Deciphering the assembly of the *Yersinia* type III secretion injectisome

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The assembly of the *Yersinia enterocolitica* type III secretion injectisome was investigated by grafting fluorescent proteins onto several components, YscC (outer-membrane (OM) ring), YscD (forms the inner-membrane (IM) ring together with YscJ), YscN (ATPase), and YscQ (putative C ring). The recombinant injectisomes were functional and appeared as fluorescent spots at the cell periphery. Epistasis experiments with the hybrid alleles in an array of injectisome mutants revealed a novel outside-in assembly order: whereas YscC formed spots in the absence of any other structural protein, formation of YscD foci required YscC, but not YscJ. We therefore propose that the assembly starts with YscC and proceeds through the connector YscD to YscJ, which was further corroborated by co-immunoprecipitation experiments. Completion of the membrane rings allowed the subsequent assembly of cytosolic components. YscN and YscQ attached synchronously, requiring each other, the interacting proteins YscK and YscL, but no further injectisome component for their assembly. These results show that assembly is initiated by the formation of the OM ring and progresses inwards to the IM ring and, finally, to a large cytosolic complex.

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Introduction

The type III secretion (T3S) apparatus, also called injectisome, allows bacteria to export effector proteins on contact with eukaryotic cell membranes (Cornelis and Wolf-Watz, 1997; Galan and Collmer, 1999; Cornelis and Van Gijsegem, 2000). Effectors (called Yops in *Yersinia*) display a large repertoire of biochemical activities and modulate the function of crucial host regulatory molecules to the benefit of the bacterium (Alfano and Collmer, 2004; Mota and Cornelis, 2005; Grant *et al.*, 2006). In *Yersinia* spp., the injectisome is built when temperature reaches 37°C and export of the

Yops can be artificially triggered, in the absence of cell contact, by Ca^{2+} chelation (Cornelis, 2006).

About 25 proteins (called Ysc in *Yersinia*) are needed to build the injectisome. Most of these are structural components, but some are ancillary components that are only involved during the assembly process and are either shed afterwards (e.g. the molecular ruler) or kept in the cytosol (e.g. chaperones). In contrast to the large diversity observed among effectors, the core proteins forming the injectisome (YscC, J, N, Q, R, S, T, U, V, and, to a lesser extent, YscD in *Yersinia*) are well conserved (Van Gijsegem *et al.*, 1995; Cornelis, 2006).

A number of injectisome proteins copurify as a complex cylindrical structure, resembling the flagellar basal body. This structure, called the needle complex, consists of two pairs of rings that span the inner membrane (IM) and outer membrane (OM) of bacteria, joined together by a narrower cylinder and terminated by a needle, a filament, or a pilus (Kubori *et al.*, 1998; Blocker *et al.*, 1999; Kimbrough and Miller, 2000; Daniell *et al.*, 2001; Jin and He, 2001; Sekiya *et al.*, 2001; Morita-Ishihara *et al.*, 2006; Sani *et al.*, 2007; Hodgkinson *et al.*, 2009; Schraidt *et al.*, 2010). The needle is a hollow tube assembled through helical polymerization of a small protein (around 150 copies of YscF in *Yersinia*) (Cordes *et al.*, 2003; Deane *et al.*, 2006). It terminates with a tip structure serving as a scaffold for the formation of a pore in the host cell membrane (Mueller *et al.*, 2005). The ring spanning the OM (hereafter called OM ring) and protruding into the periplasm consists of a 12–14mer of a protein from the YscC family of secretins (Koster *et al.*, 1997; Kubori *et al.*, 2000; Tamano *et al.*, 2000; Blocker *et al.*, 2001; Marlovits *et al.*, 2004; Burghout *et al.*, 2004b; Spreter *et al.*, 2009). The lower ring spanning the IM is called MS ring and made of a lipoprotein (YscJ in *Yersinia*, MxiJ in *Shigella*, PrgK in *Salmonella enterica* SPI-1) proposed to form a 24-subunit ring (Kimbrough and Miller, 2000; Crepin *et al.*, 2005; Yip *et al.*, 2005; Silva-Herzog *et al.*, 2008; Hodgkinson *et al.*, 2009). A protein from the less-conserved YscD family (MxiG in *Shigella*, PrgH in *S. enterica* SPI-1), which has the same general fold as the components of the two rings, is proposed to participate in MS ring formation and possibly connect the rings in the two membranes (Spreter *et al.*, 2009).

Besides these proteins forming a rigid scaffold, the injectisome contains five essential integral membrane proteins (YscR, S, T, U, V), which are believed to recognize export substrates (Sorg *et al.*, 2007) and form the export channel across the IM. Some of them, if not all, are likely to be inserted in a patch of membrane enclosed within the MS ring, but this could not be shown so far. We will refer to these proteins as to the ‘export apparatus’. At the cytosolic side of the injectisome, an ATPase of the AAA⁺ family (YscN) forms a hexameric ring that is activated by oligomerization (Woestyn *et al.*, 1994; Pozidis *et al.*, 2003; Muller *et al.*, 2006; Zarivach *et al.*, 2007) and resembles the flagellar ATPase FliI

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(Abrahams *et al*, 1994; Imada *et al*, 2007). The ATPase is associated with two proteins (YscK, L) (Jackson and Plano, 2000; Blaylock *et al*, 2006), one of them (YscL) probably exerting a control on the ATPase activity as was shown for FliH in the flagellum (Minamino and MacNab, 2000; Gonzalez-Pedrajo *et al*, 2002; McMurry *et al*, 2006). The ATPase is strikingly similar to the α and β subunits of the stator of the F_0F_1 ATP synthase (Abrahams *et al*, 1994), suggesting an evolutionary relation. This assumption is reinforced by the sequence similarity observed between YscL_{N-term} and the b subunit of the F-type ATPase, and between YscL_{C-term} and the δ subunit of the same ATPase (Pallen *et al*, 2006). A function of the ATPase, characterized in *S. enterica* Typhimurium SPI-1, is to detach some T3S substrates from their cytoplasmic chaperone before their export and to unfold the exported proteins in an ATP-dependent manner (Akeda and Galan, 2005). It is likely that the ATPase also directly energizes export, but the proton motive force is also involved (Wilharm *et al*, 2004; Minamino and Namba, 2008; Paul *et al*, 2008).

In the flagellum, the most proximal part of the basal body is the 45–50 nm C ring (for cytosolic) made of FliM and FliN (Driks and DeRosier, 1990; Khan *et al*, 1992; Kubori *et al*, 1997; Young *et al*, 2003; Thomas *et al*, 2006). Together with FliG, it forms the switch complex reversing the rotation of the motor, but in its absence, no filament appears, indicating that it is also involved in the export of distal constituents (Macnab, 2003). However, recent reports (Konishi *et al*, 2009; Erhardt and Hughes, 2010) showed that in C ring mutants, the export function can be partially restored by overexpression of the ATPase or the master regulator. No such C ring could be visualized so far by electron microscopy in a needle complex, but proteins of the YscQ family, which are essential components of all injectisomes, have a significant similarity to FliN and FliM. In *Pseudomonas syringae*, the orthologue of YscQ even appears as two products called HrcQ_A and HrcQ_B, which interact with each other, and the overall fold of HrcQ_B is remarkably similar to that of FliN (Fadoulglou *et al*, 2004). This suggests that injectisomes do have a C ring, although they have not been reported to rotate. YscQ and its homologues have been shown to bind the ATPase complex (Jackson and Plano, 2000) as well as substrate-chaperone complexes (Morita-Ishihara *et al*, 2006). The C ring would, therefore, form a platform at the cytoplasm/IM interface for the recruitment of other proteins. In agreement with this assumption, immunogold-labelling experiments have shown that the *Shigella* orthologue of YscQ (Spa33) localizes to a lower portion of the injectisome (Morita-Ishihara *et al*, 2006). A list of homologues in the flagellum and the various archetypal T3S systems is given in Supplementary Table 3.

The assembly of the flagellum is for the most part linear and sequential, proceeding from more proximal structures to more distal ones. The proposed scenario is that the plasma membrane ring (called the MS ring) formed by FliF assembles first, followed by periplasmic components, OM components, and finally components that lie in the cell exterior (Kubori *et al*, 1992; Macnab, 2003). The C ring (FliG, FliM, FliN) is thought to appear immediately after the MS ring, because it forms spontaneously when its components are overexpressed in the presence of FliF even in the absence of any other component (Kubori *et al*, 1997; Lux *et al*, 2000; Young *et al*, 2003).

Less is known about the assembly steps of the injectisome. The heterologous overexpression of the *S. enterica* SPI-1 MS ring components PrgH and PrgK in *Escherichia coli* leads to stable ring structures (Kimbrough and Miller, 2000). The same is true for the *Yersinia* secretin YscC together with its pilotin YscW (Koster *et al*, 1997). This suggests that the transmembrane rings might form independently. It has thus been proposed (Kimbrough and Miller, 2000) that the first step consists in the assembly of the MS ring, possibly along with the recruitment of the transmembrane proteins forming the export apparatus. In parallel, the secretin ring would form in the OM. Afterwards, the two rings would join by an unknown mechanism, allowing the assembly of the remaining machinery, which then exports the distal components, including the needle and the needle tip. The exact order of these later steps of the injectisome assembly remains largely unknown. A similar model was put forward based on the genetic analysis of the requirements for needle complex formation in *S. enterica* SPI-1 (Sukhan *et al*, 2001).

In this paper, we systematically investigate the whole assembly process of the *Yersinia* injectisome by combining four functional fluorescent hybrid proteins covering different parts of the machinery with an array of deletions. We conclude that the assembly starts from the secretin, the outermost and most stable ring, and sequentially proceeds inwards through YscD and YscJ. After completion of the membrane rings, an ATPase-C ring complex formed by YscK, YscL, YscN, and YscQ joins the machinery. All of the four participating proteins, but not the ATPase activity of YscN are required for the formation of this structure.

Results

Various substructures of the *Yersinia* injectisome including the C ring can be monitored using functional fluorescent fusion proteins

To visualize the injectisome and its subunits, the wild-type alleles of *yscC*, *yscD*, and *yscQ* on the virulence plasmid of *Y. enterocolitica* E40 were replaced by hybrid genes encoding the fluorescent proteins YscC-mCherry, EGFP-YscD, and EGFP-YscQ. Further, a non-polar complete deletion of *yscN* was constructed and complemented *in trans* with a plasmid encoding EGFP-YscN. The fusion proteins were expressed at near wild-type levels; no proteolytic release of the fluorophore was detected (Supplementary Figure 1).

To test the functionality of the fusion proteins, the pattern of proteins secreted into the supernatant in secretion-permissive medium (BHI-Ox) was analysed 3 h after induction of the system. YscC-mCherry, EGFP-YscN, and EGFP-YscQ were fully functional, whereas the strain expressing EGFP-YscD secreted a lower amount of effector proteins (Figure 1B). All fusion proteins allowed the formation of needles, which could be visualized by transmission electron microscopy (data not shown).

The localization of the hybrid proteins was analysed by fluorescence microscopy. Three hours after induction of synthesis of the injectisome, fluorescent spots were observed at the cell periphery for all labelled proteins (Figure 1A, three-dimensional view in Supplementary data). The formation of these spots was independent of the Ca^{2+} concentration in the medium, showing that their appearance was not directly

Figure 1 Fluorescently labelled Ysc proteins are functional and allow visualization of the injectisome. **(A)** Fluorescence deconvolution microscopy showing the formation of fluorescent spots at the bacterial membrane of *Y. enterocolitica* bacteria grown in secretion-non-permissive (BHI + Ca²⁺) and secretion-permissive medium (BHI-Ox): 1—E40(pYV40) [wild type], 2—E40(pMA4005) [YscC-mCherry], 3—E40(pAD4050) [EGFP-YscD], 4—E40(pAD4136) (pAD182) [Δ YscN + pBAD-*egfp-yescN*], 5—E40(pAD4016) [EGFP-YscQ]. All fusion proteins except for EGFP-YscN are encoded under their native promoter on the pYV virulence plasmid. Upper lane: mCherry fluorescence for strain 2, EGFP fluorescence for other strains; lower lane: corresponding DIC picture. All fluorescence pictures were taken 3 h after the induction of the T3S system by temperature shift to 37°C. Scale bars: 2 μm. **(B)** Analysis of the Yop proteins secreted in secretion-permissive conditions. The tagged strains are fully functional for effector secretion, except for the strain expressing EGFP-YscD (lane 3), which shows reduced secretion. Culture supernatants were separated on a 12% SDS-PAGE gel and stained with Coomassie Brilliant Blue. Strains as listed in **(A)**, 6—E40(pMAAD4006) [EGFP-YscQ, YscC-mCherry], 7—E40(pAD4051) [Δ YscD, negative control]. Bottom line: Needle formation (+/–) in the tested strains (data not shown). **(C)** Fluorescence microscopy showing the colocalization of EGFP-YscQ with YscC-mCherry in E40(pMAAD4006) bacteria. Fluorescent pictures were obtained as described in **(A)**. **(D)** Model of the *Yersinia* Ysc injectisome. Fluorescently labelled proteins are shown in bold print. OM, outer membrane; PP, periplasm; IM, inner membrane.

linked to the secretion of Yop proteins by the T3S system (Figure 1A).

To ascertain that the membrane spots correspond to assembled basal bodies, we constructed a strain expressing both YscC-mCherry and EGFP-YscQ, and monitored the localization of the green fluorescence from EGFP-YscQ and the red fluorescence from YscC-mCherry. As visible in Figure 1C, the green and red spots largely colocalized, with small deviations because of chromatic aberrations of the microscope. We thus assumed that the fluorescent spots correspond to assembled basal bodies. In a minority of cells, a polarly localized YscC-mCherry spot without

EGFP-YscQ equivalent could be observed in addition to the colocalizing spots. We assumed that these polar spots consist of misassembled YscC-mCherry proteins. Colocalization of spots was also observed for EGFP-YscD and EGFP-YscN with YscC-mCherry (data not shown). To test for colocalization of the needle with the basal body components, bacteria producing EGFP-YscQ were analysed by immunofluorescence with purified antibodies directed against the needle subunit. Overlays of the resulting pictures with the EGFP-YscQ fluorescence revealed that the majority of spots for YscF and YscQ colocalized (Supplementary Figure 2). A fraction of YscQ spots did not correspond to YscF spots.

Most likely, the needles of these basal bodies were detached during the immunofluorescence procedure. We conclude from all these experiments that the fluorescent spots correspond to functional injectisomes.

Assembly of the injectisome starts from the secretin ring in the OM and proceeds inwards through stepwise assembly of YscD and YscJ

As earlier work has shown that secretins can insert in the OM provided they are assisted by their pilotin (Burghout *et al*, 2004a; Guilvout *et al*, 2006), the fluorescent YscC-mCherry and its pilotin YscW were expressed *in trans* in *Y. enterocolitica* E40 (pMA8)(pRS6), in the absence of the pYV virulence plasmid encoding the T3S components. YscC-mCherry localized in membrane spots (Figure 2A), as observed before for PulD, the secretin involved in a type II secretion pathway (Buddelmeijer *et al*, 2009). These data thus confirm earlier results showing that YscC only requires its pilotin for assembly in the OM (Burghout *et al*, 2004a). In the absence of YscW, the majority of YscC-mCherry clustered in spots at the bacterial pole (Supplementary Figure 3). This phenotype was clearly distinguishable from the membrane spot formation in

the presence of YscW, and confirmed the function of YscW in proper localization and oligomerization of YscC (Burghout *et al*, 2004a).

Not surprisingly, mutants lacking any of the structural ring proteins YscC, YscD, or YscJ failed to assemble the cytosolic injectisome components YscN and YscQ (Table I), showing that establishment of the membrane-spanning structure formed by YscC, YscD, and YscJ is at the beginning of injectisome formation. To test for the assembly order of these proteins, we combined the *egfp-yscD* allele on the pYV plasmid with non-polar deletions in *yscC* and *yscJ*. Although the absence of YscC clearly abolished the formation of EGFP-YscD spots at the bacterial membrane, the absence of YscJ did not affect this assembly (Figure 2B). This implies that YscC assembles first, followed by YscD, and finally YscJ.

To confirm this order of assembly, we performed co-immunoprecipitation assays using strains in which the wild-type alleles of *yscD* or *yscJ* on the virulence plasmid were replaced by *his-flag-yscD* or *yscJ-flag-his*, respectively. The affinity tagged proteins were functional for effector secretion (data not shown) and hence assumed to assemble in the same way as wild type. They were further combined

Figure 2 YscC assembly only requires its pilotin; YscD assembly requires the presence of YscC, but not of YscJ. Copurification of the three structural ring proteins suggests the stepwise assembly order YscC–YscD–YscJ. (A) Fluorescence microscopy showing the formation of secretin spots [YscC-mCherry] at the bacterial membrane in a strain lacking the virulence plasmid pYV, after *in trans* expression of YscC-mCherry and YscW (plasmids pMA8, pRS6) for 3 h at 37°C. Scale bars: 2 μm. (B) Fluorescence microscopy showing the formation of YscD spots at the bacterial membrane in strains E40(pAD4050) [EGFP-YscD], E40(pMAAD4018) [EGFP-YscD, ΔYscC], and E40(pAD4080) [EGFP-YscD, ΔYscJ]. YscD remains cytosolic in the absence of YscC, whereas it assembles in membrane spots in the absence of YscJ. (C) Analysis of the copurification of YscC and YscD after affinity purification of YscJ. Deletion of either YscC or YscD abolishes the copurification of the respective other protein with YscJ-FLAG-His. Bacteria were incubated for 3 h at 37°C, spheroplasted, and lysed. Proteins were purified by FLAG affinity, separated on 4–12% gradient SDS-PAGE, and analysed by immunoblot with the respective anti-YscC, -YscD, or -YscJ antibodies. All strains were ΔYadA to facilitate cell lysis: 1—E40(pLJM4029) [WT], 2—E40(pAD4054) [YscJ-FLAG-His], 3—E40(pAD4109) [YscJ-FLAG-His, ΔYscC], 4—E40(pAD4110) [YscJ-FLAG-His, ΔYscD], 5—E40(pAD4112) [YscJ-FLAG-His, ΔYscJ]. (D) Analysis of the copurification of YscC and YscJ after affinity purification of YscD. Whereas deletion of YscC abolishes copurification of YscJ with His-FLAG-YscD, YscJ is not required for the interaction between YscC and YscD. Samples were obtained as described for (C). All strains were ΔYadA to facilitate cell lysis: 1—E40(pLJM4029) [WT], 2—E40(pAD4055) [His-FLAG-YscD], 3—E40(pADMA4101) [His-FLAG-YscD, ΔYscC], 4—E40(pAD4089) [His-FLAG-YscD, ΔYscJ].

Table 1 Formation of fluorescent spots in various injectisome mutants

Protein missing	Family/function	Localization	YscC-mCherry fluorescence	EGFP-YscD fluorescence	EGFP-YscN fluorescence	EGFP-YscQ fluorescence
All (pYV ⁻)			+ (pMA8 + pRS6)	ND	ND	ND
YscC	Secretin	OM		– (pMAAD4018)	– (pADMA4156)	– (pADMA4151)
YscD	MS ring	IM	ND		ND	– (pAD4052)
YscJ	MS ring	IM	+ (pADMA4082)	+ (pAD4080)	– (pAD4139)	– (pADMA4082)
YscN	ATPase	Cytoplasmic, IM associated	+ (pADMA4137)	ND		– (pAD4104)
YscK	ATPase associated	Cytoplasmic, IM associated	ND	ND	– (pAD22840)	– (pAD22723)
YscL	ATPase associated	Cytoplasmic, IM associated	ND	ND	– (pAD4141)	– (pAD4039)
YscQ	C ring	Cytoplasmic, IM associated	+ (pMA4007)	+ (pAD4061)	– (pAD4142)	
YscR	Export machinery	IM	ND	ND	ND	+ (pAD4032)
YscS	Export machinery	IM	ND	ND	ND	+ (pAD4034)
YscT	Export machinery	IM	ND	ND	ND	+ (pAD4036)
YscU	Export machinery ^a	IM	ND	ND	ND	+ (pAD4026)
YscV (LcrD)	Export machinery	IM	+ (pMA4011)	ND	ND	+ (pAD4038)
YscRSTUV	Export machinery	IM	ND	ND	+ (pAD4143)	+ (pAD4108)
YscF	Needle subunit	Extracellular	+ (pMA4015)	ND	+ (pAD4157)	+ (pAD4020)
LcrV	Needle tip	Extracellular	ND	ND	ND	+ (pAD4042)
YscH	Unknown ^b	Exported	ND	ND	ND	+ (pAD22769)
YscI	Unknown ^b	Exported	ND	ND	ND	+ (pAD4022)
YscO	Unknown	Exported	ND	ND	ND	+ (pAD4024)
YscX	Unknown	Exported	ND	ND	ND	+ (pAD4027)
YscY	Chaperone of YscX	Cytoplasmic	ND	ND	ND	+ (pAD4040)
YopN	Ca ²⁺ plug	Cytoplasmic, IM associated	ND	ND	ND	+ (pAD4043)
LcrG	Ca ²⁺ plug	Cytoplasmic, IM associated	ND	ND	ND	+ (pAD4041)

The formation of fluorescent spots was checked for YscC-mCherry, EGFP-YscD, EGFP-YscN, and EGFP-YscQ in combination with deletions of different proteins. + : Spot formation at the bacterial membrane; – : diffuse cytosolic fluorescence. The virulence plasmids of the corresponding strains are given in brackets (see Supplementary Table 1 for strain details). ND: not determined.

^aSubstrate specificity switch.

^bProposed inner rod.

with non-polar deletions in *yscC*, *yscD*, or *yscJ*. In each of the strains, the adhesin *YadA* was removed to facilitate cell lysis. Synthesis of the injectisome in these strains was induced under secretion-non-permissive conditions. Mild crosslinking was performed, spheroplasts were created, and the bacteria were lysed by the addition of detergent (see Material and methods). Afterwards, a one-step affinity purification was performed, and the (co-)purification of YscC, YscD, and YscJ was tested. YscJ-FLAG-His copurified YscC and YscD from complete injectisomes, and removal of YscQ, a protein thought to act further downstream in the assembly process, did not affect this copurification. In contrast, removal of YscC prevented copurification of YscD with YscJ-FLAG-His, and removal of YscD prevented copurification of YscC (Figure 2C). Likewise, His-FLAG-YscD copurified YscC and YscJ from complete injectisomes. However, although removal of YscC prevented copurification of YscJ with His-FLAG-YscD, removal of YscJ still allowed the copurification of YscC (Figure 2D). The amount of purified His-FLAG-YscD was reduced in the absence of YscC, most likely as a consequence of decreased cellular YscD levels, either because of its mislocalization in the absence of YscC or because of a lower expression level. Taken together, these data indicate (i) that the insertion of the secretin ring in the OM is required for the subsequent association of YscD and YscJ and (ii) that YscD makes the link between YscC and YscJ. Hence, the OM ring is the first ring of the injectisome to be assembled.

This assembly step is followed by the attachment of YscD, which then allows the completion of the MS ring by YscJ.

The C ring only assembles in the presence of the membrane rings, YscN, YscK, and YscL

To determine at which stage the C ring forms during the assembly process, we combined the *egfp-yscQ* allele with an array of deletions in most injectisome genes (Table I; Supplementary Table 1 for details of strains). Deletion of any of the membrane ring proteins (YscC, YscD, or YscJ) completely abolished the formation of membrane spots and led to an increased diffuse cytoplasmic fluorescence (Figure 3). This indicates that the C ring forms after the YscCDJ ring structure. Removal of the ATPase YscN or any of its two interacting proteins YscK and YscL (Jackson and Plano, 2000; Blaylock *et al*, 2006) also fully prevented C ring formation (Figure 3), indicating that assembly of the C ring additionally requires YscN as well as YscK and YscL.

Removal of individual proteins YscR, S, T, U, or V from the export apparatus as well as a complete deletion of all these proteins did not completely abolish the formation of the C ring. However, in the absence of YscR, YscS, or YscV, the number of spots was reduced, indicating that these proteins are either beneficial (but not absolutely required) for C ring formation, or have a stabilizing effect on fully assembled injectisomes.

As expected from the fact that YscF, YscI, YscO, YscX, and YopN are substrate proteins exported by the injectisome

Figure 3 C ring formation requires both transmembrane rings and the ATPase complex, but not the export apparatus or any secreted substrate. (A) Fluorescence microscopy pictures of bacteria expressing EGFP–YscQ combined with deletions of different genes. Micrographs were taken 3 h after induction of the T3S system. (For the control strains and the strains with deletions in the proteins required for C ring formation—upper lane: EGFP fluorescence, lower lane: corresponding DIC picture). Scale bars: 2 μm. (B) Schematic representation of the injectisome showing the components required (bold, dark) and not required (normal, light) for C ring formation. For information about the used strains, refer to Table I.

itself, their absence also did not prevent the formation of the C ring. Likewise, deletion of LcrG, a regulatory protein (Nilles *et al*, 1997; Torruellas *et al*, 2005), had no effect on assembly of the C ring (Figure 3; see Table I for additional strains).

ATPase assembly not only requires the presence of the YscCDJ platform, but also needs YscK, YscL, and YscQ

Finally, assembly of the ATPase YscN was tested. As replacement of the wild-type allele on the virulence plasmid by a gene encoding a fluorescent fusion protein decreased the expression of downstream genes in the *virB* operon, whereas a complete deletion of *yscN* was non-polar (Figure 1B), *egfp-yscN* was cloned in a pBAD vector and used to complement *in trans* double deletions in *yscN* and several other genes. Induction of synthesis of EGFP-YscN with 0.05% arabinose led to YscN protein levels similar to the native level (Supplementary Figure 1), and to effector secretion at wild-type levels (data not shown). As shown in Figure 4, YscN assembly required the presence of YscC (secretin), YscJ (MS ring), YscK and YscL (two proteins known to interact with the ATPase), and YscQ (the C ring). In contrast, even the complete deletion of the IM export proteins YscR, S, T, U, V still allowed formation of YscN spots, albeit again in a reduced number (Figure 4).

These data suggest that the cytosolic components of the injectisome form a single large ATPase-C ring complex, requiring all of its components YscK, L, N, Q to assemble.

In agreement with the essential function of YscQ for the ATPase assembly, we did not observe any needle formation in strains that lack YscQ, but overexpress YscN, in contrast to recent results obtained with the flagellum (Konishi *et al*, 2009) (data not shown).

ATPase activity of YscN is not required for the assembly of the ATPase-C ring complex at the injectisome

To determine whether assembly of the C ring requires YscN for its ATPase activity or as a structural component, a deletion of *yscN* in an *egfp-yscQ* background was examined. As expected, the resulting strain secreted neither Yops nor the ruler and needle subunits. Secretion could be complemented *in trans* by a wild-type *yscN* allele, but not by an *yscN* allele encoding YscN_{K175E} altered in the Walker box (Figure 5B and C). Interestingly, however, although YscN_{K175E} was not functional, it did restore the formation of the C ring spots (Figure 5A), implying that the YscN requirement for the formation of the ATPase-C ring complex is exclusively structural.

After assembly of the ATPase-C ring complex, needle formation and effector secretion take place rapidly

The kinetics of C ring formation in a strain expressing EGFP-YscQ was followed in a time-course experiment. Pictures were taken every 20 min up to 2 h after induction of the *ysc-yop* regulon (Cornelis *et al*, 1989) in a Ca²⁺-depleted medium. Weak diffuse cytoplasmic fluorescence could be observed 20 and 40 min after the temperature shift, suggest-

Figure 4 The assembly of the ATPase requires both transmembrane rings, YscK, YscL, and YscQ, but not the export apparatus. Fluorescence microscopy pictures of YscN null mutants complemented with EGFP-YscN combined with deletions of different genes. Wild-type protein levels were established by EGFP-YscN induction with 0.05% arabinose. Micrographs were taken 3 h after induction of EGFP-YscN and the T3S system. Scale bars: 2 μm. For information about the used strains, refer to Table I.

Figure 5 The structure, but not the ATPase activity of YscN, is required for the formation of the C ring. (A) Fluorescence microscopy showing the formation of C ring spots in E40(pAD4104) [EGFP–YscQ, ΔYscN], complemented with plasmids encoding wild-type YscN or the catalytically inactive YscN(K175E), 3 h after induction of the type III secretion system. Scale bars: 2 μm. (B) Upper part: Analysis of Yop protein secretion in strain E40(pAD4104) complemented with wild type, or catalytically inactive YscN. Expression of YscN was either not induced (–) or induced with 0.05% arabinose (+). Culture supernatants were separated on a 12% SDS–PAGE gel and stained with Coomassie Brilliant Blue. Lower part: Expression of YscN in the corresponding strains. Cell pellets were separated on a 12% SDS–PAGE gel and analysed by immunoblot with anti-YscN antibodies. (C) Export of different classes of substrates in strains expressing EGFP–YscQ, and the different YscN variants. Culture supernatants were separated on a 15% SDS–PAGE gel and analysed by immunoblot with the respective antibodies.

ing that synthesis of YscQ was turned on directly after the shift, and that EGFP folds rapidly in the *Yersinia* cytosol (Figure 6A). The rapid synthesis of YscQ was also confirmed by immunoblotting (data not shown). The first membrane spots could, however, only be observed 60 min after induction. Although the fluorescence intensity of single spots seemed to increase over time, the number of spots stayed roughly constant up to 3 h after induction (Figure 6A). Interestingly, the timeframe of appearance of the C ring was approximately the same as the timeframe of appearance of the needles (Figure 6B) and secretion of the effector proteins

(Figure 6C), suggesting that needle formation and effector secretion occur within a short time after establishment of the ATPase–C ring complex. A model of injectisome assembly that incorporates the above mentioned results is depicted in Figure 7.

Discussion

The assembly of the T3S injectisome is a complex process that engages >20 different proteins, and results in the

Figure 6 Formation of the C ring occurs about 60 min after induction of the T3S system. It directly precedes needle formation and effector secretion. (A) Fluorescence microscopy showing the formation of C ring spots [EGFP-YscQ] in strain E40(pAD4016) at various time points after induction of the synthesis of the T3S system by temperature shift to 37°C (upper lane: EGFP fluorescence; lower lane: corresponding DIC picture). Scale bars: 2 µm. (B) Time course of needle formation in wild-type strain E40(pYV40). Needle formation was monitored by SDS-PAGE analysis of purified needles. The needle pellet was separated on a 15% gel and analysed by immunoblot with anti-LcrV and anti-YscF antibodies, respectively. (C) Time course of Yop protein secretion by wild-type strain E40(pYV40). Culture supernatants were separated on a 12% SDS-PAGE gel and stained with Coomassie Brilliant Blue. All time-course experiments were performed in secretion-permissive conditions (Ca²⁺-depleted medium).

Figure 7 Model of assembly of the *Yersinia* injectisome. Formation of the injectisome is initiated by formation of the secretin ring in the outer membrane. Next, YscD attaches to YscC, which allows the subsequent completion of the MS ring by attachment of YscJ. After the formation of the membrane ring structures, the ATPase-C ring complex, consisting of YscN, K, L, and Q assembles at the cytoplasmic side of the injectisome. The exact time point of the integration of the IM proteins YscR, S, T, U, and V is unclear. Afterwards, the needle consisting of YscF and LcrV can be assembled. Bold font denotes protein names, normal font denotes functional subunits. The global structure of YscC, D, J is derived from Spreter *et al* (2009).

formation of a nanomachine spanning both bacterial membranes and protruding outside the bacterium. So far, little is known about this process. On the basis of the observation that heterologously overexpressed *S. enterica* MS ring components PrgH and PrgK form large rings in the absence of any other T3S component, a model was proposed (Kimbrough and Miller, 2002) in which the IM ring assembles first, and then fuses with the secretin ring in the OM. This model suggests the same general assembly scheme as the one that has been proposed for the flagellum (Kubori *et al*, 1997; Macnab, 2003), but does not explain how the two membrane

rings find each other. The subsequent steps of assembly could not be examined so far.

To gain better insight into the assembly process and the functional relations between the proteins, we constructed strains in which a number of injectisome constituents were fused to fluorescent proteins. To minimize artefacts because of non-native expression levels or timing of the fusion proteins, we replaced the wild-type allele on the pYV virulence plasmid by the hybrid allele in the case of *yscC*, *yscD*, and *yscQ*. The hybrid *yscN* was plasmid borne, but it was expressed at wild-type level.

All recombinant injectisomes were functional, and in all cases, fluorescent spots appeared at the bacterial membrane, distributed all over the bacterial body. Colocalization of the fluorophores confirmed that the fluorescent spots correspond to injectisomes. The brightness of the spots likely results from the multimeric nature of the tagged proteins, although at this stage, one cannot conclude that each spot corresponds to only one injectisome. A fluorescence quantification based on external standards, presently in progress, will address this question.

We observed that the membrane ring-forming proteins YscC, YscD, and YscJ are required for assembly of any cytosolic structure. Importantly, by monitoring the formation of YscC–mCherry and EGFP–YscD spots, we observed that YscC assembles independently of YscD and YscJ and that YscD assembles independently of YscJ, but not of YscC. Co-immunoprecipitation assays confirmed that YscJ requires YscD to become attached to YscC. All this implies that the assembly of the injectisome is initiated by formation of the secretin ring in the OM and proceeds inwards through stepwise assembly of YscD and YscJ. These data contradict the earlier report that PrgH and PrgK, the *Salmonella* homologues of YscD and YscJ, can form a ring alone (Kimbrough and Miller, 2000). The discrepancy might result from the fact that the earlier study was based on heterologously overexpressed proteins, whereas this study is based on functional proteins produced in their natural environment at native expression levels. These data are also at odd with the report indicating that MxiD and MxiJ, the *Shigella* homologues of YscC and YscJ, interact even in the absence of the connector (Schuch and Maurelli, 2001). However, this interaction was observed in the absence of the pilot protein. In this case, the majority of secretin proteins are mislocalized to the IM (Koster *et al*, 1997), which might lead to non-native interaction with MxiJ. Interestingly, it was shown recently that the assembly of two ring-forming IM components of the *Vibrio cholerae* type II secretion complex also depends on the presence of the OM secretin (Lybarger *et al*, 2009), suggesting conservation or convergent evolution of the formation process in these two prokaryotic export systems. Taken together, our results show that the order of assembly of the OM and IM rings differs between the injectisome and the flagellum. We do not see any obvious reason for this, but this observation indicates that the two nanomachines differ more than is often thought. The flagellum is indeed significantly more complex than the injectisome because it rotates, which implies not only a motor but also bushings in the peptidoglycan and the OM. The P and L rings, having this function, are precisely replaced by a very stable secretin ring in the injectisome. This basic structural difference might explain the different order of assembly of the two nanomachines.

The outside-in assembly order consistently shown by co-immunoprecipitation and fluorescence microscopy further implies that YscD is the connector between the two membrane rings, which is coherent with recent crystal structure and modelling data (Spreter *et al*, 2009). Our biochemical data allow to assess the recent models to integrate the crystal structures of the membrane ring proteins into the overall shape generated by electron microscopy averaging of purified injectisomes (Hodgkinson *et al*, 2009; Spreter *et al*, 2009). The electron density between the membranes would be generated by YscD. This in turn places YscJ in the IM, as proposed by manual fits (Moraes *et al*, 2008; Hodgkinson

et al, 2009; Spreter *et al*, 2009), but not by the best automated fit (Hodgkinson *et al*, 2009).

After assembly of the OM and IM membrane rings, cytosolic components can assemble onto the structure. The observation that the proposed C ring component YscQ assembles in membrane spots colocalizing with the other components shows that the C ring is an integral component of the injectisome, confirming an assumption so far mainly based on immunogold-labelling experiments (Morita-Ishihara *et al*, 2006). Our data indicate that a large cytosolic complex consisting of the ATPase YscN, the two interacting proteins YscK and YscL, and the C ring component YscQ is formed, requiring all of its components, but not the ATPase activity of YscN for assembly. This differs again from the situation in the flagellum. There, FlhM, FlhN, and FlhG (together forming the C ring) appear in significant amount in the membrane fraction in the presence of FlhF (MS ring), but in the absence of FlhI (ATPase) or FlhH (homologue to YscL). This suggests that the flagellar C ring forms in the absence of the ATPase complex (Kubori *et al*, 1997), in agreement with the observation that it forms on overexpression of FlhM, FlhN, and FlhG together with FlhF (Lux *et al*, 2000; Young *et al*, 2003). Although the heterologous overexpression of the proteins in these studies might account for the different observations, these results can also be the consequence of functional differences between the two nanomachines. As the constraint of rotation and spatial separation of the C ring and ATPase does not exist for the injectisome, the apparatus could be optimized for secretion. A tighter contact between the ATPase complex and the C ring might be a consequence of this optimization. The fact that we could not overcome the requirement of YscQ for secretion by overexpression of the ATPase is consistent with the essential function of YscQ for assembly of the complete ATPase–C ring complex.

Our results are also in perfect agreement with earlier results showing interactions between YscK, YscL, YscN, and YscQ (Jackson and Plano, 2000). However, the hypothesis that YscQ recruits the ATPase should be revised: YscK, L, N, and Q would rather assemble in one step. The proposed function of YscL as a negative regulator of ATPase activity (Blaylock *et al*, 2006) as well as its direct interaction with YscN and YscQ (Jackson and Plano, 2000) is consistent with the presence of YscL in this complex. Less is known about the function of YscK. As it interacts with YscQ, but not with YscN and weakly at the most with YscL (Jackson and Plano, 2000; Blaylock *et al*, 2006), it might act at the interface of the ATPase–C ring complex.

Formation and assembly of the ATPase–C ring complex did not depend on any of the five proteins forming the export apparatus, even though the number of membrane spots was reduced when YscR, YscS, YscV, or the five proteins YscRSTUV were missing. This implies that a YscKLNQ complex docks onto the IM ring rather than onto the export apparatus, which agrees with the observations made with the flagellum (Kubori *et al*, 1997). As currently, little is known about stoichiometry, localization, and function of the export apparatus, its function in the assembly process remains unclear.

In conclusion, this work shows that the assembly of the injectisome starts with the formation of the stable secretin ring in the OM, and proceeds inwards through discrete attachment steps of YscD and YscJ at the IM. Afterwards,

the components of the cytosolic ATPase-C ring complex assemble at the cytosolic side of the injectisome in one step, which allows the subsequent fast steps leading to needle formation and effector secretion.

Materials and methods

Bacterial strains, plasmids, and genetic constructions

Y. enterocolitica strains and plasmids are listed in Supplementary Table 1.

E. coli Top10, used for plasmid purification and cloning, and *E. coli* Sm10 λ pir, used for conjugation, were routinely grown on LB agar plates and in LB broth. Ampicillin was used at a concentration of 200 μ g/ml to select for expression vectors. Streptomycin was used at a concentration of 100 μ g/ml to select for suicide vectors. Plasmids were generated using either Phusion polymerase (Finnzymes, Espoo, Finland) or Vent DNA polymerase (New England Biolabs, Frankfurt, Germany). The oligonucleotides used for genetic constructions are listed in Supplementary Table 2. Mutators for modification or deletion of genes in the pYV plasmids were constructed by overlapping PCR using purified pYV40 plasmid as template, leading to 200–250 bp of flanking sequences on both sides of the deleted or modified part of the respective gene. As an exception, pKEM5 was constructed by introduction of the deletion through religation of the 5' phosphorylated internal oligonucleotides. For the mutator strains introducing EGFP, a precursor mutator vector was created as described above. Subsequently, the EGFP gene was inserted in frame from plasmid pEGFP-C1 into the digested precursor vectors. The respective regions containing the flanking sequences were subcloned into the pKNG101 suicide vector. For pMA12, insert 2 was created by overlapping PCR using oligos 5017 and 5087 to amplify mCherry from vector pRVCHYC-5 (Thanbichler *et al*, 2007), and oligos 5088 and 5068 to amplify the downstream flanking region from the pYV plasmid. Afterwards, ligation of *Sall*/*XhoI* digested insert 1, containing the upstream flanking region, *XhoI*/*XbaI* digested insert 2, and *Sall*/*XbaI* digested pKNG101 suicide vector lead to the mutator pMA12. All constructs were confirmed by sequencing using a 3100-Avant genetic analyser (Applied Biosystems, Rotkreuz, Switzerland). The allelic exchange was selected by plating diploid bacteria on sucrose (Kaniga *et al*, 1991). pAD166 expressing YscN_{K175E} was generated by overlapping PCR using internal primers encoding for the modified protein sequence, and selected by colony PCR and sequencing. For pAD182 expressing EGFP-YscN, a precursor vector was generated and EGFP was introduced from pEGFP-C1, as described above.

Y. enterocolitica cultures for secretion and microscopy analysis

Induction of the *yop* regulon was performed by shifting the culture to 37°C, either in BHI-Ox (secretion-permissive conditions) or in BHI + 5 mM CaCl₂ (secretion-non-permissive conditions) (Cornelis *et al*, 1987). Expression of the inducible YscN constructs was induced by adding 0.05% L-arabinose to the culture just before the shift to 37°C. The carbon source was glycerol (4 mg/ml) when expressing genes from the pBAD promoter, and glucose (4 mg/ml) in the other cases.

Yop secretion

Total cell and supernatant fractions were separated by centrifugation at 20 800 g for 10 min at 4°C. The cell pellet was taken as total cell fraction. Proteins in the supernatant were precipitated with trichloroacetic acid 10% (w/v) final for 1 h at 4°C.

Secreted proteins were analysed by SDS-PAGE; in each case, proteins secreted by 3×10^8 bacteria were loaded per lane. Total secreted proteins were analysed by Coomassie staining of 12% SDS-PAGE gels. Detection of specific secreted proteins by immunoblotting was performed using 15% SDS-PAGE gels. For detection of proteins in total cells, 2×10^8 bacteria were loaded per lane, if not stated otherwise, and proteins were separated on 15% SDS-PAGE gels before detection by immunoblotting.

Immunoblotting was carried out using rabbit polyclonal antibodies against LcrV (MIPA220; 1:2000), YscF (MIPA223; 1:1000), YscN (MIPA189; 1:1000), YscP (MIPA57; 1:3000), or YopE (MIPA73; 1:1000). Detection was performed with secondary antibodies

directed against rabbit antibodies and conjugated to horseradish peroxidase (1:5000; Dako), before development with ECL chemiluminescent substrate (Pierce).

Needle purification

Needles were purified from cultures incubated under secretion-permissive conditions. At the given time points, 48 ml bacteria were removed from the 500 ml culture, harvested by centrifugation (5 min at 4000 g) and resuspended in 1 ml 20 mM Tris-HCl, pH 7.5. Needle detachment was increased by repeated pipetting through a 1 ml pipet tip. Cells were pelleted by centrifugation (5 min at 4000 g), and the supernatant containing the needles was passed through a 0.45 μ m mesh filter (cellulose acetate membrane) and then centrifuged for 60 min at 20 800 g. The resulting pellet was resuspended in 20 μ l Laemmli buffer, 15 μ l of which were analysed by SDS-PAGE followed by immunoblotting (Mueller *et al*, 2005).

Fluorescence microscopy

For fluorescence imaging, cells were placed on a microscope slide layered with a pad of 2% agarose dissolved in water or PBS. A Deltavision Spectris optical sectioning microscope (Applied Precision, Issaquah, WA) equipped with an UPlanSApo 100 $\times$ /1.40 oil objective (Olympus, Tokyo, Japan) and a coolSNAP HQ CCD camera (Photometrics, Tucson, AZ) was used to take differential interference contrast (DIC) and fluorescence photomicrographs. To visualize GFP and mCherry fluorescence, GFP filter sets (Ex 490/20 nm, Em 525/30 nm) and mRFP filter sets (Ex 560/40 nm, Em 632/60 nm), respectively, were used. DIC frames were taken with 0.3 s and fluorescence frames with 1.0 s exposure time. Per image, a Z-stack containing 20 frames per wavelength with a spacing of 150 nm was acquired. The stacks were deconvoluted using softWoRx v3.3.6 with standard settings (Applied Precision, WA). The DIC frame at the centre of the bacterium and the corresponding fluorescence frame were selected and further processed with ImageJ software.

Co-immunoprecipitation of YscC, YscD, and YscJ

Y. enterocolitica cultures were grown in secretion-non-permissive conditions to an OD₆₀₀ of 1.5–2.2. Protein complexes were then stabilized by crosslinking with 0.25% formaldehyde for 15 min at 37°C. Cells were harvested by centrifugation (15 min at 1500 g, 25°C) and resuspended in 1/5 volume of PBS. After a second crosslinking step (0.4% formaldehyde, 15 min, 25°C) and harvesting as before, spheroplast generation and lysis was performed as described by Kubori *et al* (1997) and Blocker *et al* (2001). In short, cells were resuspended in 1/5 original volume of ice-cold spheroplasting buffer (0.75 M sucrose, 50 mM Tris, pH adjusted with HCl to 7.8, 0.6 mg/ml lysozyme, 6 mM EDTA), and incubated at 25°C up to 90 min, until complete spheroplast formation could be observed. Cells were lysed by addition of 1% Triton X-100 and subsequent incubation at 4°C for 15 min. After addition of 15 mM MgCl₂, unlysed cells were removed by centrifugation (20 min at 6000 g, 4°C); 300 μ l of anti-FLAG M2 affinity gel (Sigma-Aldrich, Buchs, Switzerland) were added to the supernatant, and the proteins were purified in batch according to the manufacturer's protocol. The elution fractions were recentrifuged to completely remove resin, and separated on 12% SDS-PAGE gels or 4–12% gradient SDS-PAGE gels (Serva, Heidelberg, Germany). Immunoblotting was carried out using rabbit polyclonal antibodies against YscC (MIPA250, 1:1000), YscD (MIPA232, 1:1000), and YscJ (MIPA66, 1:5000), as described above.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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