A reversibly dissociable ternary complex formed by XpsL, XpsM and XpsN of the *Xanthomonas campestris* pv. *campestris* type II secretion apparatus

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The cytoplasmic membrane proteins XpsL, XpsM and XpsN are components required for type II secretion in *Xanthomonas campestris* pv. *campestris*. We performed metal-chelating chromatography to partially purify the His₆-tagged XpsM (XpsMh)-containing complex. Immunoblot analysis revealed that both XpsL and XpsN co-eluted with XpsMh. The co-fractionated XpsL and XpsN proteins co-immune precipitated with each other, suggesting the existence of an XpsL–XpsM–XpsN complex. Ternary complex formation does not require other Xps protein components of the type II secretion apparatus. Further purification upon size-exclusion chromatography revealed that XpsN is prone to dissociate from the complex. Reassociation of XpsN with the XpsL–XpsMh complex immobilized on a nickel

column is more effective than with XpsMh alone. Membrane-mixing experiments suggested that the XpsL–XpsMh complex and XpsN probably dissociate and reassociate in the membrane vesicles. Comparison of the half-lives of the XpsL–XpsMh–XpsN and XpsL–XpsMh complexes revealed that XpsL dissociates from the latter at a faster rate than from the former. Dissociation and reassociation between XpsL and XpsM were also demonstrated with membrane-mixing experiments. A dynamic model is proposed for the XpsL–XpsM–XpsN complex.

Key words: dynamic complex, general secretory pathway, type II secretion.

INTRODUCTION

The type II secretion pathway, also known as the general secretory pathway (GSP) [1], is widely utilized by microbial pathogens to secrete toxins or hydrolytic enzymes to the milieu [2–5]. The process is completed in two steps. The substrate proteins with an N-terminal signal peptide are initially directed to, and exported through, the Sec apparatus located in the cytoplasmic membrane, to reach the periplasm (the space between the cytoplasmic membrane and outer membrane). Subsequently the substrate protein, now lacking the signal peptide, is secreted across the outer membrane through a type II secretion apparatus constituted of 12–15 proteins [2,5]. Intriguingly, most of the components of the apparatus are located in the cytoplasmic membrane. The best characterized protein, GspO, is responsible for cleavage and methylation at the N-terminus of five other components, GspG, GspH, GspI, GspJ and GspK [6,7]. These five proteins share sequence similarity with the type IV prepilin, the processed form of which is a constituent of the type IV pilus, so they are termed pseudopilins [1]. They have been proposed to form a pilus-like structure across the envelope and serve as a piston or guide for the delivery of secreted proteins through the pore located in the outer membrane [5,8]. A pilus-like structure containing PulG of *Klebsiella oxytoca* has recently been demonstrated on the surface of *Escherichia coli* which were over-expressing all the Pul components from plasmid-encoded genes [9].

In addition to GspO and the pseudopilins, five other cytoplasmic membrane proteins have been identified in the type II secretion apparatus. Complex formation has been demonstrated, by co-immune precipitation, for the GspL and GspM pair in several cases [10–12], the XpsL and XpsN and the XpsM and

XpsN pairs in *X. campestris* pv. *campestris* [12], and the OutL and OutF pair in *Erwinia chrysanthemi* [13]. Furthermore, the abundance of *K. oxytoca* PulL was significantly reduced when the *pulC* or the *pulM* gene was mutated [11]. This suggested possible complex formation between PulL and PulC or PulM proteins. However, whether these cytoplasmic membrane proteins form a single complex has not yet been resolved.

Besides the pseudopilins and the cytoplasmic membrane proteins, other protein components, localized in different compartments, are required for type II secretion. GspD, which belongs to a protein family termed secretins that includes proteins required for type III protein secretion, type IV pilus assembly and filamentous phage morphogenesis, forms a homo-multimeric complex in the outer membrane [14–17]. Electron microscopic analysis of purified secretins revealed ring-like structures with inner diameters of 76–95 Å (1 Å = 0.1 nm), a pore size large enough to allow through folded proteins or assembled phage [18–20]. Conversely, a passage of this size presumably requires a strict gating mechanism to prevent material from leaking out of the cells. Interactive partners of the secretins include secreted substrate and the cytoplasmic membrane proteins GspC and XpsN [17,21–23]. Any one, or combinations of them, could be responsible for gating the pore opening. Ion-conducting activity has also been shown for secretins in planar lipid bilayers [20,24,25]. A second outer membrane protein GspS, required for correct localization and assembly of secretins in two cases, was also demonstrated to be a minor component of the pore [16,17,20,26].

The only cytoplasmic protein component, GspE, was shown to associate with the cytoplasmic membrane through its interaction with GspL protein [11,27,28]. A nucleotide-binding motif that is conserved in all GspE homologues was shown to be

Abbreviations used: ECL[®], enhanced chemiluminescence; Ni-NTA, nickel-nitriloacetic acid; TCA, trichloroacetic acid; XpsMh, His₆-tagged XpsM protein.

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essential by mutational analysis [27,29–31]. The purified EpsE protein of *Vibrio cholerae* exhibits autokinase activity [27]. Assays for ATPase activity, however, have so far been unsuccessful.

In contrast with the secretins and the GspE homologues, the function of the cytoplasmic membrane protein components is not so well defined. A TonB-like energy transducer role, to provide energy for opening the pore, has been proposed for the EpsL and EpsM pair of *V. cholerae*, XcpP (C homologue) of *Pseudomonas aeruginosa*, and XpsL, XpsM and XpsN proteins of *X. campestris* pv. *campestris* [10,12,22]. However, a gating or a signalling role would also agree with current knowledge of these proteins. In this study, by carrying out metal chelating chromatography followed by a co-immunoprecipitation experiment, we demonstrated clearly that the XpsL, XpsM and XpsN proteins form a ternary complex. Further experiments revealed that the complex is reversibly dissociable in a hierarchical fashion.

MATERIALS AND METHODS

Bacterial strains and plasmids

Both mutants of *X. campestris* pv. *campestris* used in this study are as described previously; XC1714 was mutated in the *xpsM* gene [12] and XC17433 was deleted for the entire *xps* gene cluster [32]. The plasmids encoding XpsM alone (pST109), XpsN alone (pNC2), and XpsL, XpsM and XpsN together (pCPP-LMN), were also as described previously [12,23]. The plasmid pST116 encoding His₆-tagged XpsM (XpsMh) was constructed in two steps. Site-directed mutagenesis was employed to replace an *Mlu*I site upstream of the *xpsM* gene with a *Hind*III site and to introduce an *Xho*I site immediately upstream of the stop codon of the *xpsM* gene. The *Hind*III–*Xho*I fragment containing the entire *xpsM* gene was then cloned into a broad-host-range vector, in which the sequence encoding a C-terminal His₆ tag had been introduced. The plasmid pRT40 encoding XpsL and XpsMh was derived from pST116 in two steps. The *xpsMh* gene, excised from pST116 with *Xba*I and *Eco*RI, was cloned into a broad-host-range vector pBBR1MCS-5 [33], giving rise to pJRHism. The partial *xpsL* gene located upstream of the *xpsMh* gene in pJRHism was then replaced with a *Hind*III–*Mfe*I fragment, resulting in a complete *xpsL* gene upstream of the *xpsMh* gene.

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 124200 kPa (18000 lbf/in²). Unbroken cells were removed by centrifugation at 4300 *g* for 10 min. Membrane vesicles, precipitated upon ultracentrifugation at 23000 *g* for 45 min, were extracted by incubating with buffer A [20 mM phosphate buffer, pH 7.4, 2% (v/v) Triton X-100, 150 mM NaCl, 20% (v/v) glycerol and 1 mM PMSF] 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 23000 *g* for 30 min.

Nickel affinity chromatography

Triton X-100 extracts of membrane proteins were loaded on to a nickel-nitriloacetic acid (Ni-NTA) column (Qiagen), which had been pre-equilibrated with buffer B [20 mM phosphate buffer, pH 7.4, 0.5% Triton X-100, 300 mM NaCl, 10% (v/v) glycerol and 1 mM PMSF]. 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. All procedures were performed at 4 °C.

Co-immunoprecipitation

The procedures of Lee et al. [23] were followed with slight modifications. Fractions eluted from the Ni-NTA column were pooled and divided into four parts. Into each, antiserum against the XpsL or XpsN or their pre-immune sera was added and incubated at 4 °C for 3 h with gentle shaking. The mixtures were then incubated with Protein A–Sepharose CL-4B at 4 °C for 1 h followed by five washes with buffer D [20 mM phosphate buffer, pH 8.0, 0.5% Triton X-100, 150 mM NaCl, 10% (v/v) glycerol and 1 mM PMSF].

Size-exclusion FPLC

The procedures of Chen et al. [15] were followed, with the exception that the column was pre-equilibrated with buffer D.

Pull-down experiment

Membrane extracts prepared from XC17433(pRT40) or XC17433(pST116) were loaded on Ni-NTA columns. The immobilized XpsL–XpsMh or XpsMh was washed thoroughly with 20 vol. of wash buffer, followed by 2 vol. of buffer B, before equivalent amounts of the membrane extract prepared from XC17433(pNC2) were passed through seven times at a flow rate of approx. 0.33 ml/min. The column was then washed (flow rate of 0.5 ml/min) with 20 vol. of wash buffer followed by elution with 2.5 vol. of elution buffer. Except for the flow-through and the first wash fractions, which were loaded at 1 × concentration, all other samples were concentrated 5 × by trichloroacetic acid (TCA) precipitation and analysed by SDS/PAGE followed by immunoblotting.

Membrane-mixing experiment

To mimic the protein–protein interactions of membrane proteins *in vivo*, we designed a so-called ‘membrane-mixing experiment’. It was hypothesized that by mixing two strains of bacteria before cell breakage, we might be able to generate ‘mixed’ membrane vesicles containing membrane proteins from both strains. The appropriate two strains were mixed before harvest. Following cell breakage and ultracentrifugation as described above, the precipitated membrane vesicles were suspended in 0.05 × culture volume of buffer E (20 mM phosphate buffer, pH 7.4, and 1 mM PMSF). To increase the opportunity for the membrane proteins to interact with each other in the ‘mixed’ membrane vesicles, we kept them incubated at 4 °C for 3 h with gentle shaking. Subsequently, Triton X-100, NaCl and glycerol were added to final concentrations of 2%, 150 mM and 20%, respectively, in 0.1 culture volume and incubated at 4 °C for another 30 min. After ultracentrifugation at 23000 *g* for 30 min, the supernatant was collected as the membrane extract and loaded on a Ni-NTA column. In the control experiment named ‘mixing after extraction’, the same steps described above were followed for preparing individual Triton X-100 extract of each strain. Subsequent to solubilization, the separately prepared extracts were mixed and incubated at 4 °C for 30 min followed by nickel affinity chromatography.

Complex-dissociation assay

The XpsMh-containing complex was immobilized on Ni-NTA resin by batch binding at 4 °C for 30 min, followed by column packing and recirculating the column four times with membrane extract. The column was then washed with 20 vol. of wash buffer and 5 vol. of buffer C (buffer B without glycerol). The washed resin was subsequently resuspended in buffer C and divided into five parts. All were incubated at 4 °C with gentle shaking. At each time point, the resuspended resin was centrifuged at 4300 *g* for 2 min. The supernatant was collected as flow-through in a fresh tube and concentrated with 10% TCA. The resin-bound proteins were eluted by incubating with elution buffer at 4 °C for 5 min with gentle shaking. Centrifugation and TCA precipitation were performed as described previously. Densitometric determination of the protein amounts detected on immunoblot was carried out using TINA 2.09e software. Summation of protein amounts detected in the flow-through and elution fractions was used as denominator in calculating the percentage of XpsL or XpsN in the flow-through.

RESULTS

XpsL–XpsM–XpsN complex

Complex formation of three cytoplasmic membrane protein components of the type II secretion apparatus of *X. campestris* pv. *campestris* XpsL, XpsM and XpsN was suggested by their mutual dependence for normal steady-state protein levels and by co-immunoprecipitation results [12]. However, it was not clear if the three protein components form a ternary complex. To answer this question, we constructed an *xpsMh* gene encoding a C-terminally His₆-tagged XpsMh protein on a broad-host-range vector pCPP30. By introducing the recombinant plasmid pST116 into the *xpsM* gene knockout strain XC1714, and analysing for α -amylase secretion, we found that XpsMh is functional (results not shown). We performed metal chelating chromatography on Triton X-100 extracts of membrane proteins prepared from the complemented strain XC1714(pST116), followed by immunoblot analysis of the collected fractions. Both XpsL and XpsN co-fractionated with XpsMh (Figure 1A). In the control experiment, where untagged XpsM was expressed from plasmid pST109, neither XpsL nor XpsN was detected in the elution fractions (Figure 1A). Co-immune precipitation of the pooled E2 and E3 fractions from XC1714(pST116) with either anti-XpsL or anti-XpsN antiserum showed co-precipitation of XpsL and XpsN (Figure 1B, lanes 1 and 5), indicating that the XpsL and XpsN that co-fractionate with XpsMh are associated with each other. In other words, the three form a ternary complex.

To find out if other components of the type II secretion apparatus are required for XpsL–XpsM–XpsN complex formation, we used the mutant strain XC17433, in which the entire *xps* gene cluster was deleted [32]. The plasmid pRT40, which encodes XpsL and XpsMh, and the plasmid pNC2, which encodes XpsN, were introduced into XC17433. Nickel affinity chromatography followed by co-immunoprecipitation was performed as before. Similar to the earlier analysis, XpsL and XpsN co-eluted with XpsMh (results not shown). Furthermore, they co-immunoprecipitated with each other (Figure 1C), suggesting that the protein components that are missing in XC17433 are not required for XpsL–XpsM–XpsN complex formation.

Instability of the XpsL–XpsM–XpsN complex upon size-exclusion chromatography

To characterize the ternary complex further, we attempted to purify the XpsL–XpsMh–XpsN complex from XC1714(pST116)

Figure 1 (A) and (B) XpsL–XpsMh–XpsN complex detected in XC1714 (pST116); (C) XpsL–XpsMh–XpsN complex detected in XC17433(pRT40, pNC2)

(A) Elution profiles from nickel affinity chromatography. His₆-tagged XpsMh is encoded by pST116 (top panel). Untagged XpsM is encoded by pST109 (bottom panel). Samples of the flow-through (FT) and the first wash fraction (W1) were loaded at one-fifth the concentration of those in the last wash (Wf) and the elution fractions (E1–E4), eluted with 250 mM imidazole and collected as 1 ml aliquots. (B) Co-immunoprecipitation of pooled E2 and E3 fractions from the top panel of (A). (C) XC17433 is a mutant strain, in which the entire *xps* gene cluster is deleted. The plasmid pRT40 encodes the XpsL and XpsMh; the plasmid pNC2 encodes XpsN. Pooled E2 and E3 fractions from nickel affinity chromatography were analysed by co-immunoprecipitation as described previously. Only co-immunoprecipitation results with negative controls are shown here. Antibodies used in co-immunoprecipitation are indicated above each lane. Antibodies used in immunoblots are indicated on the left. The arrows mark the positions of XpsL and XpsN.

Figure 2 Size-exclusion chromatography of the partially purified XpsL–XpsMh–XpsN complex

The XpsL–XpsMh–XpsN, purified from XC1714(pST116) upon nickel affinity chromatography, was applied on the Sephadex HR-200 size-exclusion column equilibrated with buffer D. Fractions (0.5 ml aliquots) collected were concentrated 25-fold by TCA precipitation. The samples were subsequently separated by SDS/PAGE followed by immunoblotting with antibody against XpsL, XpsM and XpsN, respectively. XpsL expressed alone fractionated at 24–25 min, whereas the XpsM expressed alone fractionated in two peaks, one at 20 min, the other at 23 min.

by size-exclusion chromatography following nickel affinity chromatography. However, upon separation on the size-exclusion column Superdex HR200, the complex appeared to fall apart, as suggested by partial dissociation of XpsN from the other two proteins. While the XpsL and XpsMh co-fractionated at 24 to 25 min, the major peak of XpsN appeared at 21 to 24 min (Figure 2). XpsN seems to be less stably maintained in the complex than XpsL and XpsMh.

Figure 3 Pull-down of XpsN by XpsL–XpsMh or XpsMh immobilized on a Ni-NTA column

Membrane extracts in Triton X-100 from XC17433(pRT40 encoding XpsL and XpsMh) or XC17433(pST116 encoding XpsMh) were loaded on a Ni-NTA affinity column and washed with 20 vol. of wash buffer followed by 2 vol. of buffer B. A membrane extract of XC17433(pNC2 encoding XpsN) was subsequently applied (seven times) to the column and washed with 20 vol. of wash buffer. The flow-through from the final loading, the first and the last wash fractions are labelled as FT, W1 and Wf, respectively. The nickel-bound proteins eluted with 250 mM imidazole were collected in 1 ml fractions (designated as E1–E5) and concentrated 5-fold by TCA precipitation before immunoblot analysis.

Pull-down of XpsN by XpsL–XpsMh, immobilized on a Ni-NTA column, is more effective than by immobilized XpsMh only

Since XpsN is prone to dissociate from the XpsL–XpsM complex, we asked if the two could reassociate. The XpsL–XpsM complex (lacking XpsN) was immobilized on a Ni-NTA column. After thorough washing, the Triton X-100 extract of XC17433(pNC2), which expressed XpsN alone, was repeatedly passed through the column, washed with 20 mM imidazole and eluted with 250 mM imidazole. In addition to the flow-through (FT) and the first wash (W1) fractions, XpsN was detected in the second (E2) elution fraction (Figure 3A). It is possible that some of the XpsMh captured on the nickel column might be present as the free form. To determine how much of the co-eluted XpsN was associated with the free form XpsMh, a control experiment was conducted where an equivalent amount of XpsMh alone was immobilized on the Ni-NTA column. In this case, XpsN was detected mainly in the FT and W1 fractions (Figure 3B). A weak signal could be detected in the E2 fraction when the immunoblot was visualized by the more sensitive ECL[®] (enhanced chemiluminescence) method (results not shown). However, its proportion relative to the total XpsN detected in all fractions was much lower than that in the case where XpsL–XpsMh had been immobilized, implying that reassociation occurs between XpsN and the XpsL–XpsMh complex.

As a result of the weak binding of XpsMh with nickel, small amounts of XpsMh were detected in the final wash fraction (Wf) in both cases. Nevertheless, the absence of XpsN in the Wf in both cases indicates that the XpsN detected in the E2 fraction

Figure 4 Membrane mixing between XC17433(pCPP-LMN) and XC17433(pRT40)

XC17433(pCPP-LMN) expresses XpsL, XpsM, XpsN; XC17433(pRT40) expresses XpsL and XpsMh. XC17433 is a mutant strain, in which the entire *xps* gene cluster is deleted. For the set labelled 'Membrane mixing', the two strains were mixed before cell breakage and extraction (see the Materials and methods section). For the control experiment labelled 'Mixing after extraction', the same steps were followed for each strain individually up to the Triton X-100 extraction step. Subsequent to extraction, the separately prepared extracts were mixed and incubated at 4 °C for 30 min followed by nickel affinity chromatography. Fractions labelled as FT (flow-through), W1 (first wash fraction), Wf (last wash fraction), E2–E5 (eluted fractions 2–5) were collected and analysed by immunoblot with antibody against XpsL, XpsM and XpsN. Samples loaded in FT and W1 are at one-fifth the concentration of those in Wf and E1–E5. His₆-tagged XpsMh migrated slightly slower than un-tagged XpsM.

is not due to non-specific binding of XpsN with nickel. These results suggest that the XpsN protein is able to associate with the XpsL–XpsM complex more effectively than with XpsM alone. The domain to which XpsN binds is probably a composite of XpsL and XpsM. Alternatively the conformation of XpsM alone may differ from that of the XpsL–XpsM complex.

Reassociation between XpsL–XpsMh and the XpsN co-expressed with XpsL and XpsM upon membrane mixing

The previous observations showing dissociation of XpsN from, as well as reassociation with, the XpsL–XpsM complex were made with solubilized proteins. To find out if such dissociation and reassociation also occurs in the membrane, we conducted membrane-mixing experiments, in which two different strains were mixed prior to cell breakage. To permit protein–protein interactions between the two strains, we incubated the mixed membrane vesicles at 4 °C for 3 h before extracting membrane proteins with Triton X-100. A control experiment, in which the two strains were mixed after the Triton X-100 extraction step, was performed in parallel. To determine whether XpsN dissociates from, and reassociates with, the XpsL–XpsM complex in the membrane, strains XC17433(pCPP-LMN), which expresses XpsL, XpsM and XpsN, and XC17433(pRT40), which expresses XpsL and XpsMh were mixed. As shown in Figure 4 (comparing XpsN in the E2 fractions), XpsN co-eluted with XpsMh upon metal chelating chromatography, but only when the two strains were mixed before detergent extraction. This result suggests that a small, but detectable, portion of the XpsN protein co-expressed with XpsL and XpsM becomes associated with the XpsL–XpsMh upon membrane mixing. The XpsN that co-eluted with XpsMh could have dissociated from the XpsL–XpsM–XpsN complex as a result of its weak association with the other two components. Alternatively it could represent a constant dissociation and reassociation cycle. Regardless, such XpsN is able to re-associate with preassembled XpsL–XpsMh. Slower migration of the XpsN band, with a 'twisted' appearance, was often observed in FT and W1 fractions whenever the membrane concentration was high. This was probably due to membrane lipid that remained associated with XpsN in these

Figure 5 Dissociation rate of XpsL from the XpsL-XpsMh or the XpsL-XpsMh-XpsN complex

The XpsL-XpsMh complex [from XC17433(pRT40)] and the XpsL-XpsMh-XpsN complex [from XC17433(pRT40, pNC2)] immobilized on 0.5 ml of Ni-NTA resin were washed sequentially with 20 vol. of wash buffer and 5 vol. of buffer C. The resin was resuspended in 0.5 ml of buffer C and divided into five equal parts. Into each part 0.9 ml of buffer C was added and incubated at 4 °C with gentle shaking. Beginning at 0 h, one sample was centrifuged every 3 h. The supernatant, precipitated with 10% TCA, was saved as flow-through (FT). The resin-bound protein was eluted with 1 ml of elution buffer followed by centrifugation and TCA precipitation of the supernatant (E).

fractions. XpsM and XpsMh are separable by SDS/PAGE. It is interesting to note that XpsM did not co-elute with XpsMh in either case (Figure 4, bottom panel, E2 fractions). The meaning of this observation is addressed in the Discussion. On the other hand, lack of reassociation between XpsM and XpsMh strongly suggests that reassociation between XpsN and XpsMh is not due to non-specific binding.

Stabilizing effect of XpsN on the XpsL-XpsM complex

To determine the consequence of XpsN dissociation on the XpsL-XpsM complex, we compared the stability of immobilized XpsL-XpsMh and XpsL-XpsMh-XpsN complexes by analysing the dissociation rate of XpsL. The appearance of XpsL in the flow-through fraction (FT in Figure 5), collected in the absence of imidazole, indicated dissociation of XpsL from the complex, while the XpsL in the imidazole-eluted fraction (E in Figure 5) remained bound to XpsMh. It is apparent that association between XpsL and XpsMh in the XpsL-XpsMh complex is significantly weaker than in the XpsL-XpsMh-XpsN complex (Figure 5). The fraction of the XpsL in the flow-through from the XpsL-XpsMh rose to 16% in 3 h and to 34% in 12 h. In

Figure 6 Membrane mixing between XC17433(pCPP-LM) and XC17433(pST116)

XC17433(pCPP-LM) expresses XpsL and XpsM, and XC17433(pST116) expresses XpsMh. The experiments were performed as described in the legend of Figure 4. Fractions collected from nickel chelating chromatography are labelled as FT (flow-through), W1 (first wash fraction), Wf (last wash fraction), and E2-E5 (eluted fractions). Immunoblots of each fraction were detected with antibody against XpsL or XpsM. Samples loaded in FT and W1 are at one-fifth concentration of those in Wf and E1-E5.

contrast, no XpsL was released from the XpsL-XpsMh-XpsN complex after 3 h and only 2% was released after 12 h, by which time 6% of the XpsN had also dissociated from the complex. These results indicate strongly that the presence of XpsN in the complex significantly strengthens the association between XpsL and XpsM.

Reassociation of XpsMh with XpsL co-expressed with XpsM upon membrane mixing

Since the XpsL-XpsM complex in Triton X-100 extracts is prone to dissociate in the absence of XpsN, we asked whether the XpsL-XpsM complex also dissociates and reassociates in the membrane. Again we performed membrane-mixing experiments with XC17433(pCPP-LM) that expresses the XpsL-XpsM and XC17433(pST116) that expresses the XpsMh, followed by nickel affinity chromatography. Only when the two strains were mixed before extraction did XpsL co-elute with XpsMh, albeit in small amounts (Figure 6 top panel, E fractions). Apparently, some of the XpsL co-expressed with XpsM had dissociated from the XpsL-XpsM complex and reassociated with XpsMh. XpsL-XpsMh complex formation probably occurs in the membrane before detergent extraction, because it was not detectable if mixing was performed after Triton X-100 extraction. Dissociation of the XpsL-XpsM complex could be the result of its instability caused by the absence of XpsN. Alternatively the XpsMh-bound XpsL could reflect a constant cycle of dissociation and association with XpsM. In contrast, XpsM did not co-elute with XpsMh (Figure 6 bottom panel, E2 fractions).

DISCUSSION

By performing nickel affinity chromatography, followed by co-immune precipitation, we demonstrated that the cytoplasmic membrane proteins XpsL, XpsM and XpsN form a ternary complex. However, the complex is rather unstable upon size-exclusion chromatography. Sequential dissociation and reassociation appeared to occur in membrane vesicles before extraction. Based on observations made during this study, we propose a model (Scheme 1) showing a hierarchically dynamic relationship among the three components of the XpsL-XpsM-XpsN complex. XpsN is prone to detach from the complex before dissociation of the other two components. Conversely, XpsN preferentially binds to XpsL-XpsM rather than to XpsM alone. Moreover, XpsL and XpsN do not co-immune precipitate in the absence of XpsM [12]. The presence of XpsN in the complex

Scheme 1 Proposed model for reversible dissociation of the XpsL–XpsM–XpsN complex

probably strengthens the association between XpsL and XpsM, because the absence of XpsN in ternary complex favours dissociation of the XpsL–XpsM complex. Dissociation and reassociation of the XpsL–XpsM complex probably occur in membrane vesicles as well.

Previous observations indicated that, *in vivo*, protein abundances of the XpsL and XpsM are mutually dependent, as well as XpsN-dependent. Nevertheless, the reliance on XpsN was alleviated when the expression level of either XpsL or XpsM was raised [12]. Presumably, association between XpsL and XpsM is possible without XpsN. However, in such situations the XpsL–XpsM tends to dissociate, leading to protein degradation. Raising the protein concentration of either XpsL or XpsM is probably favourable to their reassociation, thus preventing their degradation even in the absence of XpsN. In spite of this, XpsN is indispensable for normal secretion [12].

The XpsN homologue was neither present nor required for the type II secretion pathway in other micro-organisms [4,11]. Conversely, another monotopic cytoplasmic membrane protein, GspC, required in all other micro-organisms is absent from *X. campestris* pv. *campestris* and the closely related plant pathogen *Xylella fastidiosa* [34]. We hypothesise that the GspC of other micro-organisms may play the same role and possess the same interactive relationships with other proteins as XpsN. Consistent with this hypothesis, the presence of PulC in the PulL–PulM complex of *K. oxytoca* was suggested by co-immunoprecipitation experiments [11]. Moreover, GspC of *P. aeruginosa* was shown to modulate the stabilizing activity of GspM on GspL [35].

XpsN interacts with the secretin XpsD as demonstrated by co-immunoprecipitation [23]. On the other hand, XpsL protein, by analogy with EpsL protein of *V. cholerae* [27], XcpY of *P. aeruginosa* [28] and OutL of *E. chrysanthemi* [31], probably interacts with the cytoplasmic protein XpsE. Thus the ternary complex, containing XpsL, XpsM and XpsN, may form a bridge between the putative energy generator and the secretion pore, serving as an energy transducer between the two. Similarly, the TonB protein, which serves as the energy transducer in iron-siderophore and vitamin B12 uptake, forms a complex with two cytoplasmic membrane proteins ExbB and ExbD and interacts with the iron-siderophore transporters, FhuA or FepA, located in the outer membrane [36,37]. Recently, a gating role was proposed for the TonB protein, as suggested by results from X-ray crystallography of the TonB-dependent iron transporters FecA and FhuA, bound or unbound to ligand [38,39]. The GspC protein, paired with the secretin, has also been proposed to serve as the ‘gatekeeper’ of the type II secretion apparatus [40]. Further analysis of how the dynamic state of the GspL–GspM–GspN(GspC) complex is related to interactions between GspC and GspD or between GspL and GspE should lead to a better understanding of the secretion process.

Stoichiometry of each component of the ternary complex is not known at this stage. EpsL or EpsM of *V. cholerae* singly expressed in *E. coli* forms a homodimer, as suggested by size-exclusion chromatography [10]. Consistent with this observation, when XpsM and XpsMh were co-expressed in XC17433 (Δxps genes) or in XC1714 (*xpsM* mutant), they co-eluted upon nickel affinity chromatography (results not shown). Although in the latter XpsL and XpsN also co-eluted with XpsMh, without further experiment we could not be certain if all four are present in the same complex. Our observations that the XpsM did not co-elute with XpsMh upon membrane mixing could be explained in at least two ways. XpsM is probably present in the ternary complex as monomer. Alternatively, it could exist in more than one copy in the complex, however as tightly associated oligomer (including dimer).

Concurrent overproduction of both XpsL and XpsM in a wild-type strain inhibited normal secretion. Furthermore, the secretion inhibition was enhanced by simultaneous overproduction of XpsN [12]. Imbalanced stoichiometry between the ternary complex and its interactive components, XpsD and XpsE, could be the cause of the secretion inhibition. Alternatively, when all three proteins concentrations are raised in the cell, the XpsL–XpsM–XpsN complex perhaps becomes less dissociable and hence unfavourable to secretion. We speculate that the dissociation status of the ternary complex may play a crucial role for normal operation of the secretion apparatus.

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