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A Novel Autotransporter Adhesin Is Required for Efficient Colonization during Bubonic Plague[∇]

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Many proteins secreted by the type V secretion system (autotransporters) have been linked to virulence in gram-negative bacteria. Several putative conventional autotransporters are present in the *Yersinia pestis* genome, but only one, YapE, is conserved in the other pathogenic *Yersinia* species. Here, we introduce YapE and demonstrate that it is secreted via a type V mechanism. Inactivation of *yapE* in *Y. pestis* results in decreased efficiency in colonization of tissues during bubonic infection. Coinfection with wild-type bacteria only partially compensates for this defect. Analysis of the host immune response suggests that YapE is required for either efficient colonization at the inoculation site or dissemination to draining lymph nodes. YapE also demonstrates adhesive properties capable of mediating interactions with bacteria and eukaryotic cells. These findings support a role for YapE in modulating host-pathogen interactions that are important for colonization of the mammalian host.

Yersinia pestis is a gram-negative zoonotic pathogen that causes bubonic and pneumonic plague in humans (44). The bacterium is primarily transmitted via the bite of an infected flea and proceeds to colonize the proximal lymph node (5). Bacterial growth and host inflammation at these sites lead to the development of a swollen, painful bubo, the hallmark manifestation of bubonic plague. Without treatment, *Y. pestis* escapes the immune response in the lymph nodes and disseminates through the bloodstream to colonize other tissues such as the spleen, liver, and lungs (33). Colonization of the lungs can lead to the development of a pneumonic infection (secondary pneumonic plague) and person-to-person transmission (primary pneumonic infection) when patients aerosolize the bacteria during coughing (43). Infection progresses rapidly, with mortality rates approaching 100% in untreated pneumonic patients.

Y. pestis recently evolved from *Y. pseudotuberculosis* but still shares virulence factors with its enteric progenitor, including the pCD1 (pYV) virulence plasmid essential for successful colonization of the mammalian host (1, 12). Encoded on pCD1 are the Ysc type III secretion apparatus and the Yop effector proteins that are translocated by this system (for a review, see reference 54). During infection, the Yops are translocated directly into host cells and disrupt normal cellular functions. The Yops have been shown to inhibit phagocytosis by disrupting actin polymerization, suppress host cytokine responses, and trigger apoptosis in macrophages (3, 9, 18, 31, 45). The combination of these actions allows *Y. pestis* to circumvent the early innate immune response and rapidly disseminate throughout the host. Mutational analysis in *Y. pseudotuberculosis* emphasizes the importance of the Ysc/Yop system by demonstrating

that many of the Yops encode redundant functions, suggesting selection for an efficient, multifaceted dysregulation of the innate immune response (39).

During the evolution from an enteric to a vector-borne pathogen, *Y. pestis* lost factors important for *Y. pseudotuberculosis* virulence, including inactivation of the adhesins *inv* and *yadA* (12). However, other adhesins have been identified in *Y. pestis* that appear to be important for virulence. The *psa* locus encodes components of a fimbrial structure that promotes binding to respiratory epithelial cells (15, 38, 57). Furthermore, *Y. pestis* lacking the fimbrial subunit PsaA is deficient in dissemination during bubonic infection and, to a lesser degree, during pneumonic plague (11). A second potential adhesin, Pla, is encoded on the pPCP1 plasmid. Pla is a member of the ompTn surface protease family and cleaves host plasminogen and components of the complement pathway (53). Independent of this protease activity, Pla binds to the extracellular matrix component laminin and promotes invasion of endothelial cells (35). Inactivation of *pla* severely attenuates *Y. pestis* during bubonic infection (47, 52); however, a *pla* mutant is still lethal during intranasal or intravascular infection (37). In addition to PsaA and Pla, there is evidence that *Y. pestis* has other adhesins. Liu et al. demonstrated that in the absence of PsaA and the Caf1 capsule, *Y. pestis* still bound to epithelial cells and induced invasion (38). The *Y. pestis* protein(s) responsible for these interactions has yet to be identified, but any cryptic adhesin may prove to be important in *Y. pestis* pathogenesis.

Autotransporters are a family of secreted proteins found in gram-negative bacteria that encode a variety of virulence functions (for reviews, see references 28 and 30). Proteins that use this system for secretion (type V secretion) are unique in that the secreted protein encodes all of the necessary information to mediate translocation across the bacterial membranes. All autotransporters share a conserved domain structure: an N-terminal signal peptide, a central domain encoding the function of the mature protein (referred to as the passenger

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domain), and a C-terminal beta domain that mediates translocation of the passenger domain across the outer membrane.

The importance of the trimeric autotransporter YadA in the pathogenesis of *Y. pseudotuberculosis* and *Y. enterocolitica* has been evident through several studies. YadA has been implicated in autoaggregation, host cell binding, and serum resistance and is required for enteric infection (8, 21, 42); however, YadA does not appear to contribute to *Y. pestis* virulence, since it is a pseudogene in all of the *Y. pestis* biovars (51). Recently, at least 10 additional putative autotransporters have been identified in the genomes of *Y. pestis* (58, 29; M. B. Lawrenz, J. D. Lenz, and V. L. Miller, unpublished data). These novel autotransporters have been named Yaps for *Yersinia* autotransporter proteins. The Yaps demonstrate limited sequence similarity to other characterized proteins, but three appear to have some adherence properties (23, 58). *Escherichia coli* expressing YapN and YapK agglutinate red blood cells, and YapC demonstrates autoagglutination and adherence to epithelial cells. Although many autotransporters from other bacteria have been implicated as important virulence factors, it has yet to be determined whether the Yaps contribute to *Y. pestis* virulence.

In this study, we introduce the YapE autotransporter of *Y. pestis* and demonstrate its importance in efficient colonization of the mammalian host. In addition, we analyzed the cytokine response of infected animals to help decipher possible defects in $\Delta yapE$ colonization. Finally, we demonstrate that YapE can mediate binding to eukaryotic cells in an apparent cell-type-specific manner.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and animals. Wild-type (WT) *Y. pestis* strain CO92 was provided by the U.S. Army, Fort Dietrich, MD (19). The Δpla , $\Delta yapE$, $\Delta lacZ$, and $\Delta psaA \Delta caf1$ mutants have in-frame deletions in the indicated genes generated using the *lambda red* recombinase method essentially as described previously (11, 16, 37). The $\Delta yapE$ mutant was complemented with *yapE* plus 600 bp of upstream DNA at the *attTn7* site using the mini-Tn7 system developed by Choi et al. (14). The kanamycin resistance cassette was removed from the mutant strains using the FRT recombinase provided by a version of pLH29 which has been modified by replacing the chloramphenicol resistance gene with an ampicillin resistance gene. Ampicillin and kanamycin sensitivity was confirmed prior to passage in animals. A tetracycline-inducible *yapE* strain was generated in the avirulent WT pCD1⁽⁻⁾, WT pCD1⁽⁻⁾ Δpla , and WT pCD1⁽⁻⁾ $\Delta psaA \Delta caf1$ strains using the mini-Tn7-based system described by Latham et al. (37). For adherence assays using *E. coli*, *yapE* was introduced between the KpnI and HindIII sites of the plasmid pLP-PROTet-6xHN (Clontech, Palo Alto, CA) downstream of the tetracycline promoter and transformed into *E. coli* DH5 α PRO (Clontech) or the $\Delta ompT$ strain UT5600 (New England Biolabs, Ipswich, MA) containing the plasmid pVM1286 harboring the tetracycline repressor (Clontech). *Y. pestis* and *E. coli* were cultivated on brain heart infusion (BHI) or Luria-Bertani (LB) (Difco, Sparks, MD) plates or broth, respectively, with the addition of kanamycin (50 μ g ml⁻¹), ampicillin (50 μ g ml⁻¹), spectinomycin (100 μ g ml⁻¹) or X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; 100 μ g ml⁻¹) when needed. No alterations in the *in vitro* growth characteristics of the *Y. pestis* mutant strains compared to the parental strain were observed.

All animal experiments were approved by the Animal Studies Committee of Washington University (protocol 20050189). Four- to six-week-old female C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were maintained in a barrier facility and allowed free access to sterilized food and water. Animals were anesthetized prior to infection with a mixture of ketamine-HCl (2 mg per mouse) and xylazine-HCl (0.32 mg per mouse) by intraperitoneal injection. Mice were inoculated with *Y. pestis* subcutaneously ($\sim 10^2$ CFU) or intranasally ($\sim 10^5$ CFU) as described previously (11, 36). At the indicated time points after inoculation, mice were euthanized by an intraperitoneal injection of sodium pentobarbital at a dose of 75 mg/kg of body weight. Tissues were aseptically harvested

and macerated, and the bacterial load of each tissue was determined by plating serial dilutions.

RNA isolation and reverse transcription-PCR (RT-PCR). An overnight culture of WT *Y. pestis* was subcultured into BHI to an optical density at 600 nm (OD₆₀₀) of 0.2 and grown for ~ 8 h at 26°C to an OD₆₀₀ of 3.5. RNA was isolated by using the RiboPure-Bacteria kit (Ambion, Austin, TX). Contaminating DNA was removed from the samples with the DNA-free DNase kit (Ambion), and cDNA was generated from 2.0 μ g of total DNase-treated RNA by using Super-script III reverse transcriptase (Invitrogen, Carlsbad, CA).

Surface proteolysis and Western blot analysis. To determine the surface localization of YapE, *E. coli* cultures were grown overnight, diluted 1:50 in fresh media, and grown for 2 h at 37°C. *Y. pestis* cultures were grown overnight, diluted 1:25 in fresh media, and grown for 3 h at 26°C. YapE expression was induced by the addition of anhydrous tetracycline (Sigma, St. Louis, MO) at 10 ng ml⁻¹ (*E. coli*) or 100 ng ml⁻¹ (*Y. pestis*), and cultures were grown for an additional 2 h. Samples from induced cultures equivalent to an OD₆₀₀ of 1.0 were harvested, washed twice with 1 \times phosphate-buffered saline (PBS)-5 mM MgCl₂ (pH 7.5) (resuspension buffer), and resuspended in 450 μ l of the same buffer. Then, 50 μ l of proteinase K (4 mg ml⁻¹) was added to each sample, followed by incubation on ice for 30 min, and 50 μ l of 50 mM phenylmethylsulfonyl fluoride was added to inhibit further proteolysis. Samples were washed twice with resuspension buffer, and cell pellets were resuspended in Laemmli buffer containing 10% β -mercaptoethanol. Samples were boiled for 10 min, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to Immobilon-P membranes (Millipore, Billerica, MA) for Western blot analysis. Anti-YapE serum was generated in rabbits (Cocalico Biological, Reamstown, PA) against the passenger domain (amino acids 42 to 709) of YapE, preabsorbed against *E. coli* lysates, and used at a concentration of 1:1,000. Anti-MalE rabbit serum (New England Biolabs) was used at a dilution of 1:10,000. Proteins from culture supernatants were isolated from induced cultures as described previously (55, 59).

Host cytokine response. To determine host cytokine response, superficial cervical lymph nodes from either naive or mice infected with WT or the $\Delta yapE$ mutant were harvested at 24, 36, and 48 h postinfection ($n = 5$ for each time point), directly immersed into RNAlater (Ambion), and stored at 4°C. RNA was isolated from the lymph nodes by using the RiboPure RNA isolation system (Ambion). Contaminating DNA was removed from the samples and cDNA generated as described above. Quantitative RT-PCR was performed to determine cytokine transcript levels using SYBR Green (Qiagen, Valencia, CA) and 900 nM gene-specific primers (26), and data were normalized to the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) transcript. The relative fold change was calculated by using the $\Delta\Delta C_T$ method (4) comparing infected to naive lymph nodes.

Competitive index. $\Delta lacZ$ (fully virulent) and $\Delta yapE$ strains were grown in BHI for 15 h at 26°C. A 1:1 mixture of $\Delta lacZ$ and $\Delta yapE$ strains was made and diluted to $\sim 10^3$ CFU/ml in 1 \times PBS. Next, 100 μ l of the diluted mixture was plated on BHI agar containing X-Gal to differentiate between the $\Delta lacZ$ strain (white colonies) and the $\Delta yapE$ strains (blue colonies), and the ratio between the two strains was determined (CFU of $\Delta yapE$ /CFU of $\Delta lacZ$ = input ratio). Mice were challenged with 100 μ l of the mixture subcutaneously, and tissues were harvested as described above. Serial dilutions of macerated tissues were plated on BHI agar containing X-Gal to determine the recovered ratio (CFU of $\Delta yapE$ per tissue/CFU of $\Delta lacZ$ per tissue). A competitive index score was generated by dividing the recovered ratio by the input ratio. A score of less than zero indicates that the recovered ratio differs from the input ratio and, specifically, fewer $\Delta yapE$ bacteria are present in the tissues. Similar infections were performed with a mixed infection of the $\Delta lacZ$ and *yapE* complemented $\Delta yapE$ strains.

Bacterial settling and adherence assays. To analyze settling of *E. coli* expressing YapE in broth cultures, bacteria were grown overnight, diluted 1:50 in fresh medium, and grown for 2 h at 37°C on a roller drum. YapE expression was induced by the addition of 10 ng of anhydrous tetracycline ml⁻¹, and cultures were grown for an additional 2 h. Cultures were removed from the rotator and allowed to sit statically, and samples were harvested at a specific depth to determine the changes in absorbance at 600 nm over time.

For adherence assays, wells of a 24-well plate were seeded with 4 $\times 10^5$ or 2 $\times 10^5$ A549 human lung or HEp-2 human epithelial cells, respectively, and grown overnight at 37°C with 5% CO₂ to obtain ca. 80% confluent monolayers. YapE expression was induced in *E. coli* as described above with either 10 or 20 ng of anhydrous tetracycline ml⁻¹. For *Y. pestis*, YapE expression was induced as described above with 100 ng of anhydrous tetracycline ml⁻¹. Bacteria were diluted to an OD₆₀₀ of 0.3 (*E. coli*) or 0.003 (*Y. pestis*) ml⁻¹ in fresh tissue culture medium, and 1 ml of diluted culture was added to each well. Plates were centrifuged at 200 $\times g$ for 5 min to initiate bacterium-cell interactions and

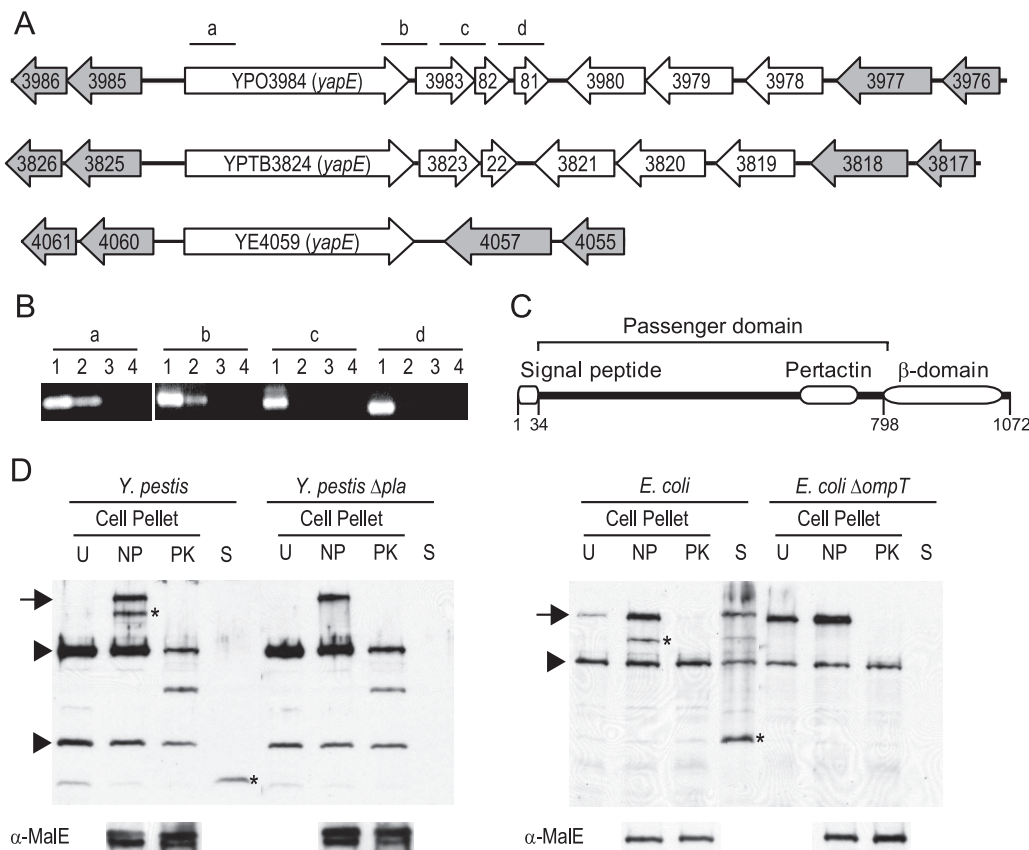


FIG. 1. Characterization of *yapE*. (A) *yapE* and surrounding genes from *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* (top to bottom, respectively). Gray arrows represent flanking genes that are conserved in all three species. Bars with lowercase letters represent regions amplified by RT-PCR. (B) RT-PCR to determine whether *yapE* is in an operon. Lowercase letters correspond to regions in panel A amplified by RT-PCR. Lanes: 1, DNA; 2, RNA; 3, No RT; 4, no template. (C) Diagram showing the conserved domains in YapE predicted by Pfam. Numbers indicate amino acid positions of predicted domains within YapE. (D) Western blot analysis demonstrating the localization of YapE in *E. coli* and *Y. pestis*. The bottom panel demonstrates the stability of MalE during protease digestions. Protein samples from cell pellets (U, uninduced; NP, induced, no proteinase K; PK, induced, + proteinase K) and culture supernatants from induced, no-proteinase-K samples (S) were probed with anti-YapE serum. Arrows denote surface-exposed mature-length YapE. Asterisks denote omptin-processed YapE fragments. Arrowheads denote nonspecific proteins that are recognized by the anti-YapE serum.

incubated at 37°C for either 2 h (*E. coli*) or 30 min (*Y. pestis*). To remove nonadherent bacteria, the culture medium was removed, and wells were washed four times with PBS. Adherent bacteria were recovered by treatment with 0.02% trypsin for 10 min and 1% Triton for 5 min (to lyse the eukaryotic cells). Serial dilutions of recovered bacteria were plated on agar. To calculate the percentage of adherent bacteria, the number of adherent bacteria was divided by the number of total bacteria as determined from an independent well. For microscopy, cells were fixed and stained by using the Diff Quick Stain kit (IMEB, Inc., San Marcos, CA).

RESULTS

Characterization of the *Yersinia* autotransporter YapE. The *Y. pestis* CO92 genome encodes 10 conventional autotransporters (Lawrenz et al., unpublished data) but only *yapE* (YPO3984) is conserved in the enteric pathogens *Y. pseudotuberculosis* IP32953 (YPTB3824, 97% amino acid identity) and *Y. enterocolitica* 8081 (YE4059, 65% amino acid identity). YapE orthologs are also found in the other *Y. pestis* biovars, *Y. pestis* subspecies *Microtus* and *Pestoides* F (100% amino acid identity for both), *Y. mollaretii* (62% amino acid identity), and *Y. bercovieri* (60% amino acid identity). Outside the *Yersinia* genus, YapE has little similarity to other proteins, with the

highest degree of similarity occurring between the C-terminal end of the protein and β-domains of other putative autotransporters. A comparison between the *yapE* loci of the human pathogenic species is shown in Fig. 1A. The presence of conserved open reading frames (ORFs) flanking *yapE* in all three species suggests the gene was acquired prior to the divergence of *Y. pseudotuberculosis* and *Y. enterocolitica*. In the case of the *Y. pseudotuberculosis* lineage, five ORFs are present between *yapE* and the 3' flanking conserved gene YPTB3818 (in *Y. pestis* an extra ORF is present due to a duplication of YPO3982). These ORFs do not have homologs in the *Y. enterocolitica* genome and are likely a more recent acquisition by *Y. pseudotuberculosis* or loss by *Y. enterocolitica*. In *Y. pestis*, there are only 92 bp separating *yapE* from YPO3983, suggesting *yapE* may be in an operon with the downstream ORFs.

To determine whether *yapE* is transcribed in an operon with these ORFs, we isolated RNA from WT CO92 and performed RT-PCR with primers bridging the genes of the putative operon (Fig. 1B). We obtained PCR products for the region spanning YapE and YPO3983 but not for the regions spanning YPO3983-YPO3982 or YPO3982-YPO3981. This suggests

that *yapE* is transcribed monocistronically with YPO3983 but not the two downstream ORFs. However, it is possible that YPO3982 and YPO3981 are also part of the operon, but our RT-PCRs yielded negative results due to decreased stability of the transcript at the 3' end.

Sequence analysis of YapE reveals the presence of three putative domains that are important for autotransporter translocation. Computational analysis predicts an N-terminal signal peptide, an autotransporter conserved pertactin domain (Pfam ID PF03212), and a C-terminal β -domain, which is required for translocation of all autotransporters (Fig. 1C) (25). The presence of these domains, particularly the signal peptide and the β -domain, strongly suggests that YapE is an autotransporter. To demonstrate experimentally that YapE is a secreted autotransporter, we determined the accessibility of the protein to proteinase K. Proteinase K is unable to diffuse across the outer membrane of gram-negative bacteria and can only cleave outer surface proteins in intact bacteria. Therefore, if YapE is a functional autotransporter, it should be accessible to proteolysis by proteinase K. *E. coli* and *Y. pestis* containing inducible *yapE* were incubated with or without proteinase K as described in Materials and Methods. An inducible system was used because we were unable to visualize YapE protein expressed from its native promoter by Western blotting (data not shown). Induction of YapE resulted in the appearance of two additional proteins that are recognized by the anti-YapE antibody (Fig. 1D). The largest protein migrated near the predicted molecular mass (~ 108 kDa) for YapE. We did not observe the presence of reactive bands indicating trimerization of YapE. Within 30 min of incubation with proteinase K, both proteins were completely digested in *E. coli*, and a truncated protein, likely representing the β -domain (protected from digestion) and a small region of the passenger domain, remained in the *Y. pestis* samples. As a control, we determined the stability of the periplasmic protein MalE after proteinase K treatment. The levels of MalE did not change after protease treatment, indicating that the bacteria remained intact throughout the experiment.

The presence of two proteins upon induction of YapE expression suggests that YapE is processed after translocation. Protein processing has been observed for several autotransporters and is a mechanism to secrete portions of the passenger domain into the environment. Many autotransporters require outer surface proteases such as ompTins for processing, but a select group have autocatalytic mechanisms for release (17). To determine whether the smaller protein in the induced samples resulted from cleavage of the larger protein, we harvested culture supernatant from YapE induced cultures and looked for the presence of YapE in these samples by Western blotting. In both *E. coli* and *Y. pestis* cultures, we discovered an ~ 37 -kDa reactive protein (Fig. 1D). This protein was not present in uninduced cultures (data not shown), indicating that, at least in vitro, YapE can be processed after translocation and secreted. Furthermore, deletion of the ompTins *ompT* in *E. coli* or *pla* in *Y. pestis* resulted in the loss of both the smaller protein in the cell pellet and the ~ 37 -kDa protein from the supernatant (Fig. 1D). Taken together with the surface proteolysis results, these data confirm that YapE is a functional, translocated autotransporter that can be processed by members of the ompT family of proteases.

YapE is required for *Y. pestis* virulence during bubonic plague. The presence of YapE in all three human pathogenic *Yersinia* suggests that it may be a conserved virulence factor. To determine the contribution of YapE to *Y. pestis* virulence, we generated an in-frame deletion of the *yapE* gene in the fully virulent CO92 strain and compared its ability to colonize mice to the WT parental strain. Compared to the WT, the $\Delta yapE$ mutant was defective in efficiently colonizing the proximal lymph nodes, spleen, and lungs via the bubonic route of infection. We observed a noticeable delay in the colonization of the lymph nodes by the $\Delta yapE$ mutant beginning as early as 36 h postinfection (Fig. 2A). Significantly more WT-infected animals had detectable bacteria present in their cervical lymph nodes at 36 and 48 h postinfection. Furthermore, the median bacterial load was consistently lower in the lymph nodes of $\Delta yapE$ strain-infected animals, with a $>10^6$ -fold difference in the median recovered CFU at 60 h postinfection.

We also observed a difference in the ability of the mutant to efficiently disseminate or survive in the spleens and lungs of infected animals. By 60 h, all mice infected with WT had bacteria in their spleens, but 35% of the $\Delta yapE$ -infected mice had spleens without detectable bacteria (Fig. 2B). As observed in the lymph nodes, bacterial loads were also significantly lower in spleens of $\Delta yapE$ -infected mice at 60 h, with $>10^3$ -fold more bacteria in tissues of WT-infected animals. A similar delay in colonization by the mutant was observed in the lungs, with more organs colonized, and to higher levels, by WT at 60 h (Fig. 2C). Virulence could be restored to the $\Delta yapE$ mutant by complementing the mutant with a functional copy of the *yapE* gene, indicating that attenuation was specific for the *yapE* mutation.

While *Y. pestis* is primarily a vector-borne pathogen, pneumonic infection is a secondary route of transmission associated with rapid progression of disease and severe mortality rates. To determine whether YapE contributes to pathogenesis during pneumonic plague, we inoculated mice intranasally with $\sim 10^5$ CFU of the $\Delta yapE$ mutant and compared colonization of the lungs and dissemination to the spleen with a WT infection. Unlike the bubonic route, deletion of *yapE* did not significantly alter the ability of CO92 to efficiently colonize mice (Fig. 3A). $\Delta yapE$ strain-infected mice had bacterial counts in their lungs by 12 h postinfection that were similar to WT-infected counterparts. The mutant also appeared to replicate effectively in the lungs, reaching levels comparable to those of the WT throughout the course of the experiment. By 48 h postinfection, the spleens of animals infected with both strains of *Y. pestis* were colonized, with a slightly lower bacterial load in mutant-infected animals ($P = 0.0078$), but similar levels were reached by 60 h postinfection (Fig. 3B). These results suggest YapE has a greater impact on the virulence of *Y. pestis* during bubonic infection than intranasal inoculation. Interestingly, the mutant still caused a lethal infection and had a 50% lethal dose similar to that of the parental WT strain (<10 CFU) during bubonic infection.

$\Delta yapE$ mutant does not stimulate an earlier immune response in infected lymph nodes. Attenuation in colonization of the lymph nodes by the $\Delta yapE$ mutant during bubonic infection could be a result of either poor dissemination from the inoculation site or a reduced ability to survive the immune response in the lymph nodes. WT *Y. pestis* is able to delay early recog-

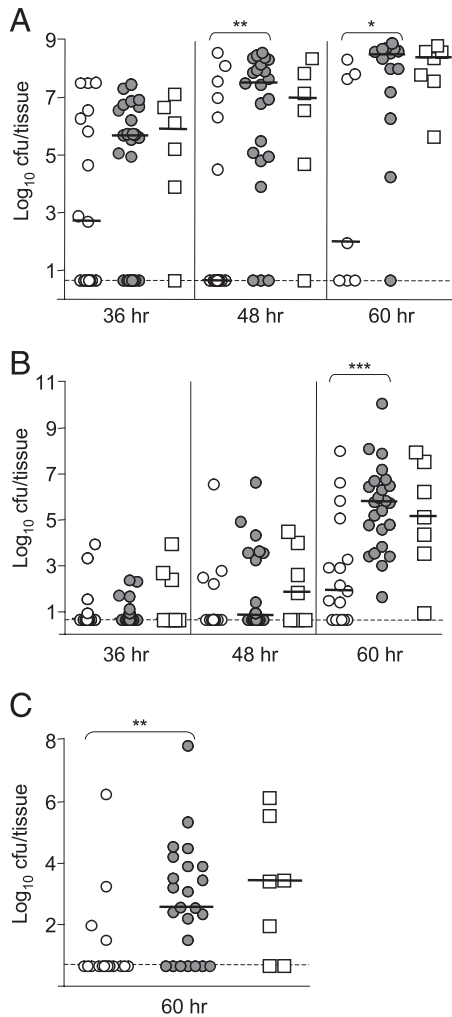


FIG. 2. Dissemination of the $\Delta yapE$ mutant during bubonic infection. Mice were infected subcutaneously in the neck with $\sim 10^2$ CFU of the $\Delta yapE$ mutant (open circles), WT (gray circles), or the $yapE$ complemented mutant (open squares). Mice were sacrificed at 36, 48, and 60 h postinfection, and the colonization of the superficial cervical lymph nodes (A), spleen (B), and lungs (C) was determined. Each symbol represents an individual animal. Black bars correspond to the median CFU/tissue for each group. The dashed line indicates the limit of detection. Asterisks denote significant differences between the median CFU/tissue (Mann-Whitney t test with a two-tailed nonparametric analysis: *, $P \leq 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.0005$). The data represent the composite of three independent experiments. The number (n) of tissues harvested for each indicated time point is given in parentheses in the format “time point = (n) $_{\Delta yapE}$, (n) $_{WT}$, (n) $_{\text{complemented mutant}}$ ” as follows: lymph nodes (36 h = 17, 24, 6; 48 h = 17, 22, 6; 60 h = 7, 14, 7), spleen (36 h = 17, 24, 7; 48 h = 17, 24, 7; 60 h = 17, 24, 7), and lungs (60 h = 17, 24, 7).

nition by the innate immune system, but as the infection progresses the host eventually mounts a strong inflammatory response (10, 36, 46; M. B. Lawrenz and V. L. Miller, unpublished data). We postulated that monitoring cytokine and chemokine expression in the lymph nodes may provide insight into the reason(s) that the $\Delta yapE$ mutant is attenuated in lymph node colonization. In WT-infected lymph nodes, expression of the proinflammatory cytokines interleukin-6 (IL-6), IL-1 α , and IL-1 β was low at 24 h postinfection, but by 48 h all three were

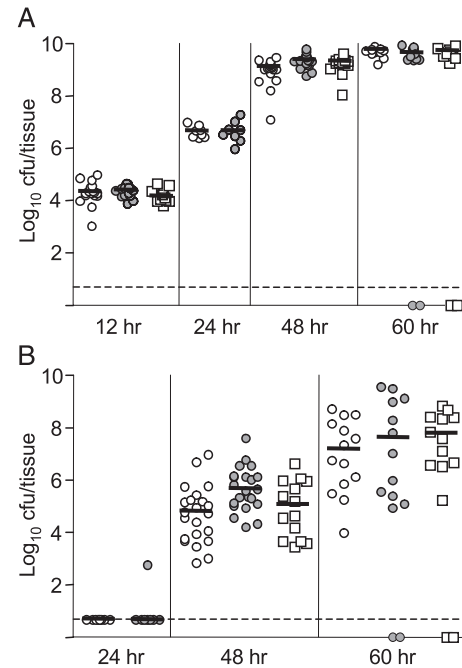


FIG. 3. Dissemination of the $\Delta yapE$ mutant during pneumonic infection. Mice were infected intranasally with $\sim 10^5$ CFU of $\Delta yapE$ mutant (open circles), WT (gray circles), or the $yapE$ complemented mutant (open squares). Mice were sacrificed at 12, 24, 48, and 60 h postinfection, and colonization of the lungs (A) and spleen (B) was determined. Each symbol represents an individual animal, and black bars correspond to the median CFU/tissue for each group. The dashed line indicates the limit of detection. Symbols on the dashed line represent animals with CFU below the limit of detection, and symbols on the x-axis represent animals that succumbed to infection. The number (n) of tissues harvested for each time point is given in parentheses in the format “time point = (n) $_{\Delta yapE}$, (n) $_{WT}$, (n) $_{\text{complemented mutant}}$ ” as follows: lungs (12 h = 18, 17, 11; 24 h = 8, 8, 0; 48 h = 21, 20, 14; 60 h = 13, 13, 13) and spleen (24 h = 8, 8, 0; 48 h = 21, 21, 14; 60 h = 12, 14, 12).

dramatically induced compared to uninfected controls (Fig. 4A). In contrast, during the $\Delta yapE$ mutant infection transcription of these cytokines remained low through 36 h postinfection. When cytokine levels began to increase in the $\Delta yapE$ mutant-infected mice at 48 h, expression was dramatically lower than that seen in WT-infected tissues. These differences correlated with less bacteria in the lymph nodes of $\Delta yapE$ mutant-infected animals at these time points (Fig. 4C) and suggest that a similar immune response occurred in both cases but was delayed in $\Delta yapE$ mutant-infected mice.

Previous work suggests that gamma interferon (IFN- γ), tumor necrosis factor alpha (TNF- α), and IL-12 are protective against *Y. pestis* infection (6, 40, 41). We hypothesized that the $\Delta yapE$ mutant may be attenuated in lymph node colonization because it stimulates the expression of these cytokines more effectively than does the WT. Earlier induction could in turn lead to a more effective immune response and increased killing of the mutant. However, we did not observe a significant difference in the expression of IFN- γ , TNF- α , and IL-12 transcripts in the lymph nodes of mice infected with the $\Delta yapE$ mutant compared to uninfected samples (Fig. 4B). These data suggest that the $\Delta yapE$ mutant does not stimulate an earlier immune response in the lymph nodes.

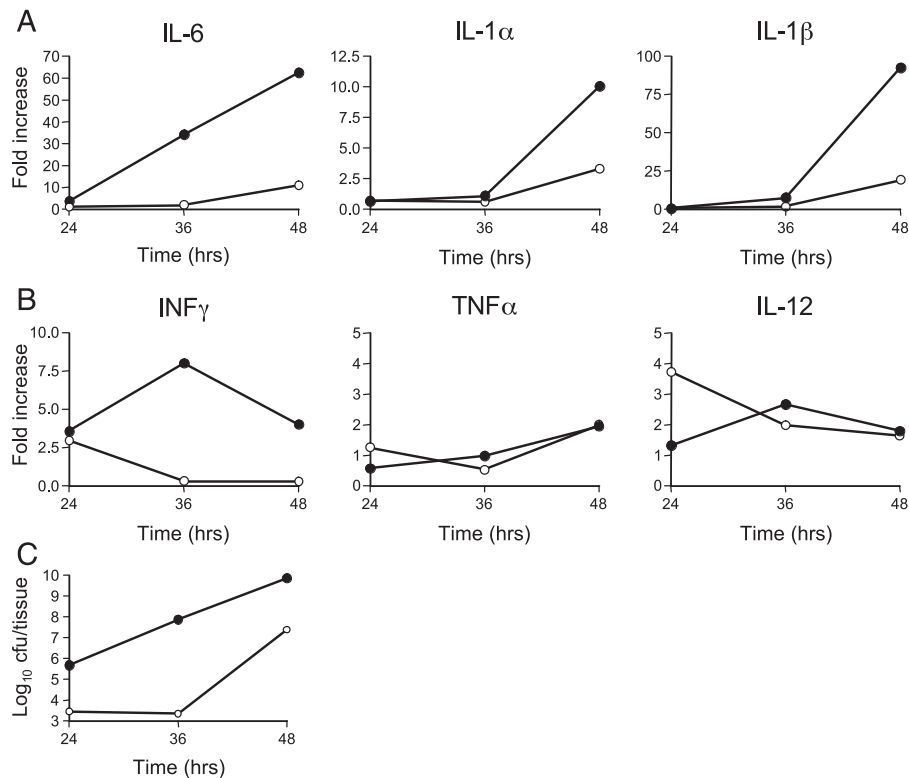


FIG. 4. Cytokine response in the lymph nodes during bubonic infection of mice. Mice were infected subcutaneously in the neck with $\sim 10^2$ CFU of WT (black circles) or the $\Delta yapE$ mutant (open circles), and RNA was harvested from the superficial cervical lymph nodes of five mice at 24, 36, and 48 h postinfection. The expression of IL-6, IL-1 α , and IL-1 β (A) or INF- γ , TNF- α , and IL-12 (B) was determined by quantitative RT-PCR and is represented as the fold difference over levels from uninfected lymph nodes. (C) Two mice were sacrificed at each time point to determine the approximate CFU/tissue.

Coinfection with WT does not fully complement attenuation of the $\Delta yapE$ mutant. Competitive infections are often used to better understand the impact of factors on virulence. In many cases, coinfection can enhance the attenuation of the mutant (7, 39); however, it has been speculated that WT bacteria could complement the defects of some mutants in *trans*, especially mutants in secreted virulence factors (13). To determine the effect of the WT on the ability of the $\Delta yapE$ mutant to cause disease, we infected mice subcutaneously with a 1:1 mixture of

a $\Delta lacZ$ derivative of WT and the $\Delta yapE$ mutant and monitored the colonization of the lymph nodes, spleen, and lungs by determining the competitive index (Fig. 5). In contrast to the phenotype we observed in single infections, the $\Delta yapE$ mutant efficiently colonized the lymph nodes during coinfection to levels comparable to WT, represented by a competitive index score of ≥ 1.0 at all time points. However, we recovered fewer $\Delta yapE$ bacteria in the spleens and lungs of coinfecting mice at 60 h postinfection (median competitive index scores of 0.006

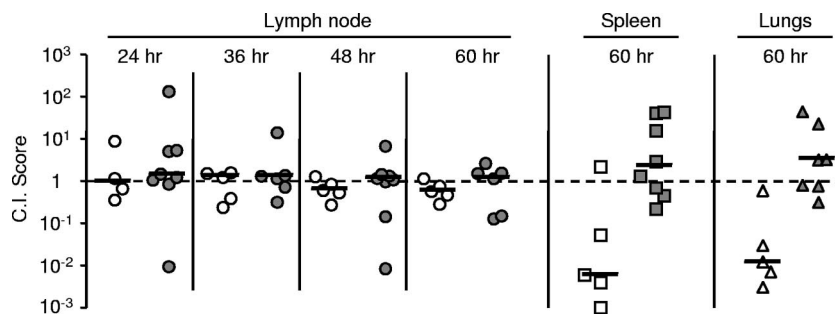


FIG. 5. Colonization by the $\Delta yapE$ mutant during mixed infection with WT CO92. Mice were infected subcutaneously in the neck with a 1:1 mixture of the $\Delta yapE$ mutant and $\Delta lacZ$ WT (open symbols) or the *yapE* complemented mutant and $\Delta lacZ$ WT (gray symbols). Lymph nodes (circles), spleen (squares), and lungs (triangles) were harvested at 24, 36, 48, and 60 h postinfection. Serial dilutions were plated on agar containing X-Gal to differentiate between the test and $\Delta lacZ$ WT strains, and CFU of each were determined. The ratio of recovered mutant bacteria to $\Delta lacZ$ WT was compared to the equivalent ratio of the inocula to generate a competitive index (C.I.) score. Median competitive index scores are represented by black bars. Scores less than one indicate attenuation in colonization by the test strain.

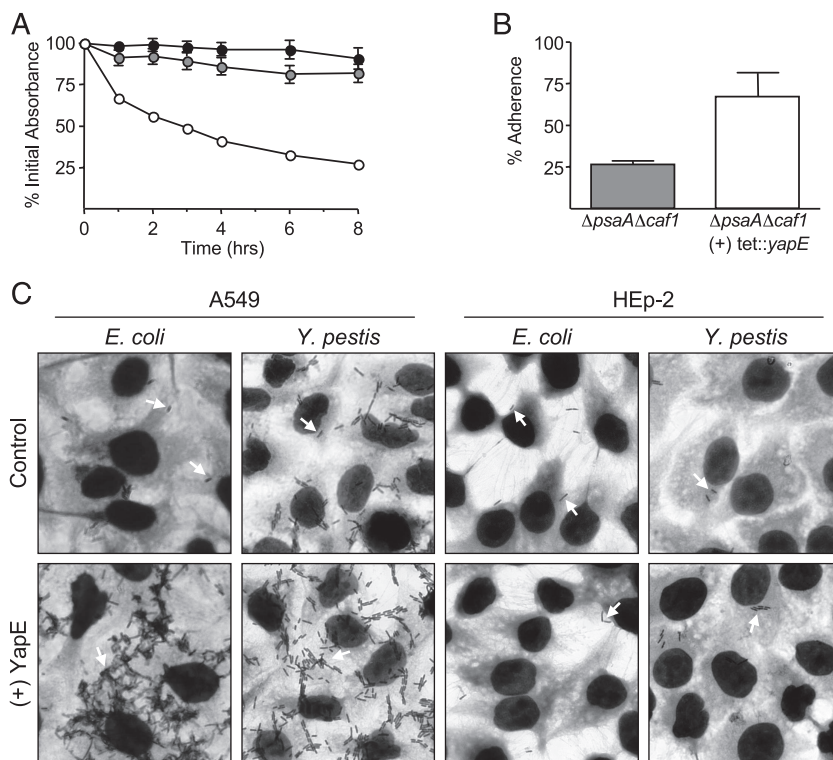


FIG. 6. Autoaggregation and adherence to eukaryotic cells by YapE-expressing bacteria. (A) YapE expression was induced in *E. coli* (open circles), and changes in the absorbance of static cultures were monitored over time and compared to vector only (gray circles)- and YapF (black circles)-expressing *E. coli* cultures. The data represent the mean percentage of the absorbance \pm the standard error of the mean at 0 h ($n = 6$). The standard errors of the mean for YapE data were calculated but are smaller than the symbols. (B and C) YapE expression was induced in bacterial cultures, and bacteria were incubated with eukaryotic cells. Nonadherent bacteria were washed away, and the percentage of *Y. pestis* $\Delta psaA \Delta caf1$ that adhered to A549 cells was determined (B). (C) Microscopy demonstrating YapE-mediated binding to A549 but not HEp-2 cells. White arrows denote bacteria.

and 0.012, respectively), indicating that the mutant is still attenuated in the colonization of these tissues. Complementation of the $\Delta yapE$ strain with *yapE* in the Tn7 *att* site restored virulence and resulted in no differences in colonization of tissues during coinfection. These results indicate that the WT is able to complement the virulence defect of the $\Delta yapE$ mutant in the lymph nodes but not during dissemination to and/or colonization of the spleen or lungs of coinfecting mice.

YapE mediates autoaggregation and adherence to eukaryotic cells. During our localization analyses, we observed that induction of YapE resulted in the formation of a ring at the fluid-air interphase on test tubes and settling of *E. coli* to the bottom of the cultures (data not shown). Similar phenotypes have been reported for other autotransporters and are indicative of protein-protein interactions between bacteria (27, 49, 50). To determine whether YapE mediates autoaggregation, YapE expression was induced in *E. coli* for 2 h, and samples were harvested from static cultures to monitor the bacterial settling (Fig. 6A). We observed that while the absorbance of YapF expressing and vector control strains did not decrease significantly over the course of the assay, the absorbance of the YapE-expressing culture decreased quickly and was ca. 50% of the starting OD₆₀₀ within 3 h. We observed no differences in the number of recovered viable CFU between any of the strains at 8 h, indicating that protein induction was not harming the YapE-expressing bacteria (data not shown). These data

indicate that YapE mediates autoaggregation in *E. coli* and suggest that YapE has adhesive properties.

Since other autotransporters demonstrating autoaggregation also interact with eukaryotic cells (34), we hypothesized that YapE may promote adherence of *Y. pestis* to eukaryotic cells. Previous work demonstrated that *Y. pestis* adheres to various eukaryotic cells in vitro and that some of these interactions are mediated by cryptic adhesins (23, 35, 38). To determine whether YapE mediates adherence to epithelial cells, we incubated either *E. coli* or *E. coli* expressing YapE with two epithelial cell lines (Fig. 6C). *E. coli* poorly adhered to both cell lines; however, expression of YapE resulted in specific adherence to A549 cells but not HEp-2 cells. To demonstrate in *Y. pestis* that YapE promotes adherence to A549 cells, we generated a *Y. pestis* strain with an inducible copy of *yapE* that lacked PsaA (a known major adhesin) and Caf1 (shown to mask other adhesins) (38). YapE expression in *Y. pestis* resulted in adherence to A549 but not to HEp-2 cells (Fig. 6B and C). These data suggest YapE promotes adherence to eukaryotic cells and that these interactions occur through a receptor present on A549 but not HEp-2 cells.

DISCUSSION

Autotransporters have been associated with virulence in many gram-negative pathogens (28). Analysis of the *Y. pestis*

CO92 genome revealed the presence of several putative conventional autotransporters. YapE is unique in this group since it is the only one that is also found in *Y. pseudotuberculosis* and *Y. enterocolitica* and appears to have been acquired prior to the divergence of these species. Its conservation in other *Yersinia* species suggests that YapE provides a selective advantage for the genus, perhaps during infection. Furthermore, because the vector-borne lifestyle of *Y. pestis* differs considerably from its enteric counterparts, the functions of YapE may not be specific to an enteric lifestyle.

One advantage of the type V secretion mechanism is that while all autotransporters use the same basic mechanism to migrate across the bacterial cell envelope, the system imposes little restriction on the final localization of the passenger domain (17). Some autotransporters remain associated with the bacterium, either as intact polypeptides or through noncovalent interactions with the outer membrane after proteolytic processing. Others are released after translocation and are free to diffuse away from the bacterium. In the case of YapE, we demonstrated that the passenger domain primarily remains associated with the bacterium, but some polypeptides are processed, resulting in the release of a portion of the passenger domain into the supernatant. YapE does not appear to autoproteolytically cleave its passenger domain, as has been shown for several members of the SPATE family of autotransporters (reviewed in reference 17), but is processed by outer membrane omptins (Pla in *Y. pestis* and OmpT in *E. coli*). Omptin cleavage is not unique to YapE; autotransporters from other bacteria are processed by omptins (32). It is unclear at this time how omptin processing affects YapE. Pla processing may be part of the normal maturation process of YapE, resulting in a functional protein. Conversely, cleavage may allow *Y. pestis* to release itself from YapE-mediated interactions with eukaryotic cells. A similar release mechanism has been proposed for the Hap adhesin of *Haemophilus influenzae*, although Hap relies on autoproteolysis for cleavage (24).

Omptin processing may also determine the surface localization of YapE. In *Shigella flexneri* cleavage of the autotransporter IcsA by the omptin SopA is required for polar localization of the protein and actin-based intracellular motility (20, 48). Location-specific cleavage could explain the presence of both full-length and processed versions of YapE in *Y. pestis*. Finally, it is possible that increased expression of YapE with our inducible system may artificially promote omptin cleavage. However, other *Yersinia* autotransporters expressed by this system are not sensitive to OmpT cleavage (unpublished data). Therefore, we believe that omptin processing of YapE is not a result of overexpression.

We have demonstrated that a *yapE*-null mutant is significantly attenuated in colonizing tissues during bubonic infection compared to WT. Attenuation is first observed in the draining lymph nodes. Three scenarios may explain this delay in colonization: (i) the $\Delta yapE$ mutant is defective in colonization of the subcutaneous tissue, (ii) the mutant is defective for dissemination to the proximal lymph node, or (iii) the mutant disseminates to the tissue at the same rate but is less successful at surviving in the tissue. These scenarios are not necessarily mutually exclusive, but at this time, our data support either of the first two as more likely to be occurring. First, in animals with detectable bacteria in the lymph nodes, the mutant colo-

nizes the tissue to levels comparable to the WT. Second, if the mutant was killed more efficiently in the lymph nodes, one might expect that antigen presentation and activation of the innate system would be stimulated earlier. In contrast to this hypothesis, we did not observe an earlier or more intense immune response in mutant-infected lymph nodes. In fact, the proinflammatory response was delayed in mutant-infected mice, likely because the mutant arrives at the lymph node later than the WT. Furthermore, once initiated, the inflammatory response to the $\Delta yapE$ mutant appears similar to the WT infection.

In addition to delayed lymph node colonization, we observed that the $\Delta yapE$ mutant was attenuated in dissemination to the spleen and lungs. Colonization of the lymph nodes is thought to be a prerequisite for infection of other tissues; therefore, the defect in dissemination to deeper tissues could be a direct consequence of delayed colonization of the lymph nodes. However, mixed infections demonstrated that the $\Delta yapE$ mutant was still attenuated in efficient colonization of spleens and lungs even when the lymph nodes were colonized. Similarly, we did not observe differences in colonization of the initial tissue during intranasal inoculation but detected subtle defects in colonization of the spleens by the $\Delta yapE$ mutant. We also observed that some mice succumbed by 60 h to infections with WT and *yapE* complemented strains but not with the $\Delta yapE$ mutant. Together, these results suggest that YapE may contribute independently both to colonization of the lymph nodes and to dissemination to deeper tissues, possibly through distinct functions; it is not unprecedented for autotransporters to be multifunctional (2, 28).

Dissemination defects similar to those we observed for the $\Delta yapE$ mutant have been previously reported for another *Y. pestis* virulence factor, the surface protein Pla (52, 56). Inactivation of *pla* does not alter colonization of the inoculation site during subcutaneous infection but results in delayed spleen colonization. It has been speculated that Pla activates host plasminogen to degrade fibrin deposits, allowing *Y. pestis* to disseminate from the inoculation site. We have shown that Pla processes YapE and, due to similar defects in dissemination by the two mutants, it is possible that lack of the processed form of YapE in the Δpla mutant may contribute to the attenuated dissemination phenotype of the Δpla mutant. However, we believe that Pla likely also contributes to virulence independent of YapE. This is evidenced by the significantly higher 50% lethal dose of the Δpla mutant (52) and its decreased ability to colonize the lungs during intranasal infection (37). In the future, we will determine the effect of *yapE* inactivation on colonization of the subcutaneous tissue during bubonic infection. Comparing the abilities of the $\Delta yapE$, Δpla , and $\Delta yapE \Delta pla$ mutants to colonize the inoculation site will help clarify the role of Pla processing of YapE in virulence.

The passenger domain of YapE shares little similarity to other described autotransporters, making it difficult to predict the function(s) of the protein. We have demonstrated that YapE mediates interactions between bacteria and with eukaryotic cells. Adhesins are common virulence factors in many bacteria, suggesting that an adherence defect in the YapE mutant could be responsible for attenuation in the mouse model. Identification of a novel adhesin in *Y. pestis* is of particular interest due to its lack of the major enteric *Yersinia*

adhesins. In *Y. pestis*, the adhesins *inv* and *yadA* are both pseudogenes, containing naturally occurring mutations. It has been proposed for *Y. enterocolitica* and *Y. pseudotuberculosis* that *YadA* and *Inv* binding promotes efficient translocation of Yop effectors into mammalian cells (22, 45). If similar intimate interactions are required for Yop translocation by *Y. pestis*, then other adhesins must be responsible. In addition to YapE, pH6 antigen (*PsaA*), *Pla*, and *YapC* have been shown to contribute to adherence in *Y. pestis* (23, 35, 38). It is unclear at this time whether these adhesins promote Yop translocation, but one could envision that decreased Yop translocation could be responsible for the attenuation we observed for the YapE mutant. Furthermore, the specificity of YapE binding suggests that YapE mediates interactions with specific cells and/or tissues. Our future studies will seek to determine the YapE receptor on eukaryotic cells and define the role of YapE in Yop translocation in the context of the other known *Y. pestis* adhesins.

Mixed infections demonstrated that attenuation in the *ΔyapE* mutant can be at least partially complemented by WT bacteria, restoring colonization of the lymph nodes by the mutant. To our knowledge, this is the first report of *trans*-complementation in bacterial coinfections. More surprising was that the WT could complement a mutation in a bacterium-associated adhesin. However, as stated earlier, autotransporters can encode multiple functions. For example, *YadA* has been shown to mediate adherence, invasion, and resistance to killing by complement (21). We have not ruled out the possibility that YapE may also be multifunctional. Furthermore, the secreted and bacterial associated proteins may contribute independent virulence functions. It is possible that the secreted portion of YapE is responsible for the delayed colonization of the lymph nodes, while systemic dissemination is more dependent on the cell surface form of YapE. This would explain why WT bacteria can *trans*-complement colonization of the lymph node but not later stages of infection by the mutant. As we begin to better understand the interactions of YapE with the mammalian host, new functions for YapE may be revealed.

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