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

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Total character count (with spaces): 46289 (without table and refs)

Running title: Assembly of the *Yersinia* injectisome

Subject category: Microbiology & Pathogens

ABSTRACT

The assembly of the *Yersinia enterocolitica* type III secretion injectisome was investigated by grafting fluorescent proteins onto several components, YscC (outer-membrane ring), YscD (forms the inner-membrane 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: while 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 via 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 demonstrate that assembly is initiated by formation of the outer-membrane ring and progresses inwards to the inner-membrane ring and, finally, to a large cytosolic complex.

Keywords: microbial pathogenesis / nanomachine / protein complex assembly / protein transport

INTRODUCTION

The type III secretion (T3S) apparatus, also called injectisome, allows bacteria to export effector proteins upon contact with eukaryotic cell membranes (Cornelis & Van Gijsegem, 2000; Cornelis & Wolf-Watz, 1997; Galan & Collmer, 1999). 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 & Collmer, 2004; Grant et al, 2006; Mota & Cornelis, 2005). 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, D, J, N, Q, R, S, T, U, V in *Yersinia*) are well conserved (Cornelis, 2006; Van Gijsegem et al, 1995).

A number of injectisome proteins co-purify 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 (IM) and outer (OM) bacterial membranes, joined together by a narrower cylinder and terminated by a needle, a filament or a pilus (Blocker et al, 1999; Daniell et al, 2001; Hodgkinson et al, 2009; Jin & He, 2001; Kimbrough & Miller, 2000; Kubori et al, 1998; Morita-Ishihara et al, 2006; Sani et al, 2007; Schraidt et al, 2010; Sekiya et al, 2001). The needle is a hollow tube assembled through helical polymerisation 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 (Blocker et al, 2001; Burghout et al, 2004b; Koster et al, 1997; Kubori et al, 2000; Marlovits et al, 2004; Spreter et al, 2009; Tamano et al, 2000). 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 (Crepin et al, 2005; Hodgkinson et al, 2009; Kimbrough & Miller, 2000; Silva-Herzog et al, 2008; Yip et al, 2005). 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 inner membrane. 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 (Muller et al, 2006; Pozidis et al, 2003; Woestyn et al, 1994; Zarivach et al, 2007) and resembles the flagellar ATPase FliI (Abrahams et al, 1994; Imada et al, 2007). The ATPase is associated with two proteins (YscK, L) (Blaylock et al, 2006; Jackson & Plano, 2000), one of them (YscL) probably exerting a control on the ATPase activity as was shown for FliH in the flagellum (Gonzalez-Pedrajo et al, 2002; McMurry et al, 2006; Minamino & MacNab, 2000). 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 $YscL_{N\text{-}term}$ and the b subunit of the F-type ATPase, and between $YscL_{C\text{-}term}$ 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 & Galan, 2005). It is likely that the ATPase also directly energizes export, but the proton motive force is also involved (Minamino & Namba, 2008; Paul et al, 2008; Wilharm et al, 2004).

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 & DeRosier, 1990; Khan et al, 1992; Kubori et al, 1997; Thomas et al, 2006; Young et al, 2003). 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 (Erhardt & Hughes, 2009; Konishi et al, 2009) 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 ortholog 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 (Fadouloglou et al, 2004). This suggests that injectisomes do have a C ring, although they have not been reported to rotate. YscQ and its homologs have been shown to bind the ATPase complex (Jackson & 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* ortholog of YscQ (Spa33) localizes to a lower portion of the injectisome (Morita-Ishihara et al, 2006). A list of homologs 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, outer membrane 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 over-expression of the *S. enterica* SPI-1 MS ring components PrgH and PrgK in *E. coli* leads to stable ring structures (Kimbrough & Miller, 2000). The same is true for the *Yersinia* secretin YscC together with its pilotin YscW (Kimbrough & Miller, 2000; Koster et al, 1997). This suggests that the transmembrane rings might form independently. It has thus been proposed (Kimbrough & 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* (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 via 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

In order 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 (Suppl. Fig. 1).

To test the functionality of the fusion proteins, the pattern of proteins secreted into the supernatant in secretion-permissive medium (BHI-Ox) was analyzed three hours after induction of the system. YscC-mCherry, EGFP-YscN, and EGFP-YscQ were fully functional, while the strain expressing EGFP-YscD secreted a lower amount of effector proteins (Fig. 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 analyzed by fluorescence microscopy. Three hours after induction of synthesis of the injectisome, fluorescent spots were observed at the cell periphery for all labelled proteins (Fig. 1A, three-dimensional view in Suppl. Online Material). The formation of these spots was independent of the Ca^{2+} concentration in the medium, showing that their appearance was not directly linked to the secretion of Yop proteins by the T3S system (Fig. 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 Fig. 1C, the green and red spots largely colocalized, with small

deviations due to 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 analyzed 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 (Suppl. Fig. 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 outer membrane and proceeds inwards via stepwise assembly of YscD and YscJ

Since previous 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 (Fig. 2A) as observed before for PulD, the secretin involved in a type II secretion pathway (Buddelmeijer et al, 2009). These data thus confirm previous results showing that YscC only requires its pilotin for assembly in the outer membrane (Burghout et al, 2004a). In the absence of YscW however, the majority of YscC-mCherry clustered in spots at the bacterial pole (Suppl. Fig. 3). This phenotype was clearly distinguishable from the membrane spot formation in the presence of YscW, and confirmed the role 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*. While 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 (Fig. 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 like wild-type. They were further combined 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 (Fig. 2C). Likewise, His-FLAG-YscD copurified YscC and YscJ from complete injectisomes. However, while removal of YscC prevented copurification of YscJ with His-FLAG-YscD, removal of YscJ still allowed the copurification of YscC (Fig. 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 due to a lower expression level. Taken together, these data indicate (i) that the insertion of the secretin ring in the outer membrane 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; Suppl. 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 (Fig. 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 (Blaylock et al, 2006; Jackson & Plano, 2000) also fully prevented C ring formation (Fig. 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 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 itself, their absence did also 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 (Fig. 3, see Table I for additional strains)

ATPase assembly requires the presence of the YscCDJ platform, but also needs YscK, YscL, and YscQ

Finally, assembly of the ATPase YscN was tested. Since replacement of the wild-type allele on the virulence plasmid by a gene encoding a fluorescent fusion protein decreased expression of downstream genes in the *virB* operon, while a complete deletion of *yscN* was non-polar (data not shown), *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 (Suppl. Fig. 1), and to effector secretion at wild-type levels (data not shown). As shown in Fig. 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 (Fig. 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 (Fig 5BC). Interestingly however, although $YscN_{K175E}$ was not functional, it did restore the formation of the C ring spots (Fig. 5A), implying that the YscN requirement for 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 twenty minutes up to two hours after induction of the *ysc-yop* regulon (Cornelis et al, 1989) in a Ca^{2+}-depleted medium. Weak diffuse cytoplasmic fluorescence could be observed 20 min and 40 min after the temperature shift, suggesting that synthesis of YscQ was turned on directly after the shift, and that EGFP folds rapidly in the *Yersinia* cytosol (Fig. 6A). The rapid synthesis of YscQ was also confirmed by immunoblotting (data not shown). The first membrane spots could however only be observed 60 minutes after induction. While the fluorescence intensity of single spots seemed to increase over time, the number of spots stayed roughly constant up to three hours after induction (Fig. 6A). Interestingly, the timeframe of appearance of the C ring was approximately the same as the timeframe of appearance of the needles (Fig. 6B) and secretion of the effector proteins (Fig. 6C), suggesting that needle formation and effector secretion occur within a short time after establishment of the ATPase – C ring complex.

DISCUSSION

The assembly of the T3S injectisome is a complex process that engages more than 20 different proteins, and results in the formation of a nanomachine spanning both bacterial membranes and protruding outside the bacterium. So far, little is known about this process. Based on 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 & 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 due to 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. Co-localisation 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 via stepwise assembly of YscD and YscJ. These data contradict the previous report that PrgH and PrgK, the *Salmonella* homologues of YscD and YscJ, can form a ring alone (Kimbrough & Miller, 2000). The discrepancy might result from the fact that this study was based on heterologously overexpressed proteins, while the present 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 & 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 differ between the injectisome and the flagellum. We do not see any obvious reason therefor, 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 ring, playing this role, 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-immunprecipitation 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 therefore be generated by YscD. This in turn places YscJ in the IM, as proposed by manual fits (Hodgkinson et al, 2009; Moraes et al, 2008), 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 demonstrates 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, FliM, FliN and FliG (together forming the C ring) appear in significant amount in the membrane fraction in the presence of FliF (MS ring), but in the absence of FliI (ATPase) or FliH (homolog 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 upon overexpression of FliM, FliN and FliG together with FliF (Lux et al, 2000; Young et al, 2003). While 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. Since 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 and a tighter contact between the ATPase complex and the C ring might be a consequence of this optimisation. The fact that we could not overcome the requirement of YscQ for secretion by overexpression of the ATPase is consistent with the essential role of YscQ for assembly of the complete ATPase – C ring complex.

Our results are also in perfect agreement with previous results showing interactions between YscK, YscL, YscN, and YscQ (Jackson & 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 & Plano, 2000) is consistent with the presence of YscL in this complex. Less is known about the role of YscK. As it interacts with YscQ, but not with YscN and weakly at the most with YscL (Blaylock et al, 2006; Jackson & Plano, 2000), it might act at the interface of the C ring – ATPase 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 observations made with the flagellum (Kubori et al, 1997). As currently, little is known about stoichiometry, localization, and function of the export apparatus, its role 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 via 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.

MATERIAL AND METHODS

Bacterial strains, plasmids and genetic constructions

Y. enterocolitica strains 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 μg/ml to select for expression vectors, Streptomycin was used at a concentration of 100 μg/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' phoshorylated 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. 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 *Sal*I/*Xho*I digested insert 1, containing the upstream flanking region, *Xho*I/*Xba*I digested insert 2, and *Sal*I/*Xba*I digested pKNG101 suicide vector lead to the mutator pMA12. The respective regions containing the flanking sequences were subcloned into the pKNG101 suicide vector. All constructs were confirmed by sequencing using a 3100-Avant genetic analyzer (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°, either in BHI-Ox (secretion-permissive conditions) or in BHI + 5 mM $CaCl_2$ (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 analyzed by SDS–PAGE; in each case, proteins secreted by 3 x 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 done using 15% SDS-PAGE gels. For detection of proteins in total cells, 2 x 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 μm mesh filter (cellulose acetate membrane) and then centrifuged

for 60 min at 20,800 g. The resulting pellet was resuspended in 20 μl Lämmli buffer, 15 μl of which were analyzed 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, United States) equipped with an UPlanSApo 100x/1.40 oil objective (Olympus, Tokyo, Japan) and a coolSNAP HQ CCD camera (Photometrics, Tucson, AZ, United States) 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 OD_{600} of 1.5-2.2. Protein complexes were then stabilized by crosslinking with 0.25% formaldehyde for 15 min at 37°. Cells were harvested by centrifugation (15 min at 1500 g, 25°) and resuspended in 1/5 volume of PBS. After a second crosslinking step (0.4% formaldehyde, 15 min, 25°) 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° 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° for 15 min. After addition of 15 mM $MgCl_2$, unlysed cells were removed by centrifugation (20 min at 6000 g, 4°). 300 μl 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.

ACKNOWLEDGEMENTS

We thank K. Maylandt and I. Stainier for providing strains pKEM5, pKEM4001, pSI51 and pSI4006. This work was supported by the Swiss National Science Foundation (grant 310000-113333/1) to GC.

LEGEND TO THE FIGURES

Fig. 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 + Ca^{2+}) 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) [ΔYscN + pBAD-*egfp-yscN*], 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 three hours after the induction of the T3S system by temperature shift to 37°. 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) [ΔYscD, negative control]. Bottom line: Needle formation (+ / -) in the tested strains (data not shown).

C: Fluorescence microscopy showing the co-localization 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.

Fig. 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 hours at 37°. 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 hours at 37 °C, spheroplasted and lysed. Proteins were purified by FLAG affinity, separated on 4-12% gradient SDS-PAGE and analyzed 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, ΔYscQ].

D: Analysis of the copurification of YscC and YscJ after affinity purification of YscD. While 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].

Fig. 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 three hours 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 italics, dark) and not required (normal, light) for C ring formation.

For information about the used strains, refer to Table I.

Fig. 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 three hours after induction of EGFP-YscN and the T3S system. Scale bars: 2 μm. For information about the used strains, refer to Table I.

Fig. 5: The structure, but not the ATPase activity of YscN are 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), three hours 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 analyzed 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 analyzed by immunoblot with the respective antibodies.

Fig. 6: Formation of the C ring occurs about 60 minutes 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° (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 analyzed by immunoblot with anti-LcrV and anti-YscF antibodies, respectively.

C: Timecourse 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 (Ca^{2+} depleted medium).

Fig. 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. Following 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 timepoint 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.

The global structure of YscC, D, J is derived from Spreter *et al*. (2009).

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Table I: 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)	*n.d.*	*n.d.*	*n.d.*
YscC	Secretin	OM ring		- (pMAAD4018)	- (pADMA4156)	- (pADMA4151)
YscD	MS ring	IM	*n.d.*		*n.d.*	- (pAD4052)
YscJ	MS ring	IM	+ (pADMA4082)	+ (pAD4080)	- (pAD4139)	- (pADMA4082)
YscN	ATPase	cytoplasmic, IM-associated	+ (pADMA4137)	*n.d.*		- (pAD4104)
YscK	ATPase-associated	cytoplasmic, IM-associated	*n.d.*	*n.d.*	- (pAD22840)	- (pAD22723)
YscL	ATPase-associated	cytoplasmic, IM-associated	*n.d.*	*n.d.*	- (pAD4141)	- (pAD4039)
YscQ	C ring	cytoplasmic, IM-associated	+ (pMA4007)	+ (pAD4061)	- (pAD4142)	
YscR	Export machinery	IM	*n.d.*	*n.d.*	*n.d.*	+ (pAD4032)
YscS	Export machinery	IM	*n.d.*	*n.d.*	*n.d.*	+ (pAD4034)
YscT	Export machinery	IM	*n.d.*	*n.d.*	*n.d.*	+ (pAD4036)
YscU	Export machinery, *1)*	IM	*n.d.*	*n.d.*	*n.d.*	+ (pAD4026)
YscV(LcrD)	Export machinery	IM	+ (pMA4011)	*n.d.*	*n.d.*	+ (pAD4038)
YscRSTUV	Export machinery	IM	*n.d.*	*n.d.*	+ (pAD4143)	+ (pAD4108)
YscF	Needle subunit	extracellular	+ (pMA4015)	*n.d.*	+ (pAD4157)	+ (pAD4020)
LcrV	Needle tip	extracellular	*n.d.*	*n.d.*	*n.d.*	+ (pAD4042)
YscH	unknown	exported	*n.d.*	*n.d.*	*n.d.*	+ (pAD22769)
YscI	unknown, *2)*	exported	*n.d.*	*n.d.*	*n.d.*	+ (pAD4022)
YscO	unknown	exported	*n.d.*	*n.d.*	*n.d.*	+ (pAD4024)
YscX	unknown	exported	*n.d.*	*n.d.*	*n.d.*	+ (pAD4027)
YscY	Chaperone of YscX	cytoplasmic	*n.d.*	*n.d.*	*n.d.*	+ (pAD4040)
YopN	Ca^{2+} plug	cytoplasmic, IM-associated	*n.d.*	*n.d.*	*n.d.*	+ (pAD4043)
LcrG	Ca^{2+} plug	cytoplasmic, IM-associated	*n.d.*	*n.d.*	*n.d.*	+ (pAD4041)

1) Substrate specificity switch; 2) Proposed inner rod

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 I for strain details). n.d.: not determined.

Fig. 1

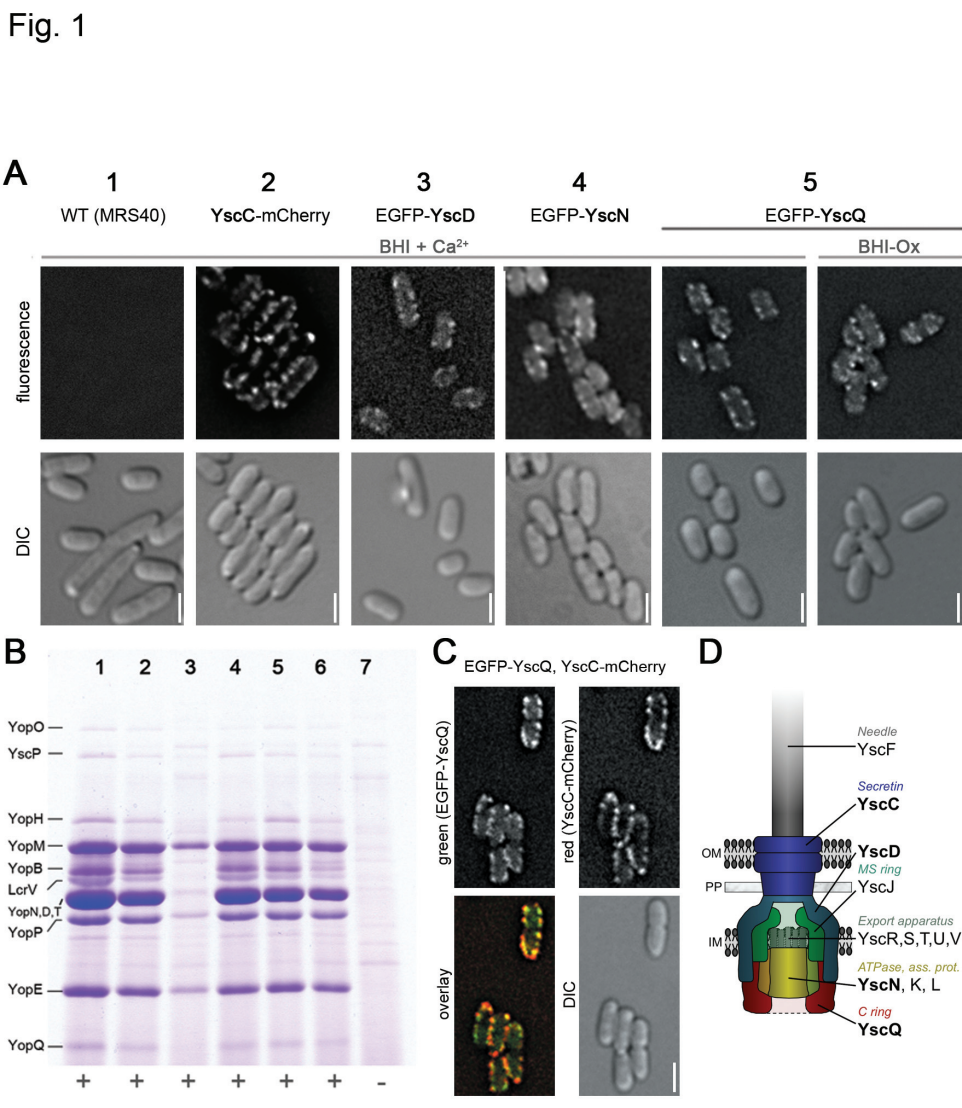

A
1
WT (MRS40)
2
YscC-mCherry
3
EGFP-YscD
4
EGFP-YscN
5
EGFP-YscQ
BHI + Ca2+
BHI-Ox
fluorescence
DIC
B
1 2 3 4 5 6 7
YopO
YscP
YopH
YopM
YopB
LcrV
YopN,D,T
YopP
YopE
YopQ
+ + + + + + -
C
EGFP-YscQ, YscC-mCherry
green (EGFP-YscQ)
red (YscC-mCherry)
overlay
DIC
D
Needle
YscF
Secretin
YscC
OM
YscD
MS ring
PP
YscJ
Export apparatus
IM
YscR,S,T,U,V
ATPase, ass. prot.
YscN, K, L
C ring
YscQ

Fig. 2

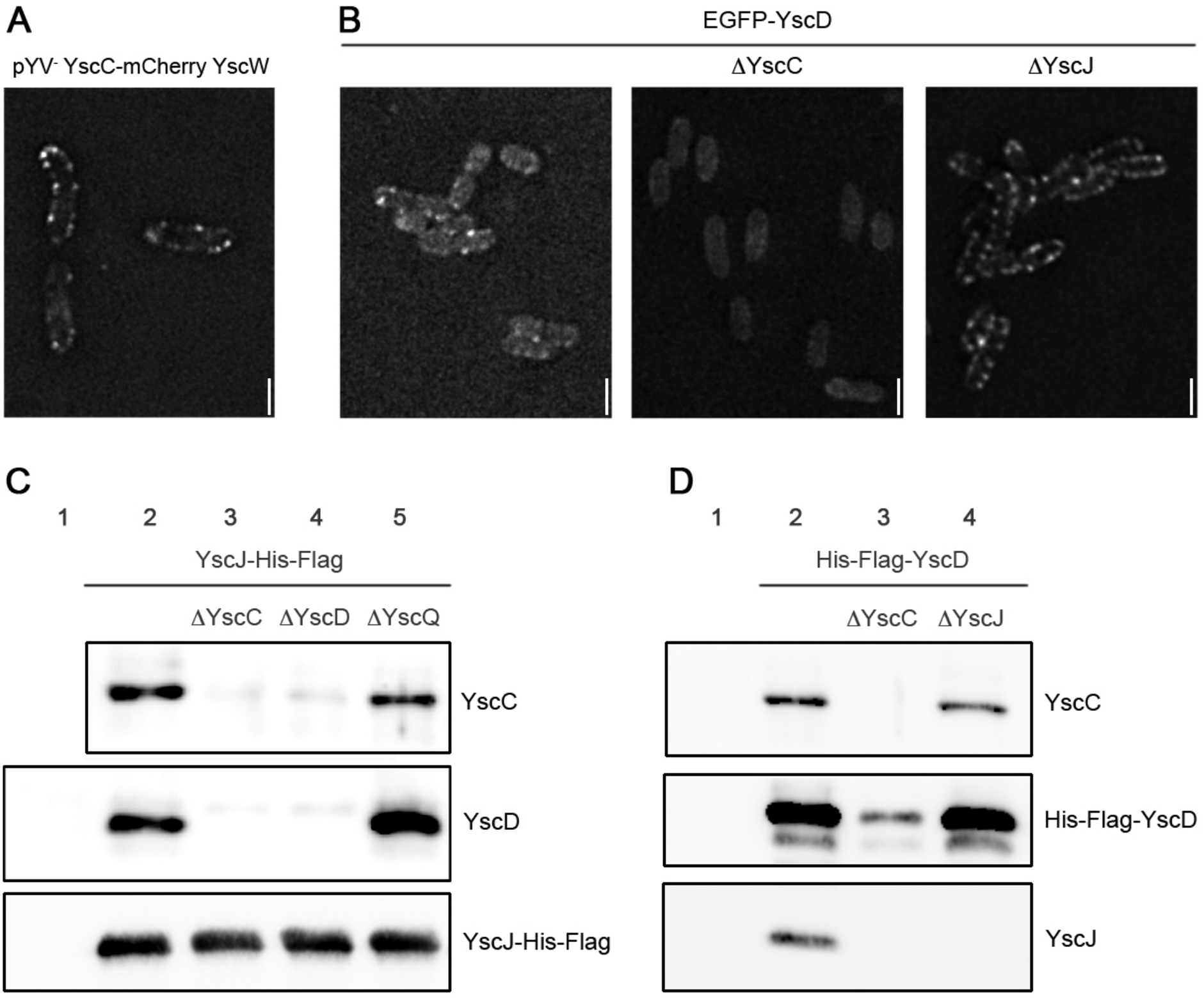

A
pYV- YscC-mCherry YscW
B
EGFP-YscD
ΔYscC
ΔYscJ
C
1 2 3 4 5
YscJ-His-Flag
ΔYscC ΔYscD ΔYscQ
YscC
YscD
YscJ-His-Flag
D
1 2 3 4
His-Flag-YscD
ΔYscC ΔYscJ
YscC
His-Flag-YscD
YscJ

Fig. 3

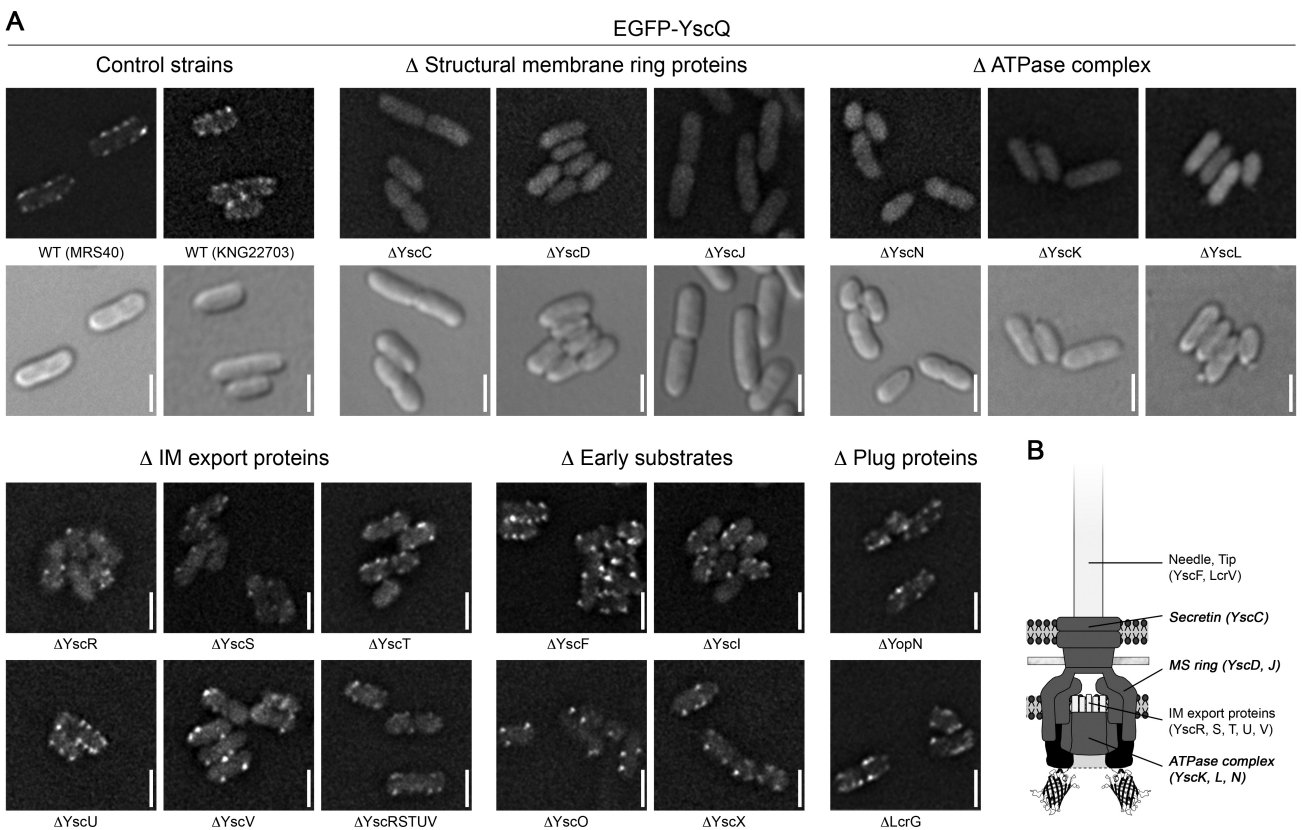

A
EGFP-YscQ
Control strains
Δ Structural membrane ring proteins
Δ ATPase complex
WT (MRS40)
WT (KNG22703)
ΔYscC
ΔYscD
ΔYscJ
ΔYscN
ΔYscK
ΔYscL
Δ IM export proteins
Δ Early substrates
Δ Plug proteins
ΔYscR
ΔYscS
ΔYscT
ΔYscF
ΔYscI
ΔYopN
ΔYscU
ΔYscV
ΔYscRSTUV
ΔYscO
ΔYscX
ΔLcrG
B
Needle, Tip
(YscF, LcrV)
Secretin (YscC)
MS ring (YscD, J)
IM export proteins
(YscR, S, T, U, V)
ATPase complex
(YscK, L, N)

Fig. 4

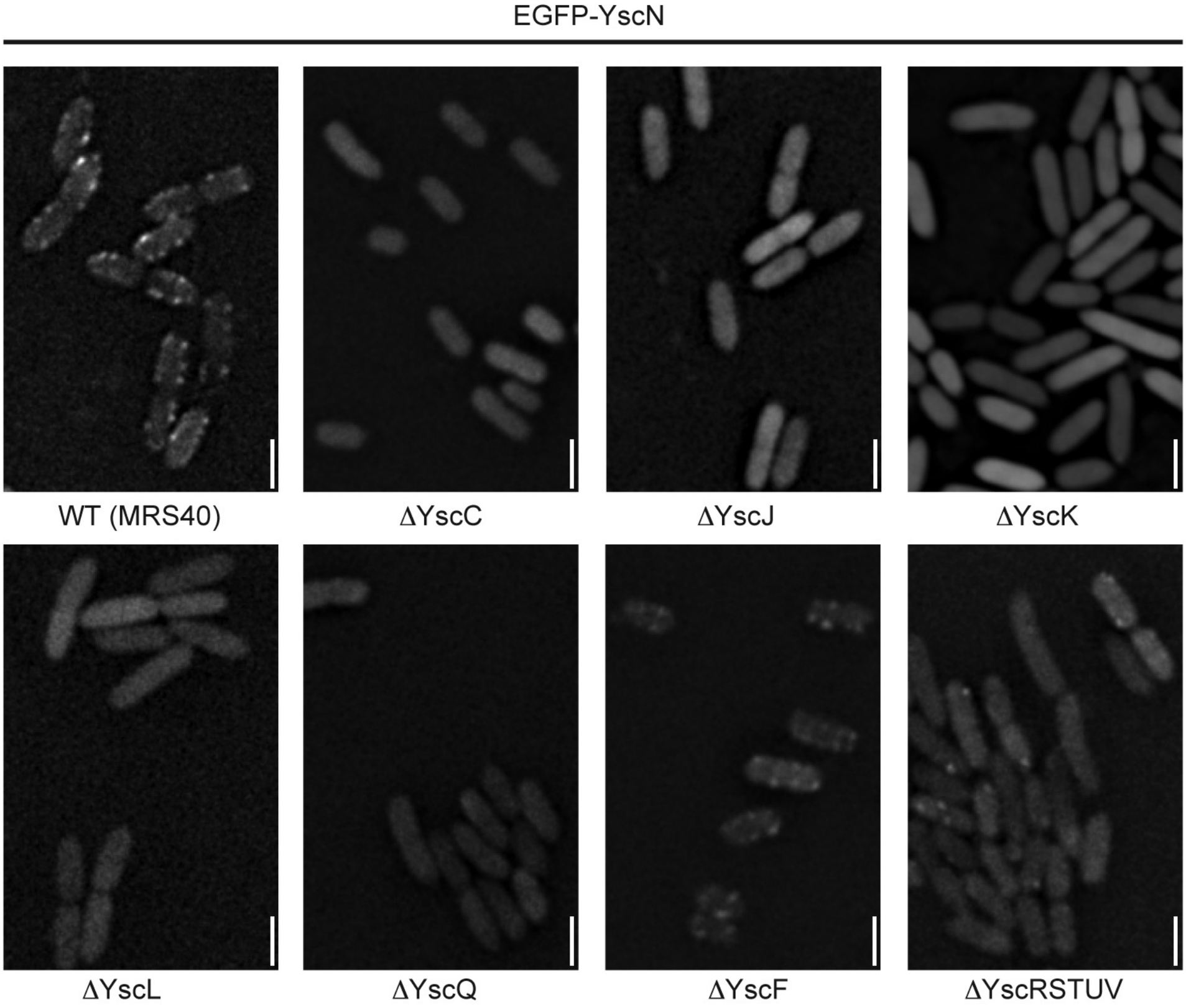

EGFP-YscN
WT (MRS40)
ΔYscC
ΔYscJ
ΔYscK
ΔYscL
ΔYscQ
ΔYscF
ΔYscRSTUV

Figure 5

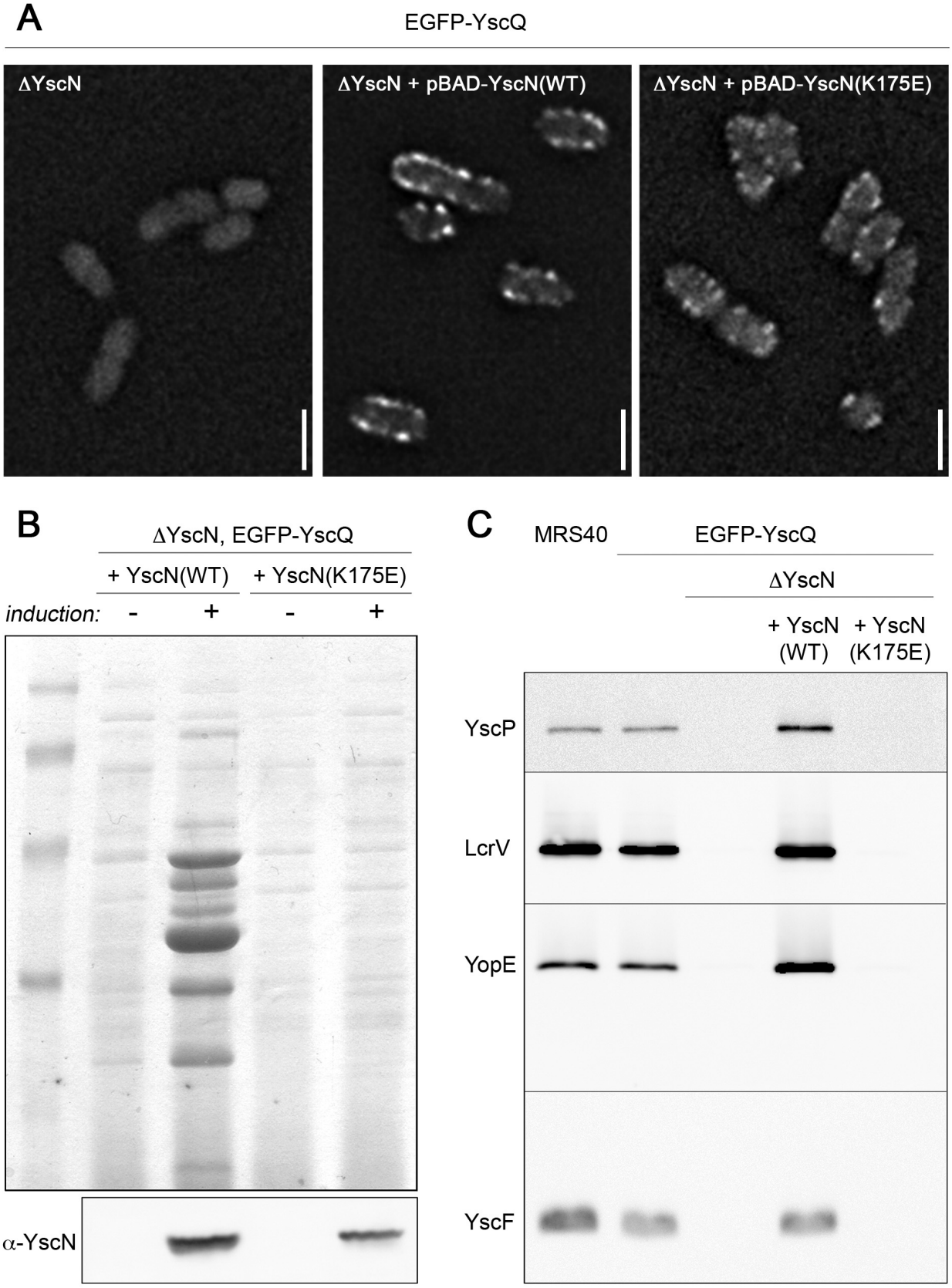
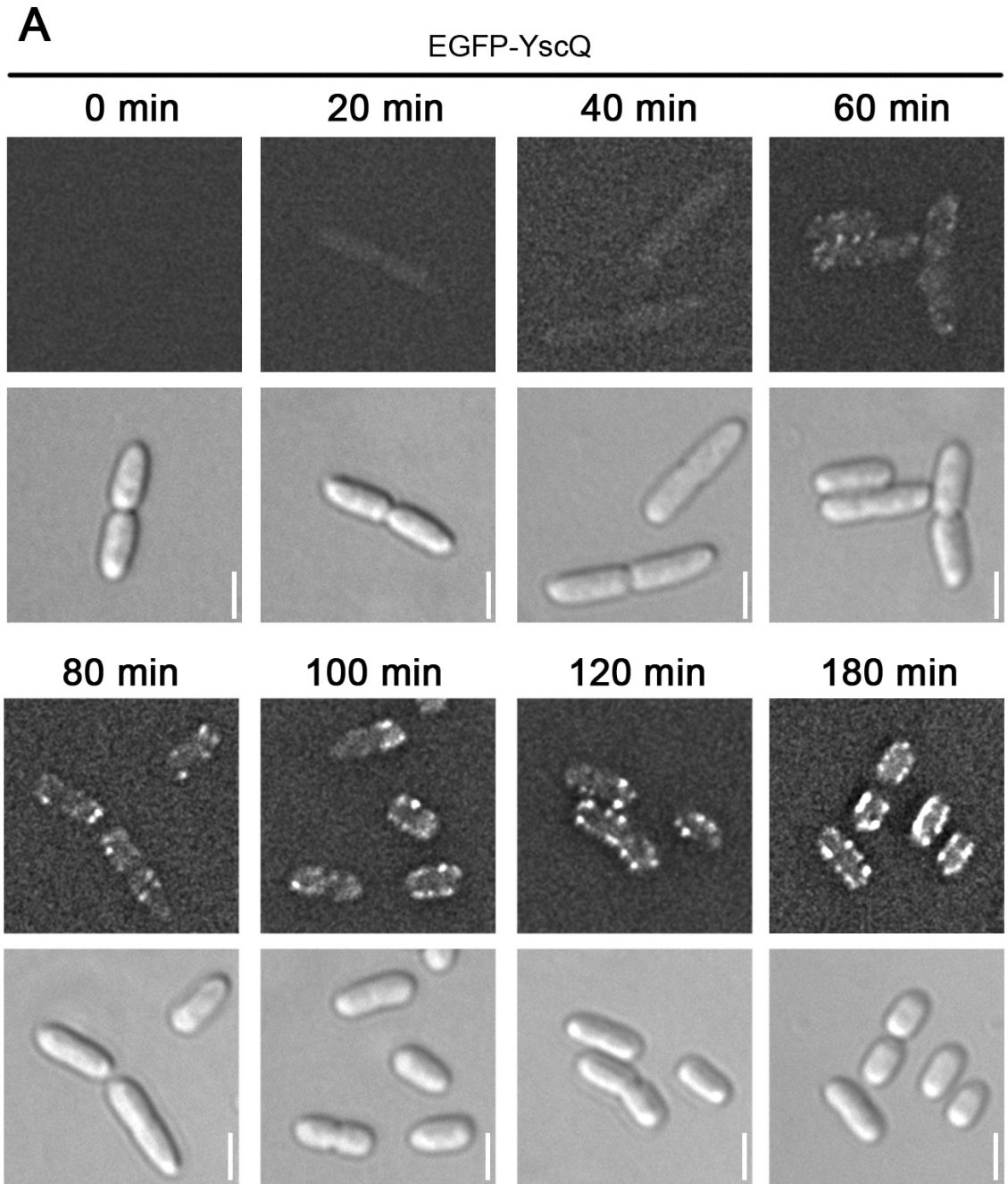

A
EGFP-YscQ
ΔYscN
ΔYscN + pBAD-YscN(WT)
ΔYscN + pBAD-YscN(K175E)
B
ΔYscN, EGFP-YscQ
+ YscN(WT)
+ YscN(K175E)
induction:
-
+
-
+
α-YscN
C
MRS40
EGFP-YscQ
ΔYscN
+ YscN (WT)
+ YscN (K175E)
YscP
LcrV
YopE
YscF

Fig. 6


A
EGFP-YscQ
0 min
20 min
40 min
60 min
80 min
100 min
120 min
180 min

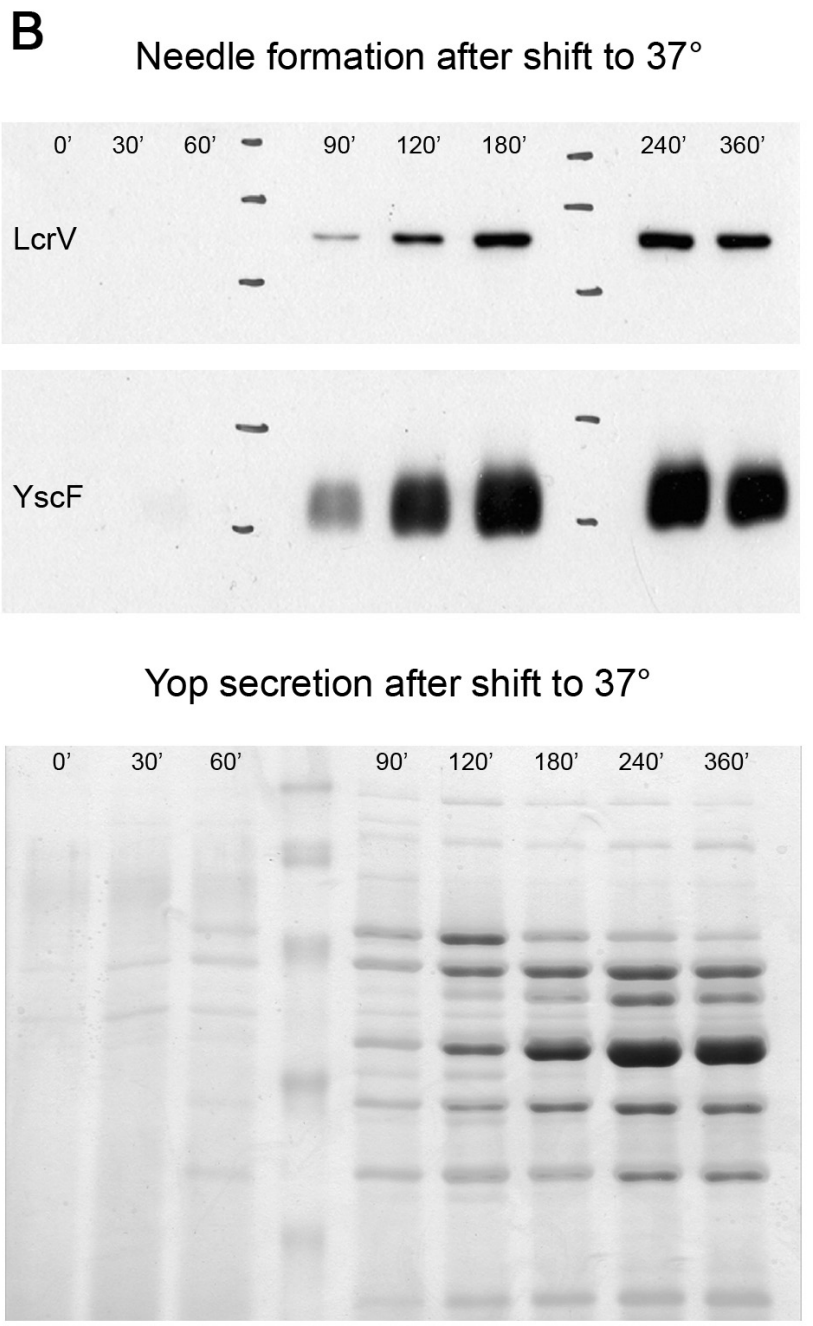
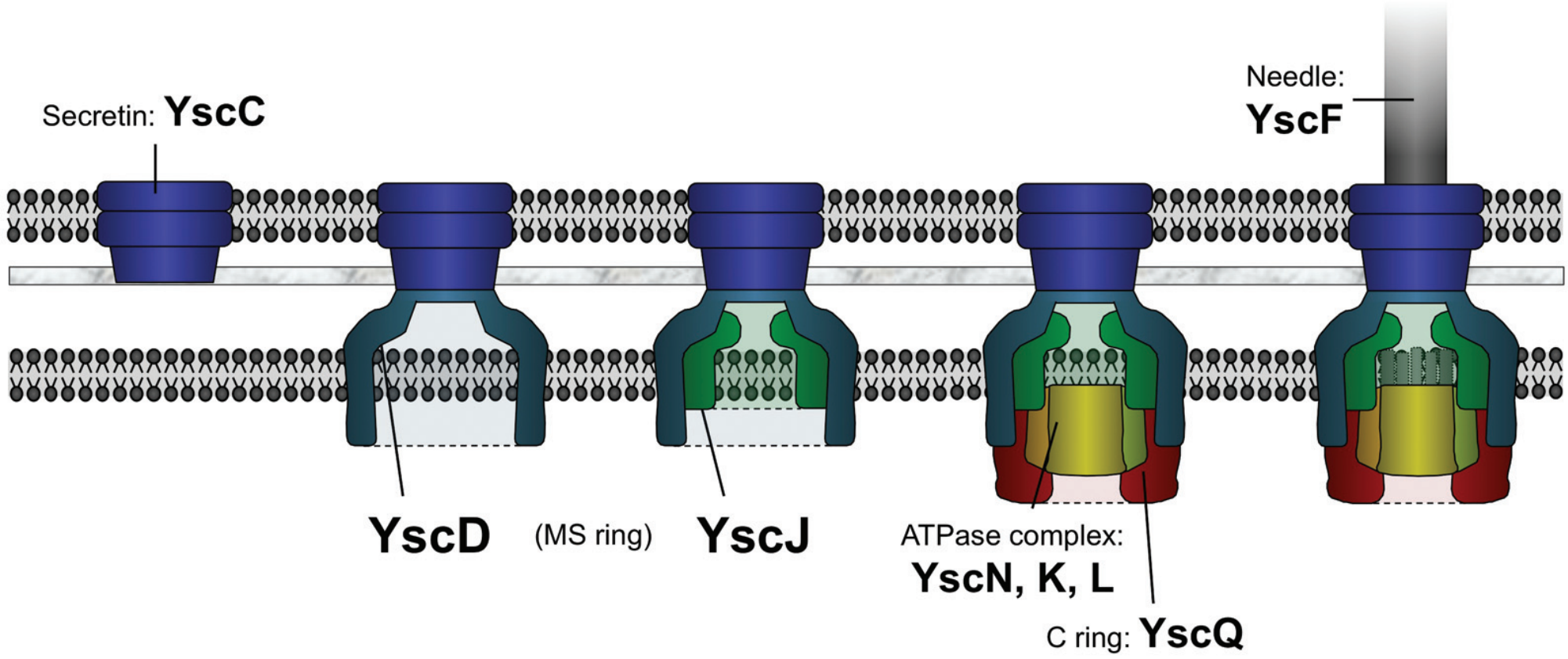

B
Needle formation after shift to 37°
0'
30'
60'
90'
120'
180'
240'
360'
LcrV
YscF
Yop secretion after shift to 37°
0'
30'
60'
90'
120'
180'
240'
360'

Fig. 7


Secretin: YscC
Needle:
YscF
YscD
(MS ring)
YscJ
ATPase complex:
YscN, K, L
C ring: YscQ