Interactions between type III secretion apparatus components from Yersinia pestis detected using the yeast two-hybrid system

ARTICLE *in* FEMS MICROBIOLOGY LETTERS · JUNE 2000

Impact Factor: 2.12 · DOI: 10.1016/S0378-1097(00)00121-X · Source: PubMed

2 AUTHORS, INCLUDING:

Gregory V Plano
University of Miami

65 PUBLICATIONS **2,972** CITATIONS

SEE PROFILE

ELSEVIER

FEMS Microbiology Letters 186 (2000) 85–90

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Interactions between type III secretion apparatus components from *Yersinia pestis* detected using the yeast two-hybrid system

Michael W. Jackson, Gregory V. Plano *

Department of Microbiology and Immunology, University of Miami School of Medicine, P.O. Box 016960 (R-138), Miami, FL 33101, USA

Received 18 January 2000; received in revised form 29 February 2000; accepted 3 March 2000

Abstract

Interactions among the *Yersinia* secretion (Ysc) proteins of *Yersinia pestis* were explored using the yeast two-hybrid system. Various pairwise combinations of the *yscEFGHIKLN* and *Q* genes fused to the DNA-binding or activation domain of the yeast GAL4 gene were introduced into yeast, and expression of a reporter gene encoding β-galactosidase was detected. Combinations of *yscN* and *yscL*, *yscL* and *yscQ*, and *yscQ* and *yscK* resulted in high levels of reporter gene activation. These results suggest that YscL interacts with both YscN and YscQ, and that YscQ interacts with both YscL and YscK. Three-hybrid analyses using plasmid pDELA to target a third hybrid protein to the yeast nucleus was used to detect the formation of ternary protein complexes. Using the three-hybrid system, YscQ expressed from plasmid pDELA was able to bring together the YscK and YscL fusion proteins. In a similar manner, YscL expressed from plasmid pDELA was able to bring together the YscN and YscQ fusion proteins. Together, these results suggest that a complex composed of YscN, YscQ, YscK and YscL is involved in the assembly and/or function of the *Y. pestis* type III secretion apparatus.

Keywords: Protein secretion; Protein interaction; Yeast two-hybrid; Yeast three-hybrid; *Yersinia pestis*

1. Introduction

Several animal and plant Gram-negative bacteria use a specialized protein secretion system, termed type III, to manipulate eukaryotic cells [1]. These systems are activated upon contact with a eukaryotic cell and function to translocate effector proteins from the cytosol of the bacterium directly into the cytosol of the eukaryotic cell [2]. Translocated effector proteins function to destroy or subvert the targeted cell. The type III secretion system of the human pathogenic Yersiniae (*Yersinia enterocolitica*, *Yersinia pseudotuberculosis* and *Yersinia pestis*) is an archetype of these systems [3]. It consists of a secretion apparatus composed of approximately 21 Ysc proteins and a set of 12 *Yersinia* out proteins (Yops) that are exported by the secretion apparatus.

Genes encoding the *Yersinia* type III secretion apparatus are clustered within several large transcriptional units which include *yscBCDEFGHIJKLM* [4], *yscNOPQRSTU* [5], *yscW* [6], and *yopNtyeAsycNyscXYVlcrR* [7,8]. Mutational inactivation of any of the *ysc* genes (with the exception of *yscB* and *yscH*) prevents Yop secretion. A recent study shows that the YscC protein forms a pore-like structure in the *Yersinia* outer membrane, and that the outer membrane lipoprotein YscW is also required to form this pore-like structure [6]. The *yscD*, *yscJ*, *yscR*, *yscS*, *yscT*, *yscU* and *yscV* gene products are predicted to be integral inner membrane proteins with at least one hydrophobic membrane-spanning region [4,5,7]. The *yscE*, *yscF*, *yscG*, *yscI*, *yscK*, *yscL*, *yscN*, *yscQ* and y*scY* gene products are predicted to be cytoplasmic or peripheral membrane proteins [4,5,9], whereas several recent studies show that a portion of the *yscO*, *yscP* and *yscX* gene products are secreted in vitro [9–11]. Together, these proteins are thought to assemble into a large multiprotein secretory complex that spans both the bacterial inner and outer membranes. A large multiprotein complex corresponding to the type III secretory machinery of *Salmonella typhimurium* has recently been visualized by electron microscopy [12]. Although the former study and a similar study in *Pseudomonas syringae* pv. tomato [13] suggest that the individual components of the type III secretory apparatus assemble into a stable multiprotein secretory complex, no comprehensive attempts to identify the indi-

* Corresponding author. Tel.: +1 (305) 243-6310; Fax: +1 (305) 243-4623; E-mail: gplano@med.miami.edu

0378-1097/00/$20.00
PII: S0378-1097(00)00121-X

vidual protein interactions in this complex have been attempted.

We chose to take a genetic approach based upon the yeast two-hybrid system [14] in order to identify protein–protein interactions involved in the assembly of the *Y. pestis* type III secretion apparatus. The yeast GAL4 transcriptional activator contains two separable domains, a DNA binding domain and a transcriptional activation domain. When physically separated, neither domain alone can activate transcription. However, when fused to proteins that interact, the domains of GAL4 can be rejoined into a transcription-competent complex. In this study we examined the interaction of several Ysc proteins using the yeast two-hybrid systems.

2. Materials and methods

2.1. Bacterial and yeast strains, and growth conditions

Y. pestis KIM5 was grown at 30°C in heart infusion broth or on tryptose blood agar base plates as described previously [7]. *Escherichia coli* XL1 blue and *E. coli* BL21 were routinely grown at 37°C in LB medium or on LB agar plates. *Saccharomyces cerevisiae* strains SFY526 (*MAT*α, *gal*4-542, *gal*80-538, *URA*3::*GAL*1-*lac*Z, *can*^{r}, *ura*3-52, *his*3-200, *ade*2-101, *lys*2-801, *trp*1-901, *leu*2-3, 112) and BY3161 (*MAT*α, *leu*2-3, *trp*1-901, *his*3-200, *ura*3-52, *ade*2-101, *gal*4-542, *gal*80-538, *GAL*1-*lac*Z, *GAL*1-*His*3) were grown at 30°C in YPD medium or on yeast minimal synthetic dropout (SD) agar plates as described previously [15].

2.2. Construction of yeast plasmids

The coding sequences for the *yscE*, *yscF*, *yscG*, *yscH*, *yscI*, *yscK*, *yscL*, *yscN* and *yscQ* genes were obtained using plasmid pCD1 [16] as template for the polymerase chain reaction (PCR) with *Pfu* DNA polymerase (Stratagene). Full-length PCR products (ATG start codon to stop codon) for each of these genes were amplified using synthetic oligonucleotide primers tailed with an *Eco*RI or *Pst*I restriction endonuclease site. The resultant PCR products containing a 5′-*Eco*RI site and a 3′-prime *Pst*I site were ethanol precipitated, digested with *Eco*RI and *Pst*I, and inserted in-frame with the DNA encoding the GAL4 DNA binding domain of *Eco*RI and *Pst*I-digested pGBT9 or in-frame with the DNA encoding the transcriptional activation domain of *Eco*RI and *Pst*I-digested pGAD424. The resultant plasmids designated pGAD-YscE, pGAD-YscG, pGAD-YscH, pGAD-YscI, pGAD-YscK, pGAD-YscL, pGAD-YscN, pGAD-YscQ, pGBT-YscE, pGBT-YscG, pGBT-YscH, pGBT-YscI, pGBT-YscK, pGBT-YscL, pGBT-YscN and pGBT-YscQ were used to identify protein interactions using the yeast two-hybrid system (Clontech, Palo Alto, CA). Full-length PCR products corresponding to *yscL* and *yscQ* were also amplified using synthetic oligonucleotide primers tailed with a *Bam*HI or *Eco*RI restriction endonuclease site. *Bam*HI and *Eco*RI-digested PCR products were inserted in-frame with the DNA encoding the SV40 Large T antigen nuclear localization sequence of *Bam*HI and *Eco*RI-digested pDELA [17], generating plasmids pDELA-YscL and pDELA-YscQ. The pDELA plasmids were used to target a third fusion protein to the yeast nucleus for yeast three-hybrid experiments.

2.3. Yeast two- and three-hybrid assay of Ysc protein interactions

Derivatives of plasmids pGAD424 and pGBT9 constructed as described above were introduced into yeast SFY526 carrying a β-galactosidase reporter gene for yeast two-hybrid experiments. Derivatives of plasmids pGAD424, pGBT9 and pDELA were introduced into yeast BY3161 for yeast three-hybrid experiments. Expression of the *lacZ* gene product in SFY526 and BY3161 was measured by both colony lift and quantitative liquid β-galactosidase assays as described previously [15]. The optical densities at 420 nm of *o*-nitrophenol released from the substrate *o*-nitrophenyl-β-galactoside was normalized by the cell density of yeast cultures measured photometrically at 600 nm and were expressed in Miller units [18].

2.4. Construction of pMAL-c2 maltose-binding protein (MBP) fusion plasmids for expression of MBP-YscK, MBP-YscL, MBP-YscN and MBP-YscQ hybrid proteins

Derivatives of plasmid pMAL-c2 (New England Biolabs, Beverly, MA) encoding full-length YscK, YscL, YscN, YscQ or truncated versions of YscL fused to the carboxyl-terminus of the *E. coli* MBP were constructed to facilitate stable high-level expression of the encoded proteins. DNA fragments corresponding to the full-length *yscK*, *yscL*, *yscN* or *yscQ* open-reading frames or to truncated versions of the *yscL* gene encoding amino acid residues 1–150, 1–100, or 1–50 of YscL were amplified using oligonucleotide primers that introduced an *Eco*RI site at the 5′ end and a *Pst*I site at the 3′ end of each fragment. *Eco*RI and *Pst*I digested fragments were inserted into *Eco*RI and *Pst*I digested pMAL-c2, generating plasmids pMBP-YscK, pMBP-YscL, pMBP-YscN, pMBP-YscQ, pMBP-YscL$_{1-150}$, pMBP-YscL$_{1-100}$, and pMBP-YscL$_{1-50}$. Plasmid pMBP-YscL$_{\Delta 39-60}$, which contains an internal deletion in *yscL* eliminating the DNA sequence encoding amino acid residues 39–60 of YscL, was constructed by the PCR-ligation-PCR technique [19]. Derivatives of pMAL-c2 were introduced into *E. coli* BL21, and high-level expression of the encoded fusion proteins was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

2.5. Construction of pFLAG-CTC plasmids for expression of FLAG epitope-tagged YscK, YscL, YscN and YscQ proteins

Plasmids pYSCK-FLAG, pYSCL-FLAG, pYSCN-FLAG and pYSCQ-FLAG, which express carboxyl-terminal FLAG-tagged YscK, YscL, YscN and YscQ, respectively, were constructed by inserting full-length (ATG start codon to last codon preceding stop codon) PCR amplified fragments of *yscK, yscL, yscN* and *yscQ* tailed with *Hin*dIII and *Bgl*II restriction endonuclease sites into *Hin*dIII and *Bgl*II digested pFLAG-CTC (Sigma, St. Louis, MO). The recombinant YscK-FLAG, YscL-FLAG, YscN-FLAG and YscQ-FLAG proteins expressed from plasmids pYSCK-FLAG, pYSCL-FLAG, pYSCN-FLAG and pYSCQ-FLAG are predicted to be expressed with an additional three amino-terminal residues (Met-Lys-Leu), and an additional 11 carboxyl-terminal residues (Arg-Ser-Val-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) which includes the FLAG epitope (underlined). Derivatives of plasmid pFLAG-CTC were introduced into *E. coli* BL21, and high-level expression of the encoded fusion proteins was induced by the addition of 0.5 mM IPTG. Cell lysates from *E. coli* BL21 carrying pYSCQ-FLAG were prepared from 20 ml of IPTG-induced culture harvested at an OD_{620} of approximately 1.0. Cell pellets were collected by centrifugation at $8000 \times g$ for 5 min at 4°C, washed with 20 ml of TBS buffer (20 mM Tris–HCl, 100 mM NaCl, pH 7.4) and resuspended in 2 ml of TBS buffer. Cells were lysed at 4°C by a single passage through a French pressure cell at 20 000 psi. Unlysed cells and large debris were removed by centrifugation at $8000 \times g$ for 5 min at 4°C. The cleared cell lysate was stored at 4°C.

2.6. Binding of YscQ-FLAG to MBP-YscL on membrane filters

Whole cells from IPTG-induced cultures of *E. coli* BL21 carrying pMAL-c2 or derivatives of this plasmid were mixed with an equal volume of 2×SDS solubilization buffer (2% SDS, 10% β-mercaptoethanol, 50% glycerol, 0.25 M Tris–HCl, pH 6.8), boiled for 5 min, and separated by SDS-PAGE on 12% acrylamide gels as previously described [7]. SDS-PAGE-separated proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) and nonspecific binding of proteins blocked with 5% non-fat milk in TBS buffer. After washing the membranes with TBS buffer, a cell lysate containing FLAG-tagged YscQ (2 ml) was diluted into 50 ml of TBS buffer containing 1% non-fat milk and incubated with the membrane for 1 h at room temperature. After washing the membrane with TBS buffer, bound FLAG-tagged YscQ was detected with the FLAG M2 monoclonal antibody according to the manufacturer's instructions.

3. Results and discussion

3.1. Pairwise tests for interactions between individual Ysc proteins

The genes encoding YscE, YscF, YscG, YscH, YscI, YscK, YscL, YscN and YscQ of *Y. pestis* plasmid pCD1 [16] were fused to DNA encoding the GAL4 DNA-binding domain of pGBT9 and then again to DNA encoding the GAL4 transcriptional activation domain of pGAD424. Entire structural genes were included in the constructs. These gene fusion plasmids were introduced into yeast strain SFY526, which contains a GAL4-dependent *lac*Z reporter gene. Plasmids were introduced separately and in every possible pairwise combination. Initially, β-galactosidase activity was determined by colony lift assay. SFY526 carrying pGBT-YscL activated a low level of transcription in the absence of the GAL4 transcriptional activation domain (data not shown). The results of the pairwise tests are summarized in Table 1. Several strong two-hybrid interactions between various pairs of Ysc proteins were identified as dark blue colonies on filters exposed to 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL). For example, SFY526 cells that coexpressed a GAL4 activation domain fusion to YscG and a GAL4 binding domain fusion to YscE produced a dark blue color in colony lift assays. The YscG–YscE interaction has recently been examined further in a separate study [9] and is believed to represent a specific chaperone (YscG) substrate (YscE) interaction. In addition to the YscG–YscE interaction, several SFY526 strains that coexpressed combinations of the GAL4 activation domain and GAL4 binding domain fusions to the YscK, YscL, YscN and YscQ proteins produced dark blue (or light blue) colonies in colony lift assays. Liquid β-galactosidase assays were also performed on SFY526 strains carrying every possible pairwise combination of the YscK, YscL, YscN and YscQ GAL4 activation domain fusions and GAL4 binding domain fusions. These results suggest pairwise interactions exist between YscK and YscQ, YscQ and YscL, and between YscL and YscN. Each of these interactions was reciprocal, for example, YscL and YscN interacted when YscL was fused to the GAL4 activation domain and YscN was fused to the GAL4 DNA binding domain, or vice versa. SFY526 carrying pGBT-YscQ and pGAD-YscK produced only low levels of β-galactosidase (2.2 U), compared to the reciprocal combination of pGBT-YscK and pGAD-YscQ (24.4 U); however, even the low level of β-galactosidase expressed by SFY526 carrying pGBT-YscQ and pGAD-YscK was significantly above the background β-galactosidase activity (< 0.2 U). No interaction of YscN, YscQ, YscK or YscL with the other *ysc* gene products tested was found.

Table 1
Results of yeast two-hybrid tests

	pGBT9	pGBT-YscE	pGBT-YscF	pGBT-YscG	pGBT-YscH	pGBT-YscI	pGBT-YscK	pGBT-YscL	pGBT-YscN	pGBT-YscQ
pGAD424	− (<0.2)	−	−	−	−	−	−	+/−	−	−
pGAD-YscE	−	−	−	+	−	−	−	+/−	−	−
pGAD-YscF	−	−	−	−	−	−	−	+/−	−	−
pGAD-YscG	−	+	−	−	−	−	−	+/−	−	−
pGAD-YscH	−	−	−	−	−	−	−	+/−	−	−
pGAD-YscI	−	−	−	−	−	−	−	+/−	−	−
pGAD-YscK	−	−	−	−	−	−	− (<0.2)	+/− (0.9 ± 0.3)	− (<0.2)	+/− (2.2 ± 0.6)
pGAD-YscL	−	−	−	−	−	−	− (<0.2)	+/− (1.1 ± 0.1)	+ (51.6 ± 1.6)	+ (48.8 ± 4.7)
pGAD-YscN	−	−	−	−	−	−	− (<0.2)	+ (66.2 ± 2.7)	− (<0.2)	− (<0.2)
pGAD-YscQ	−	−	−	−	−	−	+ (24.5 ± 2.2)	+ (23.7 ± 1.3)	− (<0.2)	− (<0.2)

Plasmids pGAD424, pGBT9 and derivatives of these plasmids were introduced in pairs into the yeast reporter strain SFY526 for two-hybrid testing. The relative intensity of the blue color developed after exposure of permeabilized cells to X-GAL for 1 h are indicated by +: (dark blue), +/−: (light blue) or −: (white) signs. Quantitative liquid β-galactosidase assays were performed according to the method of Miller [18]. The values (Miller units) given in parentheses represent averages (± standard deviations) assayed in triplicate.

3.2. Yeast three-hybrid analysis of YscK, YscL, YscN and YscQ interactions

Yeast two-hybrid analyses predicted YscQ to interact with both YscK and YscL. Three-hybrid analyses using plasmid pDELA [17] to target a third hybrid protein to the yeast nucleus was used to test whether YscK and YscL interact at the same or independent sites on YscQ. The third protein was expressed as a fusion to the SV40 T-antigen nuclear localization sequence. Using the three-hybrid system, YscQ expressed from plasmid pDELA was able to bring together the YscK and YscL GAL4 fusion proteins (Table 2), suggesting that YscK and YscL interact at independent sites on YscQ. In a similar manner, YscL fused to the SV40 T-antigen nuclear localization sequence was able to bring together the YscN and YscQ GAL4 fusion proteins. Together, these results suggest that a complex composed of YscN, YscQ, YscK and YscL is involved in the assembly and/or function of the *Y. pestis* type III secretion apparatus.

3.3. Binding assays of YscQ-FLAG to MBP-YscK, MBP-YscL, MBP-YscN and MBP-YscQ

In vitro protein binding assays of FLAG epitope-tagged YscK, YscL, YscN and YscQ to SDS-PAGE-separated MBP, MBP-YscK, MBP-YscL, MBP-YscN and MBP-YscQ were used in an attempt to confirm the interactions identified using the yeast two- and three-hybrid systems. Unfortunately, although each of the FLAG-tagged proteins was expressed in *E. coli* BL21, only the YscQ-FLAG protein was recovered in the soluble fraction following cell lysis and centrifugation to remove unlysed cells and large debris. The YscQ-FLAG protein bound specifically to the MBP-YscL protein, but not to MBP, MBP-YscK, MBP-YscN or MBP-YscQ (Fig. 1A). The failure to detect binding to MBP-YscK could be due to any one of several problems associated with this procedure. For example, the MBP-YscK protein may be irreversibly denatured following boiling in the presence of SDS. Alternatively, the addition of the MBP domain to YscK or the addition of the FLAG epitope to YscQ may have sterically blocked the YscQ–YscK interaction.

To localize the region of the 210 amino acid residue YscL protein recognized by YscQ, a series of in-frame fusions between the *malE* gene and portions of the *yscL* gene encoding amino acid residues 1–150 (MBP-$YscL_{1-150}$), 1–100 (MBP-$YscL_{1-100}$), 1–50 (MBP-$YscL_{1-50}$) and an internal deletion eliminating amino acid residues 39–60 (MBP-$YscL_{\Delta 39-60}$) (Fig. 1B) were con-

Table 2
Yeast three-hybrid analysis of YscK, YscL, YscN and YscQ interactions

PGAD424[a]	pGBT9[a]	pDELA[a]	Colony lift assay[b]	β-GAL activity[c]
pGAD-YscL	pGBT-YscQ	pDELA	+	15.34 ± 2.22
pGAD-YscL	pGBT-YscK	pDELA	−	0.83 ± 0.02
pGAD-YscL	pGBT-YscK	pDELA-YscQ	+	8.46 ± 0.44
pGAD-YscN	pGBT-YscL	pDELA	+	7.38 ± 0.37
pGAD-YscN	pGBT-YscQ	pDELA	−	0.45 ± 0.01
pGAD-YscN	pGBT-YscQ	pDELA-YscL	+/−	2.04 ± 0.02

[a]The BY3161 reporter strain was transformed with derivatives of the indicated plasmids.
[b]+ and − signs indicate the relative intensity of the blue color developed after exposure of lysed cells to X-GAL for 1 h.
[c]Expression of the reporter lacZ gene was measured by the method of Miller [18]. The given values represent averages (± standard deviation) assayed in triplicate.

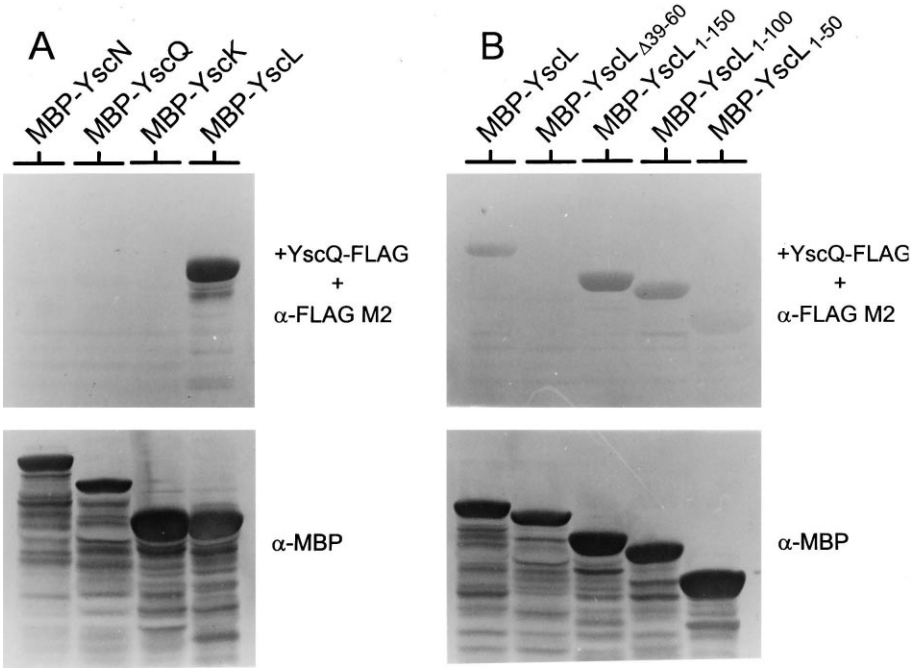

Fig. 1. Binding of YscQ-FLAG to MBP-YscL. Immobilon-P membranes containing SDS-PAGE-separated cell pellet fractions from BL21 expressing (A) MBP-YscN, MBP-YscQ, MBP-YscK and MBP-YscL or (B) truncated or internally deleted MBP-YscL hybrid proteins. MBP derivatives were detected with an antiserum specific for MBP (α-MBP). Bound YscQ-FLAG was detected with the FLAG M2 monoclonal antibody.

structed and transformed into *E. coli* BL21. FLAG-tagged YscQ bound strongly to MBP-YscL, MBP-$YscL_{1-150}$, and MBP-$YscL_{1-100}$; however, YscQ-FLAG did not bind or bound only very weakly to MBP-$YscL_{1-50}$, indicating that the carboxyl-terminal 110 residues of YscL are not required for interaction with YscQ. Interestingly, a predicted coiled-coil region was found between amino acid residues 31 and 60 of YscL [20]. An in-frame deletion eliminating the coding region for residues 39–60 of YscL (MBP-$YscL_{\Delta 39-60}$) was not recognized by YscQ, suggesting that the predicted coiled-coil domain in YscL is essential for the interaction of YscQ with YscL.

3.4. Homology of YscK, YscL, YscN and YscQ to proteins from other bacteria

The YscL, YscN and YscQ proteins are each members of well conserved families of type III secretion components [1]. Each of these proteins also exhibit significant amino acid similarity to one or two flagellar export proteins: YscL to FliH, YscN to FliI, and YscQ to FliN and FliM [1]. YscK only exhibits significant amino acid similarity to the closely related PscK protein of *Pseudomonas aeruginosa* [21]. Members of the YscN family of proteins are ATPases that are related to the catalytic α and β subunits of the F_oF_1 proton translocating ATPases [1]. Interestingly, YscL exhibits amino acid similarity [22] with subunit e of the proton-translocating ATPase of *Methanococcus jannaschii* (accession no. E64327; 22% identity and 43% similarity over a 131 amino acid alignment) and with subunit e of the vacuolar-ATPase of *Desulfurococcus* spp. (accession no. U96487; 19% identity and 46% similarity over 198 amino acid alignment). The e subunit has been shown to form a stable cytoplasmic complex with the α and β subunits of the V1 component of the V_oV_1 proton-translocating ATPase [23]. Thus, YscN and YscL may represent two components of an ATPase-like complex that may also involve the YscQ and YscK proteins. All four of these interacting proteins are predicted to be cytoplasmic or peripheral membrane proteins, suggesting that the YscK-YscQ-YscL-YscN complex may be a peripheral membrane multi-protein complex, a location similar to that found with the F_1 and V_1 multiprotein complexes.

Acknowledgements

We thank Jef Boeke for the BY3161 yeast strain, and Dr. Jie Zhang and Guilford Pharmaceuticals for plasmid pDELA. This study was supported by Public Health Service Grant AI39575.

References

[1] Hueck, C.J. (1998) Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev. 62, 379–433.
[2] Rosqvist, R., Magnusson, K. and Wolf-Watz, H. (1994) Target cell contact triggers expression and polarized transfer of the *Yersinia* YopE cytotoxin into mammalian cells. EMBO J. 13, 964–972.
[3] Cornelis, G.R. and Wolf-Watz, H. (1997) The *Yersinia* Yop virulon:

a bacterial system for subverting eukaryotic cells. Mol. Microbiol. 23, 861–867.
[4] Allaoui, A., Schulte, R. and Cornelis, G.R. (1995) Mutational analysis of the *Yersinia enterocolitica virC* operon: characterization of *yscE, F, G, I, J, K* required for Yop secretion and *yscH* encoding YopR. Mol. Microbiol. 18, 343–355.
[5] Bergman, T., Erickson, K., Galyov, E., Persson, C. and Wolf-Watz, H. (1994) The *lcrB* (*yscN/U*) gene cluster of *Yersinia pseudotuberculosis* is involved in Yop secretion and shows high homology to the *spa* gene clusters of *Shigella flexneri* and *Salmonella typhimurium*. J. Bacteriol. 176, 2619–2626.
[6] Koster, M., Bitter, W., de Cock, H., Allaoui, A., Cornelis, G.R. and Tommassen, J. (1997) The outer membrane component, YscC, of the Yop secretion machinery of *Yersinia enterocolitica* forms a ring-shaped multimeric complex. Mol. Microbiol. 26, 789–797.
[7] Plano, G.V. and Straley, S.C. (1993) Multiple effects of *lcrD* mutations in *Yersinia pestis*. J. Bacteriol. 175, 3536–3545.
[8] Viitanen, A.-M., Toivanen, P. and Skurnik, M. (1990) The *lcrE* gene is part of an operon in the *lcr* region of *Yersinia enterocolitica* 0:3. J. Bacteriol. 172, 3152–3162.
[9] Day, J.B. and Plano, G.V., unpublished results.
[10] Payne, P.L. and Straley, S.C. (1998) YscO of *Yersinia pestis* is a mobile core component of the Yop secretion system. J. Bacteriol. 180, 3882–3890.
[11] Payne, P.L. and Straley, S.C. (1999) YscP of *Yersinia pestis* is a secreted component of the Yop secretion system. J. Bacteriol. 181, 2852–2862.
[12] Kubori, T., Matsushima, Y., Nakamura, D., Uralil, J., Lara-Tejero, M., Sukhan, A., Galan, J.E. and Aizawa, S.I. (1998) Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. Science 280, 602–605.
[13] Roine, E., Wei, W., Yuan, J., Nurmiaho-Lassila, E.-L., Kalkkinen, N., Romatschuk, M. and He, S.Y. (1997) Hrp pilus: an hrp-dependent bacterial surface appendage produced by *Pseudomonas syringae* pv. tomato DC3000. Proc. Natl. Acad. Sci. USA 94, 3459–3464.
[14] Fields, S. and Song, O. (1989) A novel genetic system to detect protein–protein interactions. Nature 340, 245–246.
[15] Day, J.B. and Plano, G.V. (1998) A complex composed of SycN and YscB functions as a specific chaperone for YopN in *Yersinia pestis*. Mol. Microbiol. 30, 777–788.
[16] Perry, R.D., Straley, S.C., Fetherston, J.D., Rose, D.J., Gregor, J. and Blattner, F.R. (1998) DNA sequencing and analysis of the low-Ca^{2+}-response plasmid pCD1 of *Yersinia pestis* KIM5. Infect. Immun. 66, 4611–4623.
[17] Zhang, J. and Lautar, S. (1996) A yeast three-hybrid method to clone ternary protein complexes. Anal. Biochem. 242, 68–72.
[18] Miller, J. (1992) Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[19] Ali, S.A. and Steinkasserer, A. (1995) PCR-ligation-PCR mutagenesis: a protocol for creating gene fusions and mutations. Biotechniques 18, 746–750.
[20] Wolf, E., Kim, P.S. and Berger, B. (1997) MultiCoil: A program for predicting two- and three- stranded coiled coils. Protein Sci. 6, 1179–1189.
[21] Frank, D.W. (1997) The exoenzyme S regulon of *Pseudomonas aeruginosa*. Mol. Microbiol. 26, 621–629.
[22] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
[23] Doherty, R.D. and Kane, P.M. (1993) Partial assembly of the yeast vacuolar H(+)-ATPase in mutants lacking one subunit of the enzyme. J. Biol. Chem. 268, 16845–16851.