Calcium-Regulated Type III Secretion of Yop Proteins by an *Escherichia coli hha* Mutant Carrying a *Yersinia pestis* pCD1 Virulence Plasmid

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A series of four large deletions that removed a total of ca. 36 kb of DNA from the ca. 70-kb *Yersinia pestis* pCD1 virulence plasmid were constructed using lambda Red-mediated recombination. *Escherichia coli hha* deletion mutants carrying the virulence plasmid with the deletions expressed a functional calcium-regulated type III secretion system. The *E. coli hha*/pCD1 system should facilitate molecular studies of the type III secretion process.

Yersinia pestis is the etiologic agent of plague, an often fatal disease of both animals and humans (6). The pathogenicty of *Y. pestis* results largely from its ability to evade the defenses of its mammalian hosts. This ability is dependent upon the presence of a 70-kb plasmid designated pCD1 in *Y. pestis* KIM (18, 28). Plasmid pCD1 and related plasmids (2) in the enteropathogenic yersiniae (*Yersinia enterocolitica* and *Yersinia pseudotuberculosis*) encode a set of secreted antihost proteins termed *Yersinia* outer proteins (Yops) and a unique delivery system, classified as a type III secretion system (T3SS) (13). The type III secretion apparatus, or "injectisome" as it is sometimes called, spans the bacterial cell envelope and allows extracellular yersiniae to inject Yops directly into host phagocytic cells (15, 29, 32). Injected Yops prevent bacterial engulfment and block production of proinflammatory cytokines (26, 27, 34).

The type III secretion process is activated by contact between a bacterium and the surface of a eukaryotic cell (33). Following cell contact, effector Yops are injected into the eukaryotic cell and are not found in substantial amounts in the extracellular environment, indicating that the type III secretion process is activated only at the point of contact between the two cells. In vitro, Yop secretion is blocked in the presence of millimolar amounts of extracellular calcium and is activated during growth at 37°C in the absence of calcium (23, 35). Secretion of Yops in vitro is accompanied by cessation of bacterial growth, an event termed growth restriction (3).

To determine the minimum number of pCD1 genes required to express a functional calcium-regulated T3SS, we created four large deletions in plasmid pCD1 that together removed more than one-half of the genetic material carried on the plasmid (Fig. 1A). The four deletions eliminated pCD1 sequences at positions 41,640 to 52,022 (Δ1), 3,740 to 15,602 (Δ2), 61,430 to 70,331 (Δ3), and 55,568 to 60,495 (Δ4). In general, the deletions were designed to remove the genes encoding the six effector Yops (*yopE*, *yopH*, *yopJ*, *yopM*, *yopT*, and *ypkA*), two chaperones (*sycE* and *sycT*), *ylpA*, *yopK*, *yadA*, all uncharacterized open reading frames, and essentially all transposable elements. The four deletions did not disrupt the low-calcium response (Lcr) region (positions 15,801 to 41,540), the origin of replication, the partitioning region, or the *sycH* gene.

Lambda Red-mediated recombination was used to delete each specified region of pCD1 and to simultaneously insert an FLP recognition target-flanked kanamycin resistance (*kan*) cassette or a *dhfr* cassette essentially as described by Datsenko and Wanner (8). PCR products used to construct the gene replacements were generated using plasmid pKD4 (*kan*) or *dhfr* (EZ::TN<DHFR>; Epicentre, Madison, WI) as a template for PCR. The oligonucleotide primers used in this study are listed in Table 1. Deletions were confirmed by PCR analysis. Each individual deletion was initially constructed in plasmid pCD1 of *Y. pestis* KIM8 (pMT1⁺, pCD1⁺, and pPCP1⁻), which generated kanamycin-resistant (Kmʳ) strains KIM8 Δ1, KIM8 Δ2, and KIM8 Δ3, as well as trimethoprim-resistant (Tmʳ) strain KIM8 Δ4. Plasmid pCP20, which encodes the FLP recombinase (8), was electroporated into KIM8 Δ1 to facilitate removal of the FLP recognition target-flanked *kan* cassette, generating strain KIM8 Δ1s. Plasmids pKD46 and pCP20, which carry temperature-sensitive origins of replication, were cured from the kanamycin-sensitive strain by overnight growth at 39°C (8). The presence of the deletions and the absence of pKD46 and pCP20 were confirmed by PCR analysis and by agarose gel electrophoresis of plasmids isolated by the method of Kado and Liu (21).

To construct a pCD1 variant that had all four deletions, we used lambda Red-mediated recombination (8) to sequentially delete three additional regions (Δ2, Δ3, and Δ4) from pCD1 of KIM8 Δ1s. PCR products used to construct the sequential gene replacements were generated using pKD4 (*kan*), pKD3 (*cat*), or *dhfr* as the template for PCR. The Δ2::*kan*, Δ3::*cat*, and Δ4::*dhfr* insertional deletions were confirmed by PCR analysis and by agarose gel electrophoresis of plasmids isolated

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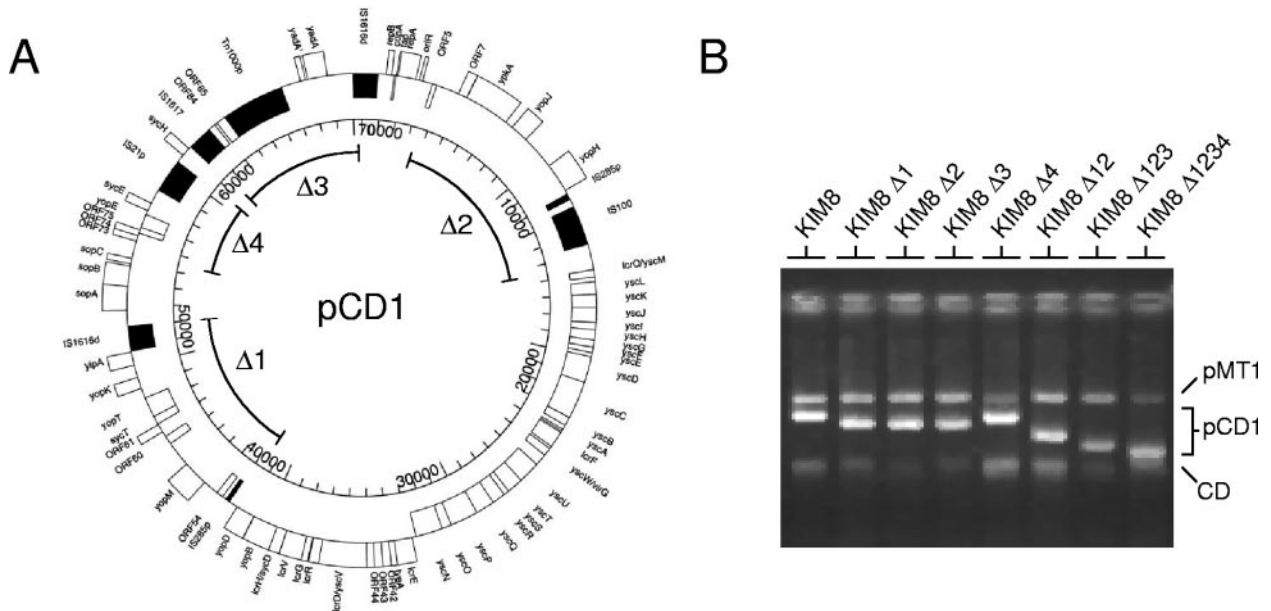
FIG. 1. Construction of plasmid pCD1-Δ1234. (A) Map of the whole pCD1 plasmid of *Y. pestis* KIM5. The outer circle shows the positions of identified pCD1 genes and open reading frames (open boxes). Genes located on the outside of the circle are transcribed in a clockwise direction, whereas genes located on the inside of the circle are transcribed in a counterclockwise direction. Transposable elements are indicated by solid boxes. The pCD1 sequences removed by deletions Δ1, Δ2, Δ,3 and Δ4 are indicated by the labeled lines. Reprinted from reference 28 with permission of the publisher. (B) Agarose gel electrophoretogram of plasmid pCD1 and derivatives of this plasmid. Plasmid DNA was isolated from *Y. pestis* KIM8, KIM8 Δ1, KIM8 Δ2, KIM8 Δ3, KIM8 Δ4, KIM8 Δ12, KIM8 Δ123, and KIM8 Δ1234 by the procedure of Kado and Liu (21), separated on 0.7% agarose gels, and stained with ethidium bromide. CD, chromosomal DNA.

by the method of Kado and Liu (21). The resultant multiple-deletion pCD1 plasmids pCD1-Δ12 (*kan*), pCD1-Δ123 (*kan*$_{Δ2}$ *cat*$_{Δ3}$), and pCD1-Δ1234 (*kan*$_{Δ2}$ *cat*$_{Δ3}$ *dhfr*$_{Δ4}$), as well as the original single-deletion plasmids pCD1-Δ1 (*kan*), pCD1-Δ2 (*kan*), pCD1-Δ3 (*kan*), and pCD1-Δ4 (*dhfr*), were isolated and electroporated into *Y. pestis* KIM10 (pMT1$^+$, pCD1$^-$, pPCP1$^-$), generating *Y. pestis* KIM8 Δ1 (Km^r), KIM8 Δ2 (Km^r), KIM8 Δ3 (Km^r), KIM8 Δ4 (Tm^r), KIM8 Δ12 (Km^r), KIM8 Δ123 (Km^r Cm^r), and KIM8 Δ1234 (Km^r Cm^r Tm^r) in a clean genetic background (Fig. 1B). The resultant strains were used in all subsequent studies.

Secretion of Yops by *Y. pestis* KIM8, KIM8 Δ1, KIM8 Δ12, KIM8 Δ123, and KIM8 Δ1234 was measured following growth of the bacteria in TMH medium for 5 h at 37°C in the presence

and absence of calcium. Concentrated culture supernatant proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie blue R-250 (Fig. 2A). All five strains secreted Yops in the absence of calcium but not in the presence of calcium. *Y. pestis* KIM8 Δ1234, which had all four deletions, expressed and secreted YopB, YopD, YopN, and LcrV but none of the effector Yops. In addition to the deletions in plasmid pCD1 mentioned above, a deletion (Δ5) eliminating the pCD1 sequence at positions 55,568 to 70,331 from pCD1 of *Y. pestis* KIM8 was constructed using primers Δ4-P1 and Δ3-P2. *Y. pestis* KIM8 carrying plasmid pCD1-Δ5, in which the *sycH* gene was deleted, expressed and secreted only low levels of Yops (data not shown), confirming that SycH is required for efficient

TABLE 1. Oligonucleotides used for lambda Red-mediated recombination

| Deletion	Template	Primer	Oligonucleotide sequence (5′ to 3′)
Δ1::*kan*	pKD4 (*kan*)	Δ1-P1	TGATAGAGCTTATCTATATAAGGTATAAGGTGCTGAAAATGTGTAGGCTGGAGCTGCTTC
		Δ1-P2	TTACATGTTGAATTTGAAGGCGAGTGGACCGGACCTTATCATATGAATATCCTCCTTAGT
Δ2::*kan*	pKD4 (*kan*)	Δ2-P1	AGGTGAAATATGACGACACGGTATTCTCGTAGTGAACGGTGTGTAGGCTGGAGCTGCTTC
		Δ2-P2	GAGTCTGCTCCTCATATAAATTGAGAGAATTAGGATGAACATATGAATATCCTCCTTAGT
Δ3::*kan*	pKD4 (*kan*)	Δ3-P1	GTTTAATTCTATAAAAGAAAAACGTACGATATCCATTAATGTGTAGGCTGGAGCTGCTTC
		Δ3-P2	CAAAGATTTGATGGGGAACTCGCTCATCAACACGGCTGCCATATGAATATCCTCCTTAGT
Δ4::*dhfr*	EZ::TN<DHFR>[a]	Δ4-P1	TTTTTGATAGCGATATCCATCCCGCCAGGTTGGATACGGATAGACGGCATGCACGATTTG
		Δ4-P2	TAACTTTTACTGAGCGAAATCTGATATTACTGGCACCACCGCATCCAATGTTTCCGCCAC
Δ2B::*kan*	pKD4 (*kan*)	Δ2B-P1	AGGTGAAATATGACGACACGGTATTCTCGTAGTGAACGGCGCTGCCGCAAGCACTCAGGG
		Δ2B-P2	GAGTCTGCTCCTCATATAAATTGAGAGAATTAGGATGAAGTCCCGCTCAGAAGAACTCGT
Δ3B::*cat*	pKD3 (*cat*)	Δ3B-P1	TGACTTCGGGCAAATTCTGCCGGAGTCAGGTTATTTAACCTTCGGAATAGGAACTTCATT
		Δ3B-P2	CGTTTAATCTGCGCCCTGAGCGACTGGATTTCACGTTGCGGCGCGCCTACCTGTGACGGA
Δ*hha*	pKD4 (*kan*)	Δ*hha*-P1	TATTTAATGCGTTTACGTCGTTGCCAGACAATTGACACGTGTGTAGGCTGGAGCTGCTTC
		Δ*hha*-P2	ATTCATGGTCAATTCGGCGAGGCGGTGATCTGCGGCTGACATATGAATATCCTCCTTAGT

a Obtained from Epicentre.
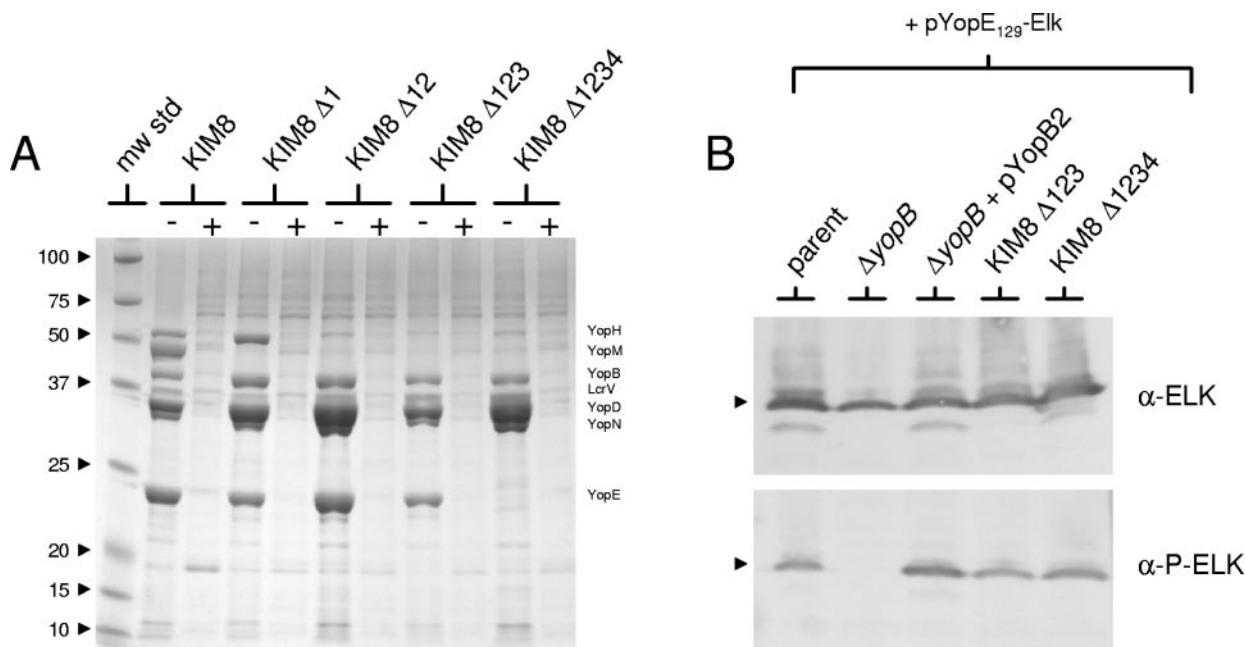
FIG. 2. Secretion and translocation of Yops by *Y. pestis* strains carrying plasmid pCD1 or versions of this plasmid with deletions. (A) *Y. pestis* KIM8, KIM8 Δ1, KIM8 Δ12, KIM8 Δ123, and KIM8 Δ1234 were grown in TMH medium with (lanes +) or without (lanes −) 2.5 mM CaCl$_2$ for 5 h at 37°C. Volumes of trichloroacetic acid-precipitated culture supernatant proteins corresponding to equal numbers of bacteria were resolved by SDS-PAGE and stained with Coomassie blue. The positions of different Yops are indicated on the right, and the molecular masses (in kilodaltons) of protein standards are indicated on the left. (B) Translocation of YopE$_{129}$-ELK into HeLa cells. *Y. pestis* KIM8 Δ123, KIM8 Δ1234, KIM8-3002.P40 (ΔyopE ΔyopJ; parent), KIM8-3002.41 (ΔyopE ΔyopJ ΔyopB), and KIM8-3002.41 complemented with pYopB2, all carrying pYopE$_{129}$-ELK, were used to infect HeLa cell monolayers at a multiplicity of infection of 30. After 3 h of incubation at 37°C, infected monolayers were solubilized with SDS-PAGE sample buffer and analyzed by SDS-PAGE and immunoblotting with anti-Elk antipeptide antibodies (α-ELK) and phosphospecific anti-Elk antipeptide antibodies (α-P-ELK). The position of YopE$_{129}$-ELK is indicated by arrowheads.

T3SS gene expression (4). These studies confirmed that with the exception of *sycH*, all of the genes required to express a functional calcium-regulated T3SS are encoded in the Lcr region of plasmid pCD1. Similar results were obtained by Trulzsch et al. (36), who inserted the entire Lcr region of the *Y. enterocolitica* pYV virulence plasmid into a cosmid vector (mini-pYV). In this case, the mini-pYV plasmid directed secretion and translocation of effector Yops encoded on expression plasmids provided in *trans*. Although the mini-pYV plasmid encoded a functional T3SS, it did not carry the *sycH* gene; therefore, LcrQ (YscM)-dependent regulation of T3SS gene transcription was not reconstituted with this construct. SycH, the chaperone for YopH and LcrQ, is required for efficient export of the negative regulatory component LcrQ in the absence of calcium (4, 37). Deletion of *sycH* prevents secretion of LcrQ and results in repression of T3SS gene transcription (30, 31).

The injection of Yops into a eukaryotic cell is essentially a three-step process that involves (i) attachment of the bacterium to a eukaryotic cell via specific bacterial adhesins, (ii) secretion of effector Yops across the bacterial membranes, and (iii) translocation of Yops across the eukaryotic membrane. The ability of *Y. pestis* KIM8 Δ123 and KIM8 Δ1234 to inject Yops into a eukaryotic cell was evaluated using the ELK tag reporter system described previously (9). *Y. pestis* KIM8 Δ123, KIM8 Δ1234, KIM8-3002.P40 (ΔyopE ΔyopJ), KIM8-3002.41 (ΔyopE ΔyopJ ΔyopB), and KIM8-3002.41 carrying pYopB2 were transformed with plasmid pYopE$_{129}$-ELK (9). The re-

porter plasmid encodes a truncated YopE protein with a C-terminal ELK tag that is phosphorylated upon translocation into a eukaryotic cell. Cultured HeLa cells were infected at a multiplicity of infection of 30 for 3 h at 37°C, and the infected monolayers were analyzed by SDS-PAGE and immunoblot analysis (Fig. 2B). The total YopE$_{129}$-ELK expressed during the infection was detected with anti-ELK antipeptide antibodies, whereas YopE$_{129}$-ELK translocated into the HeLa cells was phosphorylated and detected with phospho-specific anti-peptide antibodies (Cell Signaling Technology).

Immunoblot analysis with anti-ELK antipeptide antibodies demonstrated that all the strains expressed comparable amounts of the YopE$_{129}$-ELK protein (Fig. 2B, top panel). *Y. pestis* KIM8-3002.P40 (parent), KIM8 Δ123, and KIM8 Δ1234 translocated approximately equal amounts of YopE$_{129}$-ELK into the HeLa cells (Fig. 2B, bottom panel). In contrast, the translocation-defective ΔyopB strain was unable to inject the YopE$_{129}$-ELK protein into the HeLa cells. Providing complementing plasmid pYopB2 in *trans* completely restored the ability of the *yopB* deletion mutant to translocate the YopE$_{129}$-ELK protein. These studies confirmed that the pCD1-Δ1234 plasmid encodes a functional T3SS capable of injecting Yops into eukaryotic cells.

The calcium-regulated secretion of Yops by *Yersinia* spp. is dependent upon at least 30 different pCD1 genes; however, the role of chromosomal genes in this process is not fully understood. Recently, several chromosomal genes required for efficient expression of the *Yersinia* spp. T3SS have been identified
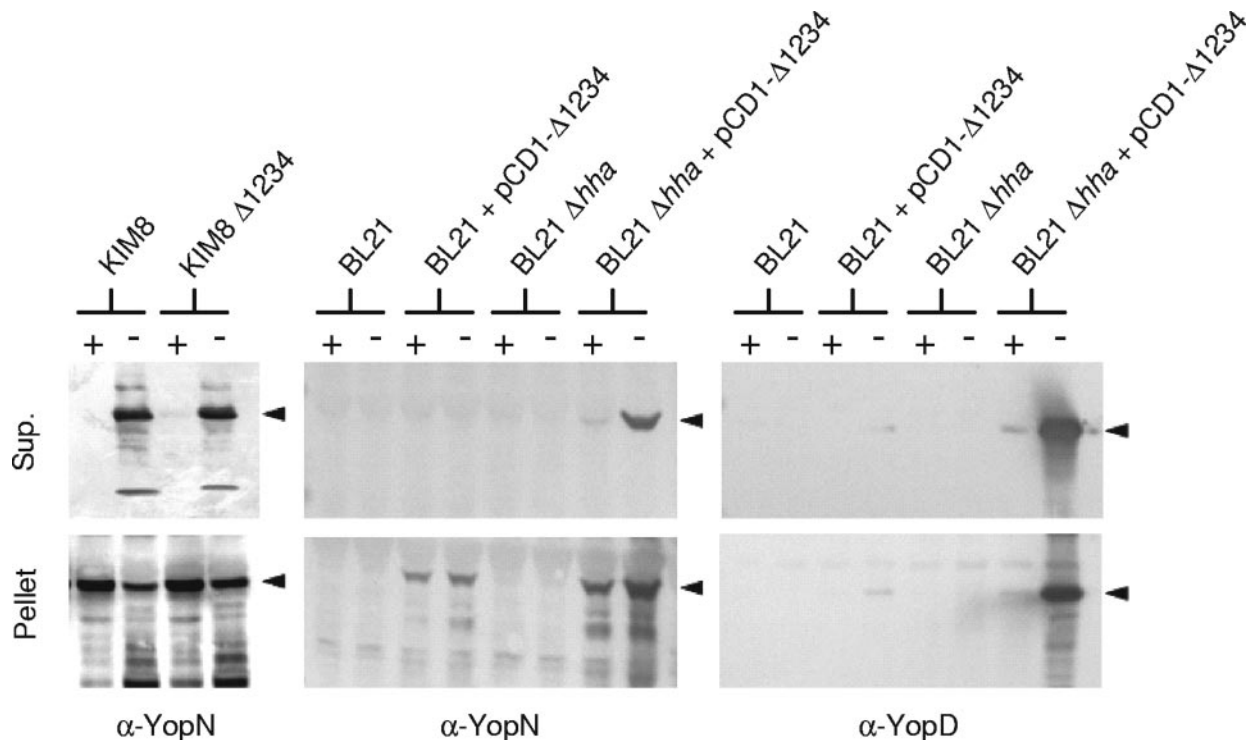
FIG. 3. Expression and secretion of Yops by an *E. coli hha* deletion mutant carrying pCD1-Δ1234. *E. coli* BL21 and BL21 *hha* with and without plasmid pCD1-Δ1234 were grown in TMH medium with (lanes +) or without (lanes −) 2.5 mM CaCl$_2$ for 5 h at 37°C. Cultures of *Y. pestis* KIM8 and KIM8 Δ1234 were grown under identical conditions. Volumes of culture supernatant (Sup.) proteins and cell pellet (Pellet) fractions corresponding to equal numbers of bacteria were analyzed by SDS-PAGE and immunoblotting with antisera specific for YopN or YopD. The locations of YopN and YopD are indicated by arrowheads.

(7, 10, 19, 20); however, the gene products identified thus far do not appear to be intimately involved in the type III secretion process per se. To determine if the *Y. pestis* chromosome carries genes that are essential for expression of a functional calcium-regulated T3SS, we moved the pCD1-Δ1234 construct into *E. coli* DH5α and BL21. Only low levels of Yop expression and no Yop secretion were observed in *E. coli* DH5α or BL21 carrying plasmid pCD1-Δ1234, indicating that pCD1 T3SS genes are not efficiently expressed in *E. coli* (17) (Fig. 3 and data not shown).

Transcription of T3SS genes in *Yersinia* spp. is negatively regulated by a small histone-like protein designated YmoA (7). *E. coli* strains express a protein homologous to YmoA, designated Hha (24), which functions with H-NS to thermoregulate the transcription of several virulence-related genes (22, 25). To examine the role of Hha in regulating the expression of pCD1-Δ1234 T3SS genes in *E. coli* DH5α and BL21, we used lambda Red-mediated recombination to delete the *hha* locus and to insert a *kan* cassette at the site of the deletion. The deletion of *hha* in both DH5α and BL21 was confirmed by PCR analysis. Plasmid pKD46 was cured from the *hha* deletion mutants, and the pCD1-Δ1234 plasmid was electroporated into the pKD46-cured *hha* deletion strains.

Expression and secretion of YopN and YopD by *Y. pestis* KIM8 and KIM8 Δ1234 and by *E. coli* BL21, BL21 Δ*hha*, DH5α, and DH5α Δ*hha* with and without plasmid pCD1-Δ1234 were determined following growth of the bacteria at 37°C in TMH medium in the presence and absence of calcium

(Fig. 3). The *E. coli* BL21 and DH5α *hha* deletion strains carrying plasmid pCD1-Δ1234 secreted YopN and YopD in the absence of calcium but not in the presence of calcium. The amounts of YopN and YopD secreted by these strains were comparable to or slightly less than the amounts secreted by *Y. pestis* KIM8 or KIM8 Δ1234. These studies confirmed that plasmid pCD1-Δ1234 contains the complete complement of genes unique to the yersiniae that are required to express a functional T3SS. Interestingly, the *E. coli hha* strains carrying pCD1-Δ1234 secreted high levels of Yops but did not exhibit growth restriction (data not shown), suggesting that the physiological determinants that link growth and secretion are unique to the yersiniae.

E. coli DH5α and BL21 are avirulent bacteria that do not express the surface adhesins required for attachment to the surface of eukaryotic cells (5). Therefore, the *E. coli* strains that express a functional T3SS should not be able to attach to HeLa cells and inject Yops. Indeed, *E. coli* DH5α Δ*hha* and BL21 Δ*hha* carrying pCD1-Δ1234 and pYopE$_{129}$-ELK expressed the YopE$_{129}$-ELK protein but did not translocate the protein into cultured HeLa cells (data not shown).

Previous studies have suggested that Hha and YmoA, which exhibit 82% amino acid sequence identity, are interchangeable (1, 11). Our results indicate that Hha in *E. coli*, but not YmoA in *Y. pestis*, prevents essentially all pCD1 T3SS gene expression at 37°C. The reason for the difference is unclear; however, we hypothesize that it could be related to the amount of YmoA or Hha present in the relevant bacterial cells at 37°C. We have
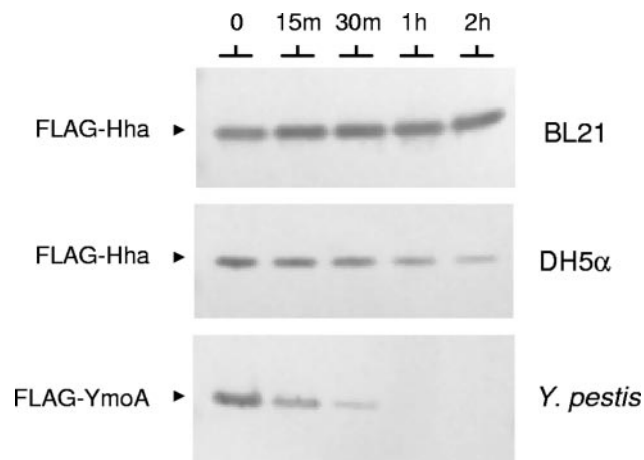
FIG. 4. Stability of FLAG-Hha in *E. coli* DH5α and BL21. *E. coli* DH5α and BL21, both carrying pFLAG-Hha, as well as *Y. pestis* KIM5-3001 carrying pFLAG-YmoA, were grown in heart infusion broth containing 2.5 mM CaCl$_2$ for 3 h at 37°C. Chloramphenicol (final concentration, 40 μg/ml) was added to block protein synthesis. An aliquot of each culture was removed at the times indicated at the top (15m, 15 min; 30m, 30 min). Whole-cell protein extracts from equivalent numbers of bacteria were separated by SDS-PAGE, transferred to Immobilon-P membranes, and probed with antibodies specific for the FLAG epitope tag.

demonstrated previously that YmoA is rapidly degraded by the Lon protease at 37°C in *Y. pestis*, a process that is essential to trigger T3SS gene transcription in *Y. pestis* (20). It is possible that Hha is more resistant to Lon-mediated proteolysis and is not rapidly degraded at 37°C in *E. coli*. To begin to examine this possibility, we constructed plasmid pFLAG-Hha, which encodes the Hha protein with an N-terminal 14-amino-acid tag (N-MDYKDDDDKVKLLE-Hha). Plasmid pFLAG-Hha was electroporated into *E. coli* DH5α and BL21, whereas plasmid pFLAG-YmoA was electroporated into *Y. pestis* KIM5-3001 (20). The stability of FLAG-Hha and FLAG-YmoA at 37°C was determined by determining the amounts of these proteins after new synthesis was blocked by addition of chloramphenicol (40 μg/ml) to the cultures, as described previously (20). Samples corresponding to equal numbers of bacteria were removed at specific times and analyzed by SDS-PAGE and immunoblot analysis with the FLAG M2 antibody (Sigma, St. Louis, MO) (Fig. 4). As previously demonstrated, FLAG-YmoA was rapidly degraded at 37°C in *Y. pestis*. FLAG-Hha was stable at 37°C in *lon*-deficient *E. coli* BL21 (14), but it was degraded in *E. coli* DH5α. These results suggest that Hha, like YmoA, is degraded in a Lon-dependent manner; however, the half-life of FLAG-YmoA in *Y. pestis* (19.3 min) was significantly less than the half-life of FLAG-Hha in DH5α (40.3 min). These results suggest that the degradation of Hha in *E. coli* and the degradation of YmoA in *Y. pestis* are to some extent different, and the difference may account for the ability of Hha to suppress pCD1 gene transcription at 37°C in *E. coli*. Alternate explanations are also possible, and further studies are required to determine the mechanism by which Hha represses T3SS gene transcription at 37°C in *E. coli*.

The *E. coli* Δ*hha*/pCD1-Δ1234 strain-plasmid combination provides a system to study the *Yersinia* spp. type III secretion

process in a heterologous host. Previously, only the *Erwinia chrysanthemi* T3SS has been shown to function in a heterologous bacterial host (16). The combination of the pCD1-Δ1234 virulence plasmid lacking the genes encoding all six translocated effector Yops with an avirulent *E. coli* host provides a reliable and safe system to analyze the molecular events involved in the *Y. pestis* type III secretion process. The procedures for preparation and manipulation of membrane vesicles suitable for in vitro secretion studies have been developed and fine-tuned in *E. coli* (12). Thus, the *E. coli* Δ*hha*/pCD1-Δ1234 system should facilitate the development of in vitro secretion assays that are required to dissect the molecular events involved in the type III secretion process.

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Editor: J. B. Bliska