Involvement of the XpsN Protein in Formation of the XpsL-XpsM Complex in *Xanthomonas campestris* pv. *campestris* Type II Secretion Apparatus

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The *xps* gene cluster is required for the second step of type II protein secretion in *Xanthomonas campestris* pv. *campestris*. Deletion of the entire gene cluster caused accumulation of secreted proteins in the periplasm. By analyzing protein abundance in the chromosomal mutant strains, we observed mutual dependence for normal steady-state levels between the XpsL and the XpsM proteins. The XpsL protein was undetectable in total lysate prepared from the *xpsM* mutant strain, and vice versa. Introduction of the wild-type *xpsM* gene carried on a plasmid into the *xpsM* mutant strain was sufficient for reappearance of the XpsL protein, and vice versa. Moreover, both XpsL and XpsM proteins were undetectable in the *xpsN* mutant strain. They were recovered either by reintroducing the wild-type *xpsN* gene or by introducing extra copies of wild-type *xpsL* or *xpsM* individually. Overproduction of wild-type XpsL and -M proteins simultaneously, but not separately, in the wild-type strain of *X. campestris* pv. *campestris* caused inhibition of secretion. Complementation of an *xpsL* or *xpsM* mutant strain with a plasmid-borne wild-type gene was inhibited by coexpression of XpsL and XpsM. The presence of the *xpsN* gene on the plasmid along with the *xpsL* and the *xpsM* genes caused more severe inhibition in both cases. Furthermore, complementation of the *xpsN* mutant strain was also inhibited. In both the wild-type strain and a strain with the *xps* gene cluster deleted (XC17433), carrying pCPP-LMN, which encodes all three proteins, each protein coprecipitated with the other two upon immunoprecipitation. Expression of pairwise combinations of the three proteins in XC17433 revealed that the XpsL-XpsM and XpsM-XpsN pairs still coprecipitated, whereas the XpsL-XpsN pair no longer coprecipitated.

The type II secretion pathway is used by a wide range of pathogenic gram-negative bacteria for the secretion of extracellular proteins (29, 33, 35). The secreted protein possesses a typical N-terminal signal peptide, which is cleaved by the signal peptidase upon its export across the cytoplasmic membrane through the Sec pathway. A maximum of 14 genes is required for the second step of the type II secretion pathway. Mutation in these genes causes accumulation of the secreted protein in the periplasm. Of the 14 protein components, two were located in the outer membrane, as suggested by sucrose gradient sedimentation analysis (9, 10, 15). The D homologues were demonstrated in various cases to form multimers, by either sucrose gradient sedimentation, gel filtration chromatography, or electron microscopy (3, 8, 18, 22, 24, 25). They were postulated to be the secretion channel. The PulS protein of *Klebsiella oxytoca* was shown to be copurified with the PulD protein at a 1:1 ratio, presumably as a component of the secretion channel (24). Of the remaining protein components, at least four (G, H, I, and J) have an N-terminal sequence similar to that of the type IV prepilin protein. They were shown to be processed by another protein, designated O except in *Pseudomonas aeruginosa*, in which it is called XcpA or PilD (2, 11, 16, 30, 40, 41). Disrup-

tion of the O protein also caused the secreted protein to accumulate in the periplasm (40). The four pilin-like proteins have been postulated to form pilus-like multimeric structures; thus, they are named pseudopilins. Recently, a pilus-like structure was demonstrated for the PulG protein of *K. oxytoca* upon electron microscopy by overexpressing the entire *pul* operon in *Escherichia coli* (39). The similarity between a fifth pseudopilin, the K protein, and the other pseudopilins is not so obvious (5). The remaining protein components, all of which but one (the C, F, L, M and N proteins) possess at least one putative membrane-anchoring sequence, are cytoplasmic membrane proteins. The last of all, the E protein, is predicted to be a cytoplasmic protein; however, it is associated with the cytoplasmic membrane through the L protein (36).

The cytoplasmic protein EpsE, which exhibited autokinase activity in vitro, was shown to associate with the cytoplasmic membrane via the EpsL protein in *Vibrio cholerae* (37). Interaction between the OutE and the OutL proteins of *Erwinia chrysanthemi* was also observed in the yeast two-hybrid system (31). Furthermore, overproduction of a truncated protein composed of the cytoplasmic domain of the OutL protein in the wild-type strain of *E. chrysanthemi* is inhibitory to normal secretion. Such inhibition was alleviated by overproduction of the wild-type OutE protein, suggesting interaction between the cytoplasmic domain of the OutL and the OutE protein. A nucleotide-binding motif, the Walker A box, with the sequence GXXGXGKT is conserved in all E proteins. Mutation in the

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nucleotide-binding motif has been shown to eliminate extracellular protein secretion in *K. oxytoca*, *P. aeruginosa*, *V. cholerae*, and *E. chrysanthemi* (26, 31, 36, 42). Moreover, autokinase activity of the EpsE protein was abolished as a result of mutation in the nucleotide-binding motif (36). In another case, the mutated OutE protein of *E. chrysanthemi* no longer exhibited OutL-dependent conformational change detected as proteinase K sensitivity (31).

An interactive relationship between the L and the M proteins was suggested primarily by the observation that XcpY (the L homologue) and XcpZ (the M homologue) of the secretion apparatus in *P. aeruginosa* are mutually required for normal steady-state protein levels (23). Mutual stabilization of the XcpY-XcpZ pair expressed in *E. coli* was also suggested by the observation that the abundance of each protein was increased by coexpression of the other one (23). Likewise, proteolytic degradation of the EpsL protein of the secretion apparatus of *V. cholerae* expressed in *E. coli* was reduced by coexpression of the EpsM protein (37). Although the L and the M proteins in these two microorganisms showed different degrees of instability in the absence of their partners, both observations suggested that the L and the M proteins probably stabilize each other through complex formation. Direct interaction between the EpsL and EpsM proteins of the secretion apparatus in *V. cholerae* was demonstrated by Sandkvist et al. (37) by coimmunoprecipitation. By exchanging various regions of the EpsL protein of *V. cholerae* with homologous regions of the ExeL protein of *Aeromonas hydrophila*, Sandkvist et al. (38) constructed a series of EpsL-ExeL and ExeL-EpsL chimeric proteins. They further demonstrated that the region between amino acid residues 216 and 296 of the EpsL protein is likely to be involved in its complex formation with the EpsM protein. A typical transmembrane sequence predicted within this region is detected at similar positions in all L proteins. It was proposed that complex formation between EpsL and EpsM probably occurs through the transmembrane regions in each protein. A transmembrane sequence is predicted at the N terminus of all M proteins. Coimmunoprecipitation between the PulL and the PulM proteins of *K. oxytoca* was also observed (28).

The *xpsM* gene is located upstream of the *xpsN* gene (19), which is required for extracellular protein secretion in *Xanthomonas campestris* pv. *campestris*. It encodes a protein of 213 amino acid residues (accession number M81111). A putative transmembrane segment is predicted at its N terminus (residues 10 to 28). Further upstream is the *xpsL* gene, which encodes a protein of 373 amino acid residues (GenBank accession number L02630). A putative membrane-anchoring sequence is predicted at residues 215 to 233. To understand the interactive relationship of the XpsM protein with other protein components in *X. campestris* pv. *campestris*, we constructed a chromosomal nonpolar mutant strain with a mutation in the *xpsM* gene. By monitoring the abundance of other Xps proteins with available antibodies, we detected all but XpsL in the mutant strain. Further analysis revealed mutual dependence for normal steady-state levels of the XpsL and -M proteins. Moreover, neither protein was detected in the $\Delta xpsN$ mutant strain. Recovery of both was made possible by reintroducing the wild-type *xpsN* gene or by introducing extra copies of the *xpsL* or the *xpsM* gene alone. Coexpression of all three proteins

from plasmid-borne genes in the secretion-positive strain caused inhibition. Complementation of the chromosomal mutation in each of the three genes with a plasmid-borne wild-type gene was also inhibited by coexpression with the other two. We further demonstrated that each protein coimmunoprecipitated with the other two when all three were coexpressed. Our interpretation of these observations is discussed below.

MATERIALS AND METHODS

Bacterial strains, plasmids, and antisera. *E. coli* BL21(DE3) was a kind gift from F. W. Studier. *X. campestris* pv. *campestris* XC1701 was originally isolated as a spontaneous Rif^r mutant from a wild isolate, XC17. XC17433 is a secretion mutant strain in which the entire *xps* gene cluster (*xpsEFGHIJKLMND*) was deleted (14). XC1709 and XC1712 are chromosomal nonpolar mutant strains with in-frame deletions of *xpsN* and *xpsL*, respectively (19; Leu et al., unpublished data). All plasmids used in this study are listed in Table 1. All antisera were prepared from rabbits. Antiserum against XpsN was prepared as described previously (19) and used in immunoblot reactions at 1:250. Antiserum against purified α -amylase was used at 1:500. Antiserum against XpsL (used at 1:200) or against XpsG (used at 1:1,000) was prepared against a nickel-nitrilotriacetic acid column-purified TrxA-His₆-XpsL_N (N for N-terminal domain) fusion protein or the TrxA-His₆-XpsG fusion protein, respectively, that was overexpressed from the T7 promoter in *E. coli* BL21(DE3) (Leu et al., unpublished data; Chen et al., unpublished data). Antiserum against *E. coli* OmpA, used in detecting the OprF protein at 1:60,000, was a kind gift from U. Henning.

Production of antibody against XpsM. A truncated XpsM protein was overexpressed as a fusion protein with an N-terminal thioredoxin (TrxA) and a His₆ tag from the T7 promoter located on the T7 expression vector pET32. The overexpression plasmid pET32M was constructed by cloning a PCR-generated fragment of the *xpsM* gene encoding amino acids 41 to 185 of XpsM in frame with the *trxA* gene. The PCR primers used were Mfor (5'-CACTTGGGATCC GACGAATTGCAATCGCTG-3') and Mrev (5'-CACTTGGTGCACAGTTC GAAGGCGATGTCCAG-3'). The fusion protein TrxA-His₆-XpsM overexpressed in *E. coli* BL21(DE3) was purified by passing it through a nickel-nitrilotriacetic acid column following the procedures suggested by the supplier. Sera were prepared following the procedures of Harlow and Lane (12). Immunoblot analysis (used at 1:200) indicated that the antiserum could detect in *X. campestris* pv. *campestris* XC1701 a protein with an apparent molecular mass of approximately 25 kDa that is absent in XC17433, in which the entire *xps* gene cluster was deleted (14).

Construction of a chromosomal, nonpolar *xpsM* mutant. Following the procedures suggested by the supplier of the Altered Sites II in vitro mutagenesis system (Promega), we introduced two insertions into the *xpsM* gene located on the plasmid pST101. The primer MAPA, 5'-GCCTTGGGCCCTGTGCTGCT G-3', was used for introducing a single C insertion downstream of the 43rd base of the *xpsM* ORF, which also created an *ApaI* site. The primer MHD, 5'-GCC AACGAAGCTTGGCAATGGCTG-3', was used for inserting two T's downstream of the 525th base and creating a *HindIII* site. As a result of two frame-shifts, the amino acid sequence between the 15th and the 175th amino acid residues of XpsM was altered without generating a premature stop codon that might exert a polar effect on the downstream gene expression. Subsequently, the mutated *xpsM* gene was introduced into *X. campestris* pv. *campestris* genome following the procedures of Kamoun et al. (17) with slight modifications as described elsewhere (19). Southern blot analysis of chromosomal DNA digested with *ApaI* and with *HindIII* in combination with *EcoRI* confirmed that only the mutated *xpsM* gene was present in the genome. We designated this strain XC1714.

Triparental conjugation. A DNA fragment cloned in the broad-host-range vector pCPP30 or pCPP33 (Fig. 1) was introduced into *X. campestris* pv. *campestris* via triparental conjugation. *E. coli* DH5 α containing the recombinant plasmid served as the donor. *E. coli* HB101 containing pRK2013 served as a helper, and *X. campestris* pv. *campestris* served as the recipient. Since the conjugation frequency is quite high, we developed a simple cross-streaking method. Picked from fresh colonies, the donor and the helper were streaked onto Luria-Bertani agar plates in parallel, and the recipient was streaked across the donor and the helper in that order. After incubation at 28°C overnight, cells grown near the end of the recipient streak were streaked out for single colonies on the selection plate containing rifampin (100 μ g/ml) and kanamycin (50 μ g/ml). Streaking for single colonies was repeated to avoid possible *E. coli* contamination. Plasmid isolated

TABLE 1. Plasmids

Plasmid	Relevant characteristics	Source or reference
Plasmids used in raising antibody against XpsM		
pET32a	Amp ^r ; P _{T7} -containing expression vector for expressing N-terminal TrxA-His ₆ tag fusion	Novagene
pET32M	Amp ^r ; a PCR fragment encoding amino acid residues 41 to 185 of XpsM cloned in pET32a at <i>Bam</i> HI and <i>Sal</i> I sites	This study
Plasmids used in constructing chromosomal mutations in <i>xpsM</i>		
pSelect-1	Tet ^r ; a phagemid vector; ColE1 replicon with an <i>f1</i> (-) <i>ori</i>	Promega
pBluescript II SK(-)	Amp ^r ; a phagemid vector; ColE1 replicon with an <i>f1</i> (-) <i>ori</i>	Stratagene
pUCD4121	Cam ^r Suc ^r ; derivative of pTZ18R	C. I. Kado
pUC4K	Amp ^r Kan ^r ; <i>kan^r</i> gene cassette can be excised as a restriction fragment	Pharmacia Biotech
pKC101	Tet ^r ; a 6.4-kb <i>Eco</i> RI fragment containing the <i>xpsKLMND</i> genes cloned in pSelect-1	Wang and Hu (unpublished) data
pST102	Amp ^r ; an <i>Eco</i> RI- <i>Sal</i> I fragment of 5.8 kb containing the <i>xpsKLMN</i> genes subcloned in pSelect-1	This study
pST107	Cam ^r Suc ^r ; an <i>Eco</i> RI- <i>Sal</i> I fragment of 5.8 kb containing wild-type <i>xpsKLN</i> and the mutated <i>xpsM</i> gene subcloned at <i>Xba</i> I- <i>Sal</i> I sites in pUCD4121	This study
pST108	Kan ^r Cam ^r Suc ^r ; a 1.3-kb <i>Sal</i> I fragment containing the Kan ^r gene cassette subcloned from pUC4K at <i>Sal</i> I site in pST107	This study
Plasmids used in expressing <i>xpsM</i> in combinations with <i>xpsL</i> and <i>xpsN</i>		
pCPP30 and pCPP33	Tra ⁻ Mob ⁺ Tet ^r IncP replicon, each with different multiple cloning sites downstream of P _{lac}	D. W. Bauer 19
pNC2	<i>Sac</i> II- <i>Bcl</i> II 1.2-kb fragment carrying the <i>xpsN</i> gene subcloned at <i>Hind</i> III- <i>Bam</i> HI sites downstream of P _{lac} in pCPP30	
pCPP-30L2	2.3-kb <i>Nco</i> I- <i>Eco</i> RI fragment carrying the <i>xpsL</i> gene subcloned at <i>Kpn</i> I- <i>Eco</i> RI sites downstream of P _{lac} in pCPP30; <i>xpsM</i> was disrupted by frameshifts; a 0.7-kb fragment in the <i>xpsN</i> region was deleted during cloning	This study
pST109	1.7-kb <i>Mlu</i> I- <i>Bam</i> HI fragment containing <i>xpsM</i> subcloned at <i>Eco</i> RI- <i>Hind</i> III sites downstream of P _{lac} in pCPP33	This study
pCPP-LMN	3-kb <i>Nco</i> I- <i>Bcl</i> II fragment containing the wild-type <i>xpsLMN</i> genes subcloned at <i>Kpn</i> I- <i>Eco</i> RI sites downstream of P _{lac} in pCPP30	This study
pCPP-LM	<i>xpsN</i> in pCPP-LMN disrupted by deletion of a 0.6-kb <i>Bam</i> HI fragment	This study
pCPP-MN	<i>xpsL</i> in pCPP-LMN disrupted by an in-frame deletion of a 0.5-kb <i>Sna</i> I fragment	This study
pCPP-LN2	<i>xpsM</i> in pCPP-LMN replaced with the frameshift mutant <i>xpsM</i> gene of pST107	This study

from the putative transconjugant was examined by digestion with restriction enzymes.

Starch plate assay for α -amylase secretion. The method of Hu et al. (14) for the starch plate assay for α -amylase secretion was used.

Subcellular fractionation. For subcellular fractionation, the procedures described by Hu et al. (15) were followed with slight modifications. In brief, cells were grown in XOL with 0.4% maltose as the carbon source to an A₆₀₀ of 1. Supernatant collected upon centrifugation at 11,000 $\times$ g for 5 min was saved as the extracellular fraction. Spheroplasts were prepared upon lysozyme (3 mg/ml, wt/vol) treatment of the cells on ice for 1 h in 10 mM HEPES, pH 8.0, containing 20% (wt/vol) sucrose. Supernatant collected from centrifugation of the spheroplasts at 11,000 $\times$ g for 30 min was saved as the periplasmic fraction. The spheroplasts were gently rinsed with 10 mM HEPES (pH 8.0) containing 20% sucrose, suspended in 10 mM Tris-HCl (pH 8.0), and saved as the spheroplast fraction. The extracellular and the periplasmic fractions were concentrated upon trichloroacetic acid precipitation as described previously (15). Equivalent amounts of each fraction were loaded on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels for immunoblot analysis of α -amylase distribution.

Sucrose gradient sedimentation analysis. Total membrane was analyzed on a step sucrose gradient (25 to 61%, wt/wt) following the procedures of Lee et al. (19). Each fraction was analyzed for density, for distribution of the XpsM and OprF (OmpA homologue) proteins, and for succinate dehydrogenase activity as described previously (19).

SDS-PAGE and immunoblot analysis. For SDS-PAGE, the procedures of Hu et al. (15) were followed, with slight modifications. Separation of total protein on SDS-polyacrylamide gels (10 or 12.5% [wt/vol] acrylamide) was followed by electroblotting onto polyvinylidene difluoride (PVDF) membranes at 100 V for 60 min. At the completion of electrotransfer, the PVDF membrane was soaked

in blocking buffer (TBS buffer [10 mM Tris-HCl, pH 7.4; 0.9%, wt/vol, NaCl] plus 5% [wt/vol] nonfat milk powder) for 30 to 60 min before addition of the rabbit antiserum against the targeted protein. Subsequently the mixture was incubated at room temperature for 2 h or at 4°C overnight. Incubation with the goat anti-rabbit immunoglobulin G antibody conjugated with peroxidase for 1 to 2 h was preceded by two washes with TBS buffer (5 to 10 min each time). After repeated washing with TBS buffer, the PVDF membrane was soaked in TBS buffer containing 0.01% (wt/vol) 4-chloro-1-naphthol and 0.2% (wt/vol) H₂O₂. To minimize the cross-reactive signals on immunoblots, antisera against XpsL and XpsM were preincubated in a final volume of 500 μ l with XpsL- or XpsM-negative crude lysate (at 1.5 to 3 mg of protein/ml) at room temperature for 30 min before use. Crude lysates of *E. coli* BL21(DE3)(pET32a) and XC1712 (*xpsL*) or XC1714 (*xpsM*) were prepared by breaking cells through a French press and then collecting supernatant after centrifugation at 785 $\times$ g at 4°C for 20 min.

Immunoprecipitation. The procedures of Lee et al. (19) for immunoprecipitation were followed. Two milligrams of Triton X-100 extract was included in the immunoprecipitation mixture. The percentage of coimmunoprecipitated proteins was estimated by calibrating with immunoprecipitation efficiency of each antibody. Densitometric determination of the protein amounts detected on immunoblot was carried out using TINA 2.09e software. Only signals that fell within a linear range were used in the calculation.

RESULTS

XpsM is a cytoplasmic membrane protein required for extracellular protein secretion. By analyzing α -amylase secretion on starch plates, we observed that the clear zone produced by

FIG. 1. Expression of *xpsL*, *-M*, and *-N* genes, separately and in different combinations, from the P_{lac} promoter on broad-host-range vector pCPP30. The locations of the *xpsL*, *-K*, *-L*, *-M*, *-N*, and *-D* genes are shown on top of the restriction map. The DNA fragment cloned in each plasmid is represented by a single line. The frameshifted *xpsM* gene is designated by a shaded box flanked by A (*Apa*I site) and H (*Hind*III site). Bst/E designates an original *Bst*EII site that was filled in and ligated to an *Eco*RI linker. The other restriction enzyme sites are abbreviated as follows: Nc, *Nco*I; M, *Mlu*I; St, *Sna*I; B, *Bam*HI; Bc, *Bcl*I; Sa, *Sal*I.

the *xpsM* mutant strain XC1714 was much smaller than that produced by the parental strain (Fig. 2C). In agreement with this observation, immunoblot analysis of subcellular distribution of α -amylase also indicated that the *xpsM* gene is required for α -amylase secretion across the outer membrane. In contrast to the parental strain XC1701, where α -amylase was mainly present in the extracellular fraction, major portions of α -amylase in the mutant XC1714 were detected in the periplasmic fraction (data not shown). Introduction of the plasmid pST109, which carries a wild-type *xpsM* gene alone, complemented the secretion defect in XC1714 (Fig. 2C), suggesting that mutation in the chromosomal *xpsM* gene did not have a polar effect on its downstream gene expression. Sucrose gradient sedimentation analysis of the membrane vesicles prepared from the parental strain XC1701 followed by immunoblot analysis of XpsM indicated that XpsM cofractionated with the cytoplasmic membrane protein succinate dehydrogenase (data not shown), suggesting that it is a cytoplasmic membrane protein.

Dependence on XpsN for protein abundance of XpsL and -M expressed from chromosomal genes. Immunoblot analysis of the *xpsM* mutant strain XC1714 for other Xps proteins with antibodies available detected all but the XpsL protein. Furthermore, cross-examination of the XpsL, -M, and -N proteins in the chromosomal mutant XC1712 (*xpsL*), XC1714 (*xpsM*), and XC1709 (*xpsN*) revealed complex relationships among them. Both the XpsL and XpsM are undetectable in all strains examined (Fig. 3). The amount of XpsN was clearly reduced in the *xpsL* and *xpsM* mutant strains (Fig. 3). All samples were loaded in equal amounts, as judged by immunoblotting with

antibody against the pseudopilin XpsG protein and Coomassie blue staining of the gel (data not shown).

The XpsL protein reappeared in the complemented *xpsM* mutant strain XC1714(pST109), in which the plasmid carries the wild-type *xpsM* gene alone, as well as in the complemented *xpsN* mutant strain XC1709(pNC2), in which the plasmid carries the wild-type *xpsN* gene alone (Fig. 4A). Similarly, the

FIG. 2. Starch plate assay for α -amylase secretion. Freshly grown colonies were transferred with toothpicks in triplicate onto XOL-starch (2%, wt/vol) plates and incubated at 28°C overnight. XC1701 and XC17433 were included as secretion-positive and -negative controls, respectively. Plasmids were introduced into XC1701 (wild-type [wt] parental strain) (A), XC1712 (*xpsL* mutant) (B), XC1714 (*xpsM* mutant) (C), and XC1709 (*xpsN* mutant) (D). L, M, and N represent *xpsL*, *-M*, and *-N*, respectively. Numbers next to each strain represent the percentage of α -amylase secreted. They were determined by immunoblot analysis of α -amylase in extracellular and cellular fractions. ND, not determined. XC1701(pNC2) tends to form smaller colonies, and so the clear zones on the starch plate appear to be smaller. However, immunoblot analysis detected no α -amylase in the cellular fraction, as indicated by 100% secretion.

FIG. 3. Abundance of XpsL, XpsM, and XpsN in *xpsL*, *xpsM*, and *xpsN* mutants. *X. campestris* pv. *campestris* cells grown in LB medium to an A_{600} of ~ 1 were broken with a sonicator followed by mixing with equal volumes of $2\times$ sample buffer. Ten or fifteen microliters of sample at a cell density of $10 A_{600}$ units were loaded on SDS-PAGE gels, followed by immunoblotting with antibody against XpsL, -M, -N, and -G. WT, XC1701; N⁻, XC1709; L⁻, XC1712; M⁻, XC1714; Δxps , XC17433.

XpsM protein reappeared in the complemented *xpsL* mutant strain XC1712(pCPP30L2), in which the plasmid carries the wild-type *xpsL* gene alone. This protein was also detected in the complemented *xpsN* mutant strain XC1709(pNC2) (Fig. 4B). On the other hand, in the *xpsN* mutant strain XC1709, both XpsL and XpsM were recovered by supplementing a plasmid that carries either *xpsL* or *xpsM* (Fig. 4C). Slightly

FIG. 4. Recovery of XpsL (A) and XpsM (B) in complemented strains. (C) Recovery of XpsL and XpsM in an *xpsN* mutant strain. Samples were prepared as described in the legend to Fig. 3 and are designated as in Fig. 3. The plasmids pNC2, pST109, and pCPP30L2 carry wild-type *xpsN*, *xpsM*, and *xpsL*, respectively.

FIG. 5. Abundance of XpsL, -M, and -N expressed from different plasmids in XC1701 (wild type [WT]). Sample preparation and immunoblot analysis were conducted as described in the legend to Fig. 3. Δxps designates XC17433.

larger amounts of XpsM appeared in the strain carrying pST109 (*xpsM*) than in that carrying pNC2 (*xpsN*). Presumably, this reflects a higher *xpsM* gene copy number in the former strain. Interestingly, the increase in the XpsL protein in XC1709 (pCPP30L2) was not so obvious, but the reason for this is not clear.

Overproduction of wild-type XpsL and -M, with or without XpsN, in the wild-type strain inhibited secretion. In order to unravel the complex relationships among the XpsL, -M, and -N proteins, we constructed four plasmids, of which one carries all three genes simultaneously (pCPP-LMN) and three encode the three genes in pairs (pCPP-LM, pCPP-MN, and pCPP-LN2). Following introduction of each plasmid into the parental strain XC1701 via triparental conjugation, we examined all transconjugants on starch plates for α -amylase secretion and examined its subcellular distribution by immunoblot analysis. It is clear that none of the singly expressed XpsL, XpsM, and XpsN proteins caused inhibition of α -amylase secretion (Fig. 2A). Only pCPP-LM and pCPP-LMN resulted in inhibition. The latter (22% of total is secreted) caused more severe inhibition than the former (31% of total is secreted). pCPP-MN and pCPP-LN2 did not result in inhibition (Fig. 2A).

Examination of total lysates of these transconjugants on immunoblots for steady-state levels of the XpsL and the XpsM proteins revealed that secretion inhibition correlated with a high steady-state level of XpsL (pCPP30L2) as well as XpsM (pST109) (Fig. 5). These results suggested that overproduction of XpsL and XpsM, which was made possible only by their coexpression from the plasmid, caused secretion inhibition. The results are reminiscent of the observations that the chromosomal *xpsL* and *xpsM* genes are required for the detection of each other's protein product (Fig. 3 and 4). The steady-state level of XpsN appeared to be slightly higher when this protein was coexpressed with XpsL and XpsM (pCPP-LMN) than when it was expressed alone (pNC2) (Fig. 5). This agrees with the observation that the normal steady-state level of chromosome-encoded XpsN protein requires the simultaneous presence of XpsL and XpsM (unpublished results).

Coexpression of XpsL, -M, and -N caused negative complementation. The *xpsL* (XC1712) and *xpsM* (XC1714) mutants were complemented in α -amylase secretion by the singly expressed *xpsL* and *xpsM* genes, respectively (Fig. 2B and C).

FIG. 6. Coimmunoprecipitation. Triton X-100 extracts of membrane proteins prepared from the different strains were immunoprecipitated (IP) with antibody against XpsL, XpsM, or XpsN, followed by immunoblotting with antibody against XpsL (top panels), XpsM (middle panels), or XpsN (bottom panels). "+" and "-" designate presence and absence, respectively, of the gene. (A) XC1701 (lanes 1, 5, and 9), XC17433(pCPP30L2) (lanes 2, 6, and 10), XC17433(pST109) (lanes 3, 7, and 11), and XC17433(pNC2) (lanes 4, 8, and 12); (B) XC17433(pCPP-LMN) (lanes 1, 5, and 9), XC17433(pCPP-LM) (lanes 2, 6, and 10), XC17433 (pCPP-LN2) (lanes 3, 7, and 11), and XC17433(pCPP-MN) (lanes 4, 8, and 12).

Coexpression of each with XpsN did not affect positive complementation. However, when XpsL and XpsM were coexpressed in either mutant strain (*xpsL* or *xpsM*), negative complementation was observed (Fig. 2B and C). Coexpression of all three proteins caused a more severe negative effect (0% of total is secreted) than coexpression of XpsL and -M (36% of total is secreted). Negative complementation was also observed in the *xpsN* mutant strain XC1709 when XpsN was coexpressed with XpsL and XpsM (Fig. 2D). Complementation of the *xpsN* mutant with the singly expressed *xpsN* gene was as effective as when XpsN was coexpressed with either XpsL or XpsM (Fig. 2D). These results suggested that negative complementation of all three mutants is probably similar to secretion inhibition in the wild-type strain XC1701 in terms of its correlation with simultaneous overexpression of the XpsL, -M, and -N proteins.

Complex formation detected by coimmunoprecipitation. In order to examine the possibility of complex formation among the three proteins, we conducted coimmunoprecipitation experiments on the parental strain XC1701. When antibody against XpsL was included in the immunoprecipitation mixture, all three proteins (XpsL, -M, and -N) were detected on the immunoblot (Fig. 6A). Likewise, all three proteins were precipitated by antibody against XpsN (Fig. 6A). When anti-

body against XpsM was incubated with the Triton X-100 extract, XpsN was precipitated along with XpsM (Fig. 6A). Immunoprecipitation by antibody against XpsM was approximately three- to fourfold less efficient than that by antibody against the XpsN or XpsL. That could explain why XpsL was not detected in the anti-XpsM antibody precipitate of XC1701.

In order to determine if each pair of the three proteins coprecipitates in the absence of all other Xps proteins, we performed similar coimmunoprecipitation experiments on XC17433(pCPP-LMN), in which the XpsL, -M, and -N proteins are expressed from plasmid-borne genes without other *xps* genes. In addition, to determine the relationships within each pair of the three proteins we also conducted coprecipitation experiments on the following strains that express pairwise combinations of the three proteins in the absence of other Xps proteins, XC17433(pCPP-LM), XC17433(pCPP-MN), and XC17433(pCPP-LN2). The results indicated that each protein coprecipitated with the other two in XC17433(pCPP-LMN) (Fig. 6B, lanes 1, 5, and 9). Pairwise combinations revealed that XpsM coprecipitated with either XpsL (Fig. 6B, lanes 2 and 6) or XpsN (Fig. 6B, lanes 8 and 12). In contrast, coprecipitation between XpsL and XpsN was undetectable in the absence of XpsM (Fig. 6B, lanes 3 and 11). A minor band migrating faster than XpsN was detected when XpsN was in

high abundance (Fig. 6B, lanes 1, 9, and 12), the reason for which is not clear. We also noted that abundance of the XpsL protein was apparently lower when XpsL was coexpressed with XpsN than in other cases and vice versa (Fig. 6B, lanes 3 and 11). Semiquantitative analysis of the immunoblot from coimmunoprecipitation experiments on XC17433(pCPP-LMN) indicated that approximately 3 to 18% of each protein coprecipitated with one of the other two.

DISCUSSION

By analyzing the XpsL, -M, and -N protein steady-state levels in strains with chromosomal knockouts of each gene, we observed mutual dependence of the XpsL and XpsM for their detection in SDS-polyacrylamide gels. This is similar to the situation in *P. aeruginosa* (23). Formation of the XpsL-XpsM complex was suggested by their coimmunoprecipitation, as observed in *V. cholerae* and in *K. oxytoca* (28, 37). Furthermore, we found that the *xpsN* gene is required for maintaining both XpsL and -M proteins at normal steady-state levels. In the *xpsN* knockout strain, both XpsL and -M proteins were undetectable on the immunoblot. Both were recovered when the wild-type *xpsN* gene alone was reintroduced. Complex formation between XpsN and XpsM was supported by their coimmunoprecipitation, with or without coexpression of XpsL. Coprecipitation between XpsN and the XpsL occurs as long as XpsM is present. In its absence, both XpsN and XpsL levels were reduced. Apparently, XpsM plays a critical role in maintaining both at normal steady-state levels. Decreases in the XpsN and XpsL levels could explain why their coprecipitation was not detected when XpsM was absent. Alternatively, this result could be the consequence of a lack of complex formation due to the absence of XpsM. The number of complexes and their exact composition are not known at the present time.

Introducing extra copies of either *xpsL* (carried on pCPP30L2) or *xpsM* (carried on pST109) alone into the *xpsN* mutant strain XC1709 resulted in the reappearance of both XpsL and XpsM. This observation suggests that detection of XpsL and XpsM in the absence of the *xpsN* gene is possible if the copy number of one of the genes is raised. We hypothesize that XpsN is involved in maintaining the XpsL-XpsM pair at normal steady-state level by enhancing XpsL-XpsM complex formation or stabilizing the complex. Such a requirement becomes obsolete when the level of either member of the pair is raised, implying a weak association between the XpsL and XpsM. Reliance on XpsN for maintaining their association in normal situations could be bypassed by raising either one of the protein concentrations. Although the XpsN coimmunoprecipitated with either protein, whether XpsN forms a complex with XpsL-XpsM (XpsL-XpsM-XpsN) remains to be determined.

The *xpsN* mutant strain supplemented with the *xpsL* or the *xpsM* gene is unable to secrete (Fig. 2D), suggesting that XpsN plays other roles essential to the secretion process. An association between XpsN and the outer membrane protein XpsD was recently demonstrated with coimmunoprecipitation data (19). Interactive relationships between XpsN and the outer membrane protein on one side and the cytoplasmic membrane XpsL-XpsM complex on the other bear a resemblance to those observed for the TonB protein. TonB is a cytoplasmic membrane protein that serves as the energy transducer for the

uptake of iron-siderophore complexes and vitamin B₁₂ in *E. coli* (7). Sucrose gradient sedimentation analysis indicated that it shuttles between the cytoplasmic membrane and the outer membrane (20). Its interaction with the outer membrane through the receptors for iron-siderophore complexes was suggested by cross-linking experiments (13). In the cytoplasmic membrane, it probably forms a complex with the hexameric ExbB-ExbD complex, also suggested by cross-linking results (13). TonB-dependent activities require proton motive force (PMF) (6, 32). Both PMF and ATP were demonstrated to be required for type II secretion in *Aeromonas* species (21, 43). Similar to TonB with the ExbB-ExbD complex, the N protein along with the L-M complex probably serves as the energy transducer for the type II secretion apparatus by coupling PMF across the cytoplasmic membrane to drive protein secretion through the channel composed of the D protein in the outer membrane. An alternative (or additional) energy source for type II secretion apparatus could come from the putative "molecular motor" E protein, whose homologues contain an indispensable nucleotide-binding motif. One may speculate that energy generated from E protein could be coupled to protein secretion across the outer membrane through its interaction with L protein (36). Nevertheless, without further experiments we cannot rule out other possible roles for XpsN. For instance, it could serve to signal channel opening, as proposed for the pI protein in the assembly of filamentous phage (34).

The N homologue was not found in *E. chrysanthemi* or *P. aeruginosa*. Moreover, PulN in *K. oxytoca* is not required for the secretion process (28). In these cases, the biological roles played by XpsN in *X. campestris* pv. *campestris* could be engaged by another cytoplasmic membrane protein, for instance, the C homologue, whose molecular size and membrane topology are similar to those of the N protein. The abundance of the C homologues in *K. oxytoca* (27) and *P. aeruginosa* (4) has been shown to depend on their respective D proteins. Interestingly, C homologues were identified in the type II secretion apparatus of all microorganisms but *X. campestris* pv. *campestris*.

Contradictory to our observations, overproduction of the wild-type L protein was demonstrated to be inhibitory in *P. aeruginosa* and in *V. cholerae* (1, 23, 38). We have recently been able to raise the XpsL protein level by subcloning the *xpsL* gene into a broad-host-range vector with a copy number higher than that of pCPP30. By introducing this plasmid into a wild-type strain, we observed very weak inhibition (unpublished results). However, introduction of a second plasmid carrying the *xpsM* gene alone raised the XpsL protein level (at least 10 times) as well as the inhibitory effect significantly. These observations suggest that it is possible to cause inhibition, albeit very weak, by overproduction of XpsL alone. However, for strong inhibition, simultaneous overproduction of XpsM is necessary. Apparently, the abundance of XpsL relies strongly on XpsM. At the present time we cannot distinguish whether such strong inhibition is caused by overproduced XpsL-XpsM complex or by overproduced XpsL alone.

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