Membrane localization and topology of the *Yersinia pestis* YscJ lipoprotein

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The localization and membrane topology of the *Yersinia pestis* YscJ lipoprotein, an essential component of the type III secretion apparatus, was investigated. YscJ was demonstrated to be an inner membrane (IM) lipoprotein that is anchored to the periplasmic face of the IM via an N-terminal lipid moiety and via a C-terminal transmembrane (TM) domain. Localization of the N-terminal lipid moiety to the IM occurred regardless of the amino-acid residues found in the +2 or +3 positions. IM localization was dependent upon an intact N-terminal domain (amino acids +1 to +61), suggesting that this region plays a role in YscJ localization. In contrast, the YscJ C-terminal domain and TM domain were not required for IM localization. N-terminal sequence analysis demonstrated that a significant proportion of membrane-localized YscJ lacks *N*-acylation, the final modification required for Lol-dependent transport of a lipoprotein to the OM. Interestingly, attachment of the N-terminus to the IM was required for YscJ function; however, the YscJ secretion signal and lipo-box could be functionally replaced by the first TM domain of the YscV protein, suggesting that the mechanism of attachment to the IM was not critical.

INTRODUCTION

Bacterial pathogens are responsible for numerous diseases in higher plants, insects and animals. Some Gram-negative bacterial pathogens use type III secretion systems (T3SSs) to modulate eukaryotic cell function to their advantage (Hueck, 1998). T3SSs function to transport effector proteins from the cytoplasmic compartment of a bacterial cell directly into a targeted eukaryotic cell (Rosqvist *et al.*, 1994). These systems are essential for the virulence of many pathogens; however, T3SSs have also been identified in bacterial symbionts of both plants and insects (Troisfontaines & Cornelis, 2005). In addition, the T3S process has been demonstrated to play a critical role in the assembly of the bacterial flagellum (Macnab, 1999). Although the proteins secreted by the different T3SSs are highly divergent, the core components of the secretion apparatus and the mechanism of secretion appear to be conserved (Pallen *et al.*, 2005).

The human pathogenic yersiniae (*Yersinia enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*) utilize a virulence-plasmid-encoded T3SS to deliver effector proteins that disrupt host cell signalling pathways involved in bacterial

phagocytosis and in the production of proinflammatory cytokines (Juris *et al.*, 2002; Viboud & Bliska, 2005). These capabilities allow the yersiniae to avoid the host's innate defences and to multiply within the host's tissues. The *Yersinia* T3S apparatus is composed of 21 essential *Yersinia* secretion (Ysc) proteins that are predicted to assemble into a large secretory complex that spans both bacterial membranes (Cornelis, 2002; Yip & Strynadka, 2006). At least 11 components of this macromolecular complex are conserved in essentially all virulence-associated, as well as flagellar, T3SSs, suggesting a common core structure.

A portion of the T3S apparatus, or injectisome, of several bacterial pathogens has been isolated and visualized by transmission electron microscopy (Blocker *et al.*, 1999; Kubori *et al.*, 1998; Sekiya *et al.*, 2001). The isolated structure, termed the needle complex (NC), consists of a base structure that spans both bacterial membranes and a thin needle-like structure that extends beyond the surface of the bacterium. Not surprisingly, the NC base structure closely resembles the related flagellar basal body structure (Kubori *et al.*, 1998).

The assembly of the T3S apparatus, like that of the flagellar system, initiates with a *sec*-dependent stage that includes localization and oligomerization of members of the YscC/InvG, YscD/PrgH and YscJ/PrgK families (Kimbrough & Miller, 2000). In *Salmonella enterica*, the PrgH and PrgK proteins have been shown to form the base of the NC structure (Marlovits *et al.*, 2004). The *Yersinia* YscD and

Abbreviations: IM, inner membrane; NC, needle complex; OM, outer membrane; T3S, type III secretion; T3SS, type III secretion system; TBA, tryptone blood agar; TM, transmembrane; Ysc, *Yersinia* secretion.

Supplementary tables of strains, plasmids and primers are available with the online version of this paper.

YscJ proteins are hypothesized to form a similar base structure. The YscJ/PrgK family of proteins is highly conserved in all virulence-related T3SSs (Allaoui *et al.*, 1992). Members of this family also share homology with the N-terminal region of FlhF, the flagellar membrane/supramembrane (MS)-ring protein (Hueck, 1998; Ueno *et al.*, 1994). The *Yersinia* YscD and *Salmonella* PrgH proteins share significant primary amino-acid sequence identity (Pallen *et al.*, 2005) and have both been shown to be associated with the inner membrane (IM) (Kimbrough & Miller, 2000; Plano & Straley, 1995). YscC and InvG are members of the secretin family and have been shown to form ring-shaped structures in the bacterial outer membrane (OM) (Crago & Koronakis, 1998; Koster *et al.*, 1997). Stable expression, OM localization and oligomerization of YscC requires a lipoprotein chaperone, termed YscW, and the insertion of two DsbA-dependent disulfide bonds in the C-terminal region of the protein (Burghout *et al.*, 2004; Jackson & Plano, 1999; Koster *et al.*, 1997).

All YscJ/PrgK family members have a *sec*-dependent signal sequence with a consensus lipoprotein signal peptidase recognition/cleavage site and, with the exception of EscJ, a predicted C-terminal hydrophobic IM-spanning domain. The predicted C-terminal transmembrane (TM) domain of YscJ is immediately followed by an Arg-Lys-Lys sequence that may serve as a stop transfer signal. Yip *et al.* (2005) have provided evidence that EscJ forms a large 24-subunit 'doughnut-shaped' platform that may be anchored to the periplasmic face of the IM by its N-terminal acyl groups. In contrast, Crepin *et al.* (2005) found that EscJ was predominantly localized to the OM. Analysis of the 17 Å structure of the *S. enterica* SPI-1 NC suggests that PrgK and PrgH are both associated with the bacterial IM (Marlovits *et al.*, 2004). Thus, YscJ family members are probably IM lipoproteins that form the base of the T3S apparatus, probably serving both as a platform for the assembly of the T3S apparatus and as part of the structural link between the IM and OM components of this apparatus.

Bacterial lipoproteins are initially synthesized as precursors in the cytoplasm that can be translocated across the IM by the Sec translocation machinery (Hayashi & Wu, 1990). Subsequent processing to mature lipoproteins occurs on the periplasmic side of the IM. A consensus sequence (L-A/S-G/A[↓]C), termed the lipo-box, targets lipoprotein precursors for modification and subsequent cleavage ([↓]). The cysteine (+1 residue of the mature lipoprotein) is initially modified through the addition of a diacylglycerol molecule to its thiol group. This modification allows cleavage by the prolipoprotein signal peptidase (Tokunaga *et al.*, 1982). A third acyl chain is then added to the amino group of the modified N-terminal cysteine residue by apolipoprotein N-acyltransferase. Acylated lipoproteins are found anchored to the periplasmic leaflet of either the IM or the OM.

The machinery that transports lipoproteins to the OM has been extensively studied in *Escherichia coli*. An Asp at position +2 acts as an IM retention signal (Terada *et al.*,

2001; Yamaguchi *et al.*, 1988), whereas essentially all other residues in the +2 position allow lipoprotein transport to the OM via the Lol machinery (Tokuda & Matsuyama, 2004). The Lol transport machinery consists of the LolABCD and E proteins. The LolCDE complex is an IM ABC transporter that recognizes and delivers fully acylated mature lipoproteins to a periplasmic chaperone termed LolA (Matsuyama *et al.*, 1995; Yakushi *et al.*, 2000). The LolA-lipoprotein complex traverses the periplasmic space and delivers the lipoprotein to LolB, an OM receptor protein (Matsuyama *et al.*, 1997). The IM retention signal (Asp in the +2 position) functions as a Lol avoidance signal and prevents the recognition of lipoproteins by LolCDE, leaving the lipoprotein in the IM (Hara *et al.*, 2003). Recent studies have revealed that the IM retention of lipoproteins can also be influenced by the residues in positions +3 and +4, as well as by structural constraints of the polypeptide (Masuda *et al.*, 2002; Robichon *et al.*, 2003; Terada *et al.*, 2001). Interestingly, the HrcJ lipoprotein, a YscJ/PrgK family member, has an Asp residue in position +2; however, most YscJ/PrgK family members, including EscJ, YscJ, PrgK, PscJ, LscJ and RhoJ, have a Lys or another residue in position +2 (see Fig. 1b), suggesting that localization of YscJ/PrgK family members may occur by a unique mechanism.

In this study, we determined the topology of YscJ and have shown that periplasmic YscJ is inserted in the IM both via its lipid moiety and via a C-terminal TM domain. Localization of YscJ to the IM occurred regardless of the amino acid residues in the +2 or +3 position. Furthermore, we provide evidence that the IM localization of the YscJ lipoprotein moiety is dictated by an N-terminal domain that may function to prevent YscJ N-acylation.

METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are described in detail in Supplementary Table S1, available with the online version of this paper. All *Y. pestis* KIM strains are Pgm[−] and avirulent from peripheral routes of infection (Une & Brubaker, 1984). These strains and their derivatives were routinely grown in heart infusion broth (HIB) or on tryptose blood agar (TBA) base plates (Difco). For Yop secretion experiments, *Y. pestis* strains were grown in the presence or absence of 2.5 mM calcium chloride in the defined medium TMH (Goguen *et al.*, 1984). *E. coli* SY327 λ pir (Miller & Mekalanos, 1988) was used as a host strain for derivatives of the suicide vector pUK4134 (Skrzypek *et al.*, 1993). *E. coli* XL1 Blue (Stratagene) or DH5 α (Cambio *et al.*, 1993) was used for routine cloning experiments, and *E. coli* BL21(DE3) (Novagen) for overexpression of polyhistidine-tagged YscJ.

Construction of a *yscJ* deletion mutant. A ~2.9 kb *Bam*HI–*Cl*AI fragment of plasmid pPH2 (Haddix & Straley, 1992), encoding *yscGHIIJKL* (Fig. 1a), was inserted into *Bam*HI- and *Cl*AI-digested pBluescript SK[−] (Stratagene), generating plasmid pYscGHIIJKL. Unique *Stu*I and *Eco*RV sites flanking the region encoding amino acids 44–94 of YscJ were used to create an in-frame deletion within *yscJ*. A ~3.9 kb *Ecl*136II–*Sal*I fragment, carrying the *yscJ* deletion, was inserted into the *Eco*RV site of pUK4134, creating plasmid pUK4134.P26. This construct was utilized to move the *yscJ* deletion into plasmid pCD1 of

Fig. 1. The *yscJ* gene encodes a lipoprotein. (a) Genetic map of the *yscHIJKL* region of plasmid pCD1. *Stu*I and *Eco*RV sites were used to create an in-frame deletion in *yscJ*. (b) Model of the YscJ presecretory protein. The YscJ N-terminal secretion signal contains a consensus prolipoprotein signal peptidase recognition/cleavage site (LTG↓C). An amino-acid sequence alignment (YscJ residues -3 to +17) of various YscJ/PrpK family members is presented, which shows the diversity of amino-acid residues found in the +2, +3 and +4 positions. The predicted prolipoprotein signal peptidase cleavage site is shown by the arrow.

Y. pestis KIM8-3002 as previously described (Skrzypek *et al.*, 1993), generating *Y. pestis* KIM8-3002.P26.

Construction of a *yscC yscW* deletion mutant. A ~4.6 kb *Bam*HI fragment of pCD1 (*Bam*HI G fragment), containing the *yscW* gene, was inserted into plasmid pT7Blue (Novagen), generating plasmid pT7-YscW. Unique *B*l*p*I sites flanking sequences encoding YscW amino-acid residues 24 to 102 were used to create an in-frame deletion within *yscW*. A ~4.2 kb *E*cl136II-*Eco*RV fragment, carrying the *yscW* deletion, was inserted into the *Eco*RV site of pUK4134, creating plasmid pUK4134.P86. Plasmid pUK4134.P86 was utilized to move the *yscW* deletion into plasmid pCD1 of *Y. pestis* KIM5-3001.12 (Δ *yscC*), generating *Y. pestis* KIM5-3001.P86 (Δ *yscW* Δ *yscC*). Transformation of this strain with plasmid pLG-YscC generated a strain specifically deficient in expression of YscW.

Construction of YscJ expression plasmids. Plasmid pYscJ was constructed by inserting an 807 bp PCR-amplified fragment carrying the entire *yscJ* gene into *E*cl136II/*Bam*HI-digested pBluescript SK⁻ (Stratagene), such that the *yscJ* gene was transcribed from the vector *lac* promoter. Oligonucleotide primers YscJ1 and YscJ2 were used to amplify the *yscJ*-encoding fragment. Oligonucleotides used in this study are presented in Supplementary Table S2, available with the online version of this paper. Plasmid pYscJ was used in complementation experiments.

Plasmids pYscJ₊₂₂₆-FLAG, pYscJ₊₁₉₇-FLAG, pYscJ₊₆₁-FLAG, pYscJ₊₄₂-FLAG and pYscJ₊₂₅-FLAG express the N-terminal +226, +198, +61, +42 or +25 amino-acid residues of the mature YscJ protein with a C-terminal FLAG epitope tag. DNA fragments amplified with oligonucleotide primers YscJ-FLAG1 and YscJ-226, YscJ-197, YscJ-61, YscJ-42 or YscJ-25 were inserted into *Hind*III- and *S*alI-digested plasmid pFLAG-CTC (Sigma).

Plasmids pCDD-YscJ, pPal-CSS-YscJ, pDsbA-YscJ were constructed using the PCR-ligation-PCR technique (Ali & Steinkasserer, 1995). Internal oligonucleotide primers Pal-1, CDD-1 and DsbA-1 were used in conjunction with outside primer YscJ-*Kpn*I; primers Pal-2, CDD-2 and DsbA-2 were paired with outside primer YscJ-*Hind*III. The corresponding Pal, CDD and DsbA PCR fragments were ligated to each other and the resultant full-length fragment was amplified using primers YscJ-*Kpn*I and YscJ-*Hind*III. The analogous wild-type YscJ fragment was also generated using primers YscJ-*Kpn*I and YscJ-*Hind*III, with plasmid pCD1 as template. The amplified fragments were digested with *Kpn*I and *Hind*III and inserted into *Kpn*I- and *Hind*III-digested pBAD30 (Guzman *et al.*, 1995), generating plasmids pYscJ3, pCDD-YscJ, pPal-CSS-YscJ and pDsbA-YscJ. DNA fragments inserted in plasmids pYscJ₊₆₁, pCDD-YscJ₊₆₁ or pPal-CSS-YscJ₊₆₁ were generated with primers YscJ-*Kpn*I and YscJ₊₆₁-*Hind*III using plasmids pYscJ3, pCDD-YscJ and pPal-CSS-YscJ, respectively, as template. The resulting PCR fragments were digested

and inserted into pBAD30, generating plasmids pYscJ₃₊₆₁, pCDD-YscJ₃₊₆₁ and pPAL-CSS-YscJ₃₊₆₁.

Construction of plasmids encoding YscJ–YscW hybrid proteins. Plasmids pYscJ₃₊₅-YscW, pYscJ₃₊₂₅-YscW, pYscJ₃₊₄₂-YscW and pYscJ₃₊₆₁-YscW were constructed by the PCR-ligation-PCR technique (Ali & Steinkasserer, 1995). Internal oligonucleotide primers YscJ₃₊₅, YscJ₃₊₂₅, YscJ₃₊₄₂, YscJ₃₊₆₁ were paired with outside primer YscJ-KpnI. Internal oligonucleotide primer YscW₃₊₁ was paired with primer YscW-XbaI. The resultant YscJ₃₊₅, YscJ₃₊₂₅, YscJ₃₊₄₂ and YscJ₃₊₆₁ PCR fragments were individually ligated to the YscW₃₊₁ PCR fragment and the resultant full-length fragments were amplified using primers YscJ-KpnI and YscW-XbaI. The DNA insert in plasmid pYscW was generated using primers YscW-KpnI and YscW-XbaI. The amplified fragments were digested with KpnI and XbaI and inserted into KpnI- and XbaI-digested pBAD30 (Guzman *et al.*, 1995), generating plasmids pYscW, pYscJ₃₊₅-YscW, pYscJ₃₊₂₅-YscW, pYscJ₃₊₄₂-YscW and pYscJ₃₊₆₁-YscW.

Construction of plasmids pYscV_{TM1}-YscJ and pYscJ-YscV_{TM2}. Plasmid pYscV_{TM1}-YscJ encodes a YscV–YscJ hybrid protein consisting of residues 1–40 of YscV (TM 1 of YscV) fused to residues +2 to +226 of YscJ. The *yscV-yscJ* gene fusion was constructed by the PCR-ligation-PCR technique (Ali & Steinkasserer, 1995). Primers YscV-XbaI and YscV-2 were used to amplify a *yscV* fragment that carries upstream sequences including the *yscV* ribosome-binding site as well as the coding region for amino acids 1–40 of YscV. Primers YscJ₃₊₂-F and YscJ-HindIII were used to amplify the *yscJ* fragment encoding residues +2 to +226 of YscJ. The resultant YscV- and YscJ-encoding fragments were ligated and the gene fusion amplified with primers YscV-XbaI and YscJ-HindIII. The resulting DNA fragment was digested with XbaI and HindIII and inserted into XbaI- and HindIII-digested pBAD30, generating plasmid pYscV_{TM1}-YscJ.

Plasmid pYscJ-YscV_{TM2} encodes a mature YscJ–YscV hybrid lipoprotein consisting of YscJ residues +1 to +199 fused to residues 42 to 61 of YscV (TM 2 of YscV) terminating with the C-terminal four residues of YscJ (QRKK). The *yscJ-yscV* gene fusion was constructed by the PCR-ligation-PCR technique (Ali & Steinkasserer, 1995). Primers YscJ-XbaI and YscJ₃₊₁₉₉ were used to amplify a *yscJ* fragment that encodes the secretion signal and residues +1 to +199 of YscJ. Primers YscV-TM2 and YscV-QRKK-HindIII were used to amplify the *yscV* fragment encoding residues 42–61 of YscV with a C-terminal QRKK sequence. The resultant YscJ- and YscV-encoding fragments were ligated and the gene fusion amplified with primers YscJ-XbaI and YscV-QRKK-HindIII. The resulting DNA fragment was digested with XbaI and HindIII and inserted into XbaI- and HindIII-digested pBAD30, generating plasmid pYscJ-YscV_{TM2}.

Construction of plasmid pLG-YscC. A ~2.3 kb YscC-encoding fragment was obtained by PvuII and XmnI digestion of plasmid pYscC1 (Plano & Straley, 1995). The YscC-encoding fragment was inserted into HincII-digested pLG338 (Stoker *et al.*, 1982), generating plasmid pLG-YscC.

Construction of *yscJ::phoAlacZα* fusions. The dual *phoAlacZα* gene fusion reporter system developed by Alexeyev & Winkler (1999) was used to investigate the membrane topology of YscJ in *Y. pestis*. A SacI–NarI fragment of plasmid pMA632 (Alexeyev & Winkler, 1999), encoding the mature portion of the *E. coli* alkaline phosphatase protein and the α peptide fragment of *E. coli* β-galactosidase, was inserted into SacI- and ClaI-digested pBluescript SK[–], generating plasmid pPHO1. DNA fragments encoding YscJ amino-acids residues +1 to +131, +1 to +198, +1 to +221 and +1 to +226 of YscJ were generated by PCR with a plasmid pYscJ-specific T3 oligonucleotide primer and primers J131, J198, J221 and J226, respectively. The J131, J198, J221 and J226 primers were tailed with an ApaI site. After

digestion with SacI and ApaI, the PCR fragments were inserted in-frame with the *phoAlacZα* gene fusion of pPHO1, generating plasmids pPHO-YscJ₃₊₁₃₁, pPHO-YscJ₃₊₁₉₈, pPHO-YscJ₃₊₂₂₁ and pPHO-YscJ₃₊₂₂₆. Reporter plasmids were electroporated into the *Y. pestis yscJ* deletion mutant for the determination of alkaline phosphatase activity. Alkaline phosphatase activity was measured by determining the rate of *p*-nitrophenyl phosphate hydrolysis in permeabilized cells as previously described (Plano *et al.*, 1991).

Generation of antiserum specific for YscJ. Overexpression and purification of polyhistidine-tagged YscJ was facilitated by construction of expression plasmid pET-YscJ1. The entire *yscJ* gene was amplified by PCR using oligonucleotide primers YscJ-H1 and YscJ-H2. The oligonucleotides were tailed with NdeI and BamHI sites to facilitate insertion of the amplified fragment into the NdeI- and BamHI-digested pET15b (Novagen). Plasmid pET-YscJ1 was electroporated into *E. coli* BL21(DE3) for overexpression of polyhistidine-tagged YscJ. Purification of the polyhistidine-tagged protein was performed according to the manufacturer's protocol with the His-Bind Resin and Buffer kit (Novagen). Purified YscJ protein was used to raise antiserum specific for YscJ in female New Zealand White rabbits (Animal Pharm Services).

Expression and purification of full-length YscJ-6x-His for N-terminal sequence analysis. Plasmid pYscJ-6x-His was constructed by amplifying a *yscJ*-encoding fragment of pCD1 with primers YscJ-KpnI and YscJ-6x-His-XbaI. The YscJ-6x-His-XbaI oligonucleotide provides the coding sequence for six histidine residues and a stop codon followed by an XbaI site. The resulting fragment was digested with KpnI and XbaI and inserted into KpnI- and XbaI-digested pBAD30, generating plasmid pYscJ-6x-His. Plasmid pYscJ-6x-His was moved into *Y. pestis* KIM10 (no T3SS). The resulting strain was grown in 250 ml HIB to OD₆₂₀ 0.5. The expression of the YscJ-6x-His protein was induced by the addition of 0.2% (w/v) L-arabinose for 3 h. Chloramphenicol (20 µg ml^{–1}) was added 1 h prior to harvest to halt protein synthesis and to allow the processing of the YscJ-6x-His protein to be completed. The bacteria were harvested, and lysed by two passages through a French pressure cell. The lysates were then centrifuged at 8000 g at 4 °C for 10 min twice to remove large debris and unlysed cells. The resultant supernatant was subjected to ultracentrifugation at 160 000 g for 1 h at 4 °C to pellet the total membrane fraction. The recovered membranes were solubilized with 50 mM Tris/HCl, pH 7.4 containing 0.1% (w/v) SDS and 1% (v/v) NP-40. Insoluble material was pelleted by ultracentrifugation and the solubilized YscJ-6x-His was purified according to the manufacturer's protocol with Nickel Sepharose 6 Fast Flow resin (Amersham Bioscience). The purified YscJ-6x-His protein was analysed by SDS-PAGE, transferred to BioTrace PVDF membranes (Pall Life Sciences) and N-terminal sequence analysis of the processed YscJ-6x-His protein was performed by the Protein Core Facility at Columbia University.

SDS-PAGE and immunoblotting. Volumes of cellular fractions corresponding to equal numbers of bacteria or equal amounts of protein were mixed 1:1 (v/v) with 2× electrophoresis sample buffer and analysed by SDS-PAGE and immunoblotting essentially as described by Plano *et al.* (1991). *Y. pestis* proteins were visualized as previously described, using antibodies specific for YopE, YopM, YopN, YscV, YscC, YscW or YscJ. FLAG-tagged proteins were detected using anti-FLAG polyclonal antibody (Sigma).

Membrane fractionation. Separation of *Y. pestis* IM and OM fractions was performed by flotation sucrose density-gradient centrifugation following a modification of the procedure reported by Robichon *et al.* (2005). Briefly, *Y. pestis* cultures were grown at 27 °C for 1–2 h and then shifted to 37 °C for 3–4 h. *Y. pestis* strains carrying pBAD30 constructs were induced by the addition of 0.2%

(w/v) L-arabinose and pFLAG constructs with 0.1 mM IPTG. Bacterial cultures were harvested by centrifugation, resuspended in 25 mM HEPES, 5 mM EDTA, pH 7.4 (HE buffer) and disrupted by two passages through a French pressure cell. DNase I and RNase A were added to the lysate to degrade released DNA and RNA. The lysates were then centrifuged at 8000 *g* at 4 °C for 10 min twice to remove large debris and unlysed cells. The membranes were collected by ultracentrifugation at 160 000 *g* at 4 °C for 1 h and resuspended in 60 % (w/w) sucrose, in HE buffer. Membranes were placed at the bottom of gradients created by steps of 56 %, 53 %, 50 %, 47 %, 44 %, 41 %, 38 % and 35 % (w/w) sucrose solutions. The gradients were centrifuged in swinging-bucket rotors at 220 000 *g* for 36 h. Fractions were collected from the top of the tubes and centrifuged at 160 000 *g* at 4 °C for 1 h after dilution of the sucrose to less than 6 % (w/w). Membrane pellets were resuspended in HE buffer and analysed by SDS-PAGE and immunoblotting.

RESULTS

Secretion phenotype of the *yscJ* deletion mutant

Secretion of YopE by the parent strain *Y. pestis* KIM8-3002, the *yscJ* deletion mutant KIM8-3002.P26, and the *yscJ* deletion mutant carrying plasmid pYscJ, pYscJ₊₂₂₆-FLAG or pYscJ₊₁₉₇-FLAG was analysed by SDS-PAGE and immunoblotting (Fig. 2a). Plasmid pYscJ encodes the full-length YscJ protein (Fig. 2b). Plasmids pYscJ₊₂₂₆-FLAG and pYscJ₊₁₉₇-FLAG encode full-length and

truncated FLAG epitope-tagged versions of YscJ, respectively. The YscJ₊₁₉₇-FLAG protein lacks the C-terminal TM region and predicted RKK stop-transfer sequence. The *yscJ* deletion strain failed to secrete YopE at 37 °C in the presence or absence of calcium, whereas the parent strain, as well as the strain transformed with pYscJ or pYscJ₊₂₂₆-FLAG, showed normal calcium-regulated secretion of YopE. These data confirm that the lack of Yop secretion by the *yscJ* deletion mutant was due to the absence of the YscJ protein and not to polar effects on downstream genes. Moreover, the addition of a FLAG epitope to the C-terminus of YscJ did not prevent the assembly of a functional T3SS. In contrast, expression of the YscJ₊₁₉₇-FLAG protein, which lacks the C-terminal TM domain, failed to restore YopE secretion, indicating that this region is essential for YscJ expression, stability or function.

Polyclonal antiserum raised against a polyhistidine-tagged YscJ protein was used to identify the *yscJ* gene product in immunoblots from cell-pellet fractions of the parent strain, the *yscJ* deletion mutant and the deletion mutant transformed with plasmids pYscJ, pYscJ₊₂₂₆-FLAG or pYscJ₊₁₉₇-FLAG (Fig. 2a). The *yscJ* locus is predicted to encode a mature 226 amino acid lipoprotein with a predicted molecular mass of approximately 25 kDa (Allaoui *et al.*, 1995). A protein with an apparent molecular mass of 25 kDa was observed in the parent strain and in

Fig. 2. Analysis of YopE and YscJ expression and YopE secretion. (a) Immunoblot analysis of whole bacterial cell (WC) and culture supernatant (S) fractions from the parent strain, the *yscJ* deletion mutant and the *yscJ* deletion mutant complemented with plasmids pYscJ, pYscJ₊₂₂₆-FLAG and pYscJ₊₁₉₇-FLAG grown in the presence (+) or absence (-) of 2.5 mM calcium chloride. (b) Diagrammatic model of YscJ proteins encoded by plasmids pYscJ, pYscJ₊₂₂₆-FLAG and pYscJ₊₁₉₇-FLAG.

the deletion strain complemented with plasmid pYscJ, but not in the *yscJ* deletion strain. FLAG-tagged YscJ gene products of approximately 28 kDa and 25 kDa were identified in strains carrying plasmids pYscJ₊₂₂₆-FLAG and pYscJ₊₁₉₇-FLAG, respectively. These results indicate that the YscJ C-terminal TM region is essential for YscJ's function in the T3S process.

Construction and analysis of *yscJ::phoAlacZα* fusions

The topology of YscJ was initially analysed using a set of four *yscJ::phoAlacZα* gene fusions. The *phoAlacZα* cassette encodes the mature portion of *E. coli* alkaline phosphatase (no secretion signal) fused to the LacZ α peptide. Plasmid vectors carrying the *phoAlacZα* cassette in-frame with regions of *yscJ* encoding residues +1 to +131, +1 to +198, +1 to +221 and +1 to +226 of the mature YscJ protein were constructed as described in Methods. The *phoAlacZα* fusion joints were selected to generate YscJ-PhoA fusions in the middle of YscJ (pPHO-YscJ₊₁₃₁), just prior to the predicted TM domain (pPHO-YscJ₊₁₉₈), near the end of the TM domain (pPHO-YscJ₊₂₂₁) and at the C-terminus of YscJ following the TM domain and predicted stop-transfer sequence (pPHO-YscJ₊₂₂₆). The *phoAlacZα* fusion plasmids and control plasmid (pYscJ) were electroporated into the *Y. pestis* *yscJ* deletion mutant for the determination of alkaline phosphatase activity. Fusion of PhoA to regions of YscJ that are localized to the periplasmic space would be expected to allow export of the PhoA portion of the hybrid protein to the periplasm, resulting in high phosphatase activity. In contrast, fusions of PhoA to regions of YscJ localized on the cytoplasmic side of the membrane would be expected to have low phosphatase activity (Manoil *et al.*, 1988). No determination of β -galactosidase activity was possible in *Y. pestis* (no expression of the ω fragment of β -galactosidase). Furthermore, the *yscJ::phoAlacZα* gene fusions produced significant β -galactosidase activity in *E. coli* DH5 α , probably due to a cytoplasmic pool of unprocessed preprotein (data not shown).

The *Y. pestis* *yscJ* deletion strain had essentially no detectable background level of alkaline phosphatase activity (Table 1). Expression of YscJ-PhoA fusions with fusion joints located before the predicted TM domain (pPHO-YscJ₊₁₃₁ and pPHO-YscJ₊₁₉₈) exhibited high levels (>200 U) of enzymic activity, indicating a periplasmic localization. The PhoA fusion at residue +221, near the end of the predicted TM domain, produced an intermediate level (79.2 U) of alkaline phosphatase activity, whereas PhoA fused at the C-terminus of YscJ produced only low levels of enzymic activity (11.3 U), suggesting that the extreme C-terminus of YscJ is located in the cytoplasmic compartment. The expression of each of the YscJ-PhoA hybrid proteins in *Y. pestis* was determined by immunoblot analysis (Fig. 3). Each of the hybrid proteins was expressed; however, some proteolytic degradation of

Table 1. AP activity of *yscJ::phoAlacZα* fusions in *Y. pestis* Δ *yscJ*

Plasmid	AP activity*
None	0.0
pYscJ	0.7 $\pm$ 0.7
pPHO-YscJ ₊₁₃₁	210.6 $\pm$ 1.9
pPHO-YscJ ₊₁₉₈	201.1 $\pm$ 1.9
pPHO-YscJ ₊₂₂₁	79.2 $\pm$ 2.3
pPHO-YscJ ₊₂₂₆	11.3 $\pm$ 1.1

*Values represent means $\pm$ SD assayed in duplicate. AP, alkaline phosphatase.

all four hybrid proteins was evident. In particular, extensive degradation of the YscJ₊₂₂₆-PhoA hybrid protein was observed, providing additional evidence for the cytoplasmic localization of the PhoA domain of this hybrid protein.

Fig. 3. Analysis of YscJ and YscJ-PhoA hybrid protein expression. Immunoblot analysis of whole bacterial cell fractions from the *yscJ* deletion mutant complemented with plasmids pYscJ, pPHO-YscJ₊₁₃₁, pPHO-YscJ₊₁₉₈, pPHO-YscJ₊₂₂₁ and pPHO-YscJ₊₂₂₆ grown in the presence (+) or absence (-) of 2.5 mM calcium chloride. The location of the full-length YscJ and YscJ-Pho hybrid proteins is shown (arrowheads) as well as the location of several prominent proteolytic degradation products (*). Blots were probed with antiserum specific for YscJ. The locations of molecular mass standards (kDa) are shown.

PhoA is unable to fold properly in the reducing environment of the cytoplasmic compartment and is rapidly degraded by cytoplasmic proteases (Huang *et al.*, 2001). Overall, these results support a model that predicts YscJ to be a primarily periplasmic protein that is linked to the IM by an N-terminal lipoprotein domain and by a C-terminal TM domain.

The YscJ lipoprotein signal sequence

YscJ has previously been demonstrated to be a lipoprotein (Michiels *et al.*, 1991); however, the role of N-terminal acylation in YscJ function and membrane localization has not been investigated. The YscJ signal sequence (Fig. 1b) contains a consensus lipo-box sequence (LTG₁C) immediately preceding the cleavage site (↓). Most lipoproteins in *E. coli* and in other members of the *Enterobacteriaceae* are transported to the periplasmic face of the OM by the LolABCDE lipoprotein sorting machinery (Tokuda & Matsuyama, 2004). Lipoproteins retained in the IM

normally contain an aspartic acid residue in the +2 position, which functions as a LolCDE avoidance signal. The modified cysteine residue in position +1 of YscJ is immediately followed by the sequence K₊₂V₊₃, suggesting that YscJ should be transported to the bacterial OM; however, several studies with other YscJ family members have suggested that these proteins could be IM lipoproteins (Blocker *et al.*, 2001; Yip *et al.*, 2005). Analysis of other YscJ/PrgK family members revealed a wide variety of amino acid residues in the +2, +3 and +4 positions of these proteins (Fig. 1b), suggesting that localization of these lipoproteins may occur by a novel mechanism.

To begin to investigate the role of the YscJ lipoprotein signal sequence and sorting signals in YscJ localization and function, we generated vectors encoding several YscJ signal sequence variants (Fig. 4b) and moved these plasmids into the *yscJ* deletion mutant. The ability of these plasmids, which encode full-length versions of YscJ, to express a stable protein and to complement the defect in secretion

Fig. 4. Analysis of YopE, YopN and YscJ expression and YopE and YopN secretion. (a) Immunoblot analysis of whole bacterial cell (WC) and culture supernatant (S) fractions from the parent strain, the *yscJ* deletion mutant and the *yscJ* deletion mutant complemented with plasmids pYscJ3, pPal-CSS-YscJ, pCDD-YscJ and pDsbA-YscJ grown in the presence (+) or absence (-) of 2.5 mM calcium chloride. (b) Diagrammatic model of YscJ proteins encoded by plasmids pYscJ3, pCDD-YscJ, pPal-CSS-YscJ and pDsbA-YscJ.

associated with the *yscJ* deletion mutant was evaluated (Fig. 4a).

Plasmid pYscJ3 codes for the parent YscJ protein with the wild-type (predicted OM) sorting signal ($K_{+2} V_{+3}$); in contrast, plasmid pCDD-YscJ codes for a YscJ lipoprotein with a consensus IM sorting signal ($D_{+2} D_{+3}$). Interestingly, both the parent YscJ protein ($K_{+2} V_{+3}$) and the YscJ protein with the consensus IM sorting signal (DD-YscJ) were able to fully complement the defect in Yop secretion associated with the *yscJ* deletion mutant (Fig. 4a). These results suggest that YscJ contains an unusual lipoprotein signal sequence and sorting signal ($K_{+2} V_{+3}$) or that targeting of the YscJ lipoprotein to the IM or OM occurs independent of its $+2 +3$ sorting signal.

To further investigate the subcellular localization and sorting of YscJ, we replaced the YscJ signal sequence with the signal sequence and $S_{+2} S_{+3}$ sorting signal from the well-characterized OM-localized Pal lipoprotein (Fig. 4b). Expression of the Pal-YscJ hybrid protein (Pal-CSS-YscJ) in the *yscJ* deletion mutant restored Yop secretion (Fig. 4a), indicating that the YscJ signal sequence could be replaced with a heterologous lipoprotein signal sequence and consensus ($S_{+2} S_{+3}$) OM sorting signal.

To confirm that YscJ, which has been demonstrated to be a lipoprotein (Michiels *et al.*, 1991), actually requires a lipoprotein secretion signal for function, we replaced the YscJ lipoprotein signal sequence with the signal sequence from DsbA, a soluble, non-acylated periplasmic protein (Fig. 4b). Plasmid pDsbA-YscJ, encoding the DsbA-YscJ hybrid protein, was moved into the *yscJ* deletion mutant. The hybrid protein was expressed and processed (presumably by the type I signal peptidase) but was not functional, indicating that YscJ localization and/or function is dependent on attachment of the N-terminus to the bacterial IM or OM.

Membrane fractionation and localization of YscJ

The subcellular localization of YscJ and truncated derivatives of YscJ was investigated by membrane fractionation using sucrose density-gradient centrifugation as described in Methods. The full-length YscJ protein expressed in the presence (*Y. pestis* KIM8-3002) or absence (pYscJ in *Y. pestis* KIM10; data not shown) of a functional T3SS was found almost exclusively in the low-density (IM) fractions of the gradient, indicating an IM location for YscJ. Control IM (YscV) and OM (YscW, YscC and OMPs) proteins from the same fractions were also analysed (Fig. 5a). The IM localization of YscJ is in agreement with recent studies by Yip *et al.* (2005) with EscJ, indicating that YscJ/PrgK family members are IM lipoproteins; however, IM localization of full-length YscJ could be due to the N-terminal lipoprotein domain or to the predicted C-terminal TM domain.

To determine the subcellular localization of the YscJ N-terminal lipoprotein domain in the absence of the C-terminal TM domain, we generated a plasmid (pYscJ3₊₆₁) encoding a truncated version of YscJ that is

missing amino acids $+62$ to $+226$, which include the C-terminal TM region (Fig. 5b). YscJ₊₆₁ expressed in *Y. pestis* KIM10 fractionated with the IM-containing fractions, confirming that the wild-type YscJ signal sequence and sorting signal target YscJ₊₆₁ to the IM independent of the C-terminal TM region.

The subcellular localization of similarly truncated versions of YscJ carrying the consensus IM sorting signal (CDD-YscJ₊₆₁) or Pal signal sequence and OM sorting signal (Pal-CSS-YscJ₊₆₁) were also determined by membrane flotation sucrose gradient centrifugation (Fig. 5a). As expected, the CDD-YscJ₊₆₁ protein expressed in *Y. pestis* KIM10 consistently fractionated with the IM-containing fractions. Interestingly, the Pal-CSS-YscJ₊₆₁ protein, which was expressed at very high levels, was found primarily in the low-density IM-containing fractions, but also in a number of the high-density OM-containing fractions. These results suggest that the Pal signal sequence and $S_{+2} S_{+3}$ OM sorting signal may have had some influence on the sorting of the mature YscJ₊₆₁ protein; however, a significant portion of this protein remained associated with the IM-containing fractions. The molecular mechanism responsible for localization of full-length YscJ and Pal-CSS-YscJ, as well as YscJ₊₆₁ and Pal-CSS-YscJ₊₆₁ to the IM is unknown.

The N-terminal 61 amino acids of YscJ are required for IM localization of YscJ

Alteration of the amino-acid residues immediately following the $+1$ acylated cysteine did not modify the subcellular localization of YscJ; therefore, we hypothesized that localization of the YscJ lipoprotein moiety to the IM was directed by the formation of a stable domain as has been proposed for other lipoproteins (Robichon *et al.*, 2003). Interestingly, EscJ, another YscJ family member, has been shown to consist of two distinct domains, corresponding to YscJ residues $+1$ to $+59$ and $+85$ to $+195$, that are connected by a linker region located between amino acids $+60$ and $+85$ (Crepin *et al.*, 2005). To evaluate the role of the YscJ N-terminal domain in lipoprotein localization and to localize the minimal region of YscJ required for this localization, we generated plasmids encoding the N-terminal $+25$, $+42$ and $+61$ amino acids of YscJ fused to a C-terminal FLAG epitope tag (Fig. 6b). The pYscJ₊₆₁-FLAG, pYscJ₊₄₂-FLAG and pYscJ₊₂₅-FLAG vectors were moved into *Y. pestis* KIM10 and the subcellular location of the FLAG-tagged YscJ proteins was determined by flotation sucrose density-gradient centrifugation. As expected, the YscJ₊₆₁-FLAG protein was primarily found in the IM-containing fractions (Fig. 6a). In contrast, the YscJ₊₄₂-FLAG and YscJ₊₂₅-FLAG proteins associated primarily with the OM-containing fractions, indicating that the N-terminal 61 residues of YscJ, which are predicted to fold into a stable domain, are required for IM localization.

Truncations that prevent the formation of a properly folded protein domain often result in an increased susceptibility to proteolytic degradation. The proteolytic

Fig. 5. Localization of membrane proteins expressed in *Y. pestis*. (a) Membranes isolated from *Y. pestis* KIM8-3002 or KIM10 carrying plasmids pYscJ3, pYscJ3₊₆₁, pCDD-YscJ₊₆₁ or pPAL-CSS-YscJ₊₆₁ were separated by flotation sucrose density-gradient centrifugation. Proteins in the collected fractions were analysed by SDS-PAGE and immunoblot analysis with anti-YscJ antibodies. Control IM (YscV) and OM (YscC and YscW) proteins from *Y. pestis* KIM8-3002 were detected by immunoblot analysis with anti-YscV, anti-YscC and anti-YscW antibodies. Major OM proteins (OMPs) from *Y. pestis* KIM10 were detected by Coomassie blue staining. (b) Diagrammatic model of YscJ proteins encoded by plasmids pYscJ3, pYscJ3₊₆₁, pCDD-YscJ₊₆₁ and pPAL-CSS-YscJ₊₆₁.

stability of the YscJ₊₆₁-FLAG, YscJ₊₄₂-FLAG and YscJ₊₂₅-FLAG proteins was determined by quantifying the amount of each protein after blocking new synthesis via the addition of chloramphenicol. Samples taken immediately (time 0) and at 15 min, 30 min, 1 h and 2 h after the addition of chloramphenicol were analysed by immunoblotting with anti-FLAG antibody. The YscJ₊₆₁-FLAG protein, which contains the complete YscJ N-terminal domain, was relatively stable; in contrast, the YscJ₊₄₂-FLAG and YscJ₊₂₅-FLAG proteins were rapidly degraded, indicating that these proteins were probably not properly folded (data not shown). These results suggest that a complete and properly folded N-terminal domain is required for the retention of YscJ in the IM.

Evaluation of lipoprotein sorting using YscJ–YscW hybrid proteins

YscW is an OM lipoprotein chaperone that is required for OM localization and proper multimerization of the YscC secretin, an essential component of the T3SS (Burghout *et al.*, 2004). To further investigate the role of the YscJ

N-terminal domain in lipoprotein localization, we evaluated the ability of various YscJ N-terminal regions to target the mature domain of YscW to the OM. Constructs encoding the mature domain of YscW (amino acids +2 to +116) fused to the YscJ signal sequence and residues +1 to +5 (YscJ₊₅-YscW), +1 to +25 (YscJ₊₂₅-YscW), +1 to +42 (YscJ₊₄₂-YscW) or +1 to +61 (YscJ₊₆₁-YscW) of the mature YscJ protein were generated (Fig. 7c) and moved into a *yscW* deletion mutant. As expected, plasmid pYscW, which carries the wild-type *yscW* gene (A₊₂T₊₃), completely restored calcium-regulated Yop secretion (Fig. 7a). Likewise, plasmids expressing YscJ–YscW hybrid proteins containing the YscJ signal sequence and the N-terminal +1 to +5, +1 to +25 or +1 to +42 residues of YscJ fused to the mature domain of YscW were able to restore Yop secretion, confirming that the wild-type YscJ signal sequence and K₊₂V₊₃ sorting signal can direct the sorting of a lipoprotein to the OM in the absence of the intact YscJ N-terminal domain. In contrast, plasmid pYscJ₊₆₁-YscW expressed a stable YscJ₊₆₁-YscW hybrid protein that was unable to complement the defect in Yop secretion, most likely because the YscJ₊₆₁ discrete

Fig. 6. Localization of membrane proteins expressed in *Y. pestis*. (a) Membranes isolated from *Y. pestis* KIM10 carrying plasmids pYscJ3, pYscJ3₊₆₁, pYscJ₊₆₁-FLAG, pYscJ₊₄₂-FLAG or pYscJ₊₂₅-FLAG were separated by flotation sucrose density-gradient centrifugation. Proteins in the collected fractions were analysed by SDS-PAGE and immunoblot analysis with anti-YscJ or anti-FLAG antibodies. Major OM proteins (OMPs) from *Y. pestis* KIM10 were detected by Coomassie blue staining. (b) Diagrammatic model of YscJ proteins encoded by plasmids pYscJ3, pYscJ₊₆₁-FLAG, pYscJ₊₄₂-FLAG or pYscJ₊₂₅-FLAG.

N-terminal domain prevented sorting of the YscW domain to the OM. Flotation density-gradient centrifugation was used to determine the subcellular localization of the YscJ₊₅-YscW and the YscJ₊₆₁-YscW proteins (Fig. 7b). The functional YscJ₊₅-YscW protein primarily localized to the OM-containing fractions, whereas the non-functional YscJ₊₆₁-YscW protein localized primarily with the IM-containing fractions. These results support a role for the N-terminal YscJ domain in retaining YscJ (and the YscJ₊₆₁-YscW hybrid protein) in the IM.

Preliminary analysis of the state of YscJ acylation

As discussed above, the transport of lipoproteins to the OM by the Lol system in *E. coli* can be avoided by the presence of an aspartic acid residue in the +2 position. Recognition and transport of lipoproteins also require that the lipoprotein is fully acylated (Fukuda *et al.*, 2002; Robichon *et al.*, 2005). Previous studies using [³H]palmitic acid to specifically label lipoproteins have demonstrated that YscJ is acylated (Michiels *et al.*, 1991); however, the extent of YscJ acylation has not been determined. The final step in lipoprotein acylation is catalysed by apolipoprotein N-acyltransferase, which adds a third acyl chain directly to the amino group of the modified N-terminal cysteine residue. The presence or absence of this modification can be determined by subjecting the lipoprotein to N-terminal sequence determination, which is dependent upon the presence of a free N-terminal amino group.

A functional YscJ-6x-His protein was expressed in the *yscJ* deletion mutant and purified by nickel-affinity chromatography. The purified YscJ-6x-His protein was subjected to N-terminal sequence determination. The sequence obtained, (–) K V D L, confirmed that the YscJ-6x-His protein was processed and cleaved at the expected location. The ability to obtain an N-terminal sequence by Edman degradation depends upon the presence of a free N-terminal amino group. Thus, as pointed out previously for MxiJ by Blocker *et al.* (2001), these results indicate that a significant portion of the purified YscJ-6x-His protein was not N-acylated. The lack of N-acylation provides a possible explanation for the retention of YscJ in the IM. Thus, the processing of YscJ and other YscJ/PrGK family lipoproteins may represent a divergence from the normally efficient lipoprotein processing/sorting pathway previously characterized for *E. coli* lipoproteins.

Role of YscJ lipid moiety and C-terminal TM region in YscJ function

Previous studies (Yip *et al.*, 2005) and results presented above indicate that YscJ family members are IM lipoproteins that require at least partial acylation for function; however, the function of the N-terminal acylation has not been determined. In most instances, the lipid moiety of a lipoprotein serves simply as an anchor that attaches a proteinaceous domain to a specific cellular membrane; however, proteins intimately associated with membranes can be attached by a

Fig. 7. Localization and function of YscJ secretion/sorting signal–YscW hybrid proteins in *Y. pestis*. (a) Immunoblot analysis of whole bacterial cell (WC) and culture supernatant (S) fractions from the parent strain, a *yscW* deletion mutant, and the *yscW* deletion mutant complemented with plasmids pYscW, pYscJ₊₅-YscW, pYscJ₊₂₅-YscW, pYscJ₊₄₂-YscW and pYscJ₊₆₁-YscW grown with (+) or without (–) 2.5 mM calcium chloride. (b) Membranes isolated from the parent strain or the *yscW* deletion mutant carrying plasmids pYscJ₊₅-YscW or pYscJ₊₆₁-YscW were separated by flotation sucrose density-gradient centrifugation. Proteins were analysed by SDS-PAGE and immunoblot analysis with anti-YscJ or anti-YscW antibodies. Control IM (YscJ) and OM (YscW) proteins from the parent strain were detected by immunoblot analysis with anti-YscJ or anti-YscW antibodies. Major OM proteins (OMPs) from the *yscW* deletion mutant were detected by Coomassie blue staining. (c) Diagrammatic model of YscW and YscJ–YscW hybrid proteins encoded by plasmids pYscW, pYscJ₊₅-YscW, pYscJ₊₂₅-YscW, pYscJ₊₄₂-YscW and pYscJ₊₆₁-YscW.

variety of mechanisms including protein–protein interactions, lipid moieties or TM domains. If the YscJ lipid moiety serves simply as an IM anchor sequence it might also be possible to anchor the N-terminus of YscJ through a different mechanism. Indeed, the flagellar MS-ring protein, FliF, which shares significant amino acid sequence identity with YscJ, is not a lipoprotein, but instead has N-terminal and C-terminal TM domains (Ueno *et al.*, 1994).

To begin to examine the role of the YscJ lipid moiety, we replaced the YscJ signal sequence and +1 cysteine with the

first TM region (in-to-out) from the integral IM protein YscV (Plano *et al.*, 1991) (Fig. 8b). Similarly, to determine if the YscJ C-terminal TM domain plays a specific role in YscJ function, we replaced the C-terminal TM domain of YscJ with the second TM region (out-to-in) of YscV. Plasmids encoding the YscV_{TM1}-YscJ and YscJ-YscV_{TM2} proteins were moved into the *yscJ* deletion strain. Both plasmids encoded stable proteins that completely complemented the defect in Yop secretion (Fig. 8a). These studies suggest that the YscJ N-terminal lipid moiety and the C-terminal TM region function to attach or anchor the YscJ

Fig. 8. Analysis of YopE, YopM, YopN and YscJ expression and YopE, YopM and YopN secretion. (a) Immunoblot analysis of whole bacterial cell (WC) and culture supernatant (S) fractions from the parent strain, the *yjcJ* deletion mutant and the *yjcJ* deletion mutant complemented with plasmids pYscV_{TM1}-YscJ and pYscJ-YscV_{TM2} grown in the presence (+) or absence (-) of 2.5 mM calcium chloride. (b) Diagrammatic model of YscJ proteins encoded by plasmids pCD1, pYscV_{TM1}-YscJ and pYscJ-YscV_{TM2}.

periplasmic domains to the IM and have no separate essential function in the T3S process.

DISCUSSION

Previous studies have shown that YscJ is a lipoprotein that is required for the assembly of a functional T3S apparatus (Allaoui *et al.*, 1995; Michiels *et al.*, 1991). Recent evidence also suggests that members of the YscJ/PrgK family multimerize to form a ring-like structure analogous to the flagellar MS-ring (Yip *et al.*, 2005). The YscJ ring-like structure is probably located in the periplasmic space anchored to the bacterial IM by YscJ's N-terminal lipid moiety and C-terminal TM domain. Evidence presented here provides support for several aspects of this model. Results obtained using YscJ-PhoA-LacZα hybrid proteins indicate that the majority of YscJ is located in the periplasmic space. In contrast, a PhoA-LacZα fusion following the Arg-Lys-Lys sequence located at the

C-terminus of YscJ had extremely low alkaline phosphatase activity, indicating a cytoplasmic location. These results indicate that the YscJ C-terminal hydrophobic region is a TM domain that probably assists in anchoring the primarily periplasmic YscJ ring to the bacterial IM.

Interestingly, EscJ from enteropathogenic *E. coli* lacks a C-terminal hydrophobic domain (Crepin *et al.*, 2005), suggesting that this region, which is found in most YscJ/PrgK family members, may not be required for function. To evaluate the role of the C-terminal TM domain in YscJ expression, stability and function we created a YscJ deletion mutant that lacked this specific structural feature. A YscJ mutant lacking the C-terminal TM domain and Arg-Lys-Lys anchor sequence was non-functional. Interestingly, the C-terminal TM region of YscJ could be functionally replaced using a heterologous TM domain from another integral IM protein, indicating that the YscJ TM domain functions primarily as an anchor and does not appear to have any additional sequence-specific functions.

Multiple lines of evidence indicate that YscJ/PrgK family members are lipoproteins. These proteins all carry consensus lipoprotein signal peptidase recognition/cleavage sites, and labelling with [^{14}C]- or [^3H]palmitate has been directly demonstrated for several of these proteins, including YscJ (Allaoui *et al.*, 1992; Michiels *et al.*, 1991). Nevertheless, the exact nature of N-terminal acylation has not been determined for any YscJ/PrgK family member, and evidence suggests that these proteins as a family are not fully acylated (Blocker *et al.*, 2001). In fact, the ability to obtain N-terminal sequence information from YscJ, as well as MxiJ and PrgK (no membrane localization data are available for these proteins), indicates that the N-terminal amino group of these lipoproteins is normally not acylated (Blocker *et al.*, 2001; Kubori *et al.*, 1998). In fact, MxiJ and PrgJ obtained directly from purified assembled NCs provided N-terminal sequence information, suggesting that the absence of N-acylation is not an artefact related to overexpression of these lipoproteins. Importantly, the Lol transport system is specific for fully acylated lipoproteins; therefore, incompletely acylated lipoproteins cannot be transported to the OM (Fukuda *et al.*, 2002; Robichon *et al.*, 2005). Thus, one explanation for the IM localization of YscJ/PrgK family members is that addition of the third acyl chain by apolipoprotein N-acyltransferase is blocked by an as-yet-unidentified mechanism. If correct, this hypothesis, originally proposed by Blocker *et al.* (2001), would provide an explanation for why YscJ and other YscJ/PrgK family members are localized to the IM regardless of the amino-acid residues in positions +2 or +3. To our knowledge, YscJ, PrgK and MxiJ represent the first documented examples of a natural mechanism to prevent complete acylation of a lipoprotein.

Membrane flotation sucrose density-gradient centrifugation experiments confirmed that YscJ was localized to the *Y. pestis* IM and that IM localization was not dependent upon the C-terminal TM domain. Providing the secretion signal and OM sorting signal from the OM-localized Pal lipoprotein did not disrupt YscJ function or localization to the IM, confirming that YscJ localization occurs independent of the +2 +3 lipoprotein sorting signals. Interestingly, the localization of YscJ to the IM was dependent upon an intact YscJ N-terminal domain (residues +1 to +61). The wild-type YscJ K₊₂ V₊₃ OM sorting signal was recognized and directed the encoded lipoprotein to the OM only when provided in the context of an incomplete YscJ N-terminal domain (YscJ residues +1 to +5, +1 to +25 or +1 to +42). These results suggest that one function of the YscJ N-terminal domain may be to block or prevent complete acylation of YscJ and thus ensure that YscJ remains in the IM. As mentioned, no natural mechanism has been identified that specifically prevents N-terminal acylation of a specific lipoprotein. Thus, the mechanism by which the N-terminal domain of YscJ prevents N-terminal acylation is unknown. It is possible that the YscJ N-terminal domain could block access of apolipoprotein N-acyltransferase or direct rapid assembly of YscJ into a multimer that is no longer a substrate for this enzyme.

At this time, we have no explanation as to why YscJ/PrgK family members have a unique mechanism for localization to the IM. Indeed, a few YscJ/PrgK family proteins, such as SsaJ and HrcJ (Fig. 1b), have an Asp in position +2 and would be expected to be sorted to the IM; however, it is unknown if these lipoproteins are fully acylated. One possible explanation for this unique localization mechanism is that YscJ function or assembly may be incompatible with complete acylation. However, this explanation seems unlikely considering that the N-terminal signal sequence and consensus lipoprotein signal peptidase recognition/cleavage site of YscJ can be functionally substituted for by a TM domain from an integral IM protein. It is also unlikely that the interaction of YscJ with other T3S apparatus components plays a role in retaining YscJ in the IM, as localization of YscJ is similar in the presence or absence of plasmid pCD1, which encodes the T3SS. Understanding the mechanism of YscJ localization will require a detailed analysis of its acylation state and its interaction with the key enzymes involved in lipoprotein acylation and sorting.

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