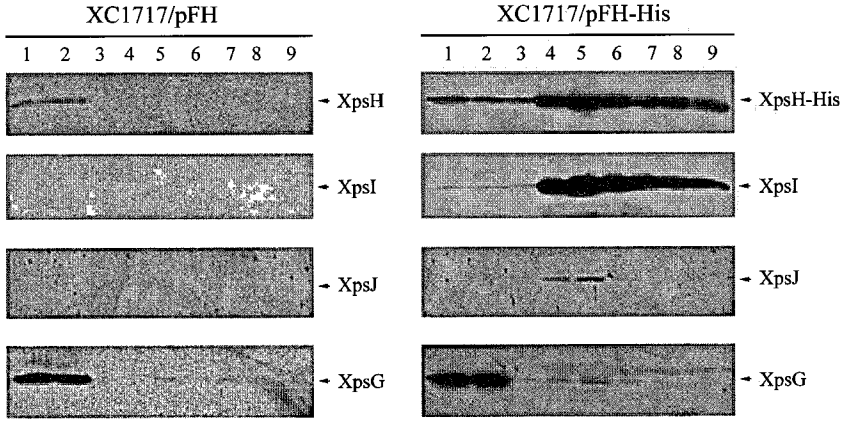

Figure 2. Metal chelating chromatography analysis of the membrane extracts from complemented strain XC1717 overexpressing XpsH or XpsH-His. French press-broken cells, XC1717/pFH (left panel) and XC1717/pFH-His (right panel) were ultracentrifuged at 56,000 rev/min for 1 h, and the collected pellet was resuspended in buffer containing Triton X-100. The supernatant collected after second ultracentrifugation at 56,000 rev/min was membrane extract and taken for Ni-NTA affinity chromatography analysis. Lanes 1, total proteins of membrane extracts; lanes 2, column flow-through; lanes 3, final washed fractions; lane 4–9, eluted fractions. All samples were analyzed by SDS/PAGE and immunoblotting with antibodies raised against the proteins as indicated.

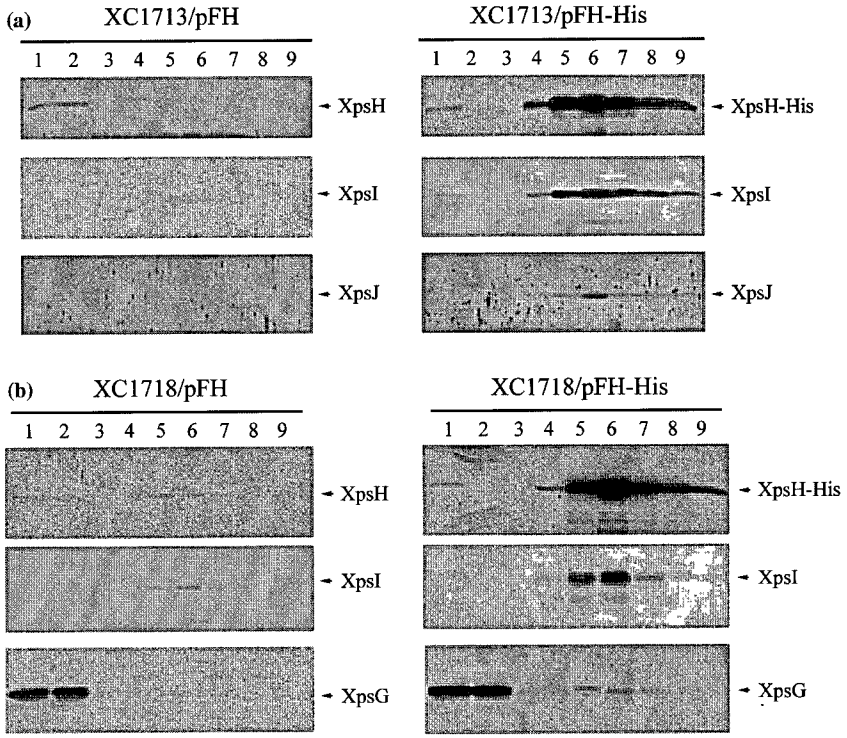

Figure 3. Metal chelating chromatography analysis of the membrane extracts from mutant strains XC1713 or XC1718 harboring plasmid pFH or pFH-His. The membrane extracts of cells, (a) XC1713/pFH (left panel) and XC1713/pFH-His (right panel), (b) XC1718/pFH (left panel) and XC1718/pFH-His (right panel), were analyzed using Ni-NTA affinity chromatography as described in Figure 2 legend. All the fractions were analyzed using SDS/PAGE and immunoblotting. The representation of each lane is the same as described in Figure 2 legend.

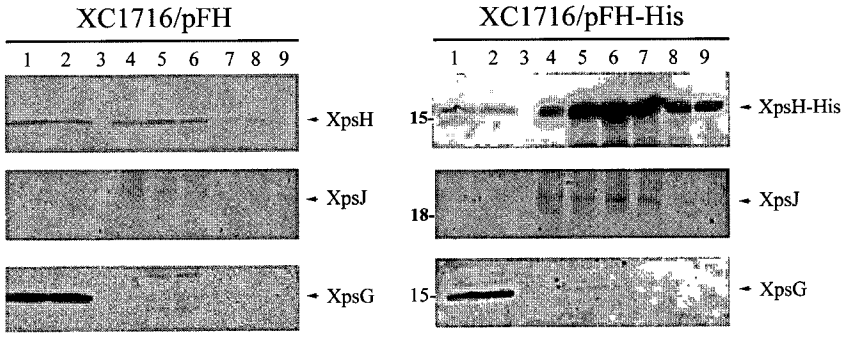

Figure 4. Metal chelating chromatography analysis of the membrane extracts from XC1716 (Δ*xpsI*) containing plasmid pFH or pFH-His. The membrane extracts of XC1716 (Δ*xpsI*) containing plasmid pFH or pFH-His were prepared for Ni-NTA affinity chromatography analysis and each lane represents as described in Figure 2 legend. Left and right panels, immunoblots detected by the antibodies against the proteins as indicated.

interaction of XpsH and XpsJ appeared a weak reaction (Figure 3a); therefore, it is suggested that some unknown proteins may involve in the association of XpsH and XpsJ. On the other hand, the association between XpsH and XpsG requires the presence of XpsI.

XpsG–XpsI–XpsH–XpsJ is the possible sequential order observed in the complex formed by pseudopilins

XpsH was demonstrated to associate directly with XpsI and with XpsJ. Does XpsH mediate the

association between XpsI and XpsJ? By introducing the plasmid encoding the His_6-tagged XpsI, or the His_6-tagged XpsJ, into XC1717 (Δ*xpsH*), we were able to determine if the association between XpsI and XpsJ require XpsH. In neither case, co-elution between XpsI and XpsJ could be detected (Figure 5a, b). When pFH-His was transformed into XC1717 (Δ*xpsH*), the coelution of XpsH with both XpsI and XpsJ can be reversed (Figure 2). This indicates that XpsI and XpsJ could not associate without the presence of XpsH.

To understand if XpsH also mediates the association of the major pseudopilin XpsG with the other two minor pseudopilins XpsI and XpsJ, we examined if lack of XpsH affects the association of XpsG with XpsI or XpsJ. We introduced the plasmids encoding XpsG, with or without the His_6-tag into XC1717 (Δ*xpsH*). Examination of the elution profiles from Ni-NTA affinity column

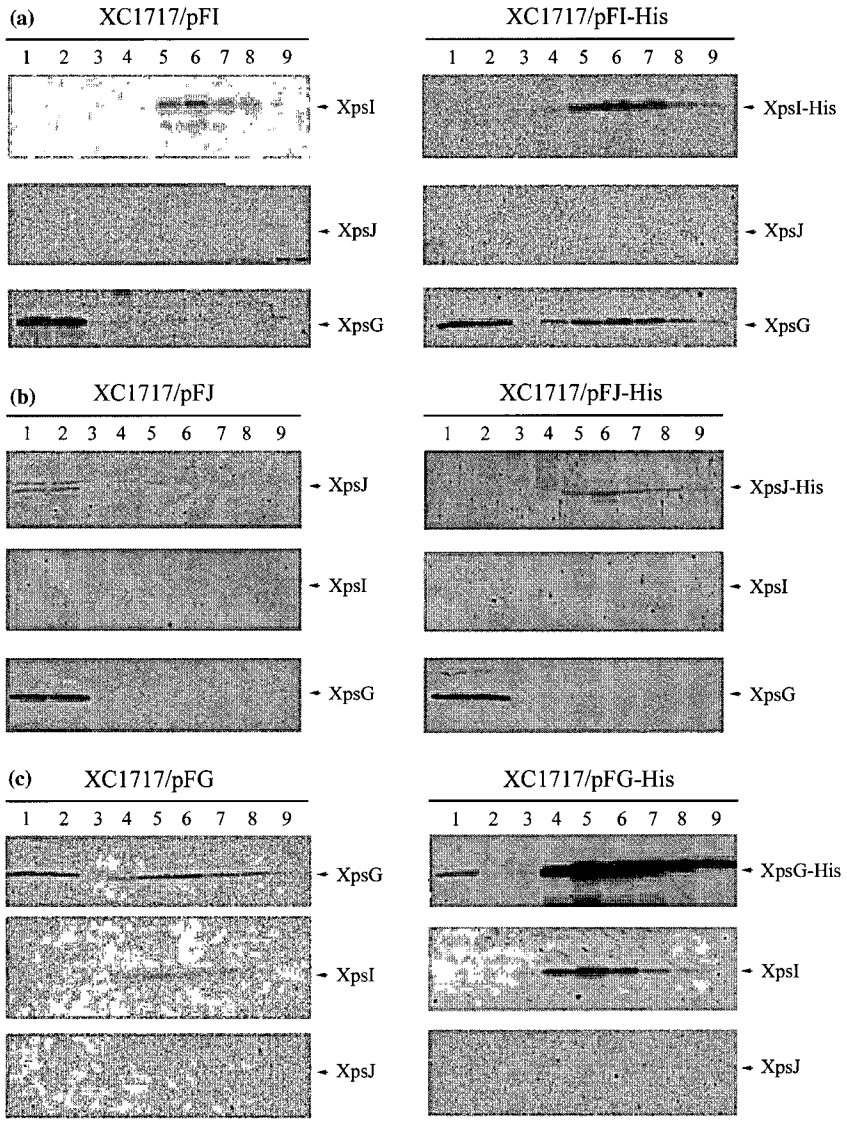

Figure 5. Metal chelating chromatography of the membrane extracts from strains XC1717 overexpressing XpsG, -I or -J as well as their His-tagged fusion proteins. The membrane extracts of XC1717 (Δ*xpsH*) containing plasmid (a) pFI or pFI-His, (b) pFJ or pFJ-His, (c) pFG or pFG-His, were prepared for Ni-NTA affinity chromatography analysis as described in Figure 2 legend. All the samples were analyzed by SDS/PAGE and immunoblotting. The representation of each lane is the same as described in Figure 2 legend.

revealed co-elution of XpsI, but not XpsJ, with the His_6-tagged XpsG (Figure 5c), suggesting that the association between XpsG and XpsI is possible without XpsH. In contrast, the association between XpsG and XpsJ appears to rely on the presence of XpsH. In agreement, XpsG co-eluted with the His_6-tagged XpsI in the absence of XpsH (Figure 5a). Taken together with the results of the requirement of XpsI for the association between XpsG and XpsH (Figure 4), and the requirement of XpsH for the association between XpsJ and XpsI, a linearly ordered interactive relationship is revealed among the four pseudopilins as follows, XpsG–XpsI–XpsH–XpsJ.

To further scrutinize the proposed scheme, we examined the requirement of XpsH for the association between XpsJ and XpsG by using a different approach. Triton X-100 extract of the membrane proteins was isolated from XC1718 (*ΔxpsJ*) complemented with a plasmid (pFJ-His) encoding the His_6-tagged XpsJ. Gel filtration chromatography followed by immunoblotting with antisera against XpsJ, XpsH or XpsG revealed two different elution profiles (Figure 6a). To our surprise, the His6-tagged XpsJ and XpsG appeared with a major peak in fractions 22–24, where hardly any XpsH was detected. On the contrary, XpsH was mainly distributed between fractions 18–21, where only small amounts of the His_6-tagged XpsJ and XpsG were detectable. These observations seem contradictory to the proposed scheme. To find out why, we collected the fractions 18–21 and fractions 22–25 as separate pools and analyzed each on Ni-NTA affinity columns. As shown in Figure 6b, appearance of XpsG, along with nickel-bound-XpsJ-His_6, in the eluted fractions was observed only in the pooled fractions 18–21, where XpsH was present (Figure 5b). In contrast, XpsG in fractions 22–25 did not co-elute with the His6-tagged XpsJ, despite the presence of minute amounts of XpsH in the eluted fractions from the nickel column. These results agree with the proposition that the possible sequence of these four pseudopilins in the complex could be XpsG–XpsI–XpsH–XpsJ.

Membrane location of XpsG, -H and -J proteins

The spanning of pilus-like structures between cytoplasmic and outer membranes of endogenous XpsG was described in our previous study [14]. The membrane locations of other pseudopilins were analyzed in XC1717/pFH , and XC1718/pFJ using sucrose gradient sedimentation. The results showed that the distribution profile of XpsH was similar to that of XpsG, which co-fractionated with outer membrane marker XpsD in fraction 26–28 and with inner membrane marker XpsN in fraction 8–12 (Figure 7a). The XpsJ appeared only in fraction 25–28 (Figure 7b) which was the distribution of the outer membrane. These results revealed that XpsH stays in both outer and inner membranes. However, XpsJ mainly stays in the outer membrane.

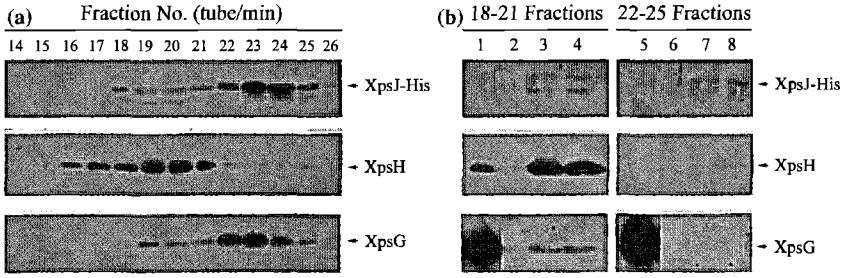

Figure 6. Gel-filtration and metal chelating chromatography analysis of the partial purified membrane extracts from XC1718 (*ΔxpJ*) containing plasmid pFJ-His. The membrane extracts of XC1718 (*ΔxpJ*) containing plasmid pFJ-His were prepared as described in Figure 2 legend. (a) The samples were taken for gel-filtration chromatography analysis on a Superdex HR-200 column (Pharmacia, 25 ml). The fractions were collected with a flow rate of 0.5 ml/min, and fractions 14–26 were collected for analysis of SDS-PAGE and immunoblotting. The column was calibrated with the following molecular mass standards: thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). (b) The fractions collected from the gel-filtration chromatography in panel (a) were pooled from 18 to 21 (left panel) and from 22 to 25 (right panel), followed by chromatographic analysis of Ni-NTA affinity column. All samples were analyzed by SDS/PAGE and immunoblotting. Lane 1 and 5 are total proteins of membrane extracts, lane 2 and 6 are the final washed fractions, lane 3, 7 and lane 4, 8 are eluted fractions.

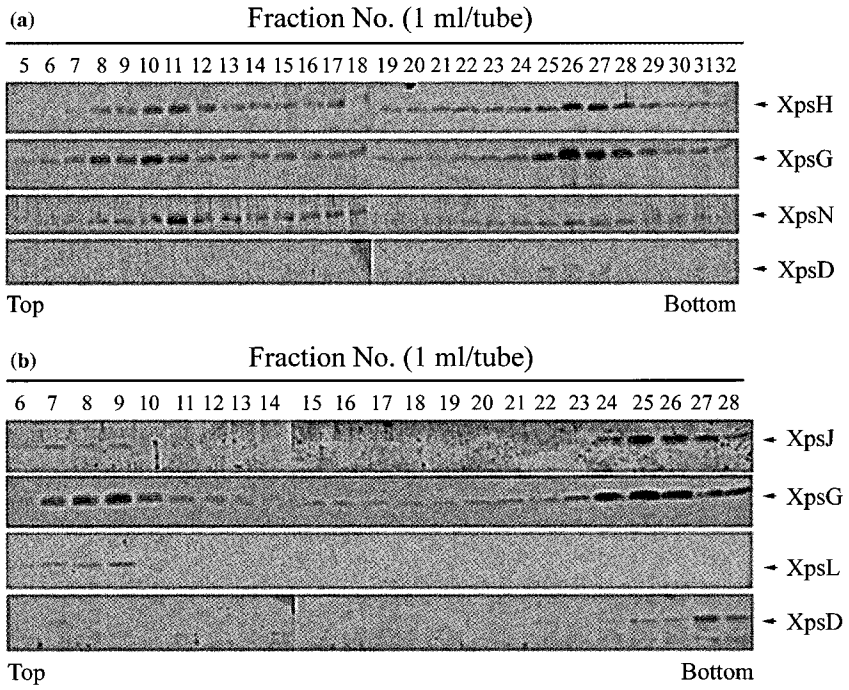

Figure 7. Sucrose gradient sedimentation analysis of XpsG, -H and -J proteins. The membrane extracts of cells, (a) XC1717/pFH, and (b) XC1718/pFJ were analyzed on a 25 to 63% (wt/wt) sucrose gradient. The fractions collected from top to bottom were analyzed for immunoblotting. The inner membrane marker XpsN is shown in fractions 8–13, XpsL in fractions 7–9 and the outer membrane marker XpsD in fractions 25–27 or 25–28.

Characterization of XpsJ mutant proteins

If XpsJ is only localized in outer membrane; the question raised is what the important functions of XpsJ are. To address this issue, XC1718 harboring various COOH-terminal deletion mutants of XpsJ were generated and their ability to secrete α-amylase was assessed. Results showed that the activity decreased from 100 to 6%, when the deleted amino acids increased, indicating the secretion function was lost by COOH- terminal deletion of XpsJ protein (Figure 8a, b). In order to determine whether the abnormal secretion function of mutants was due to the protein stability, we performed immunoblot analysis with antibody against XpsJ on total cell extracts and partially purified proteins. Although most of the data from total cell extracts were negative, positive results of Ni-NTA affinity chromatography analysis were shown in stain XC1718 carrying genes of full-length as well as mutants, XpsJ-194his and Xps-J-196his (Figure 8a), demonstrating the protein stability was not affected by the deletion mutation. These findings exclude the possibility that the disappearance of α -amylase activity was resulted from the instability of XpsJ protein. The membrane location of XpsJ-194his was analyzed using sucrose gradient sedimentation. The detection of XpsJ-194 his in fraction 8–12 indicated its localization was in the cytoplasmic-membrane (Figure 8c). This result is opposite to the location of full-length XpsJ mainly staying in the outer membrane, indicating the abnormal secretion function of XpsJ-194 his is at least partially due to the different membrane location of XpsJ-194 his from full-length XpsJ.

Effects of XpsH or XpsJ on the subcellular distribution of overexpressed XpsG

Under physiological conditions, XpsG stays inside the cell. However, the appearance of overexpressed XpsG in the extracellular fraction in XC1713 was identified in our previous work (14). Based on the results of XpsJ staying mainly in outer membrane, we want to know whether highly produced XpsJ could reduce the extracellular form of XpsG which was produced from overexpressed XpsG.

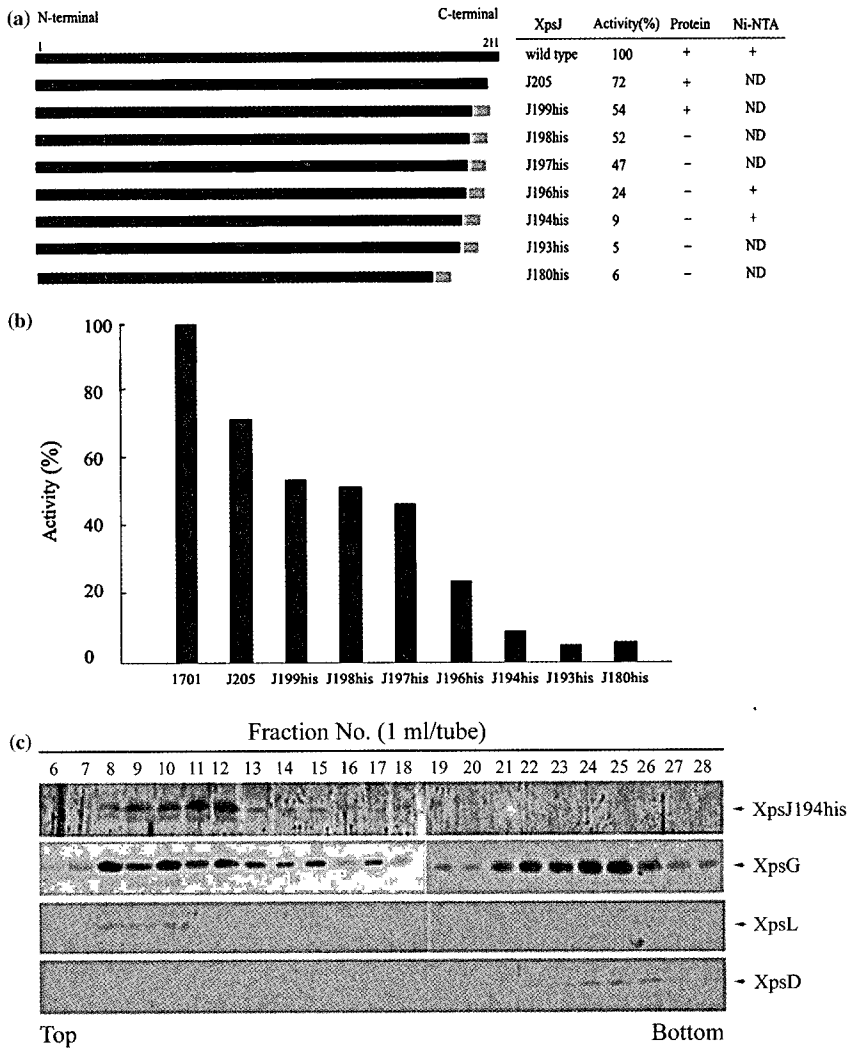

Figure 8. Function and stability of truncated XpsJ in XC1718 (Δ*xpsJ*). (a) Summary of the XpsJ truncated mutant proteins in their secretional function and protein stability. Black bars represent the wild type XpsJ protein including 211 amino acids, or truncated XpsJ proteins with different number of amino acids. Gray bars indicate the His_6-tag fused to the C-terminal of truncated proteins. The extracellular α-amylase activity of wild type XpsJ is defined as 100%, and the others are relative to that of wild type XpsJ. "+" indicates proteins which can be detected by immunoblotting. "−" indicates proteins which can not be detected. "ND" designated as not determined. (b) The relative secretion ability of α-amylase. Extracellular medium were incubated with starch solution buffer, and developed color by the addition of iodine solution, sample was diluted by distillated water for the spectrophotometric analysis at 600 nm. (c) Sucrose-gradient sedimentation analysis of mutant XpsJ-194his and XpsG proteins. The membrane extracts of the cells XC1718/pFJ-194his were analyzed by sucrose gradient sedimentation as described in Figure 7 legend.

As shown in Figure 9, when the cells were grown at OD_{600} 0.3 or 1.0, compared to XC1713 (Δ*xpsG*) overexpressing XpsG, the increasing level of XpsG in the extracellular fraction was reduced with overexpressed XpsH or XpsJ, and the reduction of the latter is more than that of the former. Additionally, the intracellular amount of XpsG was maintained at the same level in each cell strain (Figure 9). These results indicate that highly expressed XpsH and XpsJ could affect the subcellular distribution of overexpressed XpsG.

Discussion

In this study, XpsG showing a remarkable band on immunoblots is a major pseudopilin and XpsH, -I and -J, which were not detectable when

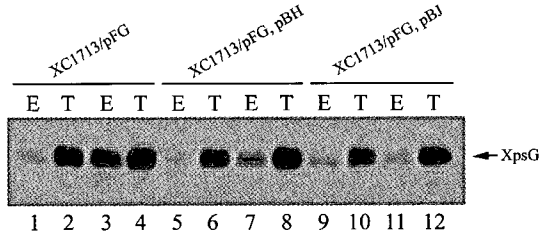

Figure 9. Immunoblot analysis of localization of XpsG protein in complemented strain XC1713/pFG with overexpression of XpsH orXpsJ. XC1713 (Δ*xpsG*) containing plasmid pFG, pBH/pFG or pBJ/pFG, were collected and divided into extracellular fraction (E) and cellular fraction (T) after centrifugation. An equivalent amount of E and T were analyzed by SDS/PAGE and immunoblotting with antibody against XpsG. Lane 1, 2, 5, 6, 9 and 10 represent the cells grown to an OD_{600} of 0.3. Lane 3, 4, 7, 8, 11 and 12 indicate the cells grown to an OD_{600} of 1.0.

expressed at the chromosomal level, are minor pseudopilins. This is consistent with the results of Nunn and Lory's study [3], indicating the relative abundance of $XcpT_G$, $XcpU_H$, $XcpV_I$, and $XcpW_J$ are approximately 16:1:1:4, respectively, in the immunoblotting results. Our recent report [14] indicated that XpsG could form a pilus-like structure spanning the inner and outer membranes. Additionally, typical Tfp is composed of the major pilin, pilA, and several minor pilin proteins, PilE, PilV, PilW, PilX, FimT and FimU. These minor proteins possess the characteristic prepilin peptidase-dependent NH_2-terminal motif similar to PilA and are required for the pilus biogenesis [19]. Therefore, we suggest that major pseudopilin XpsG forms the main body of the pilus-like structure and minor pseudopilins XpsH, -I, -J are requisite for this structure.

Type I pili, produced from uropathogenic *E. coli* and assembled via the highly conserved chaperone-usher pathway, are adhesive organelles in mediating attachment to the epithetical cells of hosts during infection [20]. The pili mediate binding to the host receptor via adhesins. Actually, fully assembled type I pili are composed of the tip fibrillum and rigid pilus rod with a heteropolymeric structure. The rod in the pili is made up of repeating major FimA subunits. The flexible fiber tip extending from the distal end of the rod is composed of FimH which acts as an adhisin to mediate interaction with the host cells [21]. FimG and FimF are two adaptors that connect adhesin FimH to the FimA rod with the sequential order FimA–FimF–FimG–FimH [22]. By analogy with the location of each subunit in type I pili [22], the pseudopilin protein complex with the linearly ordered interactive sequence, XpsG–XpsI–XpsH–XpsJ identified by Ni-NTA chromatographic analysis, coupled with the characterization of XpsJ as an outer membrane protein, implies the possible tip role of these minor pseudopilins in the pilus-like structure, which contains XpsG as the main body of pilus rod.

If the possible role of these minor pseudopilins is an adhesive tip of the pseudopilus, the question raised is what the purpose of the tip existence is. The pili, such as type I and Tfp, are adhesive organelles essential for binding to host receptors and subsequent colonization of host cells for infection. Between both of them, Tfp components present the highly structural homology to pseudopilins. Very recently, an important review article [23] about type IV pilus structure and pathogenicity mentioned that the pili use PilC, a 110-kDa adhesin protein localized to the pilus tip, and PilV, a ~14-kDa protein sharing homology with another adhesin protein in type IV pili, for adherence in the initial stage of infection. Both of the minor subunits directly interact with CD46 which was thought to be the transmembrane glycoprotein as the pilus receptor present in all human cells. In addition to the adhesion, both proteins are also required for pilus biogenesis. Therefore, these observations agree with our hypothesis that the minor pseudopilins probably play an important role of adhesion tip in the pilus-like structure in T2S.

Furthermore, the biogenesis of Tfp is processed by assembly complex proteins that are located either in the inner membrane, outer membrane or periplasm [24]. Through the biogenic interaction of these proteins, Tfp grows from the inner membrane to extrude through the cell surface. However, how the pili cross the outer membrane and how its elongation is controlled are still not clear. For the pseudopilus, it is probably too few to grow out of the outer membrane [13, 14, 25]. Since the main function of these pseudopilins is protein secretion and the prolonged pilus may occupy the gate composed of D protein [25], therefore, the length of the pilus must be monitored. Indeed, we observed that XpsJ was present in the outer membrane, and the extension of the pseudopilus formed by overexpressed XpsG beyond the cell surface was prohibited by XpsJ overexpression.

Moreover, in the study of Sauvonnet et al. [12], using *E. coli* K-12 cells carrying the 15 genes encoding the pullulanase secretion of *K. oxytoca* showed that pilus-like structure labeled by PulG antibody on the surface was still able to be observed by EM in the *pulJ*$^-$ and *pulK*$^-$ mutants, but the surface-exposed pilus was not detectable when most of other genes were missing. Based on these findings, we conjecture that XpsJ probably acts as an adhesive tip to control the growth of the pseudopilus formed by XpsG. This idea is supported by the decreased release of Xcp-dependent exoprotein extracellular elastase (LasB), when the XcpT pseudopilin was overexpressed to form the pseudopilus in *P. aeruginosa* [13]. It was also proposed that this extended pseudopilus might cross the outer membrane via XcpQ secretin to interfere protein secretion through the same channel at the same time [13]. Therefore, the maintenance of the normal pseudopilus length is probably necessary for pseudopilin to secrete proteins.

We have demonstrated the possible roles of pseudopilins in the formation of pseudopilus. Indeed, whether additional pseudopilins, such as XpsK that can be incorporated into the XpsG-containing pseudopilus is unknown, although the structure and processing of XcpX (GspK family) was discovered [26]. Additionally, the possible involvement of other T2S non-pseudopilin components (i.e. XpsE, XpsF, and XpsLMN) can not be ignored. Furthermore, under the condition of overexpressing 15 pullulanase secreton genes of *K. oxytoca*, defective expression of PulI and PulD did not result in observable pili on the surface of *E. coli* K-12 cells [12]. Our laboratory demonstrated that in spite of the lack of *xpsD* and *xpsI* genes, XpsG can still be detected in the extracellular fraction when XpsG was overexpressed in *X. campestris* pv campestris (data not shown). These findings give us a hint about the specialized function they have. More studies are needed to investigate the roles they play on the assembly of type II pseudopilus and the protein secretion machinery.

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