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Failure To Detect Binding of LcrH to the V Antigen of *Yersinia pestis*

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V antigen of *Yersinia pestis* has been reported to bind the chaperone LcrH. We were unable to demonstrate this interaction. Despite methodological differences between our study and an earlier study, we believe that the previous findings were artifactual. One likely confounding element was the tendency of LcrH to adhere on its own to metal chelation chromatographic resin.

In *Yersinia pestis*, the V antigen (LcrV) is a bifunctional protein with postulated regulatory and virulence roles (8). As a member of the low-Ca^{2+} response stimulon (also termed the *Yersinia* outer protein [Yop] virulon [1]), the V antigen operon *lcrGVHyopBD* is subject to transcriptional downregulation by millimolar concentrations of calcium at 37°C and shows maximal expression when calcium is absent in vitro or upon contact with a eucaryotic cell (5). Maximal induction in vitro is accompanied by secretion of about half of LcrV by a type III secretion mechanism (5). The LcrV remaining within the cytosol is believed to participate in ensuring maximal secretion under inductive conditions by forming a tight complex with the regulatory protein LcrG, thereby preventing LcrG from blocking secretion (4, 10, 11). Recently, it was reported that following overexpression in *Escherichia coli*, the chaperone LcrH copurifies with an LcrV-polyhistidine fusion protein, V_h, and that a protein A-LcrV fusion protein (PAV), but not the protein A portion of the fusion, binds to LcrH in an enzyme-linked immunosorbent assay (ELISA); however, saturation required 30 h at 4°C (3). It was speculated that because LcrH, also called SycD, is known to serve as a specific Yop chaperone for at least two proteins secreted by the type III mechanism (Yops B and D) (13), it might serve as a chaperone for LcrV (3). In that case, LcrV's regulatory role could come about indirectly, e.g., through competition for a common chaperone, and unchaperoned, nonsecretable Yops might exert feedback regulation (3). Workers in our laboratory had not obtained an LcrV-LcrH complex by chemical cross-linking or immunoprecipitation either from *Y. pestis* or from *E. coli* expressing *lcrGVHyopByopD′* (reference 4 and our unpublished data); however, in those studies, there always were other proteins (YopB and YopD) that potentially could have competed with LcrV for LcrH. Here we tested for the ability of LcrH and LcrV to interact in the absence of potential competing proteins and reexamined the possible existence of an LcrV-LcrH complex in *Yersinia*.

Expression of HT-′V and LcrH from linked genes in *E. coli*. We first tested for an interaction between LcrV and LcrH by using a criterion similar to one employed by Motin et al. (3): copurification of LcrH with polyhistidine-tagged LcrV in metal chelation chromatography. As in reference 3, we cloned a 5′-truncated *lcrV* gene and intact *lcrH* in their native linked configuration, and N-terminally truncated LcrV was fused at its N terminus with the polyhistidine-containing leader encoded by the vector. Our construct, pHT-′V2, was made by ligating *Eco*RV-cut *Y. pestis* DNA into *Stu*I-cut pProex-1 (Gibco BRL, Gaithersburg, Md.). This created the same truncation in *lcrV* as in pVHB62 (3), resulting in the loss of the first 67 amino acids of LcrV; however, the leader sequence containing the polyhistidine tract of HT-′V was different in composition from and shorter than that in V_h (19 in contrast to 41 amino acids) (3). The cloned DNA extended through *lcrH* and included the first 265 bases of *yopB*. Expression of *lcrV* and *lcrH* in pHT-′V2 was driven by the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *trc* promoter.

Nilles et al. had speculated (4) that the bulk-processing protocol used by Motin et al. for large volumes of high-density cultures might have been important in their observing significant coelution of LcrH with LcrV, perhaps by exposing the nickel-charged resin to more LcrH than would have been the case in the study described in reference 4 and in our unpublished experiments. To test this idea, we grew two 200-ml cultures of *E. coli* DH5α containing pHT-′V2 at 37°C to a higher density than we normally use for analyses. At a culture A_{620} of 1, IPTG was added to 0.1 mM and incubation was continued for ca. 3 h, resulting in a final A_{620} of 3. The cells from the pooled cultures were pelleted, resuspended in 10 ml of phosphate-buffered saline (PBS) (pH 7.4), and broken in a French press; the extract was clarified by low-speed centrifugation and ultracentrifugation as previously described (4). One milliliter of extract was immediately processed on a 0.5-ml bed of nickel-nitrilotriacetic acid (NTA) resin (Qiagen Inc., Studio City, Calif.) equilibrated in PBS (pH 7.4). The fractions collected were a 1-ml (2 column volumes) flowthrough (in PBS) sample, 10 1-ml samples from washes (20 column volumes) with buffer D (containing 10 mM imidazole [Qiagen]), and 6 1-ml samples from elutions (12 column volumes) with buffer E (containing 100 mM imidazole). Samples were resolved by sodium dodecyl sulfate (SDS)–12.5% (wt/vol) polyacrylamide gel electrophoresis (PAGE) (2) followed by immunoblot analysis (4, 7) with anti-histidine-tagged full-length LcrV (anti-HTV [4]) or an antipeptide antibody against amino acids 153 to 163 of LcrH (anti-LcrH [11]) as a probe. We obtained the same results whether the metal chelation chromatography and immunoblot analysis were carried out immediately or after the extracts were kept on ice for 5 days.

Figure 1A shows that the heavy loading of the column exceeded its capacity, as some of the HT-′V was present in the flowthrough and first-wash fractions (upper panel, lanes 1 and 2). Most was recovered in the elution fractions (Fig. 1A, upper panel, lanes 9 through 14). The majority of the HT-′V was

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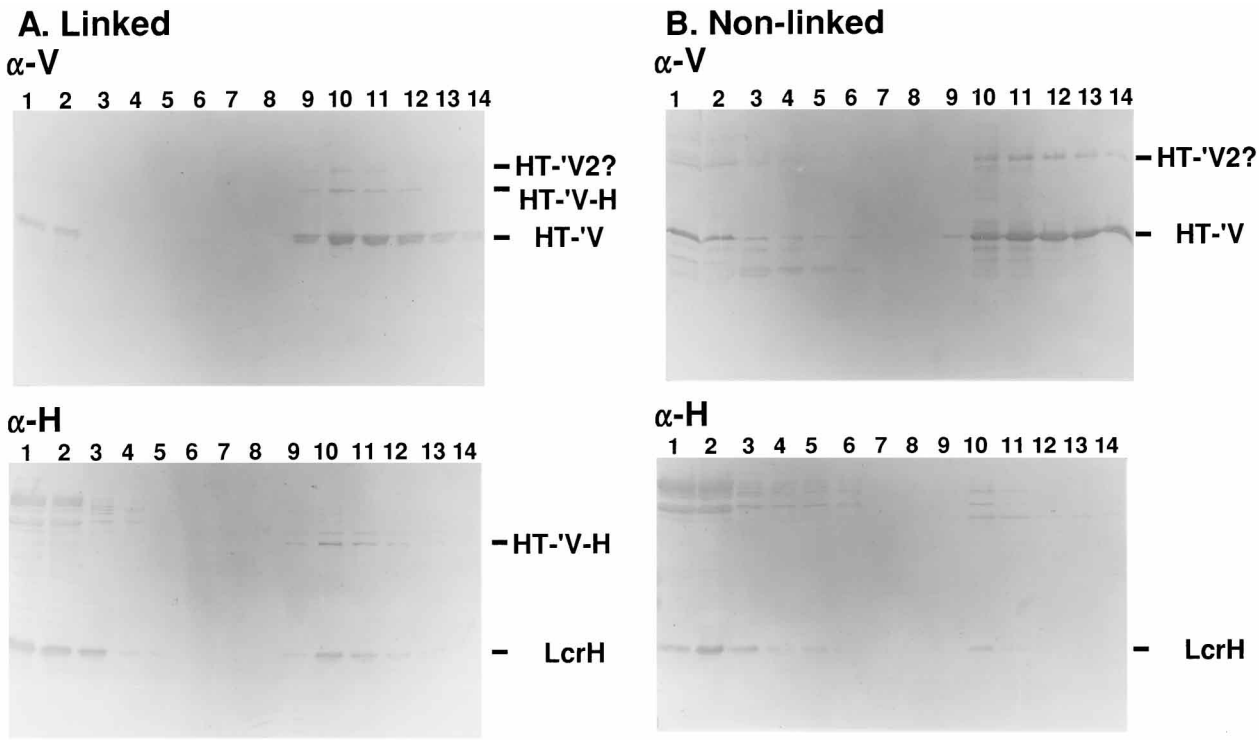

FIG. 1. Ni chelation chromatography of HT-′V and LcrH expressed in *E. coli* from linked and nonlinked genes. Histidine-tagged, N-terminally truncated LcrV (lacking its first 67 amino acids [HT-′V]) and LcrH were expressed in *E. coli* DH5α in their native linked, in-frame configuration on one plasmid (A) or on separate plasmids (B), and the bacterial extracts were subjected to Ni chelation chromatography. Fractions were analyzed by SDS-PAGE on 12.5% acrylamide gels, and HT-′V and LcrH were visualized in immunoblots probed with anti-HTV (α-V) specific for LcrV (upper panels) or anti-LcrH (α-H) (lower panels) antibody. Lanes: 1, column flowthrough fraction; 2 to 8, wash fractions 1 to 5, 7, and 10, respectively; 9 to 14, elution fractions 1 through 6, respectively. The nonlabeled slowly migrating bands in the lower panels are cross-reactive bands in the *E. coli* host, based on immunoblot analysis of a lysate of this strain lacking cloned *Yersinia* DNA. HT-′V?, species corresponding in size to two HT-′V molecules.

detected as the predicted monomer. In this study as well as previously (unpublished data), we also detected a species corresponding in size to two HT-′V molecules (Fig. 1). In addition, there was a novel ca. 52-kDa anti-HTV-reactive species (HT-′V-H) that was purified along with HT-′V (Fig. 1A). This species had also been observed in earlier experiments using smaller, lower-density cultures.

Most of the LcrH in the sample was found in the flowthrough and early-wash fractions (lower panel of Fig. 1A, lanes 1 to 4), with none being in the later washes. However, a significant amount of LcrH was eluted in the elution fractions that contained HT-′V. These fractions also contained an anti-LcrH-reactive band that was the same size as the novel anti-HTV-reactive species HT-′V-H, which was not present in fractions similarly prepared from the *E. coli* host strain lacking cloned *Yersinia* DNA (data not shown).

This experiment, like a previous study (3), showed that LcrV and LcrH copurified; it also revealed an unexpected species that reacted with both anti-HTV and anti-LcrH antibodies. This band could have resulted if HT-′V and LcrH formed a tight complex that resisted dissociation upon SDS-PAGE. Alternatively, it could represent a chimeric HT-′V–LcrH protein resulting from translational read-through of the linked *lcrV* and *lcrH* genes, as these are in the same frame and are separated by only 15 nucleotides, including the termination codon for *lcrV*. We distinguished between these possibilities by repeating the experiment whose results are depicted in Fig. 1A with *lcrV* and *lcrH* expressed in *E. coli* DH5α on separate plasmids. Although the LcrH monomer still appeared in elution fractions, the 52-kDa species was not present (Fig. 1B). Because this species was seen when *lcrV* and *lcrH* were linked but not when they were on separate replicons, we favor the hypothesis that the 52-kDa species is a chimeric HT-′V–LcrH protein resulting from translational read-through of the *lcrV* opal stop codon in *E. coli*. The UGAC sequence in *lcrV* is a notably weak translational termination signal in *E. coli* (12). Although this chimeric protein might contribute to the copurification of LcrH and HT-′V by being proteolytically cleaved during the purification procedure, its small amount is unlikely to account for the majority of the copurification seen by Motin et al. (3), and its significance is unclear, as we have not detected the chimeric protein in *Y. pestis*.

LcrH alone binds to Ni-NTA resin. In many experiments whose results are not shown, we noticed that the more LcrH we passed over Ni-NTA or Talon (Clontech, Palo Alto, Calif.) resin, the more likely we were to observe it in the elution fractions. We hypothesized that LcrH itself has some affinity for metal chelation resin. To test this idea, we cloned *lcrH* alone in the *Xho*I/*Acc*I fragment of pJIT7-1 (6), which was treated with T4 polymerase (Boehringer Mannheim Biochemicals, King of Prussia, Pa.) to make blunt ends and ligated into *Sma*I-cut pTrc99A (Pharmacia Biotech, Piscataway, N.J.). LcrH was expressed from the *trc* promoter in this vector in *E. coli* DH5α and analyzed as in the experiment whose results are shown in Fig. 1, except that 20 1-ml washes (40 column volumes) were made before elution with 100 mM imidazole. LcrH alone bound to the Ni-NTA column: initially, it passed through the column very slowly, with a significant amount still being present in the 5th wash fraction (10 column volumes of washes with 10 mM imidazole [Fig. 2, lane 5]), and it still was detect-

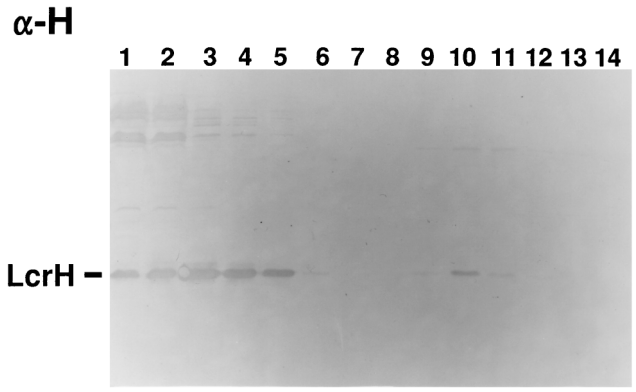

FIG. 2. LcrH binds to Ni-NTA resin. LcrH was expressed alone in *E. coli* DH5α, the cellular extract was subjected to Ni-NTA chromatography, and LcrH was detected with anti-LcrH (α-H) antibody in fractions analyzed by immunoblotting as in the experiment whose results are shown in Fig. 1. Lanes: 1, column flowthrough fraction; 2 to 8, column wash fractions 1 to 3, 5, 10, 15, and 20, respectively; 9 to 14, elution fractions 1 through 6, respectively.

able in the 10th wash fraction (lane 6). Elution with 100 mM imidazole released additional LcrH that had not been removed by washes with 40 column volumes (Fig. 2, lanes 9 to 11). This experiment indicated that the copurification of LcrH with LcrV was at least partly an artifact of metal chelation chromatography.

Test for copurification of mixed purified proteins. To further test the possibility of an interaction of HT-′V with LcrH, we purified these proteins, incubated them together for 30 h at 4°C, and then determined if they copurified in metal chelation chromatography carried out so as not to heavily expose the column to LcrH. HT-′V was purified from *E. coli* DH5α carrying pHT-′V, which contained only the 5′-truncated *lcrV* gene from pHT-′V2 in the pProex-1 vector. The *′lcrV*-containing fragment was made by using pHT-′V2 as template in a PCR (as in reference 4) with the 5′ primer from within pProex-1 and containing a *Kas*I site and the 3′ primer just within *lcrH* (nucleotides 54 to 68). This fragment was cleaved with *Kas*I, phosphorylated, and ligated into *Kas*I/*Stu*I-cut pProex-1. Polyhistidine-tagged YopD (HT-YopD) was used as a positive control protein known to bind LcrH (13). It was expressed from the vector pET-24b (Novagen, Madison, Wis.) with a polyhistidine-containing tail at the YopD C terminus (14). LcrH lacking its first 12 amino acids, ′LcrH, was expressed from pHTH, which was made by ligation of the *lcrH*-containing *Pvu*II/*Acc*I fragment (9) into *Stu*I-cleaved pProex-1. These histidine-tagged proteins were purified by metal chelation chromatography (Talon resin) to homogeneity (HT-′V and HT-YopD) or near homogeneity (HT-′H), as assessed by using silver-stained polyacrylamide gels (data not shown). HT-′H was then cleaved of its polyhistidine leader with rTEV protease (Gibco BRL) in an overnight incubation at 15°C as described by the manufacturer, leaving eight residual vector-encoded amino acids (GAHMGIQR) on the ′LcrH product, and the mixture was passed over a 1-ml Talon column. The flowthrough and wash (1 ml of PBS [pH 8]) fractions were collected, concentrated by using a Centricon 30 (Amicon, Beverly, Mass.), and dialyzed against PBS (pH 7.4). Two protein mixtures were made: (i) ′LcrH and HT-′V and (ii) ′LcrH and HT-YopD. In each case, 50 μg of each protein was present in a final volume of 351 μl of PBS (pH 7.4). After 30 h at 4°C, the mixtures were each passed over a 0.5-ml bed of Talon resin, and 1-ml fractions resulting from the flowthrough, wash, and elution treatments were collected. Samples of the fractions were analyzed by SDS-

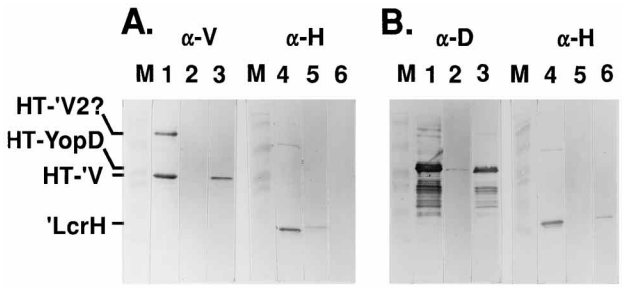

FIG. 3. ′LcrH copurifies with HT-YopD but not HT-′V in metal chelation chromatography of mixtures of purified proteins. ′LcrH (LcrH lacking its first 12 amino acids) was mixed with purified polyhistidine-tagged YopD (HT-YopD) (B) or HT-′V (A) and incubated for 30 h at 4°C before being subjected to metal chelation chromatography (Talon resin) and immunoblot analysis of resulting fractions. (A) Lanes 1 to 3, visualization of HT-′V by anti-HTV (α-V) antibody; (B) lanes 1 to 3, visualization of HT-YopD by anti-YopD (α-D). (A and B) Lanes 4 to 6, visualization of ′LcrH by anti-LcrH (α-H). Lanes 1 and 4, protein mixtures prior to chromatography; lanes 2 and 5, first column wash fraction (which contained the majority of proteins that did not bind to the resin); lanes 3 and 6, the first column elution fraction (which contained the majority of proteins that bound to the resin). M, prestained molecular mass standards (Bio-Rad Laboratories, Hercules, Calif.): 7.5, 19.2, 29, 34.1, 51.6, 86, and 118 kDa (HT-′V migrates slightly faster than does the 34.1-kDa standard). HT-′V2?, same as in Fig. 1.

PAGE and immunoblotting as described above (YopD was detected by using a rabbit polyclonal anti-YopD immunoglobulin [14]).

Figure 3A shows that ′LcrH did not copurify with HT-′V: all of the ′LcrH was in the wash fractions, and none was in the column eluate that contained all of the HT-′V. However, ′LcrH was capable of binding YopD, as all of the ′LcrH in the mixture containing YopD copurified with YopD, and none was in the column flowthrough or wash fraction (Fig. 3B). These data support the hypothesis that LcrH does not bind LcrV as a stable complex, with the caveat that if LcrH required its N-terminal 12 amino acids for binding to HT-′V but not for binding to YopD, we would have missed this interaction by our test with ′LcrH.

Test for copurification of LcrH with LcrV from *Y. pestis*. Although Nilles et al. (4) did not detect an interaction between LcrV and LcrH in *Yersinia*, we wanted to perform another test by a different method. Our approach was to metabolically label proteins in *Y. pestis* growing in the defined medium TMH lacking or containing 2.5 mM $CaCl_2$ (as in reference 4), immunoprecipitate the proteins from whole-cell extracts with antibody-coupled beads (prepared as described in reference 4), and visualize coprecipitated proteins by autoradiography. *Y. pestis* KIM5 was grown as described previously (4) in TMH having a low concentration of methionine (0.3 mM). Cultures were initiated at 26°C, shifted to 37°C at an A_{620} of 0.2, and labeled for 1 h with [^{35}S]methionine and [^{35}S]cysteine (Tran^{35}S-label; >1,000 Ci/mmol; ICN, Costa Mesa, Calif.) at 100 μCi/ml, starting at 4 h after the temperature shift. After 1 h, a 100-μl sample of the labeled culture was centrifuged to pellet the bacteria, the pellet was resuspended in 100 μl of PBS (pH 7.4), 100 μl of 10% ice-cold trichloroacetic acid (TCA) was added, and the suspension was placed on ice for ca. 2 h. The TCA-precipitated bacteria were centrifuged for 10 min in a refrigerated microcentrifuge, and the pellet was washed by centrifugation with ice-cold acetone and allowed to air dry overnight. The pellets were solubilized with 30 μl of 1% (wt/vol) SDS in 10 mM Tris-HCl (pH 8.0), heated for 10 min at ca. 90°C, and clarified by centrifugation for 5 min. For immunoprecipitation, 30 μl was added to 975 μl of Triton buffer (2% [vol/vol] Triton X-100, 50 mM Tris-HCl [pH 8.0], 0.15 mM

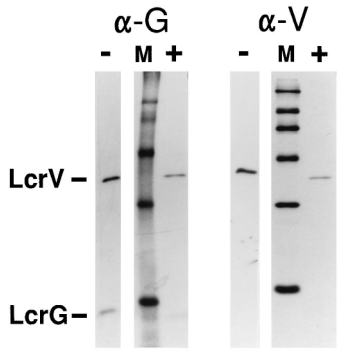

FIG. 4. LcrH does not coimmunoprecipitate with LcrV or LcrG from *Y. pestis*. *Y. pestis* KIM5 was grown at 37°C in the defined medium TMH containing (+) or lacking (−) added $CaCl_2$ (2.5 mM), labeled for 1 h with [^{35}S]methionine and [^{35}S]cysteine, washed, and TCA precipitated. Proteins in the resuspended, clarified extracts were immunoprecipitated sequentially with protein A-Sepharose beads coupled to anti-GST-G (specific for LcrG) (α-G) and then anti-HTV (α-V), and analyzed by SDS-PAGE and fluorography. M, ^{14}C-methylated molecular mass standards (Amersham Life Sciences): 14.3, 30, 46, 66, 97.4, and 220 kDa (the 220-kDa marker is not present in the α-G data set).

NaCl, 0.1 mM Na_2EDTA), and 200 μl of a slurry (10% [wt/vol] in PBS [pH 7.4]) of protein A-Sepharose beads coupled to antibody against a glutathione *S*-transferase–LcrG fusion protein (GST-G) (specific for LcrG [4]) was added. Immunoprecipitation was conducted for ca. 3 h with rocking at 4°C, the beads were pelleted for 15 s in a microcentrifuge, and the supernatant was saved for a subsequent immunoprecipitation with beads coupled to anti-HTV. The antibody-coupled beads were washed three times with PBS (pH 7.4) containing 0.05% (vol/vol) Triton X-100, suspended in 7 μl of SDS-PAGE sample buffer per 0.01 A_{620} ml of original culture (A_{620} ml is proportional to bacterial mass), and heated at ca. 90°C for 5 min; the immunoprecipitated proteins were resolved by SDS-PAGE in a 12% (wt/vol) polyacrylamide gel. The gel was treated with En^{3}Hance (New England Nuclear, Boston, Mass.) and detected by fluorography with Hyperfilm enhanced chemiluminescence (Amersham, Arlington Heights, Ill.).

Figure 4 shows that, as anticipated, the anti-GST-G immunoprecipitation recovered LcrV in addition to LcrG, due to the strong interaction previously demonstrated for these two proteins (4). No other species was detectable. Immunoprecipitation with anti-HTV brought down more LcrV, consistent with the previously observed excess of LcrV over LcrG (4), but no other protein was detectable. If LcrV interacts with LcrH in *Y. pestis*, this interaction was not stable against the handling in this experiment, even though the immunoprecipitations were quantitative and the interaction between LcrV and LcrG was preserved.

All of our experiments failed to support the existence of a specific interaction between LcrH and LcrV (or HT-′V) in *E. coli*, in *Y. pestis*, or in vitro. Motin et al. (3) employed methods with several differences from those in our study, including the 41-amino-acid leader on V_h, strong overexpression from a T7 promoter in a different *E. coli* host grown in large amounts (with a significant loss of V_h due to insolubility, which was not observed with HT-′V), the use of 5 mg of lysozyme per ml in the bacterial lysis, and different buffers and elution methods in metal chelation chromatography (including the requirement for denaturing conditions to release the majority of V_h from Ni-NTA). They also used an ELISA to test for interaction of purified proteins, whereas we tested for interaction of the proteins in solution. The ELISA configuration with LcrH bound to a solid support differs from a solution interaction assay in presenting an effectively infinite concentration of LcrH to the test protein; moreover, the LcrH protein that Motin et al. (3) bound to the solid support had previously been denatured with 6 M guanidine HCl (pH 3) to remove it (along with LcrV and lysozyme) from Ni-NTA, followed by dialysis, treatment with 8 M urea, and rechromatography on Ni-NTA, dialysis, chromatography on DEAE-cellulose, and elution by a gradient of NaCl. These methodological differences could conceivably yield different experimental outcomes, and we do not claim to have demonstrated that LcrV and LcrH never can interact under any circumstance. However, our findings do suggest a likely source of at least some of the copurification seen by Motin et al.: direct binding by LcrH to nickel chelation resin. Moreover, Motin et al. (3) did not provide evidence for an interaction of native LcrV with LcrH in *Yersinia*. We recommend waiting for such evidence before revising the model for regulation in the low-Ca^{2+} response, and we maintain the concept that LcrV is a bifunctional protein (8): it is both a cytoplasmic regulatory protein necessary for maximal expression of Yops in *Yersinia* and a secreted virulence protein.

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