Direct interaction of a chaperone-bound type III secretion substrate with the export gate

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Several gram-negative bacteria employ type III secretion systems (T3SS) to inject effector proteins into eukaryotic host cells directly from the bacterial cytoplasm. The export gate SctV (YscV in Yersinia) binds substrate:chaperone complexes such as YscX:YscY, which are essential for formation of a functional T3SS. Here, we present structures of the YscX:YscY complex alone and bound to nonameric YscV. YscX binds its chaperone YscY at two distinct sites, resembling the heterotrimeric complex of the T3SS needle subunit with its chaperone and co-chaperone. In the ternary complex the YscX N-terminus, which mediates YscX secretion, occupies a binding site within one YscV that is also used by flagellar chaperones, suggesting the interaction’s importance for substrate recognition. The YscX C-terminus inserts between protomers of the YscV ring where the stalk protein binds to couple YscV to the T3SS ATPase. This primary YscV–YscX interaction is essential for the formation of a secretion-competent T3SS.
Type III secretion systems (T3SSs; also: injectisomes, virulence-associated vT3SS) are a common device of bacterial virulence that is present in gram-negative bacteria such as Salmonella, Pseudomonas, or Yersinia. The macromolecular complex is composed of approximately 20 different protein species, many of which are present in multiple copies, and spans both bacterial membranes. Injectisomes share high structural similarity and likely a common ancestor with the secretion system found in bacterial flagella (T3SS). With the needle-like character of purified injectisomes and their capability to secrete effector proteins directly into a host cell, the T3SS can also be described as a ‘molecular syringe’ and may be a useful protein delivery tool.

Secretion through the Yersinia T3SS is strictly regulated by the inner membrane-anchored export apparatus containing YscR-STUv and can be divided into three distinct phases: (i) ‘early’ substrates, which self-assemble into the extracellular needle; (ii) ‘intermediate’ substrates, hydrophobic translocator proteins that insert themselves into the host membrane, thereby forming a direct connection between bacterial and host cytosols; and (iii) ‘late’ substrates or effector proteins that are then directly funneled through the injectisome into the host cytoplasm. Many substrates of the T3SS are transported to the export apparatus in complex with a type III secretion chaperone (T3SC), which keeps the substrate in a partially unfolded and secretion-competent conformation. The ATPase complex then separates the complex under ATP-hydrolysis and allows for the secretion of the substrate in an unfolded or partially helical state.

T3SCs can further be subdivided into three classes. Class I chaperones bind effector proteins before injection. Class II binds to hydrophobic translocator proteins. Class III is a heterogeneous group and contains chaperones of flagellin in fT3SS or the injectisomal needle subunit Scf (YscF). Class II chaperones contain three tetratricopeptide repeat (TPR) motifs where one TPR is made up of two antiparallel α-helices. The tandem arrangement of TPRs generates a convex outer side that forms extensive interactions with the translocator and a concave binding groove where a short stretch of the substrate molecule binds in an extended conformation. Additionally, a gatekeeper protein is involved to ensure timely secretion of translocators before effector proteins. For the class III chaperone of the needle component Scf, two structures (YscEFG from Yersinia and PscEFG from Pseudomonas) have been published. Both show heterotrimeric complexes of Scf with the two chaperones YscG/PscG and YscE/PscE, where Scf binds into the concave groove of the TPR protein YscG/PscG via a short α-helix. Interestingly, the second chaperone YscE/PscE, which mainly consists of two α-helices, caps the N-terminus of YscG/PscG but barely interacts with the substrate.

Injectisomes can be divided into seven families, such as the Inv-Mxi-Spa family present in Salmonella enterica, Shigella sp. or Escherichia coli, or the Ysc family associated with Yersinia sp., Pseudomonas aeruginosa, Aeromonas sp. or Photobacterium luminescens. Ysc family T3SS produce two proteins, Sci and Scy (YscX and YscY in Yersinia), that are not found in other T3SS families. Functionally, little is known about these two proteins except that they are required for the export of early substrates and hence the assembly of a functional T3SS. YscX and its Aeromonas homolog AscX are secreted with YscY acting as chaperone for YscX prior to secretion. YscX is not an early substrate, since its secretion occurs after the YscP-mediated substrate specificity switch. It has been shown that the N-terminus of YscX harbors its secretion signal, while its C-terminus is essential for the formation of a secretion-competent T3SS. While no experimental structures are available for either protein, YscY can be assigned a role as T3SC based on its sequence which predicts 3 TPRs. The structure of YscX remains elusive, given that its only homologs are other uncharacterized ScyX proteins. The binding mode appears to be conserved between different ScyX and ScyT, as heterologous complexes from different species have been observed via yeast two-hybrid assays. After separation from YscX, YscY probably serves a regulatory function within the bacterial cell due to its interaction with SycD, the chaperone escorting the hydrophobic translocator proteins YopB and YopD.

The interaction between YscX, YscY, and the major export apparatus protein YscV has been established to be a prerequisite for the timely secretion of early substrates and the tripartite complex ScvVXY is conserved between different T3SS-carrying species. ScVs belong to the FlhA superfamily and are such structurally similar to their flagellar FlhA counterparts. The export gate may receive substrates previously bound by a cytosolic sorting platform. Over the years, several structures of FlhA and ScvTs have been published (FlhA-35–38 ScvT-39–43), recently amended by the electron microscopy (EM) structure of YscVscY as a homo-nonamer. In general, ScvTs contain an N-terminal transmembrane domain followed by a linker and a large cytosolic domain. It has been shown that ScvTs form oligomeric structures in vivo that are consistent with the nonameric ring observed in different crystal and EM structures. The cytosolic domain can further be subdivided into four subdomains (SD1–SD4), where SD2 is an insertion in SD1. SD3 and SD4 are necessary for the formation of the nonameric ring. NMR data of a ternary complex composed of the cytosolic domain of Salmonella FlhA, the substrate FlcI and its chaperone Fls5 has demonstrated that for a flagellar T3SS, recognition of the substratet-chaperone complex is facilitated via the chaperone and a conserved region in SD2 of FlhA. It is noted, however, that the recognition site is not conserved in injectisomal ScvTs and deletion of the entire SD2 did not abolish secretion of translocator and tip proteins for Shigella MxiA. Therefore, the binding of substrate-chaperone complexes might occur at a different position in virulence-associated T3SS than in fT3SS.

Here, we report the structure of the YscX-YscY complex alone, and as a tripartite complex with the cytosolic domain of YscV. Our results demonstrate that the binding mode of this injectisomal substrate-chaperone complex is distinct from the binding of flagellar chaperones to the export gate and they explain why the C-terminus of YscX is essential for its function as injectional component.

Results
Structure determination of the YscX:YscY complex. To structurally characterize the two proteins YscX and YscY, we first attempted to express them independently as YscX fused to maltose-binding protein (MBP-YscX) and His6-YscY in E. coli. While YscY was expressed insolubly, MBP-YscX was observed in the soluble fraction but showed heavy precipitation after cleaving off the solubility-promoting MBP-tag. We, therefore, tried co-expression of the two constructs, which yielded soluble protein. Each protein could pull down the other during affinity chromatography and YscX and YscY co-eluted in a symmetrical peak when submitted to size exclusion chromatography. Since stored samples of the complex showed clear signs of proteolytic digestion of YscX, limited proteolysis coupled with N-terminal sequencing and mass spectrometric analysis was conducted to identify a more stable N-terminally truncated version of YscX (YscX32). Neither the C-terminal region of YscX nor YscY were sensitive towards proteolysis. A study by Day and Plano further suggested that only the region YscX32-110 is necessary for it to bind YscY, so further constructs truncating the N-terminus
(YscX\textsubscript{50}), the C-terminus (YscX\textsubscript{32–110}), or both (YscX\textsubscript{50–110}) were created.

Our crystallization constructs contained N-terminally truncated YscX\textsubscript{50} and full-length YscY with an N-terminal hexahistidine tag. The complex crystallized in space group P2\textsubscript{1}2\textsubscript{1}2 with the asymmetric unit (AU) containing two heterodimers related via translational non-crystallographic symmetry. We solved the structure by molecular replacement (MR) using a model generated by AlphaFold 2 (Supplementary Fig. 1). Data collection and refinement statistics are reported in Supplementary Table 1.

The two dimers in the AU are very similar with an r.m.s.d. value of 0.744 Å between the C\textalpha\textsubscript{2} of the complex. Differences lie mostly within YscX—where the r.m.s.d. between the two molecules is 0.904 Å—caused by a slight shift in the second helix (a2) of the protein.

**YscX and YscY form an entwined heterodimer.** The AlphaFold 2 model correctly predicted the overall fold of the complex with YscX binding to YscY at two distinct sites connected via a short linker, resulting in YscX entwining its chaperone (Fig. 1a). Similar to other T3SCs the concave face of YscY forms an interaction site to which YscX binds via a short helix. The YscX binding to YscY at two distinct sites connected via a short linker, resulting in YscX entwining its chaperone (Fig. 1a). Similar to other T3SCs the concave face of YscY forms an interaction site to which YscX binds via a short helix. The area of interaction for this site is approximately 500 Å\textsuperscript{2}. Moreover, this groove is largely lined by hydrophobic residues and allows for the binding of YscX’s L57, W58, F60, A61, and P63 into distinct binding pockets. Hydrophilic residues such as K54, R55, and D59 point toward the solvent (Fig. 1b). S53 and S56 of YscX are 3.8 and 3.9 Å apart from Y101 and Y104 of YscY, respectively, making their contribution to the binding interface via hydrogen bonds unlikely. The importance of the amphipathic character of the binding helix in YscX is further underlined by the high degree of sequence conservation for the hydrophobic residues that interact with the chaperone when compared to the otherwise low conservation of helix a1 (Fig. 2a). The linker between the binding helix and the C-terminal helices of YscX is only resolved in one of the two heterodimers in the AU, indicating the flexibility of the loop.

A second interface is formed via the interaction of YscY’s N-terminal TPR and the two longer C-terminal \alpha\textsubscript{3}-helices (a2 and a3) of YscX. The helices of YscX are rotated by about 90° relative to YscY with the binding site containing the hydrophobic residues I3, L5, F12, L13, I31, L32, A35, and L39 for YscY as well as L80, L83, L86, L87, L95, A98, L101 and L102 for YscX (Fig. 1c). The total area of interaction averages out at 1436.7 Å\textsuperscript{2} for both YscXY heterodimers (PISA\textsuperscript{39}).

The C-terminal helix of YscY extends about 26 Å further out from where it last interacts with its chaperone, as measured from L102 to H120. A hydrogen bond of Q106 to D81 is the last intramolecular interaction between a2 and a3 of YscX. Therefore, the remaining residues (about 20 Å of the C-terminal helix) are not forming interactions within the YscX:YscY heterodimer. In the crystal, this helix is stabilized by interacting with helix a3 of the neighboring molecule as well as the same helix from symmetry-related molecules, forming a 4-helix interface. B-factors for the protruding part of helix a3 are relatively high in chain B, interestingly however not in chain D despite its generally higher B-factors, especially in the poorly defined loop and helix a2 (Supplementary Fig. 2). Furthermore, K121 and V122 are not ordered in the crystal, suggesting that the C-terminus of YscX is somewhat flexible.

**The YscX N-terminus is disordered.** In addition to our structure of YscX\textsubscript{50}:YscY, we also crystallized a longer construct containing residues 32–122 of YscX (YscX\textsubscript{32–122}:YscX\textsubscript{32–122}). Crystallization of this complex required the reductive methylation of primary amines\textsuperscript{40}. The resolution was considerably poorer, with the protein...
adapting a loose packing in space group $I4_122$ with a solvent content of 64.3% (Supplementary Table 1). The overall fold is identical to that of the shorter construct and the structures align well with r.m.s.d. values of 2.033 Å between YscX$_{32}$:YscY and chain A and B of YscX$_{50}$:YscY or 1.383 Å with chain C of YscY. The latter is compared to an r.m.s.d. of 0.744 Å between the two complexes in the AU of the YscX$_{50}$:YscY crystal. In general, the r.m.s.d. values were a bit higher for YscX (1.489 Å or 1.383 Å for chain B or chain D, respectively) than for YscY (1.233 Å or 1.078 Å). The models differ in the most of the position of the loop between helices a1 and a2 of YscX and in a slightly increased bending at the C-terminus of a3 in the model for YscX$_{32}$:YscY (Fig. 1d). Even though this YscX construct is 18 residues longer than YscX$_{50}$, only L47–P49 of these are resolved, suggesting that the N-terminus is largely disordered. Moreover, the C-terminus in this model is fully resolved and is helical albeit with high B-factors for the last residues (Supplementary Fig. 2).

### Structure determination of YscX:YscY bound to nonameric YscV

We then attempted to co-purify the cytotoxic domain of YscV (YscV$_{C}$) with complexes of YscX and YscY from *E. coli*. Yields were considerably higher for the ternary complex (30 mg per liter of culture) than for the heterodimer (1–5 mg per liter). During gel filtration, the complex eluted as a species of high molecular weight. The assembly crystallized readily and conditions for crystallization were identical regardless of whether YscX construct (YscX$_{full-length}$, YscX$_{32}$, or YscX$_{50}$) was used. Diffraction measurement of the ternary complex containing YscV$_{C}$, YscX$_{32}$, and His$_6$-YscY gave data to a resolution of 4.1 Å (Supplementary Table 1). We tested approximately 100 crystals of this complex with most not diffracting further than 8 Å at all. The phase problem was solved via MR with an EM model of nonameric YscV (PDB: 7ALV). Two nonamers are stacked upside-down with their membrane-distal sides interacting in an assembly probably lacking biological significance also described by Kuhlen et al. (Fig. 3a). We then attempted to place the model of YscX$_{50}$:YscY via MR as well, which failed most likely due to its relatively low scattering mass. Instead, 18 binary complexes were placed manually into helical density present outside of the nonameric YscV$_{C}$ rings (Supplementary Fig. 3). The crystal described here is packed loosely with a solvent content of 67.2% that originates both from the central 5 nm-wide pore of the cyclic YscV$_{C}$ nonamer and the fact that the stacked nonamers hardly interact with one another, leaving a large volume of bulk solvent between the 18-mers (Supplementary Fig. 4). Instead, almost all crystal contacts occur between YscY molecules or between an YscY molecule and SD2 of YscV$_{C}$ which also points out from the ring.

### Overall structure of nonameric YscV$_{C}$

In general, the electron density was reasonably well defined for subdomains 3 and 4 (SD3 and SD4), which are mostly buried within the ring. The average area of the YscV-YscV$_{C}$ interaction is approximately 1600 Å$^2$ and is stabilized by four salt bridges between E431–R560, D511–R592, E517–R563, and R519–E531. The electron density of SD1 was
The binding of YscX:YscY to YscV C seems to involve the SD2-SD4 cleft of an individual YscV protomer. The PISA server reports interactions between the YscX:YscY complex and the SD2-SD4 cleft of an individual YscV protomer. The N-terminal truncation YscX 32 has no impact on binding, whereas deletion of the C-terminal 12 amino acids (YscX 32)—which therefore does not contain the binding site for YscV C—abolished the interaction. This was underlined by analytical gel filtrations done with mixtures of either YscV C or a truncated version lacking SD3 and SD4 (YscV SD12, residues 356–513)—which therefore does not contain the binding site for the C-terminal helix of YscX—abolished the ability of YscX C to be pulled down by MBP-YscX 32:His6-YscY in MBP pull-down assays (Fig. 6a), indicating the importance of charge neutralization for recognition of YscX by the export gate.

**YscV requires the C-terminus of YscX for its recognition.** From the ternary complex structure, it became clear that the major mode of binding between the YscX:YscY heterodimer and the nonameric YscV is via an insertion of YscX C-terminus between two YscV protomers (Fig. 3b) at the cleft formed between two adjacent SD4 (hereafter referred to as SD4–SD4 cleft). This is the major binding interface between YscV and YscX and involves SD3 and SD4 of YscV. Except for a salt bridge formed between D108 of YscX and R701 of YscV C directly at the entry point for the helix, the interaction is largely hydrophobic (Fig. 5a) with most residues involved being highly conserved (Fig. 2a). The C-terminal helix of YscX does not extend all the way into the inner hole of the YscV nonamer but instead ends contacting the neighboring YscV molecule. The negative charge of the carboxy-terminus at V122 is presumably neutralized by R551 and R669 of a second YscV molecule (Figs. 4e and 5). Mutating R551 to alanine (R551A) or glutamate (R551E) reduced or abolished the ability of YscV C to be pulled down by MBP-YscX 32:His6-YscY in MBP pull-down assays (Fig. 6a), indicating the importance of charge neutralization for recognition of YscX by the export gate.

**The C-terminus of YscX binds between YscV protomers.** The C-terminal α helix—which is the extended helix seen in the YscX:YscY structures—is inserted between two YscV C protomers (Fig. 3b) at the cleft formed between two adjacent SD4 (hereafter referred to as SD4–SD4 cleft). This is the major binding interface between YscV and YscX and involves SD3 and SD4 of YscV. Except for a salt bridge formed between D108 of YscX and R701 of YscV C directly at the entry point for the helix, the interaction is largely hydrophobic (Fig. 5a) with most residues involved being highly conserved (Fig. 2a). The C-terminal helix of YscX does not extend all the way into the inner hole of the YscV nonamer but instead ends contacting the neighboring YscV molecule. The negative charge of the carboxy-terminus at V122 is presumably neutralized by R551 and R669 of a second YscV molecule (Figs. 4e and 5). Mutating R551 to alanine (R551A) or glutamate (R551E) reduced or abolished the ability of YscV C to be pulled down by MBP-YscX 32:His6-YscY in MBP pull-down assays (Fig. 6a), indicating the importance of charge neutralization for recognition of YscX by the export gate.

**YscV requires the C-terminus of YscX for its recognition.** From the ternary complex structure, it became clear that the major mode of binding between the YscX:YscY heterodimer and the nonameric YscV is via an insertion of YscX C-terminus between two YscV protomers (Figs. 3 and 4a) with further interactions involving the chaperone and SD2 or SD4 as well as residues L47 and Y48 of YscX. To test whether the inserting helix of YscX is necessary for binding to YscV, an MBP pull-down assay was employed, where MBP-YscX:YscY complexes were immobilized from lysate either with or without YscV C present. The SDS-PAGE (Fig. 6a) clearly demonstrated that the N-terminal truncation YscX 110 has no impact on binding, whereas deletion of the C-terminal 12 amino acids (YscX 12–110:YscY or YscX 50–110:YscY) abolished the interaction. This was underlined by analytical gel filtrations done with mixtures of either YscV C or a truncated version lacking SD3 and SD4 (YscV SD12, residues 356–513)—which therefore does not contain the binding site for the C-terminal helix of YscX—with YscX 32:YscY. Since YscX 32:YscY contains the putative binding region 46RLYP49, it should be able to bind to SD1 via YscX and to SD2 via YscY.

On its own, YscX 32:YscY tends to produce a three-peak pattern during gel filtration, most likely due to the formation of oligomers in solution (Fig. 6b). At a concentration of 10 μM, YscX forms a single peak at around 60 kDa apparent molecular mass. As
discussed later, the shift from the expected weight of 40.5 kDa was attributed to dynamic assembly and disassembly of the nonameric ring during the run (Fig. 7). With a mixture of YscV C and the binary complex, a clear shift towards higher apparent molecular masses can be seen as a peak around 10 mL retention volume (Fig. 6b top, Supplementary Table 2, Supplementary Fig. 8), which we attributed to a nonameric ring of YscV C with an unknown quantity of YscX:YscY bound to it. For the ternary complex, two more peaks were observed at approximately 14 and 15 mL, the latter of which could be attributed to free YscV C in solution or a 1:1:1 ternary complex for which a molecular mass of 65 kDa would be expected. The broad and large signal at around 14 mL might also indicate other oligomeric forms and a dynamic equilibrium between different states. The shortened YscV SD12 construct elutes after approximately 16 mL, which corresponds to around 36 kDa, potentially representing a dimer (Fig. 6b, bottom). Here, no shift in mass was observed when the protein was mixed with YscX12:YscY in an equimolar fashion and thus we concluded that SD1 and SD2 alone do not bind YscX:YscY with high affinity. A construct of YscV C only containing SD3 and SD4 (residues 514–704) could not be analyzed as it was insoluble upon expression in E. coli.

The termini of YscX oppositely influence nonamORIZATION of YscV C. YscV forms higher oligomers in Yersinia regardless of whether YscX and YscY are present or not. The isolated cytosolic domain of other SctV proteins, both monomeric, as well as nonameric ones, have been observed in solution. With the major recognition site of YscX:YscY and YscV C being located at the far C-terminus of YscX and between two YscV protomers, we aimed at investigating a possible influence of YscX on the nonamORIZATION of the export gate’s cytosolic domain. Size exclusion chromatography of YscV C at different concentrations revealed a concentration-dependent dynamic equilibrium between different states with the nonameric form being stabilized at higher concentrations (Fig. 7, top). At 2.5 μM initial concentration of YscV C, the protein appears to be largely monomeric as indicated by a symmetrical peak at around the expected molecular mass of 40.5 kDa. At 10 or 25 μM, we observed a shift in the apparent mass and significant fronting, indicating the dynamic association and dissociation of higher oligomeric species on the column. At 100 μM the main peak is shifted to a high apparent molecular mass corresponding to a nonamer, but there is still notable tailing, i.e., incomplete conversion to the nonameric ring. We then compared the oligomerization behavior in the presence of two different YscX:YscY constructs, which were co-purified with YscV C. At 10 μM, YscV C:YscY showed almost-complete conversion to higher masses. Interestingly, however, the full-length YscX-containing ternary complex (YscV C:YscY) favored the monomeric species at the same molar concentration of protein (Fig. 7, middle and bottom).

Using the same concentration series as for YscV C, the nonameric ring of YscV C:YscY is stable even at an initial concentration of 2.5 μM, whereas full-length YscX seems to inhibit ring formation even at high concentrations (Fig. 7, bottom). We concluded that the N-terminus of YscX is not necessary for binding YscV, but it decreases the propensity of YscV C to nonamorize and therefore must interact in some other way.

Discussion

YscX and YscY are largely uncharacterized proteins from the T3SS of Yersinia with a yet unknown function despite being essential for a functional injectisome. With YscX belonging to a unique and elusive protein family without any homologous structures, we were unable to solve the phase problem via MR. After laborious attempts at experimental phasing, we attempted to predict the structure of the two proteins computationally using I-TASSER, I-TASSER, and
AlphaFold 2\textsuperscript{43}, YscY was always predicted as a TPR protein and the general fold was very similar between programs (Supplementary Fig. 1b). YscX on the other hand produced models that were only similar for the C-terminal $\alpha_2$ and $\alpha_3$ helices (Supplementary Fig. 1a). Finally, we attempted to predict the complex structure using the same programs but supplying both sequences fused together by a 33-residue linker. While the other predictions were disregarded, we were intrigued by how compatible the AlphaFold 2 model was to our biochemical and crystallographic observations. Indeed, we could solve the phase problem for our YscX:YscY crystals with a truncated version of the AlphaFold 2 complex model. In retrospect, models of YscY were also sufficient for MR when generated by AlphaFold 2, tFold or RoseTTAfold, but not I-TASSER.

Here, we provide the structure of a SctX-family protein. YscX and YscY depend on each other for soluble expression in \textit{E. coli}, which is understandable given how entwined the two proteins are in their complex. Lacking YscX, YscY would present a large hydrophobic face at its N-terminal TPR that could explain why it is insoluble in isolation. After secretion of its substrate, this face might be responsible for recognizing SycD, an interaction previously observed\textsuperscript{22,23}. We found that the N-terminal 31 residues of YscX are prone to proteolysis, indicating that they may not exhibit a tertiary structure. It is possible that the N-terminus carries a proteinaceous signal for YscX secretion, especially considering how conserved the sequence is among the SctX family (Fig. 2a). The smallest YscX fragment to direct secretion (amino acids 1–60) contains helix $\alpha_1$\textsuperscript{17}, leaving the possibility that YscY bound to helix $\alpha_1$ of YscX is part of the secretion signal. This does not seem to be the case for the structurally similar YscEFG complex, for which the transport of YscF to the injectisome depends on its co-chaperone YscE, which structurally fulfills the role of $\alpha_2$ and $\alpha_3$ in YscX\textsuperscript{44}. Alternatively, the structurally unresolved N-terminus of YscX might bind other T3S proteins and only fold in their presence, as was observed for YopN in presence of TyeA in the same secretion system\textsuperscript{45}. It is unclear whether YscX serves a hitherto unknown function outside the bacterial cell after its secretion or if its absence from the export apparatus signals a switch in substrate recognition, as was postulated by Day, et al.\textsuperscript{17}.

When compared to other T3S substrate:chaperone complexes, the structural similarities between YscXY and the complex of the needle protein with its two chaperones become obvious (Fig. 8a). Two structures of such a complex, one from \textit{Pseudomonas} (PscEFG, PDB: 2UWJ\textsuperscript{14}) and the other from \textit{Yersinia} (YscEFG, PDB: 2P58\textsuperscript{13}) have been published, both revealing an amphipathic Scf helix that binds into a hydrophobic groove formed by the concave side of the TPR protein YscG/PscG. The co-chaperone YscE/PscE was found to bind the hydrophobic face of the N-terminal TPR of YscG/PscG with its two helices that contribute several leucine residues to the binding interface. Thus, it appears as if YscX fulfills the roles of both “co-chaperone” and “substrate” in this assembly reminiscent of the YscEFG complex. In contrast, class II substrate:chaperone complexes—which are

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**Fig. 5 The C-terminus of YscX binds between two YscV protomers.**

\textbf{a} Surface hydrophobicity of YscV (left) and the neighboring protomer (right) from hydrophobic (red) to hydrophilic (white) with a ribbon and stick representation of YscX (cyan). Helix $\alpha_3$ inserts between two YscV molecules and binds via hydrophobic interactions. The C-terminus is pointing towards a positively charged patch of the neighboring subunit, which likely stabilizes the carboxylate.

\textbf{b} $2mF_o-DF_c$ density contoured at 1$\sigma$ at the C-Terminus of YscX suggests positioning of the carboxylate towards R551 of YscV (orange). The density of the arginines is exemplary and varies between the chains.
hydrophobic translocator proteins and their chaperones—form soluble 1:1 heterodimers like YscXY, but exhibit vastly different binding behaviors. A short stretch close to the N-terminus of the substrate binds to the groove formed by the chaperone’s concave side in extended conformation (Fig. 8b, refs. 8-11,46). The structure of *Aeromonas* AcrH with translocator protein AopB shows further interactions between the two proteins. Analogous to the YscEFG and YscXY complex, AopB binds the N-terminal TPR of AcrH via an α-helix. In addition, AopB extensively wraps around the convex side of the chaperone, a feature neither present in YscXY nor YscEFG11. Thus, despite its heterodimeric nature, the

**Fig. 6 Recognition of YscXY by YscVC depends on the C-terminus of YscX as well as SD3 and SD4 of YscVC.** A Coomassie-stained SDS-PAGE of MBP pull-downs. N- and C-terminally truncated MBP-YscX constructs were co-expressed with His6-YscY and YscVC. Lysates were loaded onto amylase resin and the washed matrix loaded onto the gel. The pull-downs generated consistent results over three replications. Source data are provided as a Source Data file. b Analytical size exclusion chromatography with 10 μM YscVC, YscVSD12 (both blue), YscX32-YscY (black), or mixtures (red) of the proteins. Absorbance at 280 nm shows a shift in retention volume for a mixture of YscVC with YscX32:YscY, but not YscVSD12 with YscX32:YscY. Chromatographies involving YscVSD12 or YscVC were performed twice or four times, respectively, with consistent results. A SDS-PAGE and Western blot analyzing contents of gel filtration peaks are shown in Supplementary Fig. 8.

**Fig. 7 Concentration-dependent nonamerization of YscVC.** For comparability, absorbances were normalized to the highest peak. From top to bottom: YscVC, a ternary complex of YscVC co-expressed with YscX50 and His6-YscY, and the same ternary complex but with full-length YscX (YscXfl) were loaded at 2.5 μM (pink) to 100 μM (blue) onto a Superdex 200 column. For reference, the expected retention volumes for the monomeric species (or 1:1:1 heterotrimer) and nonameric species (assuming 9:9:9 stoichiometry and a globular shape) are shown as dashed gray lines. The weak auto-nonamerization of YscVC is enhanced by the presence of YscX50 but severely impeded by full-length YscX. Experiments were performed three times with consistent results.
YscXY complex more closely resembles the heterotrimeric YscEFG complex, suggesting structural similarity to class III chaperones.

Since structures of FlhA were solved in 2010, several flagellar and injectosomal export gates were deposited in the PDB (FlhA<sup>26–28</sup> SctV<sup>29–33,35</sup>). Some discussion is dedicated to elucidating differences in how the subdomains are arranged relative to each other, especially considering the cleft between SD2 and SD4 that is responsible for the recognition of the flagellar FltD:FltT and FlIC:FlIS substrate:chaperone complexes<sup>38</sup>. The structure of YscV<sub>C</sub> was previously reported as adopting the “open” conformation with space between SD2 and SD4<sup>35</sup> when present without additional binding partners. Our structure of the ternary complex YscV<sub>C</sub>:YscX<sub>32</sub>:YscY exhibits the same open conformation showing that no major domain rearrangements are necessary to accommodate YscXY (Fig. 9a). Minor movements are observed at the binding site between YscY and SD4 as well as helix a3 of YscX and SD4 but these do not seem to affect the export gate globally (Supplementary Fig. 7). Our model also establishes that a highly conserved 49RLYP49 motif of YscX binds within the cavity of the ring but not reaching it. While we initially thought that this hints at YscX stabilizing an otherwise dissociation-prone YscV<sub>C</sub> nonamer in vivo, our gel filtration results indicate that full-length YscX actually disfavors nonamORIZATION of the export gate (Fig. 7 bottom). The importance of the YscX C-terminus for recognition by YscY explains why complementation of yscX deletion strains with C-terminally FLAG-tagged YscX does not produce an injectosome competent to secrete Yops<sup>17</sup>. The same construct, however, was secreted by a functional T3SS<sup>17</sup>, suggesting that the interaction of YscXY with the SD2-SD4 cleft is sufficient for the secretion of YscX.

The binding site of the ATPase stalk protein SctO, as published for the complex of <i>Chlamydia</i> CdsV:CdsO<sup>32</sup>, is likewise located in the SD4-SD4 cleft and would not allow for simultaneous binding of SctO and SctXY (Fig. 9d). It should be noted that the <i>Chlamydia</i> T3SS does not belong to the Ysc family and therefore lacks SctX and SctY. It is still poorly understood how different factors work together in the regulation of SctV. These include the binding of interacting proteins such as SctO, SepL:SepD in enteropathogenic <i>E. coli</i><sup>47</sup>, or substrate:chaperone complexes as well as local pH<sup>33</sup>, and the linker between transmembrane and cytosolic domain<sup>34,48,49</sup>. As such, further studies should investigate the potential interplay between YscV, Ysco, and YscXY. We argue that, based on the lack of rearrangement after YscXY binding and the nonamer destabilization in vitro, YscX (and its chaperone YscY) may contribute to those dynamics by destabilizing the nonameric ring of YscV, most likely via YscX’s yet uncharacterized N-terminus. This may contribute to facilitating the substrate switch from needle components to hydrophobic translocators.

**Methods**

**Cloning**. The vectors pETM-40<sub>p</sub>yscX and pACYCDuet-1<sub>p</sub>yscY were generated for the expression of YscXY,Ysc binary complexes via PCR and restriction cloning using NcoI and NotI. Furthermore, pACYCDuet-1 containing both yscY in its first and yscV56 in its second multiple cloning site was prepared using the same method but employing NdeI and XhoI. For the expression of YscV<sub>C</sub> alone, pETM-11<sub>p</sub>yscV356 was used. From this vector, a deletion mutant lacking SD3 and SD4 was generated (YscV<sub>SD12</sub>). All genes were cloned from the <i>Yersinia enterocolitica</i> pYVe227 virulence plasmid (G. Cornelis, Basel) via PCR. Deletions and mutations were introduced via Round the horn mutagenesis. For a full overview of all constructs used, refer to Supplementary Table 3.

**Expression and purification of YscX:YscY complexes**. Complexes of YscX with its chaperone YscY were expressed in <i>E. coli</i> BL21 (DE3) transformed with both pETM-40<sub>p</sub>yscX and pACYCDuet-1<sub>p</sub>yscY vectors. Cell cultures were grown at 37°C to OD<sub>600</sub> = 0.5 in LB medium and cooled to 20°C before induction with 0.25 mM isopropyl-β-D-1-thiogalactopyranoside at OD<sub>600</sub> = 0.8–0.9. After incubation over night, cells were spun down at 4000 x g and pellets stored at −20°C. Cell pellets were resuspended in 50 mM Tris pH 8, 150 mM NaCl and 10 mM β-mercaptoethanol supplemented with one tablet of cComplete protease inhibitor cocktail (Roche) and 0.6 μg DNase I per liter of expression culture and then passed through a pressure cell homogenizer (Stansted FPG12800) at 120 Mpa.

Lysates were cleared by centrifugation (60 min, 30,000 x g, 4°C). Amylose affinity chromatography (10 mL Amylose Resin HighFlow, New England Biolabs) was employed to capture MBP-YscX-His<sub>6</sub>-YscY. After 60 min of light pivoting at 4°C, the resin was washed by raising the NaCl concentration to 300 mM. The protein-loaded amylose was re-equilibrated with 50 mM Tris pH 8, 150 mM NaCl.
YscVXY superimposed with the cryo-EM structure of YscVC (gray, PDB: 7ALW), which is shown as cartoon representation. YscVC protomers are aligned with an r.m.s.d. of 0.797 Å.

Fig. 9 Superposition of export gate complexes with YscVXY. YscV, YscX, and YscY are shown in orange, cyan, and red, respectively. a) Superposition with the cryo-EM structure of YscVC (gray, PDB: 7ALW), which is shown as cartoon representation. YscVC protomers are aligned with an r.m.s.d. of 0.797 Å. b, c) Superposition with FlhA (gray) bound to the flagellar chaperone:substrate complexes (green/light green). b) FlhA:FliS:FliC (PDB: 6CH3). c) FlhA:FliF:FliD (PDB: 6CH2). Alignments were obtained using SD3 of YscVC (residues 514–600) with an r.m.s.d. of 1.194 Å (PDB: 6CH2) and 1.207 Å (PDB: 6CH3).

d) YscVXY superimposed with Chlamydia pneumoniae CdsV in complex with the stalk protein CdsO (PDB: 6WA9). The alignment was calculated using SD3 of YscVC with an r.m.s.d. of 0.854 Å. Two subunits of YscV and CdsV (gray) are shown, CdsO is colored in green.

Reductive methylation of YscX:YscY

Expression and purification of His6-YscV constructs. For the stand-alone purification of the cytosolic domain of YscV (YscV C, residues 356–704) or YscV SD12 (residues 356–513), the proteins were expressed as described above from pETM-11 with an N-terminal and TEV-cleavable hexahistidine tag. The cleared lysate was loaded onto 10 mL NiNTA resin and incubated for 60 min with 10 mM imidazole. The flow-through was collected and the resin was washed with 50 mM Tris pH 8, 300 mM NaCl, 10 mM imidazole, and 10 mM β-mercaptoethanol and then with 30 mM imidazole in the same buffer. An increase to 250 mM imidazole eluted the target protein, which was then dialyzed against 20 mM Tris pH 8, 150 mM NaCl, 1 mM DTT after adding 1:50 w/v TEV protease. Re-chromatographic NiNTA cleared the target of uncleaved His6-YscV before the protein was concentrated and polished via size exclusion chromatography on a HiLoad 16/60 Superdex 200 pg (Cytiva). Before freezing the concentrated protein complex, 5 mM TCEP was added.

Expression and purification of His6-YscV constructs

Expression and purification of YscV CXY. For the ternary complex of YscVCX:YscY with YscV C, pETM-40-yscX32 and pACYCDOuet-t_yscY-yscV356 were co-transformed into E. coli BL21 (DE3). Expression and purification steps were identical until the protein was eluted from the amylose resin after TEV-digestion. Instead of successive NiNTA affinity chromatography, the complex was loaded onto 5 mL amylose resin to remove the remaining MBP-tagged protein. The flow-through and washing fractions were combined, concentrated, and purified via size exclusion chromatography on a HiLoad 16/60 Superdex 200 pg (Cytiva).

MBP-pull-down assay. To test the binding of different YscX truncations to YscV CXY, both proteins and YscC were co-expressed as described for the ternary complex but at a scale of 100 mL culture. The cleared lysate was applied to 1 mL amylose resin and incubated for 60 min with 10 mM imidazole. The flow-through was collected and the resin was washed with 50 mM Tris pH 8, 300 mM NaCl, 10 mM imidazole, and 10 mM β-mercaptoethanol and then with 30 mM imidazole in the same buffer. An increase to 250 mM imidazole eluted the target protein, which was then dialyzed against 20 mM Tris pH 8, 150 mM NaCl, 1 mM DTT after adding 1.5 mg/mL TCEP. After concentration to 10–15 mg/mL, 5 mM TCEP was added and the protein was flash-frozen.
Limited proteolysis. YscX_{50:long}-YscY was subjected to limited proteolysis by trypsin, chymotrypsin, proteinase K, and thermolysin to identify stable constructs for expression. Purified protein (1 mg/mL) was mixed with 1:100 (w/w) protease in 50 mM Tris pH 8, 150 mM NaCl, and 1 mM DTT or 0.5 mM CaCl2 (for thermolysin) and incubated on ice for 3 h. Samples were analyzed by SDS-PAGE and sequenced at their N-terminus via Edman degradation (Proteome Factory, Berlin, DE) after transfer to PVDF membrane (Immobilon, Merck) in a semi-dry set-up. Total mass was determined in-house using MALDI-TOF after dialysis against 10 mM aceta buffer pH 4.6.

Analytical size exclusion. Size exclusion chromatography on a Superdex 200 10/300GL was utilized to analyze protein oligomerization, dissociation, and association. The proteins were thawed on ice, combined for protein mixtures, or diluted for concentration gradients, and incubated for at least 30 min on ice before centrifugation and application to the column. All runs were carried out at 0.5 mL/min flowrate with 20 mM Tris pH 8, 150 mM NaCl as running buffer.

Crystalization and data collection. Crystals of YscX_{50}-YscY grew in 0.1 M BisTris pH 6.0, 0.2 M MgCl2 and 21% PEG 3,350 with 5 mg/mL protein and a drop ratio of 0.35 μL reservoir to 0.66 μL protein solution at 20 °C in a sitting drop setting over 60 μL of reservoir. Microseeding of these into the same condition but with 13% PEG 3350 and 360 μL of reservoir. Microseeding of these into the same condition but with 13% PEG 3350 supplemented with 5% glycerol. Measurements were carried out at the BL14.2 beamline at the BESSY II electron storage ring operated by the Helmholtz-Zentrum Berlin für Materialien und Energie. Data were collected using the local installation of MxCube.

For crystallization of the methylated complex YscX_{50:long}-YscY, the protein buffer was exchanged to 10 mM Tris pH 8, 50 mM NaCl, 5% (w/v) sucrose, 2.5 mM TCEP. Crystals were obtained at 20–25 °C with 0.1 M sodium succinate pH 6–6.5, 4.2–4.4 M NaCl as reservoir solution using 5 mg/mL protein but could be optimized by the addition of 4% acetonitrile (Hampton Research Additive Screen). The drop ratio was 1 μL protein plus 0.5 or 1 μL reservoir solution. For harvesting, trays were transferred from 20°C to 4 °C, and crystals were cryoprotected with 19% w/v sucrose. Data collection took place at beamline X06SA at SLS (Villigen, Switzerland) using 1.00 Å wavelength and 0.25° of oscillation between frames. The ternary complex of YscV, YscX_{50}, and His6-YscY was crystallized in a sitting drop setting with 0.1 M Hepes pH 6.8–7.5, 1% PEG 2000 MME, and 0.7–1.0 M sodium succinate as reservoir solution. The drop contained 0.33 μL reservoir solution and 0.66 μL 5 mg/mL protein. Large crystals between 200 and 300 μm in length grew after 2–3 days of incubation at 20 °C. As these crystals were very fragile and tended to crumble when touched, the plates were transferred to 4 °C the evening before harvesting. Cryoprotection was achieved by gradually transferring the crystals to solutions containing 0.1 M Hepes, 1% PEG 2000 MME, and 1.1 M sodium succinate as well as 5, 10, and finally 22.5% glycerol. Data were collected through the local installation of MxCube at beamline P13 at DESY (Hamburg, Germany) using a helical scan with 0.066 s exposure time per image and 100% transmission.

Data reduction, model building, and refinement. Diffraction data were indexed, integrated, and scaled using XDSC59 or XDSDAP60 for YscX_{50:long}-YscY. The MR model was generated using a local installation of AlphaFold2, substituting the sequences of YscX and YscY with a 33 residue linker between them. The phase problem was initially solved for the YscX_{50}YscY data with a truncated model of the complex using Phaser39 with the INCS option disabled. After initial model building using Buccaneer45 in the CCP4 GUI, successive rounds of model building in Coot58 and refinement in phenix.refine59,66 generated the final model. TLS parameters were determined by the “find TLS” option in phenix.refine. For YscX_{50:long}-YscY a model of the shorter YscX_{50:long}-YscY construct was employed as a search model during MR. Secondary structure restraints and reference model restraints from the aforementioned structure were used during refinement in phenix.refine. The ternary complex structure was solved via MR with a cryo-EM model of YscX_{50:short} (PDB: 7ALW (YscV; nonamer)). After an initial round of refinement in phenix.refine, the YscX and YscY chains were placed manually into the density and refined with reference model restraints. To account for the low resolution data, group B-factors, secondary structure restraints, Ramachandran restraints, and torsion-angle NCS restraints were enabled in refinement. The resolution cutoff for the YscX_{50:long}-YscY dataset was determined using paired restraints as implemented in PAFREP. Manual removal of alignments were generated in PyMOL. The r.m.s.d. value of alignments was calculated in PyMOL using only Cα atoms with outlier rejection. The entire residue range was considered unless stated otherwise.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The atomic coordinates and X-ray intensities generated in this study have been deposited in the Worldwide Protein Data Bank (wwPDB) under accession codes 7QIH (YscX_{50:long}-YscY), 7Q1I (YscX_{50:long}-YscY), and 7QII (YscV_{50:long}-YscX_{50:long}-YscY). Diffraction images are available from the SB Grid Data Bank66 (data.sgird.org) with Data ID 905 (YscX_{50:long}-YscY), Data ID 906 (YscX_{50:long}-YscY), and Data ID 907 (YscV_{50:long}-YscX_{50:long}-YscY). The structure of the YscV, nonamer used as search model to solve the phase problem of the YscX_{50:long}-YscY structure is available from the wwPDB under accession code 7ALW. The structures used to generate Figs. 8 and 9 are available from the wwPDB. Figure 8a: 2P58 (YscEFG); Fig. 8b: 3WXX (AcrH-AopB); Fig. 9a: 7ALW (YscV; nonamer); Fig. 9b: 6CH1 (FliA/FliK/FliC); Fig. 9c: 6CH2 (FliA/FliH/FliD); Fig. 9d: 6WA9 (CdxV-CdxO). Source data are provided with this paper.

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