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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Yersinia pestis is the etiologic agent of plague, an often fatal disease of both animals and humans (6). The pathogenicity 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 extracellular matrix 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 (∆1), 3,740 to 15,602 (∆2), 61,430 to 70,331 (∆3), and 55,568 to 60,495 (∆4). 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 (Km r) strains KIM8 (*kan*), KIM8 ∆2, and KIM8 ∆3, as well as trimethoprim-resistant (Tm r) strain KIM8 ∆4. Plasmid pCP20, which encodes the FLP recombinase (8), was electroporated into KIM8 ∆1 to facilitate removal of the FLP recognition target-flanked *kan* cassette, generating strain KIM8 ∆1s. 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 (∆2, ∆3, and ∆4) from pCD1 of KIM8 ∆1s. PCR products used to construct the sequential gene replacements were generated using pKD4 (*kan*), pKD3 (*cat*), or *dhfr* as the template for PCR. The ∆2::*kan*, ∆3::*cat*, and ∆4::*dhfr* insertional deletions were confirmed by PCR analysis and by agarose gel electrophoresis of plasmids isolated...
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 \emph{Y. pestis} KIM10 (pMT1\(^+\), pCD1\(^-\), pPCP1\(^-\)), generating \emph{Y. pestis} KIM8 Δ1 (Km\(^n\)), KIM8 Δ2 (Km\(^n\)), KIM8 Δ3 (Km\(^n\)), KIM8 Δ4 (Tm\(^n\)), KIM8 Δ12, KIM8 Δ13 (Km\(^n\) Cm\(^n\) Tm\(^n\)) and KIM8 Δ1234 (Km\(^n\) Cm\(^n\) Tm\(^n\)) in a clean genetic background (Fig. 1B). The resultant strains were used in all subsequent studies.

Secretion of Yops by \emph{Y. pestis} KIM8, KIM8 Δ1, KIM8 Δ2, KIM8 Δ3, KIM8 Δ4, KIM8 Δ12, KIM8 Δ13, 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. \emph{Y. pestis} KIM8 Δ1234, which had all four deletions, expressed and secreted YopB, YopD, YopN, and LcrV but none of the effectors 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 \emph{Y. pestis} KIM8 was constructed using primers Δ4-P1 and Δ3-P2. \emph{Y. pestis} KIM8 carrying plasmid pCD1-Δ5, in which the syc\(H\) gene was deleted, expressed and secreted only low levels of Yops (data not shown), confirming that SycH is required for efficient secretion.

### Table 1. Oligonucleotides used for lambda Red-mediated recombination

<table>
<thead>
<tr>
<th>Deletion</th>
<th>Template</th>
<th>Primer</th>
<th>Oligonucleotide sequence (5′ to 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ1:kan</td>
<td>pKD4</td>
<td>Δ1-P1</td>
<td>TGATAGACTATCATTATATAGTAAGGTGCTGAAATTGTAGGGTGAGCTGCTTCC</td>
</tr>
<tr>
<td>Δ2:kan</td>
<td>pKD4</td>
<td>Δ2-P1</td>
<td>TTACATTGGATTTTATGAGGCGATCGCTTCCATATATGGAACACAGGGGACGTCAGGTTAAGG</td>
</tr>
<tr>
<td>Δ3:kan</td>
<td>pKD4</td>
<td>Δ3-P1</td>
<td>ATACGCTTCTCCATATATTGAGAAATGAGCTGCTGCTTCCATATATGGAACACAGGGGACGTCAGGTTAAGG</td>
</tr>
<tr>
<td>Δ4:dhfr</td>
<td>EZ::TN</td>
<td>Δ4-P4</td>
<td>TTTTTGGATGAGATTCATGCATGATATGAGAGCTGCTGCTTCCATATATGGAACACAGGGGACGTCAGGTTAAGG</td>
</tr>
<tr>
<td>Δ2B:kan</td>
<td>pKD4</td>
<td>Δ2B-P1</td>
<td>TAACTTTTACTGCAGCTTAAATTCTGAGCCAGCCGTGAAGAAAAAGGCTACTTTAAGGAGGAGCTGCTGCTTCCATATATGGAACACAGGGGACGTCAGGTTAAGG</td>
</tr>
<tr>
<td>Δ3B:cat</td>
<td>pKD3</td>
<td>Δ3B-P1</td>
<td>TAACTTTTACTGCAGCTTAAATTCTGAGCCAGCCGTGAAGAAAAAGGCTACTTTAAGGAGGAGCTGCTGCTTCCATATATGGAACACAGGGGACGTCAGGTTAAGG</td>
</tr>
<tr>
<td>Δhha</td>
<td>pKD4</td>
<td>Δhha-P1</td>
<td>TATTTTTTATTTCATGGGAGGAGCTGCTGCTTCCATATATGGAACACAGGGGACGTCAGGTTAAGG</td>
</tr>
<tr>
<td>Δhha</td>
<td>pKD4</td>
<td>Δhha-P2</td>
<td>TATTTTTTATTTCATGGGAGGAGCTGCTGCTTCCATATATGGAACACAGGGGACGTCAGGTTAAGG</td>
</tr>
</tbody>
</table>

\(^*\) Obtained from Epicentre.
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 KIM8Δ123 and KIM8Δ1234 to inject Yops into a eukaryotic cell was evaluated using the ELK tag reporter system described previously (9). Y. pestis KIM8Δ123, KIM8Δ1234, KIM8-3002.P40 (ΔyopE ΔyopD; parent), KIM8-3002.41 (ΔyopE ΔyopD ΔyopB), and KIM8-3002.41 complemented with pYopB2, all carrying pYopE129-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 YopE129-ELK is indicated by arrowheads.

![Image](https://via.placeholder.com/150)
 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-H9004 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-H9004, 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 Δhha, DH5α, and DH5α Δhha 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 Δhha and BL21 Δhha carrying pCD1-Δ1234 and pYopE129-ELK expressed the YopE129-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
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-MDYKDĐĐĐKVLLE-Hha). Plasmid pFLAG-Hha was electroporated into E. coli DH5α and BL21, whereas plasmid pFLAG-YmoA was electroporated into Y. pestis KIM3-3001 (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 Δhha/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 Δhha/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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REFERENCES


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