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Roles of YopN, LcrG and LcrV in Controlling Yops Secretion by *Yersinia pestis*

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Abstract. Control of Yops secretion in pathogenic Yersinia is achieved at several levels. These levels likely include transcriptional, post-transcriptional, translational and secretional controls. Secretion control appears to be mediated by two pathways. One pathway involves YopN and proteins that interact with YopN. The second pathway consists of LcrG and its interaction with LcrV. LcrV is a postive regulator of Yops secretion that exerts control over Yops secretion by negating the secretion blocking role of LcrG. However, the intersection of these two control pathways is not understood. Recent work has allowed the development of a speculative model that brings YopN-mediated and LcrG-LcrV-mediated control together in the context of the ability of the needle complex to respond to Ca^{2+}.

20.1 Type III Secretion in *Y. pestis*

Yersinia pestis, the etiologic agent of plague, harbors a 70-kb plasmid termed pCD1 that encodes a type III secretion system (T3SS) (Perry and Fetherson 1997). The T3SS is a group of structural proteins that comprise the T3S apparatus (YSC) and a set of secreted effector proteins called Yersinia outer proteins (Yops) (Cornelis 1999). *Y. pestis* utilizes this T3SS to inject Yops directly into the cytoplasm of host cells were they inhibit bacterial phagocytsis and block the activation of an early immune response (Cornelis 1999). *Y. pestis* grows at 26-28°C with normal growth kinetics and a generation time of approximately 90 minutes (Brubaker 1972). When grown at 37°C, i.e. mammalian body temperature, the growth of *Y. pestis* becomes dependent on the presence of Ca^{2+} ions (Straley et al. 1993). This Ca^{2+}-dependent growth phenotype is linked to the expression and secretion of Yops through the T3SS, which is expressed at 37°C and not at 26°C (Straley et al. 1993). At 37°C, the expression of the T3S genes is activated and *Y. pestis* assembles a functional T3S apparatus. When Yops secretion is blocked, the expression of the T3S genes is kept to a minimum and the bacteria grow normally without entering growth restriction (Straley and Bowmer 1986; Michiels et al. 1990).

20.1.1 Regulation of T3S in *Y. pestis*

The secretion of Yops by *Y. pestis* is not constitutive, instead Yops secretion is tightly regulated and is activated in response to environmental cues (Straley and Bowmer 1986; Straley 1988; Straley et al. 1993). In vivo, Yops secretion is thought to be blocked prior to eukaryotic cell contact and subsequently activated by

eukaryotic cell contact (Rosqvist et al. 1994). In vitro, Yops secretion is regulated by extracellular Ca^{2+} ions and nucleotides, e.g. ATP and GTP (Michiels et al. 1990; Straley et al. 1993). Maximal Yops expression and secretion are attained in vitro by growing *Y. pestis* at 37°C in media lacking Ca^{2+} (Straley and Perry 1995). Addition of millimolar concentration of Ca^{2+} or nucleotides into the growth media blocks the secretion of Yops, represses the expression of the T3S genes, and rescues the bacteria from growth restriction (Straley et al. 1993).

When *Y. pestis* is grown at 37°C, the expression of T3S genes is activated by a transcriptional activator, LcrF. LcrQ, LcrH, and YopD control the upregulation of T3S gene expression that occurs when T3S-expression is induced. Deletion of *lcrH*, *yopD* or *lcrQ* results in maximal expression of T3S genes regardless of the Ca^{2+} concentration in the growth media, however *lcrH*, *yopD* and *lcrQ* strains retain the Ca^{2+} regulation of Yops secretion (Williams and Straley 1998; Wulff-Strobel et al. 2002; Pallen et al. 2003). The mechanism used by LcrH, LcrQ, and YopD to regulate T3S expression is unknown. Post-transcriptional regulation may occur as LcrQ, YopD, and LcrH may bind to the 5' UTR of Yops mRNAs (Anderson et al. 2002; Cambronne and Schneewind 2002).

20.1.2 Regulation of Yops Secretion by YopN

Under secretion nonpermissive conditions the secretion of Yops by *Y. pestis* is blocked by the negative regulators YopN, TyeA, SycN, YscB, and LcrG; the activation signal is unknown. Recent evidence suggests the needle complex, formed by YscF, may be involved in sensing the signal for Yops secretion (Torruellas et al. 2005). Negative regulation imposed by YopN is dependant on YopN's interaction with YscB, SycN, and TyeA (Ferracci et al. 2004; Ferracci et al. 2005), while the secretion blockage by LcrG is counteracted by LcrG's interaction with LcrV (Nilles et al. 1997; Matson and Nilles 2001).

YopN is a secreted protein that is also translocated into eukaryotic cells (Forsberg et al. 1991; Day et al. 2003). A YopN null mutant constitutively secretes Yops and hypersecretes LcrV (Forsberg et al. 1991; Skrzypek and Straley 1995). YopN interacts with the cytosolic proteins SycN, YscB, and TyeA (Day and Plano 1998; Iriarte et al. 1998; Jackson et al. 1998). The inactivation of TyeA, YscB, or SycN abolishes YopN function and results in the constitutive secretion of Yops. TyeA, a cytosolic protein, binds the C-terminus of YopN and the YopN-TyeA interaction is required to block Yops secretion (Ferracci et al. 2004). The YopN/TyeA complex is thought to block the secretion of Yops from the cytosolic face of the secretion apparatus (Ferracci et al. 2004; Ferracci et al. 2005). YscB and SycN are cytosolic chaperones of YopN (Jackson et al. 1998). YscB and SycN form a complex that binds to the N-terminus of YopN and facilitates the efficient secretion of YopN (Jackson et al. 1998; Ferracci et al. 2005). The mechanism of secretion regulation by YopN and YopN's interaction partners is not completely clear. Initially studies had suggested that YopN is surface-localized and that YopN could directly sense Ca^{2+} (Forsberg et al. 1991; Iriarte et al. 1998). However recent evidence contradicts this hypothesis. New evidence suggests that the T3S needle (Kenjale et al. 2005; Torruellas et al. 2005) probably senses the extracellular signal for secretion activation. Further

strengthening a cytosolic location for YopN function, a mutant YopN that constitutively blocks Yops secretion from a cytoplasmic location in the bacteria has been described (Ferracci et al. 2005). A model for how the YopN/SycN/YscB/TyeA complex regulates Yops secretion is presented in Fig. 1 (Ferracci et al. 2005; Torruellas et al. 2005). According to this model, when conditions do not favor secretion, the YscB/SycN chaperone binds and targets the YopN/TyeA complex to the cytoplasmic face of the T3S apparatus where YopN/TyeA blocks the secretion of Yops. The mechanism of secretion blockage is not clear but partial secretion of YopN in the YopN/TyeA complex in the presence of calcium may block the T3SS. When conditions favor secretion, YopN is secreted through the T3S apparatus, which relieves the blocking activity of the YopN/TyeA complex and allows secretion of the effector Yops to occur.

20.1.3 Secretion Regulation by LcrG and LcrV

LcrV is a multifunctional protein whose activity is central for the virulence of *Y. pestis* (Perry and Fetherson 1997; Mota 2006). During a *Y. pestis* infection, LcrV mediates translocation, secretion regulation, and immunomodulation. LcrV localizes

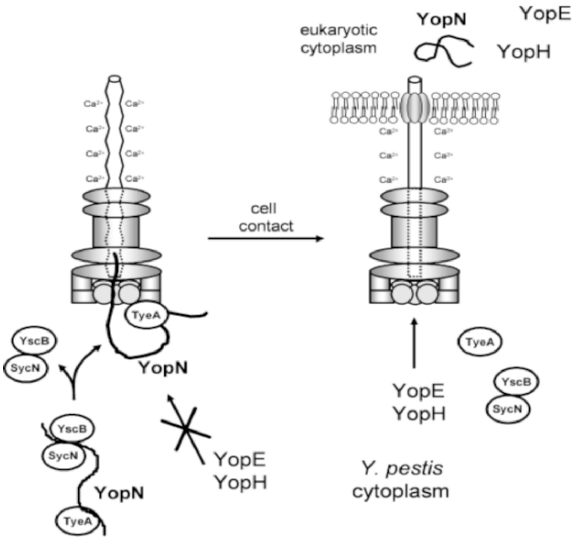

Fig. 1. A hypothetical model for the secretion regulation of Yops by YopN. In the presence of calcium, the YopN/TyeA complex is targeted to the YSC by the SycN/YscB chaperone. The YopN-TyeA complex initiates the secretion of YopN; however, secretion of YopN cannot be completed in the presence of calcium. The partially secreted YopN-TyeA complex is thought to block the T3S apparatus thus preventing the secretion of Yops. Upon secretion activation, the needle may encounter a low-calcium environment (eukaryotic cytosol or Ca^{2+} removal). YscF may sense this low Ca^{2+} environment. The YscF needle might propagate a secretion activation signal allowing YopN to be secreted. The secretion of YopN then opens the secretion apparatus. From Torruellas et al. (2005). Reprinted with permission of Blackwell Publishing Ltd.

at the bacterial surface prior to eukaryotic cell contact and forms a distinct structure at the tip of the YscF needle (Fields et al. 1999; Pettersson et al. 1999; Mueller et al. 2005). LcrV is also required along with Yops B and D for the polarized translocation of the effector Yops into the cytoplasm of targeted host cells (Nilles, Fields and Straley 1998; Fields et al. 1999). LcrV also causes immunosuppression by triggering IL-10 release by macrophages (Brubaker 2003; Overheim et al. 2005; Sing et al. 2005). Binding to the negative regulator LcrG attains regulation of Yops secretion by LcrV (Matson and Nilles 2001; Lawton et al. 2002). In addition, LcrV has been known since the mid 50's as a protective antigen and is currently being investigated in human trials as a component of a vaccine against the plague (Titball and Williamson 2004; Williamson et al. 2005).

LcrG is a small cytoplasmic protein that is required to block the secretion of Yops prior to eukaryotic cell contact or in the presence of Ca^{2+} (Skrzypek and Straley 1993; Nilles et al. 1997). An LcrG null mutant constitutively secretes Yops and hyposecretes LcrV (Skrzypek and Straley 1993; Nilles et al. 1997; Fields et al. 1999). LcrG physically interacts with LcrV in the cytosol of *Y. pestis* (Nilles et al. 1997). Disruption of the LcrG-LcrV interaction or overexpression of LcrG in a low-LcrV background both result in constitutive blockage of Yops secretion (Nilles et al. 1998; Matson and Nilles 2001). This evidence led to the LcrG-titration model (Fig. 2) that partially explains how LcrG-LcrV interaction may control Yops secretion (Matson and Nilles 2001). According to the LcrG-titration model, when culture

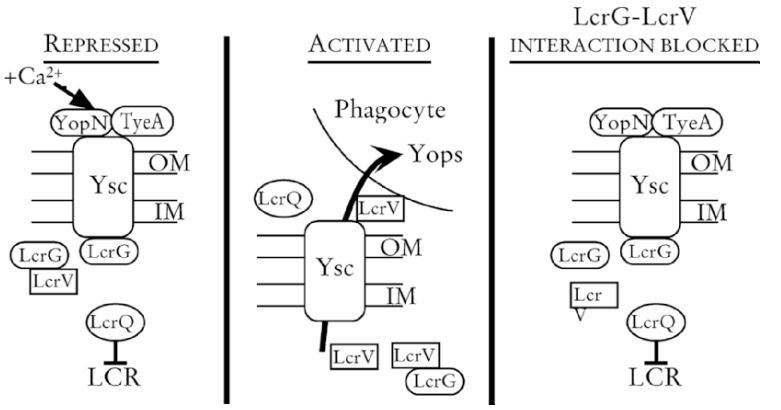

Fig. 2. LcrG titration model. In the presence of Ca^{2+}, LcrG, YopN and TyeA block the Ysc. LcrG is hypothesized to exert its blocking activity from the cytoplasm. The secretion block retains LcrQ in the cell, resulting in repression of LCR-regulated genes (Repressed). In the absence of Ca^{2+} or in the presence of eukaryotic cell contact, a block (possibly YopN) is released, allowing secretion of LcrQ (Activation). Secretion of LcrQ allows induction of LCR-regulated genes, including *lcrV*. Increased LcrV levels in the cytoplasm titrate LcrG away from the Ysc by forming a stable LcrG-LcrV complex. The removal of LcrG results in Yops and LcrV secretion and full induction of the LCR (Activated). If LcrG and LcrV cannot interact, LcrG is not titrated away from the Ysc, resulting in blockage of Yops secretion (LcrG-LcrV interaction blocked). From Matson and Nilles (2001). reprinted with permission of ASM.

conditions do not favor secretion, LcrG binds to an unidentified protein at the T3S apparatus and blocks the secretion of Yops. Upon induction of secretion LcrQ is secreted relieving the negative effects of LcrQ on Yops synthesis and allows an increase in the expression of Yops and, importantly, increases LcrV levels relative to LcrG levels. The increased LcrV binds LcrG and removes LcrG from its secretion-blocking role, resulting in the activation of Yops secretion.

20.2 Putting it all Together

A failing of the models presented for Yops secretion control by YopN in Fig. 1 and by LcrG in Fig. 2 is the lack of a link between the activities of LcrG and YopN. The development of an understanding of how LcrG, LcrV, and YopN cooperate to regulate the secretion of Yops is essential to fully understanding Yops secretion control. Recently, Hamad and Nilles demonstrated that LcrG, LcrV, and YopN are not directly involved in sensing Ca^{2+} but are required for responding to a Ca^{2+}-induced signal (Hamad 2006). These results indirectly support the proposed role of the YscF needle as the extracellular molecule involved in sensing Ca^{2+} (Torruellas et al. 2005). Interestingly, *lcrG* and *lcrGV* strains retain the ability to respond to higher Ca^{2+} concentrations by secreting reduced amounts of Yops (Hamad 2006). This ability to respond to increased Ca^{2+} is shared with a class of YscF mutants isolated by Torruellas et al. (2005). The constitutively secreting YscF mutants that respond to Ca^{2+} by secreting reduced amounts of Yops could represent a class of needle mutants that are defective in LcrG function suggesting a speculative linkage between LcrG and/or LcrV and YscF in Ca^{2+} responses. Furthermore, the ability of the *lcrG* and *lcrGV* strains to respond to high Ca^{2+} concentrations demonstrates that in the absence of LcrG, *Y. pestis* becomes less sensitive to Ca^{2+}. Therefore, if the YscF needle is involved in Ca^{2+} sensing, then LcrG may function to enhance the sensitivity of the YscF needle to extracellular Ca^{2+}. To date, no evidence suggests an LcrG association with the YscF needle complex and any linkage of LcrG to YscF function remains speculative. The analysis of YscF needle preparations by electron microscopy reveals that LcrV is found at the tip of the needles (Mueller et al. 2005) and suggests that the tip of the needle could be a site of LcrG-LcrV interaction. While LcrG has not been detected in needle preparations, that does not rule out the possibly that LcrG might regulate Yops secretion at the needle complex. Further direct experiments including immunoelectron and confocal microscopy are required to test the hypothesis that secretion control by LcrG and LcrV occurs at the tip of the YscF needle complex outside the bacterial cell. Additionally, isolation of LcrG or LcrV suppressors of YscF mutants would provide genetic evidence of a linkage between the LcrG-LcrV complex and YscF in Yops secretion control.

To dissect the roles of LcrG and LcrV in secretion control, a *ΔlcrGV3 ΔyopN* strain of *Y. pestis* was created and used as a background strain to introduce different LcrG and LcrV constructs to study how these proteins might affect the secretion of Yops in the absence of YopN (Hamad 2006; Hamad unpublished). Results obtained from this study shed light on the role of LcrG in secretion blockage relative to YopN's function. Strains lacking *lcrG* or *lcrGV* were able to respond to increased

Ca^{2+} concentrations by secreting reduced amounts of Yops only when YopN function was intact (Hamad 2006). This result suggests that when conditions do not favor secretion, LcrG contributes to secretion blockage by enhancing the blocking ability of YopN. Thus, when conditions do not favor secretion, LcrG appears to mediate secretion blockage by modulating the function of YopN. Data supporting this role of LcrG is provided by the isolation of a YopN point mutant that constitutively blocks Yops secretion (Ferracci et al. 2005). This YopN mutant's ability to block Yops secretion is dependent on TyeA but not LcrG, SycN, or YscB (Ferracci et al. 2005). Taken together the current evidence suggests that when conditions do not favor secretion, the YopN/TyeA complex serves as the molecule that physically blocks Yops secretion, while LcrG may play a role in modulating the function of the YopN/TyeA complex or LcrG may control a distinct step of the secretion process.

The mechanism by which LcrG facilitates a Yops blocking activity remains unclear. LcrG could influence YopN's function indirectly by amplifying the blocking signal induced by Ca^{2+} thus making YopN more sensitive to this signal. Another possibility is that LcrG could, directly or indirectly, modulate the interaction between YopN with interaction partners and/or the interaction between the YopN/TyeA complex with its Ysc target. When LcrG was expressed in the *ΔlcrGV3 ΔyopN* mutant, the secretion of Yops was partially blocked, suggesting that LcrG can bind a secretion-blocking target and impose a negative effect on Yops secretion in the absence of YopN. This suggests an independent role of LcrG in blocking Yops secretion. Clearly further work will be necessary to determine the influences of YopN and LcrG on each other's function as well as determining the targets of LcrG and YopN in blocking Yops secretion.

Not only are the mechanisms of secretion blockage obscure so are the mechanisms of secretion activation. When conditions favors Yops secretion, YopN's blocking role is relieved through the secretion of YopN and LcrG's role in secretion blockage is negated through the interaction of LcrG with LcrV (Nilles et al. 1998; Matson and Nilles 2001; Lawton et al. 2002; Ferracci et al. 2005). Unfortunately the mechanism that sets these events in action is unknown. According to the titration model, the removal of Ca^{2+} triggers the release of LcrQ, which results in an increase in LcrV levels (Nilles et al. 1998; Matson and Nilles 2001). This increase in LcrV levels is thought to drive the titration of LcrG by LcrV to allow for secretion activation (Nilles et al. 1998; Matson and Nilles 2001). However, the loss of LcrQ cannot simply account for secretion activation, since the secretion of Yops by an LcrQ null mutant reamins Ca^{2+} regulated (Rimpiläinen et al. 1992). In addition, inhibition of protein synthesis does not interfere with the ability of Yersinia to translocate Yops into eukaryotic cells, indicating that Yersinia is able to activate the secretion of Yops without de novo synthesis of LcrV (Lloyd et al. 2001). Thus although the interaction between LcrG and LcrV is required for secretion control, an increase in absolute LcrV levels is unlikely to be the mechanism behind LcrV's ability to bind and negate LcrG function when secretion is induced.

YopN appears to affect the function of LcrG and LcrV by influencing the interaction between LcrG and LcrV. An LcrG A16R mutant has reduced affinity for LcrV and when LcrG A16R is expressed in a *ΔlcrG3* strain the secretion of Yops is blocked (Matson and Nilles 2001; Matson and Nilles 2002), indicating that in the

presence of YopN, LcrV does not bind LcrG A16R. However, when LcrG A16R was expressed with LcrV in a *ΔlcrGV3 ΔyopN* strain, LcrV negated the blocking activity of LcrG A16R. This indicates that in the absence of YopN, LcrV was able to bind and remove the blocking activity of LcrG A16R (Hamad 2006). The interaction between LcrG and LcrV occurs in the cytosol of *Y. pestis* in the presence of YopN irrespective of Ca^{2+} (Nilles et al. 1997). Potentially the interaction between LcrG and LcrV that is required for secretion control occurs at the T3S apparatus and not in the cytosol. Since LcrG and LcrV interact in the cytosol of *Y. pestis* regardless of the presence of Ca^{2+} yet the secretion of Yops occurs only in the absence of Ca^{2+}. Thus although LcrG and LcrV interact independently of YopN's presence, YopN could influence the interaction between LcrG and LcrV at the T3S apparatus where the relevant interaction between LcrG and LcrV may occur. Unfortunately, how YopN influences the function of LcrG and LcrV remains a mystery. The only known effect that YopN has on LcrG and LcrV is at the level of LcrV secretion. *Y. pestis* hypersecretes LcrV in the absence of YopN (Skrzypek and Straley 1995; Hamad 2006). Therefore, YopN may have dual roles in blocking Yops secretion. The first role is at the level of Yops secretion, whereby YopN is required for blocking the secretion of T3S substrates when secretion is blocked. The second role of YopN is at the level of LcrV secretion, where YopN appears to negatively control the secretion of LcrV even when conditions favor Yops secretion. The significance of YopN's ability to negatively control the secretion of LcrV is unclear. Further experiments are required to determine how YopN controls the secretion LcrV and whether this event has any implications on LcrV's function in secretion control.

Based on the evidence provided above, a speculative model on how YscF, LcrG, LcrV, and YopN may control the secretion of Yops in response to Ca^{2+} is proposed (Fig. 3). In this revised model LcrG and LcrV control Yops secretion from the T3S apparatus, possibly at the tip of the YscF needle, while YopN controls secretion from the cytosolic face of the Ysc. YscF senses the presence of Ca^{2+} and transmits a blocking-signal to YopN, helping YopN to prevent the LcrG-LcrV interaction. Under these conditions LcrG's blocking role is dominant over LcrV's activating role and the YopN/TyeA complex blocks the secretion of Yops. LcrG's role in secretion blockage might possibly occur by amplifying the Ca^{2+} signal sensed by YscF, while the YopN/TyeA complex blocks the secretion of Yops from the cytosolic face of the Ysc (Ferracci et al. 2005). The removal of Ca^{2+} is sensed by the YscF needle that transmits a signal to YopN, thereby neutralizing YopN's role in preventing the LcrG-LcrV interaction. The negation of LcrG's blocking role by LcrV interaction allows the secretion of YopN, which relieves the blockage of the YopN/TyeA complex triggering Yops secretion. Current evidence largely supports this model; however further experiments are required to test the validity of this model. Future work including the identification of LcrG's and YopN's targets at the Ysc, understanding how YopN influences the function of LcrV and LcrG, and comprehending the mechanism by which the YscF needle senses and responds to the extracellular environment are required to understand the complex regulation of Yops secretion.

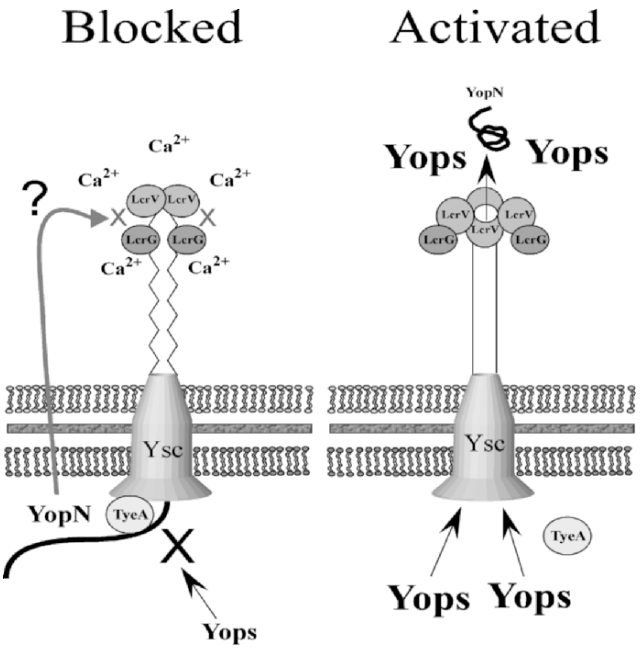

Fig. 3. A hypothetical model for the regulation of Yops secretion in *Y. pestis*. The presence of Ca^{2+} is sensed by the YscF needle that transmits a signal to YopN, allowing YopN to prevent the interaction between LcrG and LcrV. The removal of Ca^{2+} is sensed by the YscF needle that transmits a signal to YopN to neutralize YopN's role in preventing the interaction between LcrG and LcrV. Under these conditions, LcrV binds LcrG and negates LcrG's blocking role allowing the secretion of YopN. The secretion of YopN relieves the blockage of the Yopn/TyeA complex and allows for Yops secretion.

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