FRT40A/ovo^D FRT40A females with germ-line clones to *y, w hsFLP; kuz^{e29-4} ck FRT40A/CyO* males. About half of the embryos from these crosses exhibited the severe neurogenic phenotype described (Fig. 1G). The other half of the embryos exhibited an intermediate neurogenic phenotype (Fig. 1F), the same phenotype observed in all embryos produced by mating with wild-type males. The extent of the neurogenic phenotype caused by the hypomorphic *kuz* alleles varies between the wild type and the *kuz* null, but the correlation between the neural hyperplasia and the epidermal hypotrophy was always observed.

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Modulation of Virulence Factor Expression by Pathogen Target Cell Contact

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Upon contact with the eukaryotic cell, *Yersinia pseudotuberculosis* increased the rate of transcription of virulence genes (*yop*), as determined by in situ monitoring of light emission from individual bacteria expressing luciferase under the control of the *yopE* promoter. The microbe-host interaction triggered export of LcrQ, a negative regulator of Yop expression, via the Yop–type III secretion system. The intracellular concentration of LcrQ was thereby lowered, resulting in increased expression of Yops. These results suggest a key role for the type III secretion system of pathogenic bacteria to coordinate secretion with expression of virulence factors after physical contact with the target cell.

Human pathogenic *Yersinia* harbor a common 70-kb virulence plasmid encoding a set of highly regulated secreted proteins, Yops, that are key factors in the virulence process (*1–3*). When the bacteria are incubated under high Ca^{2+} conditions (≤1 mM) at 37°C, Yops are expressed at a low repressed level and no Yops are secreted to the culture medium. However, if Ca^{2+} is omitted, transcription is derepressed and high amounts of Yops are secreted to the culture medium (*1, 2, 4*). Thus, at the temperature of infection, Yop expression and secretion are regulated by external stimuli, which in vitro is constituted by the concentration of Ca^{2+}.

After infection of HeLa cells with *Yersinia pseudotuberculosis* (in vivo), the Yop effectors are found in the cytosol of the eukaryotic cell (*5–9*). This translocation process is polarized, and after intimate contact between the target cell and the pathogen has been established, the Yop effectors are secreted and translocated at the zone of contact between the bacterium and the target cell (*5–9*). Thus, the bacterium senses the contact with the eukaryotic cell surface and transmits a signal, focusing the secretion of Yop effectors to the zone of contact. How this cross talk between the eukaryotic cell and the bacterium is mediated is not known.

Yops are secreted by means of a type III secretion system (Ysc) (*10–12*), which has a high level of homology with the corresponding systems of *Salmonella* and *Shigella*. These systems are functionally conserved, allowing both the secretion and translocation of heterologous effector proteins across the target cell membrane (*12, 13*). Moreover, *Shigella* shows a target cell–induced response, manifested by the rapid release of the Ipa proteins to the culture medium (*14, 15*), and *Salmonella* rapidly induces the formation of new surface structures upon cell contact (*16*). Thus, these three human pathogens have a high degree of similarity with respect to the delivery of effector proteins attacking the target cells. Increased expression of effector proteins after microbe-host interaction has been suggested but has not yet been shown. Here, we provide evidence that physical contact between the pathogen and its target cell induces increased gene expression of virulence factors.

To determine whether Yop expression was elevated after cell contact, we measured the amount of YopE after infection of cultured HeLa cells with the wild-type strain YPIII(pIB102) of *Y. pseudotuberculosis* (Fig. 1). The total amount of YopE increased 16-fold when the bacteria made contact with the HeLa cells as compared with bacteria grown in cell culture medium alone (Fig. 1C). This increase was blocked by the addition of chloramphenicol (Fig. 1A). Similarly, the expression of the other Yop

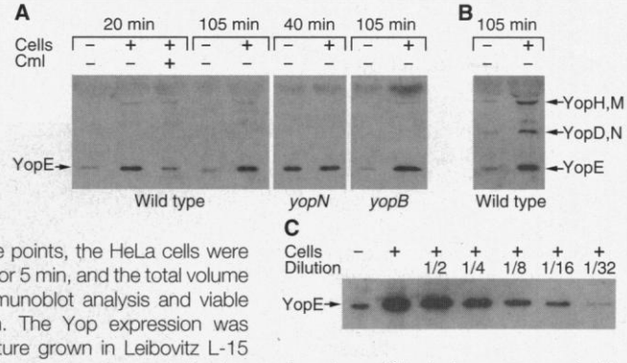

Fig. 1. HeLa cell contact stimulates increased expression of Yop proteins by *Y. pseudotuberculosis*. Confluent layers of HeLa cells in Leibovitz L-15 medium (cells +) (*24*) were infected with the wild type (YPIII/pIB102), a *yopN* mutant (YPIII/pIB82), or a *yopB* mutant (YPIII/pIB604). At the indicated time points, the HeLa cells were lysed with 0.1% Triton X-100 for 5 min, and the total volume was collected for protein immunoblot analysis and viable bacteria count determination. The Yop expression was compared with a control culture grown in Leibovitz L-15 medium alone (cells −). Protein samples corresponding to equal numbers of bacteria were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (12% gel) and transferred to nitrocellulose membranes (*25*). The blots were then probed with (**A**) a polyclonal antiserum to YopE or with (**B**) a polyclonal antiserum recognizing all Yops. (**C**) The concentrations of YopE 20 min after infection were quantified by analyzing serial dilutions of the samples (cells +). The blots were developed with an Amersham ECL-protein immunoblot kit according to instructions from the manufacturer. The amount of protein used for the blot in (C) was 10 times that used in (A). Cml = chloramphenicol.

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proteins was elevated when HeLa cells were infected (Fig. 1B). It has been suggested that the YopN protein is a bacterial surface receptor that has a negative regulatory role on Yop expression and secretion (*2*). Thus, a *yopN* mutant is derepressed in vitro for Yop expression and secretion (*6*, *17*). The *yopN* mutant strain expressed Yops at the same level in the presence or absence of HeLa cells (Fig. 1A), indicating that the behavior of the *yopN* mutant in vitro mimics its behavior in vivo. We conclude that wild-type *Y. pseudotuberculosis* senses the interaction with the target cell surface and that this contact results in increased Yop expression.

To address whether the up-regulation of Yop expression is at the transcriptional level and a result of a physical contact between the bacterium and the cell, we developed a method to monitor the level of gene expression in individual bacteria. We used the LuxAB protein of *Vibrio harveyi* as a reporter for transcription (*18*). This protein catalyzes a reaction emitting photons at a wavelength of 490 nm when the bacteria are exposed to *n*-decanal. The expression of LuxAB can thus be monitored with a light-sensitive charge-coupled device (CCD) camera coupled to an inverted microscope. Indeed, this method allowed in situ detection of gene regulation in individual bacteria infecting eukaryotic cells. A monolayer of HeLa cells was infected with strain YPIII(pIB102EL) containing a *yopE-luxAB* operon fusion (*19*). Bacteria associated with HeLa cells emitted photons at a detectable level, whereas no signal was observed from bacteria attached to the cover glass but not to HeLa cells (Fig. 2). In agreement with the above experiments (see Fig. 1), these observations demonstrate that the bacterium induced *yop* transcription only after contact with the target cell had been established, indicating that the pathogen recognizes a cell surface ligand and responds accordingly. This induction requires that a signal is transmitted from the surface to the cytosol of the bacterium, which is not a trivial task because it is likely that the eukaryotic cell surface ligand sensed by the bacterium is of macromolecular origin and, thus, is unable to cross the bacterial cell wall.

We previously identified a regulatory protein, LcrQ, that functions as a negative control for Yop expression, and a lcrQ mutant was found to be temperature-sensitive for growth and avirulent (*20*). At the time we also noticed that the LcrQ protein was possibly secreted to the culture supernatant under conditions allowing maximal expression and secretion of Yops (*20*), and we speculated that LcrQ might be secreted to lower the intracellular concentration of LcrQ and thus derepress Yop expression. To study the localization of LcrQ, we incubated the wild-type strain *Y. pseudotuberculosis* at 37°C in brain-heart infusion (BHI) medium either containing 2.5 mM Ca^{2+} (no Yop export) or depleted for Ca^{2+} by the addition of 5 mM EGTA (full Yop export). Bacteria grown under conditions permitting full Yop export (low Ca^{2+}) secreted LcrQ to the culture supernatant fraction (Fig. 3C), whereas no LcrQ was found associated with the bacteria (Fig. 3B). When the bacteria were grown in a medium suppressing Yop expression and secretion (high Ca^{2+}), the situation was reversed: all LcrQ was associated with the whole-cell fraction (Fig. 3, A to C).

We also examined the kinetics of transcription of the *lcrQ* gene using a strain carrying an *lcrQ-luxAB* operon fusion (*21*). The rate of transcription of *lcrQ* was somewhat higher when Ca^{2+} was omitted (Fig. 3D). In agreement with this result, the total amount of LcrQ was also elevated when Ca^{2+} was depleted from the growth medium as indicated in Fig. 3, B and C (*22*). Although the total amount of LcrQ is higher under inductive conditions, the intracellular concentration is lower when

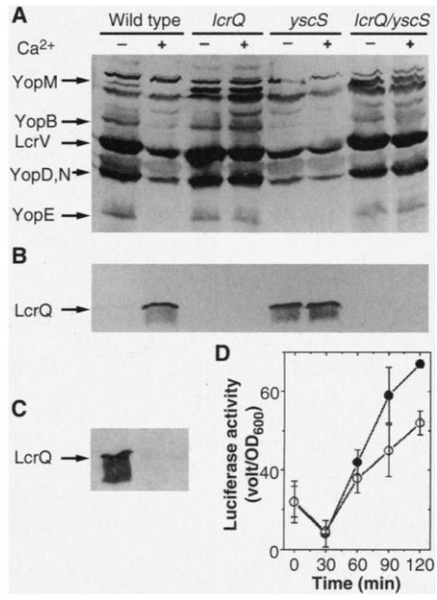

Fig. 3. Intracellular concentration of LcrQ correlates with Yop expression. Overnight cultures grown at 26°C in BHI with Ca^{2+} (+) or in BHI without Ca^{2+} (−) (*24*) were diluted to 1 × 10^8 bacteria per milliliter in the same medium and cultured at 26°C for 60 min. The cultures were then shifted to 37°C and incubated for an additional 2 hours. Proteins from bacteria and supernatant, corresponding to ~2 × 10^7 bacteria, were separated on SDS-PAGE and analyzed by ECL protein immunoblotting as described (*25*). (**A** and **B**) Bacterial pellets from the wild type and the *lcrQ* (*21*), *yscS* (*11*), and *lcrQ/yscS* (*21*) mutants and (**C**) the supernatant from the wild type were analyzed by protein immunoblotting with antiserum recognizing all Yops [panel (A)] or recognizing LcrQ [panels (B) and (C)]. (**D**) Transcriptional activity from the *lcrQ* gene was monitored in bacteria containing a fusion between *lcrQ* and the *luxAB* gene by assaying luciferase activity after a temperature shift as described (*11*). Bacteria were grown in media containing (closed circles) or depleted (open circles) of calcium. Optical density (OD) was measured at a wavelength of 600 nm.

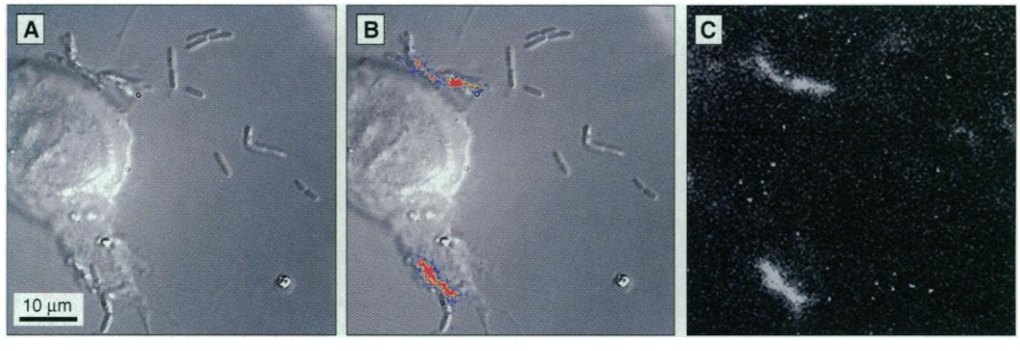

Fig. 2. The transcriptional activity of *yopE* is increased by target cell contact. (**A**) Differential interference contrast (DIC) and (**C**) emitted light images of HeLa cells infected with *Y. pseudotuberculosis* carrying the *luxAB* operon of *V. harveyi* under the control of the *yopE* promoter (YPIII/pIB102-EL) (*19*). After the DIC image was acquired, *n*-decanal was added, and images of the emitted light were obtained with a Photometrics CH250 CCD camera (1024 × 1024 pixels, 10-min exposure, −60°C, Binning 2×2, that is, 512 × 512 pixels). The resulting images were processed with Inovision ISEE software. (**B**) Color pseudo-images, reflecting the emitted light images of (C), were superimposed on the DIC images of (A) with Adobe Photoshop software. Red indicates highest intensity and blue, lowest intensity.

compared with noninduced bacteria, suggesting that the intracellular concentration of LcrQ is regulated by secretion.

Type III secretion mutants (*ysc*) have repressed Yop expression even under conditions favoring high expression and secretion in the wild-type strain (*1, 11*). The downregulation of Yop expression in *ysc* mutants could be due to a failure to secrete LcrQ; therefore, we analyzed the pattern of Yop expression and localization of LcrQ in a nonpolar *yscS* mutant defective in Yop secretion and also repressed for Yop expression (Fig. 3, A and B). Similar amounts of LcrQ were found in the bacterial pellet fraction of the *yscS* mutant irrespective of the growth conditions (Fig. 3B), and no LcrQ protein was found in the culture supernatant (*22*). Hence, LcrQ is secreted by the type III secretion system. The *yscS* mutant expressed low amounts of cell-associated Yops (Fig. 3A), whereas an *yscS/lcrQ* double mutant (*21*) expressed high amounts of Yops associated with the bacterial fraction (Fig. 3A). Thus, LcrQ exhibits a central negative role in the regulation of Yop expression, and the export of LcrQ via the type III secretion pathway is a prerequisite to achieve full induction of Yop expression.

It is evident that Yop expression in vivo is elevated after the pathogen has bound to the HeLa cell surface (Figs. 1 and 2). For LcrQ to exhibit a regulatory function by means of secretion, it is important that it is exported relatively fast after the environmental conditions have changed. We therefore analyzed the rate at which LcrQ was reduced in the cytosol in the wild-type strain after a shift from a medium with Ca^{2+} to a medium without Ca^{2+}. A decline in the intracellular concentration of LcrQ was observed as early as 3 min after the shift, and after 5 min no LcrQ protein was detected in association with the bacteria (Fig. 4). Yop expression began to increase 5 to 7 min after the intracellular concentration of LcrQ started to decline (Fig. 4), showing that LcrQ secretion precedes the derepression of Yop expression. In agreement with these in vitro results, we found that LcrQ was completely secreted after infection of HeLa cells (*22*), emphasizing the importance of LcrQ export also during in vivo conditions. After secretion of LcrQ, Yop effectors are translocated across the target cell membrane by means of a specific translocation mechanism that requires the YopB and YopD proteins (*5–9*). We found that a *yopB* mutant responded like the wild-type strain, with increased Yop expression after host cell contact (Fig. 1A), showing that translocation of LcrQ across the plasma membrane is not required to regulate the expression of Yop in vivo.

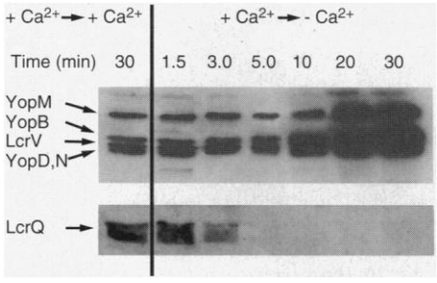

Fig. 4. Rapid decrease in the intracellular concentration of LcrQ after a shift from Ca^{2+}-containing to Ca^{2+}-depleted growth media. Wild-type *Yersinia* were grown at 37°C in BHI containing 2.5 mM Ca^{2+} for 2 hours. The cultures were then diluted 20-fold in BHI plus Ca^{2+} (2.5 mM) or BHI minus Ca^{2+}, samples withdrawn at various time points as indicated, and the bacteria harvested by centrifugation. Samples corresponding to 5×10^6 bacteria were analyzed by SDS-PAGE and ECL protein immunoblotting with antiserum recognizing all Yops or antiserum to LcrQ. The time after shift includes a 40-s centrifugation step.

In conclusion, we suggest that the type III secretion apparatus is blocked when the bacteria are present in a Ca^{2+}-containing environment. Physical contact between the pathogen and its target cell mediates the opening of the secretion channel at the zone of contact, and this event triggers LcrQ secretion. Consequently, the intracellular LcrQ concentration is lowered, leading to derepression of Yop expression. The export channels facing the culture medium are closed. This model explains how the bacterium coordinates secretion and polarized translocation with the up-regulation of Yop expression. Removal of Ca^{2+} in vitro leads to the artificial opening of all export channels, which triggers a rapid secretion of LcrQ and a subsequent derepression of Yop expression and Yop export. This model also shows striking similarities with a recent model presented for the regulation of the biosynthesis of the flagellum of *Salmonella typhimurium* (*23*). The regulation involves the negative regulator FlgM (anti-σ^{28}), which is secreted to the culture medium through the same secretion system (showing homology with the Ysc system) that mediates export of the flagellin subunits to the surface of the bacteria (*23*). Preliminary results indicate that LcrQ is not an anti-σ factor but rather an antiactivator, suggesting that LcrQ has a mode of action distinct from that of FlgM (*22*). Nevertheless, the regulatory próblem to transmit a signal from the surface to the cytosol followed by the up-regulation and export of proteins has been solved in the two different systems by using a similar approach. It is, therefore, plausible that the model proposed herein delineates a general strategy used by Gram-negative bacteria with a type III secretion system for responding to extracellular stimuli that are unable to cross the bacterial cell wall.

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