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Gain of Function Analysis Reveals Non-Redundant Roles for the Yersinia pestis Type III Secretion System Effectors YopJ, YopT, and YpkA [preprint]

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Running Head: YopJ, YopT, and YpkA Contribute to *Y. pestis* Virulence

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Abstract

Virulence of *Yersinia pestis* in mammals requires the type III secretion system, which delivers seven effector proteins into the cytoplasm of host cells to undermine immune responses. All seven of these effectors are conserved across *Y. pestis* strains, but three – YopJ, YopT, and YpkA – are apparently dispensable for virulence. Some degree of functional redundancy between effector proteins would explain both observations. Here, we use a combinatorial genetic approach to define the minimal subset of effectors required for full virulence in mice following subcutaneous infection. We found that a *Y. pestis* strain lacking YopJ, YopT, and YpkA is attenuated for virulence in mice, and that addition of any one of these effectors to this strain increases lethality significantly. YopJ, YopT, and YpkA likely contribute to virulence via distinct mechanisms. YopJ is uniquely able to cause macrophage cell death *in vitro* and to suppress accumulation of inflammatory cells to foci of bacterial growth in deep tissue, whereas YopT and YpkA cannot. The synthetic phenotypes that emerge when YopJ, YopT, and YpkA are removed in combination provide evidence that each enhances *Y. pestis* virulence, and that YopT and YpkA act through a mechanism distinct from that of YopJ.

Introduction

Yersinia pestis, causative agent of plague, is notorious for its role in the European Black Death pandemics of the Middle Ages. Its pathogenesis in the mammalian host is remarkable: following inoculation of a small number of bacteria in the dermis by the bite of an infected flea, *Y. pestis* rapidly invades distal tissues and the vasculature. The resulting dense bacteremia enhances transmission, as it allows colonization of naïve fleas that ingest a sub-microliter blood meal. Dissemination of *Y. pestis* from the dermis to the

bloodstream requires several bacterial adaptations that work in concert to achieve near-absolute suppression of the innate immune responses that would otherwise contain or clear the infection.

The *Y. pestis* type III secretion system (T3SS) is a major contributor to this innate immune evasion strategy. The T3SS transports bacterial proteins, called effectors, into the cytoplasm of target eukaryotic cells (1, 2). The bacterial translocon proteins YopB and YopD form a pore in the host cell membrane and interact with the syringe-like T3SS "injectisome" apparatus. This assembly is thought to form a continuous conduit that transports effector proteins directly from the intracellular compartment of the bacterial cell into the cytosol of target cells (2-4), though some recent data has challenged this model (5, 6).

The T3SS of the pathogenic yersiniae targets innate immune cells *in vivo* (7) and undermines a variety of antimicrobial responses in these cells, including phagocytosis, immune signaling, and the production of reactive oxygen species (ROS) (reviewed in (8)). Intoxication of these cells by the T3SS is one of the most important mechanisms underlying the innate immune evasion that is so crucial for *Y. pestis* virulence, and spontaneous loss of the pCD1 plasmid that encodes the T3SS profoundly attenuates *Y. pestis* in mammalian infection models (9-11). Mutations that compromise the type III secretion mechanism by inactivating injectisome components are likewise highly attenuating (12, 13).

Y. pestis shares a conserved set of seven T3SS effectors with the enteropathogenic yersiniae *Y. enterocolitica* and *Y. pseudotuberculosis*. Four of these effectors – YopH, YopE, YopK, and YopM – are required for full virulence of *Y. pestis* in murine infection

models (14-20), although the attenuation associated with YopM deletion seems to vary among strains (21). YopH, YopE, and YopM directly target innate immune responses: YopH and YopE inhibit the production of reactive oxygen species (ROS) (22, 23) and interfere with phagocytosis (24-27), while YopM likely enhances virulence by preventing caspase 1 signaling (28, 29) and pyrin inflammasome activation (30, 31). The attenuation of YopK mutants may result from dysregulated secretion of the other effector proteins and the translocon proteins (19, 32).

The effector YopJ profoundly deranges host cell death signaling pathways *in vitro*, and as a result YopJ has been intensively studied in all three pathogenic *Yersinia* species. YopJ induces caspase-8/RIP-1 mediated apoptosis in macrophages, inhibits transcription of pro-inflammatory cytokines by NFκB, and may also stimulate caspase-1 signaling (33-35). However, *Y. pestis* mutants lacking the *yopJ* gene have been shown more than once to retain full virulence *in vivo* (36, 37). Single knock-outs of the cysteine protease YopT and the serine/threonine kinase YpkA have not been reported to impact virulence of *Y. pestis* in mammalian infection models.

Although YopJ, YopT, and YpkA are individually dispensable for *Y. pestis* virulence, all three effectors are conserved across natural and experimental *Y. pestis* strains. Given the small number of T3SS effectors found in *Y. pestis*, especially compared with the T3SSs of many other Gram-negative pathogens, it is likely that these effectors are selectively maintained because they play a role during some stage of the natural *Y. pestis* transmission cycle. However, these three effectors share some putative targets with one another and with the other T3SS effectors at both the protein and pathway level (8). Their functions may therefore overlap sufficiently to account for the observation that

single deletion of any one does not have a measurable effect, at least in the context of standard laboratory survival studies using inbred mouse strains.

Traditional single gene knockout models are ill-suited to studying the individual contributions of the T3SS effectors, which share a high degree of interconnectedness among their putative target molecules and pathways. For example, no fewer than four effectors (YopH, YopE, YpkA, and YopT) are reported to interfere with phagocytic function of innate immune cells (reviewed in (8)). For this reason, if a mutation in a single effector fails to yield an attenuation phenotype *in vivo*, it is difficult to distinguish between true dispensability of the effector's function and possible functional redundancy with other components of the T3SS.

To determine the effects of the YopJ, YopT, and YpkA effectors, we chose instead to test for gain-of-function phenotypes when effectors were added back to a strain from which all seven effector proteins had been deleted. This combinatorial genetic approach focuses on finding synthetic phenotypes, and is therefore robust to functional redundancy. We were able to dissect the individual contribution of each effector to pathogenesis in the intact animal. All seven effector proteins enhance virulence in mice – the first clear demonstration that YopJ, YopT, and YpkA can each contribute to *Y. pestis* virulence *in vivo*. The collection of combinatorial effector knock-outs we generated also allowed us begin the process of quantifying and characterizing the non-redundant role that each effector plays during infection. While it seems likely that the contribution of YopJ results from its effective killing of macrophages, as has been reported, the mechanisms underlying the contributions of YopT and YpkA remain uncertain despite considerable knowledge of their biochemical activity.

Results

Effectors YopH, YopE, YopK, and YopM are not sufficient for full virulence *in vivo*.

To understand the functional role of each of the *Y. pestis* T3SS effector proteins during infection, we set out to find the minimal subset of effectors that were sufficient to mediate full virulence through the subcutaneous route of infection.

We approached this problem by first constructing KIM1001ΔT3SE, an unmarked strain that retains the T3SS injectisome, regulatory elements, and translocon proteins, but carries in-frame deletions in the open reading frames (ORFs) for all seven effector proteins (see Table S1 for details). This strain, constructed in the fully-virulent background KIM1001 (38), was highly attenuated through the subcutaneous route of infection. When infected with 10^3 CFU KIM1001ΔT3SE, 0 of 8 mice developed visible symptoms of disease, and all mice survived infection (Table 1).

Functional ORFs for various effectors were restored at their original loci in the ΔT3SE genetic background to generate strains expressing defined subsets of effectors. YopH and YopE are more strictly required for bacterial fitness *in vivo* than any of the other effector proteins (18). However, we found that these two effectors in combination are not sufficient for virulence. The strain KIM1001ΔT3SE::+yopHE, expressing YopH and YopE but carrying in-frame deletions in all of the remaining five effectors' ORFs, failed to sicken or kill any mice following subcutaneous infection with 10^3 CFU (n=10). By contrast, this dose is uniformly fatal with wild-type KIM1001 (Table 1).

Single deletion studies have demonstrated that YopM and YopK are also essential for full virulence of *Y. pestis* (19, 20). However, neither of these effectors was sufficient to restore virulence in the KIM1001ΔT3SE::+yopHE background. Strains expressing

only YopH, YopE, and YopM (KIM1001ΔT3SE::+yopHE**M**) or YopH, YopE, and YopK (KIM1001ΔT3SE::+yopHE**K**) remained attenuated (0 out of 6 mice killed for each group following subcutaneous infection with 10^3 CFU) (Table 1), though 2 out of 6 mice infected with KIM1001ΔT3SE::+yopHE**K** lost their fur around the injection site and developed local redness of the skin that persisted for at least 28 days.

The KIM1001ΔT3SE::+yopHE**KM** strain expresses all effectors previously reported to be necessary for full virulence, as assessed by subcutaneous infection based of single knockout mutants. This strain was substantially more virulent than the previous strains expressing subsets of these effectors, but significantly attenuated relative to the wild-type strain. KIM1001ΔT3SE::+yopHEKM killed approximately 50% of infected mice (9 out of 17) at a dose of 10^3 CFU, indicating a ~100-fold increase of LD_{50} compared to wild-type KIM1001. Simultaneously restoring functional copies of the *ypkA*, *yopJ*, and *yopT* ORFs to this strain (generating the strain KIM1001ΔT3SE::+yopHEKMAJT, genetically identical to the wild-type strain KIM1001) fully complemented its virulence defect, restoring virulence to wild-type levels as expected. This result also confirmed that the long series of genetic manipulations required for sequentially deleting and then restoring the seven effector genes did not cause unexpected or off-target modifications that alter virulence phenotypes (Figure 1 and Table 1).

Strains expressing YopK or YopM in addition to YopH and YopE induce more effective adaptive immunity. The avirulence of strains KIM1001ΔT3SE::+yopHE, KIM1001ΔT3SE::+yopHE**M**, and KIM1001ΔT3SE::+yopHE**K** led us to investigate their potential as live attenuated vaccines. To assess the degree of protection conferred by

155 infection with these strains, surviving mice were challenged 28 days after initial infection
156 with 10^3 CFU KIM1001 s.c. Mice exposed to KIM1001ΔT3SE::+yopHE**M** or
157 KIM1001ΔT3SE::+yopHE**K** uniformly survived the challenge, while 3 out of 10 mice
158 exposed to KIM1001ΔT3SE::+yopHE succumbed within 14 days (Figure 2). The full
159 protection from challenge conferred by KIM1001ΔT3SE::+yopHEK and
160 KIM1001ΔT3SE::+yopHEM suggests that each of these strains, while nonlethal, causes
161 infection that is sufficiently persistent to trigger robust involvement of the adaptive
162 immune system. The partial protection provided by exposure to
163 KIM1001ΔT3SE::+yopHE indicates a weaker or less consistent adaptive immune
164 response, suggesting that this strain is more susceptible to clearance by the innate
165 immune system.

166 **YopJ, YopT, and YpkA each contribute to virulence of *Y. pestis* following**
167 **subcutaneous infection.** The attenuation of KIM1001ΔT3SE::+yopHEKM relative to
168 KIM1001ΔT3SE::+yopHEKMAJT is strong evidence that at least one of the remaining
169 effectors (YopT, YpkA, or YopJ) functionally contributes to virulence *in vivo*. However,
170 strains deficient in any one of these effectors are not significantly attenuated (36, 37) (and
171 see Table 1).

172 We generated derivatives of the KIM1001ΔT3SE::+yopHEKM strain that
173 included a functional copy of either the *ypkA*, *yopT*, or *yopJ* gene in its original locus.
174 The resulting strains were KIM1001ΔT3SE::+yopHEKM**A**, expressing YpkA;
175 KIM1001ΔT3SE::+yopHEKM**T**, expressing YopT; and KIM1001ΔT3SE::+yopHEKM**J**,
176 expressing YopJ. Each of these strains was substantially more virulent than
177 KIM1001ΔT3SE::+yopHEKM. Although none caused 100% mortality, each was virulent

enough that the difference between survival curves for these strains and the wild-type strain KIM1001 was not statistically significant (Figure 3 and Table 1). Given the distinct biochemical activities of YopT, YpkA, and YopJ, it is curious that each of these effectors increased virulence of the KIM1001ΔT3SE::+yopHEKM approximately equally. The different targets and activities reported for these effectors suggest that they are not truly redundant at the molecular level, and may therefore enhance virulence through distinct processes.

YopJ suppresses immune cell recruitment in the liver. Evaluation of liver pathology following intravenous infection is a useful method to assay immune cell responses to *Y. pestis* (21, 39, 40). KIM1001ΔT3SE::+yopHEKM and the strains that additionally expressed YpkA, YopT, or YopJ were injected intravenously into mice. Livers were collected 48 hours after infection for histopathological analysis.

KIM1001ΔT3SE::+yopHEKM elicited robust recruitment of immune cells, whereas KIM1001ΔT3SE::+yopHEKM**AJT**, like KIM1001, effectively suppressed accumulation of inflammatory cells at foci of bacterial growth (Figure 4A-B). The addition of YopJ to KIM1001ΔT3SE::+yopHEKM appears sufficient to fully suppress immune cell recruitment in this context, as lesions caused by KIM1001ΔT3SE::+yopHEKM**J** were indistinguishable from those caused by KIM1001. By contrast, neither KIM1001ΔT3SE::+yopHEKM**A** nor KIM1001ΔT3SE::+yopHEKM**T** suppressed inflammatory cell recruitment relative to KIM1001ΔT3SE::+yopHEKM (Figure 4A-B). YopJ, therefore, appears to be uniquely essential (though likely not sufficient) for suppressing accumulation of immune cells at sites of bacterial replication *in vivo*.

YpkA and YopT are dispensable for inducing macrophage cell death and for bacterial survival in co-culture with neutrophils. The ability of the *Y. pestis* T3SS to cause apoptosis in macrophages is well established, and is considered an important function in promoting virulence. We therefore examined how various combinations of effectors influenced cell death of immortalized macrophages *in vitro*.

Some incomplete sets of *Yersinia* effectors result increased macrophage death, either via YopE induction of pyroptosis (30, 31) or as a result of dysregulated effector and translocon secretion in the absence of YopK (32, 41). However, the ΔT3SE::+yopHEKM strain expresses both YopM, which prevents YopE-mediated activation of the pyrin inflammasome (30, 31), and YopK, which prevents inflammasome activation by translocon components (41, 42). As expected, therefore, this strain resulted in minimal macrophage death (Figure S1).

Consistent with previous reports (21, 43, 44), we observed T3SS-dependent cell death induced by YopJ. The ΔT3SE::+yopHEKM**J** strain caused macrophage death at a level indistinguishable from the wild-type strain (Figure S1). This YopJ-mediated cell death may contribute to the reduced visible recruitment of innate immune cells *in vivo* (Figure 4). Addition of YopT or YpkA to a strain expressing YopE, YopH, YopK, and YopM had no effect on macrophage cell death.

In addition to undermining macrophage function, *Y. pestis* must overcome the antimicrobial host responses mediated by neutrophils. The T3SS is known to be critical for evasion of neutrophil killing *in vitro* (45) (and, to some degree, *in vivo* (20)). To determine whether any of the observed synthetic phenotypes *in vivo* result from differential ability to survive neutrophil antimicrobial responses, effector mutants were

assayed systematically for the ability to survive in co-culture with primary human neutrophils. To facilitate the effort of assaying a large number of bacterial strains simultaneously, we developed a luminescence-based assay to monitor bacterial survival in co-culture with human neutrophils that is higher-throughput than plating to measure colony-forming units (CFU), and has the additional benefit of providing a readout of bacterial metabolic activity in real time (see Methods). Survival of *Y. pestis* in co-culture with neutrophils required the T3SS, as expected (Figure S2). The T3SS injectisome in combination with either YopH or YopE is sufficient for this survival phenotype (Figure S3A), and deletion of both YopH and YopE together recapitulates the susceptibility to neutrophils observed in a T3SS-deficient strain (Figure S3B). No other effector is necessary (Figure S4) or sufficient (Figure S5) for bacterial survival in the presence of primary neutrophils *in vitro*. YopT and YpkA, therefore, are unlikely to enhance virulence *in vivo* by directly undermining neutrophil bactericidal activity.

Discussion

Since the original discovery of the type III secretion system in *Y. pestis* and *Y. pseudotuberculosis* (1, 46, 47), type III secretion systems have been found to contribute to virulence not only in the *Yersiniae* but also in diverse Gram-negative pathogens, including pathogenic species of *Salmonella*, *Pseudomonas*, *Vibrio*, *Burkholderia*, and in enteropathogenic *Escherichia coli* strains (48-54). Multifactorial virulence determinants such as these T3SSs are crucial factors in allowing fulminant pathogens to undermine host defense systems. However, complex bacterial systems are difficult to study using traditional single-knockout methods of analysis, which are confounded by apparent functional redundancy.

In this work, we used a gain-of-function approach to determine that the four effectors previously known to be required during infection – YopH, YopE, YopK, and YopM – are not sufficient to mediate full virulence of *Y. pestis*. Indeed, strains expressing only YopH, YopE, and either YopK or YopM failed to sicken or kill any mice, although infection with these strains is protective against subsequent challenge with virulent *Y. pestis*. As these strains retain the structural components of the T3SS, as well as all other non-T3SS virulence factors, they may prove to useful in the construction of live attenuated vaccines.

YopJ, YopT, and YpkA had not been shown to contribute uniquely to *Y. pestis* virulence in mammalian infection (36, 37) (and see Table 1). We initially suspected that the apparent dispensability of these effectors was due to functional redundancy between two of them, but we found instead that all three individually increase virulence when introduced to the attenuated strain expressing only YopH, YopE, YopK, and YopM. It is possible that the action of each of these three effectors contributes additively to virulence of *Y. pestis*, and that addition of any of them to the core effector set of YopH, YopE, YopK, and YopM is sufficient to pass some threshold of immune subversion that allows robust dissemination and proliferation of *Y. pestis in vivo*.

YopJ, YopT, and YpkA increase lethality approximately equally, but have different biochemical activities and targets from one another. Strains expressing each of these effectors also behave differently in more targeted assays, supporting the model that these effectors contribute to pathogenesis independently rather than redundantly. For example, addition of YopJ to the KIM1001ΔT3SE::+yopHEKM construct blocks accumulation of inflammatory cells at foci of infection in deep tissue, while addition of

either YopT or YpkA to KIM1001ΔT3SE::+yopHEKM does not produce any obvious histological signature (Figure 4). This is consistent with a recent finding by our colleagues Ratner *et al*., who report a similar phenotype in a YopJ single deletion strain (21). Ratner *et al.* also demonstrate that, when YopM is absent, YopJ is necessary for full virulence of *Y. pestis* following subcutaneous infection. The YopM-independent role for YopJ in virulence that we report here is a novel finding.

Although we have now established a functional role for YopT and YpkA in infection, we cannot yet explain how these effectors enhance the virulence of the KIM1001ΔT3SE::+yopHEKM strain. T3SS induction of macrophage cell death, while important for *Y. pestis* pathogenesis, does not appear to require either of these effectors. The work presented here is in agreement with reports that, though NLRP3/NLRC4-mediated cell death has been shown to occur in response to the needle and translocon proteins of the JG150ΔT3SE strain (21, 44), macrophage cell death mediated by the wild-type T3SS seems to depend primarily on the activity of YopJ (34, 43). The T3SS of *Y. pestis* also targets neutrophils *in vivo* (7, 20, 55), and neutrophils are key players in controlling *Y. pestis* infection (20, 56, 57). However, our work in *Y. pestis* attributes anti-neutrophil activity primarily to YopH and YopE (Figures S3-S5), consistent with previous work focusing on *Y. pseudotuberculosis* and *Y. enterocolitica* (22, 23, 58-60).

Interestingly, both YopT and YpkA interfere with Rho signaling in mammalian cells. YopT is a cysteine protease that cleaves the prenylated moiety from small GTPases of the Rho family, including RhoA and Rac1, to reduce their activity by releasing them from the cytoplasmic membrane (61). In *Y. pseudotuberculosis*, YopT also inhibits RhoG via this mechanism. This activity synergizes with YopE inhibition of RhoG to decrease

phagocytic uptake of *Yersinia* (62). YopT of *Y. enterocolitica* upregulates transcription of the anti-inflammatory GILZ protein in HeLa cells and in a monocyte cell line (63), though whether this is conserved in *Y. pestis* and functional during infection is unknown. Like YopT, YpkA inhibits Rho GTPases including RhoA, Rac1, and Rac2 (summarized in (8)). Rho GTPases are also key host targets of YopE activity. The importance of multiple effectors for deranging Rho signaling is not clear, though it is possible that differential tissue tropism or effector kinetics may play a role. Future work with the set of strains we report here may provide clues regarding the function of YopT and YpkA during infection. Promising lines of inquiry include measuring the effect of these effectors on cytokine production *in vivo*, on the kinetics of distal organ colonization following subcutaneous infection, and on the ability of *Y. pestis* to establish and maintain sufficient bacteremia to reliably infect fleas feeding on infected mammals.

In addition to refining the model of *Y. pestis* pathogenesis and creating genetic tools that will streamline further study of this T3SS in the yersiniae, this work represents a general strategy for effective and efficient genetic analysis of complex bacterial systems by unmasking the functional contributions of individual components. Genetic analysis of partially redundant systems, even those of only moderate complexity such as the seven effectors of the *Yersinia* T3SS, is difficult to perform in a comprehensive manner. Combinatorial knockouts are the traditional approach to identifying functional redundancy, but an unbiased combinatorial approach rapidly becomes unfeasible as the size of the system increases. For example, even constructing and assaying all 21 possible double knockouts of *Y. pestis* T3SS effectors is not an attractive approach, and there is no guarantee that functional redundancy is limited to only two effectors. The “bottom-up”

approach we describe here, to identify effector(s) that are sufficient rather than necessary for various phenotypes, is perhaps generalizable to other complex systems. We have shown that this approach can be particularly effective when combined with candidate-based hypothesis testing, as this limits the number of combinations that must be examined. Once such strains are generated, they can be assayed for multiple phenotypes in both *in vitro* and *in vivo* systems. These strain banks, therefore, allow for rapid systematic identification and disentanglement of apparent functional redundancy among components of complex bacterial systems.

Materials and methods

Bacterial strains and growth conditions. The genotype and source for *Y. pestis* strains are presented in Table S1. Genotype information includes the codons removed by each in-frame effector deletion: for example, the notation $yopH^{\Delta 3\text{-}467}$ denotes that the *yopH* gene harbors an in-frame deletion of codons 3-467 (inclusive). *In vivo* experiments (lethality, liver histology) were performed using strains made in the fully virulent KIM1001 background. *In vitro* experiments (macrophage cell death and bacterial survival in the presence of neutrophils) were performed in the JG150 background (equivalent to KIM5), which lacks the *pgm* locus required for iron acquisition, to permit experimentation under biosafety level 2 conditions.

Y. pestis was cultured in the rich medium TB, prepared to maximize plating efficiency as previously described (64), or in the defined Serum Nutritional Medium (SNM) (18). All *Y. pestis* cultures were supplemented with 2.5 mM $CaCl_2$ to suppress type III secretion. *E. coli* strains used in construction of *Y. pestis* mutants were cultured in Luria broth. Media were supplemented with 100 μg/ml ampicillin and/or 25 μg/ml

diaminopimelic acid as appropriate. *Y. pestis* strains are available on request to researchers with qualifying regulatory approvals (contact: Megan.Proulx@umassmed.edu).

Construction and complementation of nonpolar mutant strains of *Y. pestis*. *Y. pestis* mutants were constructed via allelic exchange with the suicide vector pRE107 ((65), gift from D. Schifferli). Primers are listed in Table S2. Deletion mutants for each gene were constructed by amplifying flanking homology to the gene using primer pairs A+B and C+D, hybridizing the resulting fragments, and cloning this "stitched" product into the pRE107 plasmid before proceeding with allelic exchange as described (65). The *E. coli* donor strain β2155 ((66), gift from B. Akerley) was used to propagate pRE107 derivatives and to introduce them into *Y. pestis* by conjugation. Complementation of effector mutants was performed *in situ*, using pRE107-based allelic exchange to replace each deleted gene with a wild-type copy amplified using the primer pair A+D for each gene. Attenuated mutants for use in *in vitro* assays were generated by screening for spontaneous loss of the *pgm* locus on HIB agar with Congo Red, verified by PCR as described (21). Luminescent derivatives were constructed by electroporation of the pML001 plasmid containing the *lux* operon from *Photorhabdus luminescens* (67) followed by selection of transformants on media supplemented with ampicillin.

Animal infections. All animal infections were conducted in conformity with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and with the review and approval of the UMass Medical School Institutional Animal Care and Use Committee (IACUC). C57BL/6 mice were infected as indicated. All bacterial cultures used for inoculation were grown at 37°C on TB agar (TB medium with 1.5% agar)

containing 2.5 mM $CaCl_2$ for one overnight prior to infection. Bacterial cells were diluted in infection-grade phosphate-buffered saline (PBS) to the desired concentration. In every case, the number of viable bacteria present in each dose was verified by dilution plating of the inoculum. Mice were monitored every twelve hours for signs of illness such as ruffled fur, shallow breathing, limping, reluctance to move, and swollen lymph nodes.

Histological analysis of T3SS mutants in liver tissue. Mice were sacrificed 48 hours following intravenous infection with 10^3 CFUs. Livers were removed, fixed in 10% neutral-buffered formalin, and embedded in paraffin for sectioning and staining. Samples were randomized and 10 lesions from each mouse were scored, blinded, for severity of inflammation in bacterial lesions. The following scale was used for scoring: 1 = free bacteria with few or no inflammatory cells; 2 = some inflammatory cells present, but free bacteria fill the majority of the lesion area; 3 = lesion area is split approximately equally between inflammatory cells and bacteria; 4 = some free bacteria are visible, but inflammatory cells fill the majority of the lesion area; 5 = abundant inflammatory cells with few or no visible free bacteria. Scoring was performed twice on scrambled, blinded samples to ensure results were consistent.

Macrophage cell death experiments. Macrophages were monitored for cell death by ethidium homodimer (EthD1) fluorescence, as described (21). Briefly, $8x10^4$ immortalized murine macrophages from C57BL/6 mice (a gift from K. Fitzgerald) were infected with $8x10^4$ CFU of various *Y. pestis* strains grown to mid-log phase in SNM supplemented with 2.5 mM $CaCl_2$. Macrophages and bacteria were added to flat-bottomed 96-well plates with black sides in DMEM supplemented with 10% ΔFBS, 10 mM HEPES, and 2 μM ethidium homodimer. Plates were centrifuged at 400 rpm for five

minutes, sealed, and incubated at 37°C in a Synergy H4 microplate reader to monitor ethidium homodimer fluorescence (645 nm emission, 530 nm excitation). Ethidium homodimer uptake data was analyzed by calculating the area under the curve (AUC) for the increasing fluorescence signal and subtracting the AUC of control wells containing uninfected macrophages.

Survival of T3SS in co-culture with primary human neutrophils. Viability assays were performed with bioluminescent *Y. pestis* strains using the plasmid pML001, which encodes the *lux* operon from *Photorhabdus luminescens* (67). Luminescence from this system requires the reduced flavin mononucleotide $FMNH_2$ as a cofactor (68). As reduced $FMNH_2$ is rapidly depleted if metabolism or the cell membrane is disrupted, bioluminescence in this system serves as a proxy for determining viability of the bacterial population in real time. CFU plating confirmed that the decreased luminescence of a T3SS-deficient strain after 4 hours in the presence of neutrophils (Figure S2) corresponded to a 50-80% reduction in bacterial viability compared to the media-only (no neutrophil) condition.

Whole blood was collected from healthy adult human volunteers in compliance with protocols reviewed and approved by the University of Massachusetts Medical School Institutional Review Board (IRB). Neutrophils were isolated from whole blood on a gelatin gradient as described (69). *Y. pestis* viability assays were performed in opaque white flat-bottomed 96-well plates that were coated for 1 hour with 10 μg/mL fibrinogen in phosphate-buffered saline (PBS) and washed twice with PBS. $5x10^5$ neutrophils were infected with luminescent strains of *Y. pestis* at MOI 0.1 in SNM supplemented with 2.5 mM $CaCl_2$, 100 μg/ml ampicillin, and 4% normal human serum. Plates were centrifuged

at 400 rpm for five minutes, sealed, and incubated at 37°C in a Synergy H4 microplate reader. Bacterial luminescence was monitored in for six hours. Three independent bacterial cultures were assayed for each *Y. pestis* strain in each experiment, and experiments were performed at least twice with neutrophils from different donors.

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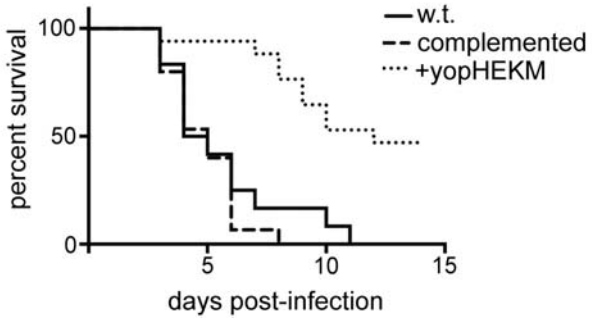
Table 1

Strain	Percent lethality (number of mice killed/total number of mice infected)	Median time to death (days)	Significance against KIM1001 (p value)
KIM1001ΔT3SE	0% (0/8)	NA	< 0.0001
KIM1001ΔT3SE::+yopHE	0% (0/10)	NA	< 0.0001
KIM1001ΔT3SE::+yopHEM	0% (0/6)	NA	< 0.0001
KIM1001ΔT3SE::+yopHEK	0% (0/6)	NA	< 0.0001
KIM1001ΔT3SE::+yopHEKM	53% (9/17)	9	< 0.0001
KIM1001ΔT3SE::+yopHEKM**A**	88% (14/16)	6	0.1305
KIM1001ΔT3SE::+yopHEKM**T**	87% (13/15)	6	0.1184
KIM1001ΔT3SE::+yopHEKM**J**	85% (22/26)	5.5	0.1031
KIM1001ΔT3SE::+yopHEKM**JT**	100% (5/5)	7	0.1501
KIM1001ΔT3SE::+yopHEKM**AJ**	100% (5/5)	7	0.4756
KIM1001ΔT3SE::+yopHEKM**AT**	80% (4/5)	7	0.1206
KIM1001ΔT3SE::+yopHEKM**AJT**	100% (15/15)	5	0.3890
KIM1001	100% (12/12)	4.5	NA

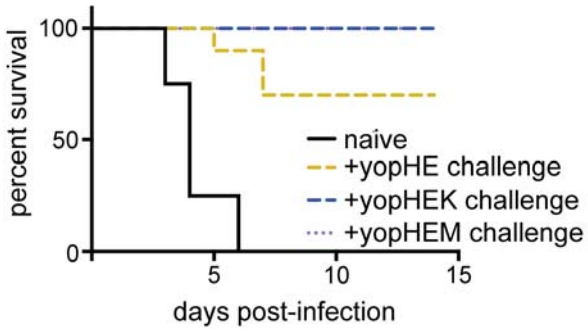
Virulence of *Y. pestis* strains expressing subsets of type III secretion effector proteins. All infection experiments were performed with a subcutaneous dose of $1x10^3$ CFU in C57BL/6 mice. Median time to death references only those mice within each group that succumbed to infection; any mice that survived the duration of the experiment were excluded from the calculation of this metric. Significance was calculated for each curve compared to the KIM1001 curve by the Mantel-Cox test.

Figure 1



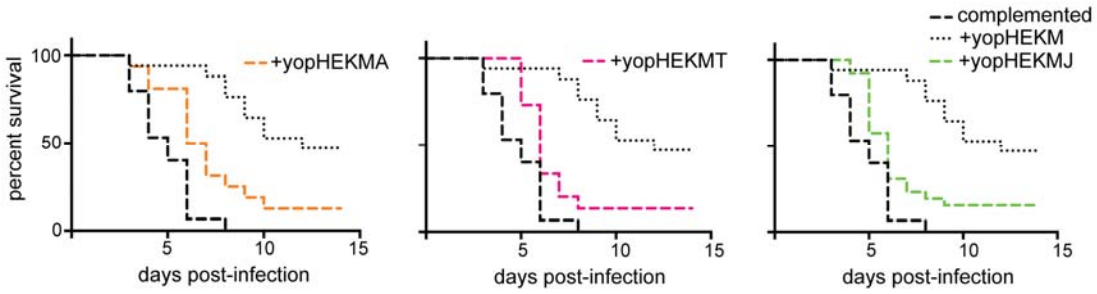
YopH, YopE, YopK, and YopM are not sufficient for full virulence. Subcutaneous infection with 1000 CFU KIM1001 (w.t.) (n=12), KIM1001ΔT3SE::+yopHEKMAJT (complemented) (n=15), or KIM1001ΔT3SE::+yopHEKM (+yopHEKM) (n=17). Mice infected with KIM1001 or with the fully complemented strain rapidly succumbed to infection, while KIM1001ΔT3SE::+yopHEKM killed 9 out of 17 mice with delayed kinetics.

Figure 2



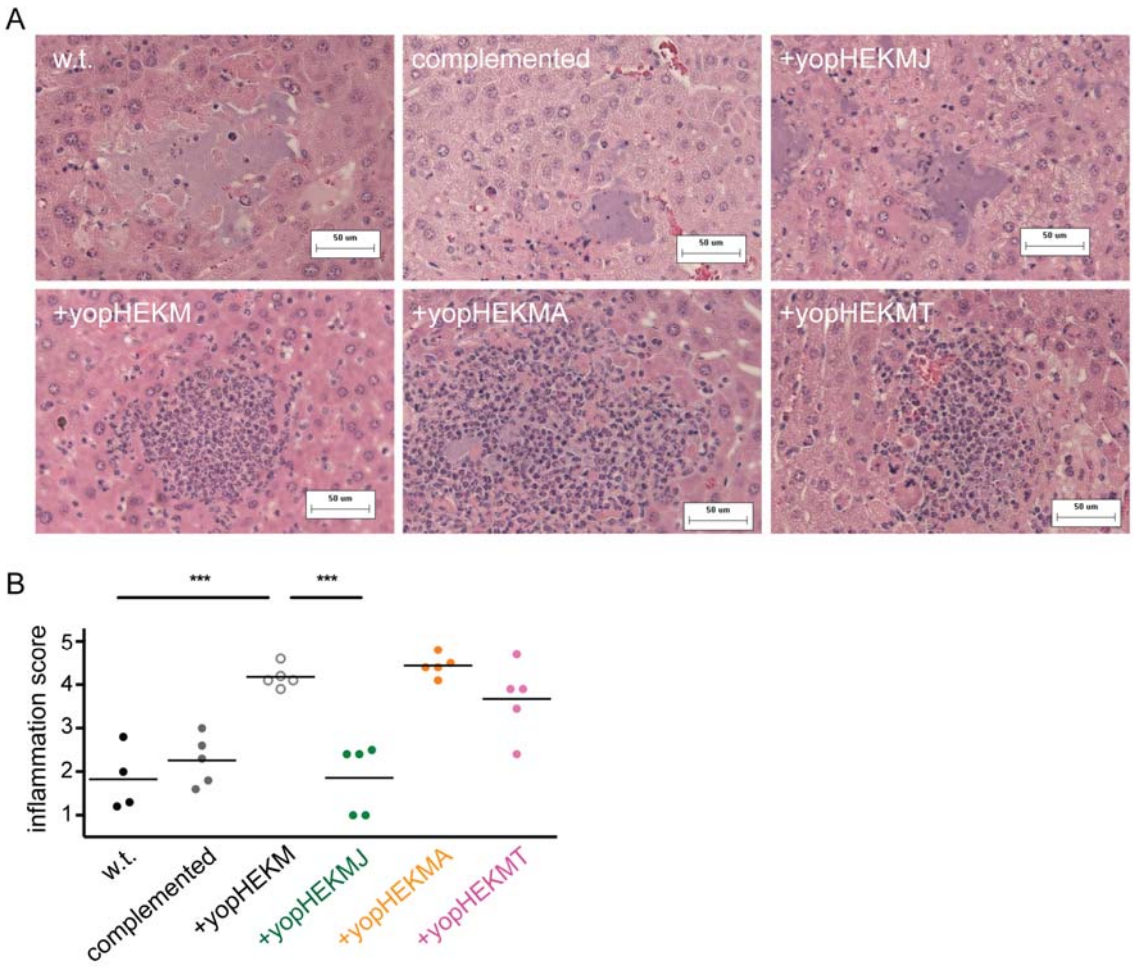
Protective immunity from exposure to *Y. pestis* mutants expressing subsets of T3SS effectors. When challenged with 1000 CFU KIM1001 28 days after initial infection, mice exposed to KIM1001ΔT3SE::+yopHEM and KIM1001ΔT3SE::+yopHEK were fully protected (n=6 mice each), while exposure to KIM1001ΔT3SE::+yopHE provided partial protection (3 of 10 mice died). Naïve mice succumbed to infection within seven days (n=8).

Figure 3



Addition of YpkA, YopT, or YopJ enhances the virulence of a *Y. pestis* strain expressing YopH, YopE, YopK, and YopM. Survival following subcutaneous infection with 1000 CFU KIM1001ΔT3SE::+yopHEKMA (+yopHEKMA) (n=16), KIM1001ΔT3SE::+yopHEKMT (+yopHEKMT) (n=15), or KIM1001ΔT3SE::+yopHEKMJ (+yopHEKMJ) (n=26), compared to the survival curves for KIM1001ΔT3SE::+yopHEKMAJT (complemented) and KIM1001ΔT3SE::+yopHEKM (+yopHEKM) from Figure 1. KIM1001ΔT3SE::+yopHEKMA killed 14 out of 16 mice, KIM1001ΔT3SE::+yopHEKMT killed 13 out of 15 mice, and KIM1001ΔT3SE::+yopHEKMJ killed 22 out of 26 mice, all with kinetics similar to KIM1001ΔT3SE::+yopHEKMAJT.

Figure 4



YopJ suppresses immune cell recruitment to foci of bacterial growth in liver tissue. (**A**) Representative liver sections stained with hematoxylin and eosin from mice 48 hours after intravenous infection with 10^3 CFU KIM1001 (w.t.), KIM1001ΔT3SE::+yopHEKMAJT (complemented), KIM1001ΔT3SE::+yopHEKMJ (+yopHEKMJ), KIM1001ΔT3SE::+yopHEKM (+yopHEKM), KIM1001ΔT3SE::+yopHEKMA (+yopHEKMA), or KIM1001ΔT3SE::+yopHEKMT (+yopHEKMT). Strains with a functional *yopJ* allele grow freely in liver tissue without attracting inflammatory cells (top row), in contrast to strains deficient in *yopJ* (bottom row). (**B**) Severity of inflammation was scored on an arbitrary scale (1 = free bacteria with few or no inflammatory cells; 5 = abundant inflammatory cells with little or no visible free bacteria). Each data point represents the average score for 10 lesions from a single mouse. Representative of two independent blinded scorings.

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