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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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Authors	Palace, Samantha G.;Proulx, Megan K.;Szabady, Rose L.;Goguen, Jon D.
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1 Gain of Function Analysis Reveals Non-Redundant Roles for the *Yersinia pestis* Type III  
2 Secretion System Effectors YopJ, YopT, and YpkA

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5 Samantha G. Palace<sup>a\*</sup>, Megan K. Proulx<sup>a</sup>, Rose L. Szabady<sup>a\*</sup>, and Jon D. Goguen<sup>a#</sup>

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7 <sup>a</sup>Department of Microbiology and Physiological Systems, University of Massachusetts

8 Medical School, Worcester, Massachusetts, USA

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

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12 # Address correspondence to Jon D. Goguen, [jon.goguen@umassmed.edu](mailto:jon.goguen@umassmed.edu)

13 \* Present addresses:

14 Samantha G. Palace: Department of Immunology and Infectious Diseases, Harvard T. H.

15 Chan School of Public Health, Boston, Massachusetts, USA

16 Rose L. Szabady: Vedanta Biosciences, Inc., Cambridge, Massachusetts, USA

17 **Abstract**

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

33 **Introduction**

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

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

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

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

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

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

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

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

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

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

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

109 **Results**

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

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

114 We approached this problem by first constructing KIM1001 $\Delta$ T3SE, an unmarked  
115 strain that retains the T3SS injectisome, regulatory elements, and translocon proteins, but  
116 carries in-frame deletions in the open reading frames (ORFs) for all seven effector  
117 proteins (see Table S1 for details). This strain, constructed in the fully-virulent  
118 background KIM1001 (38), was highly attenuated through the subcutaneous route of  
119 infection. When infected with  $10^3$  CFU KIM1001 $\Delta$ T3SE, 0 of 8 mice developed visible  
120 symptoms of disease, and all mice survived infection (Table 1).

121 Functional ORFs for various effectors were restored at their original loci in the  
122  $\Delta$ T3SE genetic background to generate strains expressing defined subsets of effectors.  
123 YopH and YopE are more strictly required for bacterial fitness *in vivo* than any of the  
124 other effector proteins (18). However, we found that these two effectors in combination  
125 are not sufficient for virulence. The strain KIM1001 $\Delta$ T3SE::*yopHE*, expressing YopH  
126 and YopE but carrying in-frame deletions in all of the remaining five effectors' ORFs,  
127 failed to sicken or kill any mice following subcutaneous infection with  $10^3$  CFU (n=10).  
128 By contrast, this dose is uniformly fatal with wild-type KIM1001 (Table 1).

129 Single deletion studies have demonstrated that YopM and YopK are also essential  
130 for full virulence of *Y. pestis* (19, 20). However, neither of these effectors was sufficient  
131 to restore virulence in the KIM1001 $\Delta$ T3SE::*yopHE* background. Strains expressing

132 only YopH, YopE, and YopM (KIM1001 $\Delta$ T3SE::+yopHEM) or YopH, YopE, and  
133 YopK (KIM1001 $\Delta$ T3SE::+yopHEK) remained attenuated (0 out of 6 mice killed for each  
134 group following subcutaneous infection with  $10^3$  CFU) (Table 1), though 2 out of 6 mice  
135 infected with KIM1001 $\Delta$ T3SE::+yopHEK lost their fur around the injection site and  
136 developed local redness of the skin that persisted for at least 28 days.

137 The KIM1001 $\Delta$ T3SE::+yopHEKM strain expresses all effectors previously  
138 reported to be necessary for full virulence, as assessed by subcutaneous infection based of  
139 single knockout mutants. This strain was substantially more virulent than the previous  
140 strains expressing subsets of these effectors, but significantly attenuated relative to the  
141 wild-type strain. KIM1001 $\Delta$ T3SE::+yopHEKM killed approximately 50% of infected  
142 mice (9 out of 17) at a dose of  $10^3$  CFU, indicating a ~100-fold increase of LD<sub>50</sub>  
143 compared to wild-type KIM1001. Simultaneously restoring functional copies of the *ypkA*,  
144 *yopJ*, and *yopT* ORFs to this strain (generating the strain  
145 KIM1001 $\Delta$ T3SE::+yopHEKMAJT, genetically identical to the wild-type strain  
146 KIM1001) fully complemented its virulence defect, restoring virulence to wild-type  
147 levels as expected. This result also confirmed that the long series of genetic  
148 manipulations required for sequentially deleting and then restoring the seven effector  
149 genes did not cause unexpected or off-target modifications that alter virulence  
150 phenotypes (Figure 1 and Table 1).

151 **Strains expressing YopK or YopM in addition to YopH and YopE induce more**  
152 **effective adaptive immunity.** The avirulence of strains KIM1001 $\Delta$ T3SE::+yopHE,  
153 KIM1001 $\Delta$ T3SE::+yopHEM, and KIM1001 $\Delta$ T3SE::+yopHEK led us to investigate their  
154 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 KIM1001 $\Delta$ T3SE::+yopHEM or  
157 KIM1001 $\Delta$ T3SE::+yopHEK uniformly survived the challenge, while 3 out of 10 mice  
158 exposed to KIM1001 $\Delta$ T3SE::+yopHE succumbed within 14 days (Figure 2). The full  
159 protection from challenge conferred by KIM1001 $\Delta$ T3SE::+yopHEK and  
160 KIM1001 $\Delta$ T3SE::+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 KIM1001 $\Delta$ T3SE::+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 KIM1001 $\Delta$ T3SE::+yopHEKM relative to  
168 KIM1001 $\Delta$ T3SE::+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 KIM1001 $\Delta$ T3SE::+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 KIM1001 $\Delta$ T3SE::+yopHEKMA, expressing YpkA;  
175 KIM1001 $\Delta$ T3SE::+yopHEKMT, expressing YopT; and KIM1001 $\Delta$ T3SE::+yopHEKMJ,  
176 expressing YopJ. Each of these strains was substantially more virulent than  
177 KIM1001 $\Delta$ T3SE::+yopHEKM. Although none caused 100% mortality, each was virulent

178 enough that the difference between survival curves for these strains and the wild-type  
179 strain KIM1001 was not statistically significant (Figure 3 and Table 1). Given the distinct  
180 biochemical activities of YopT, YpkA, and YopJ, it is curious that each of these effectors  
181 increased virulence of the KIM1001 $\Delta$ T3SE::+yopHEKM approximately equally. The  
182 different targets and activities reported for these effectors suggest that they are not truly  
183 redundant at the molecular level, and may therefore enhance virulence through distinct  
184 processes.

185 **YopJ suppresses immune cell recruitment in the liver.** Evaluation of liver pathology  
186 following intravenous infection is a useful method to assay immune cell responses to *Y.*  
187 *pestis* (21, 39, 40). KIM1001 $\Delta$ T3SE::+yopHEKM and the strains that additionally  
188 expressed YpkA, YopT, or YopJ were injected intravenously into mice. Livers were  
189 collected 48 hours after infection for histopathological analysis.

190 KIM1001 $\Delta$ T3SE::+yopHEKM elicited robust recruitment of immune cells, whereas  
191 KIM1001 $\Delta$ T3SE::+yopHEKMA**JT**, like KIM1001, effectively suppressed accumulation  
192 of inflammatory cells at foci of bacterial growth (Figure 4A-B). The addition of YopJ to  
193 KIM1001 $\Delta$ T3SE::+yopHEKM appears sufficient to fully suppress immune cell  
194 recruitment in this context, as lesions caused by KIM1001 $\Delta$ T3SE::+yopHEKM**J** were  
195 indistinguishable from those caused by KIM1001. By contrast, neither  
196 KIM1001 $\Delta$ T3SE::+yopHEKMA nor KIM1001 $\Delta$ T3SE::+yopHEKM**T** suppressed  
197 inflammatory cell recruitment relative to KIM1001 $\Delta$ T3SE::+yopHEKM (Figure 4A-B).  
198 YopJ, therefore, appears to be uniquely essential (though likely not sufficient) for  
199 suppressing accumulation of immune cells at sites of bacterial replication *in vivo*.

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

205         Some incomplete sets of *Yersinia* effectors result increased macrophage death,  
206 either via YopE induction of pyroptosis (30, 31) or as a result of dysregulated effector  
207 and translocon secretion in the absence of YopK (32, 41). However, the  
208  $\Delta$ T3SE::+yopHEKM strain expresses both YopM, which prevents YopE-mediated  
209 activation of the pyrin inflammasome (30, 31), and YopK, which prevents inflammasome  
210 activation by translocon components (41, 42). As expected, therefore, this strain resulted  
211 in minimal macrophage death (Figure S1).

212         Consistent with previous reports (21, 43, 44), we observed T3SS-dependent cell  
213 death induced by YopJ. The  $\Delta$ T3SE::+yopHEKMJ strain caused macrophage death at a  
214 level indistinguishable from the wild-type strain (Figure S1). This YopJ-mediated cell  
215 death may contribute to the reduced visible recruitment of innate immune cells *in vivo*  
216 (Figure 4). Addition of YopT or YpkA to a strain expressing YopE, YopH, YopK, and  
217 YopM had no effect on macrophage cell death.

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

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

## 236 **Discussion**

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

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

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

263 YopJ, YopT, and YpkA increase lethality approximately equally, but have  
264 different biochemical activities and targets from one another. Strains expressing each of  
265 these effectors also behave differently in more targeted assays, supporting the model that  
266 these effectors contribute to pathogenesis independently rather than redundantly. For  
267 example, addition of YopJ to the KIM1001 $\Delta$ T3SE::+yopHEKM construct blocks  
268 accumulation of inflammatory cells at foci of infection in deep tissue, while addition of

269 either YopT or YpkA to KIM1001 $\Delta$ T3SE::+yopHEKM does not produce any obvious  
270 histological signature (Figure 4). This is consistent with a recent finding by our  
271 colleagues Ratner *et al.*, who report a similar phenotype in a YopJ single deletion strain  
272 (21). Ratner *et al.* also demonstrate that, when YopM is absent, YopJ is necessary for full  
273 virulence of *Y. pestis* following subcutaneous infection. The YopM-independent role for  
274 YopJ in virulence that we report here is a novel finding.

275         Although we have now established a functional role for YopT and YpkA in  
276 infection, we cannot yet explain how these effectors enhance the virulence of the  
277 KIM1001 $\Delta$ T3SE::+yopHEKM strain. T3SS induction of macrophage cell death, while  
278 important for *Y. pestis* pathogenesis, does not appear to require either of these effectors.  
279 The work presented here is in agreement with reports that, though NLRP3/NLRC4-  
280 mediated cell death has been shown to occur in response to the needle and translocon  
281 proteins of the JG150 $\Delta$ T3SE strain (21, 44), macrophage cell death mediated by the wild-  
282 type T3SS seems to depend primarily on the activity of YopJ (34, 43). The T3SS of *Y.*  
283 *pestis* also targets neutrophils *in vivo* (7, 20, 55), and neutrophils are key players in  
284 controlling *Y. pestis* infection (20, 56, 57). However, our work in *Y. pestis* attributes anti-  
285 neutrophil activity primarily to YopH and YopE (Figures S3-S5), consistent with  
286 previous work focusing on *Y. pseudotuberculosis* and *Y. enterocolitica* (22, 23, 58-60).

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

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

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

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

### 323 **Materials and methods**

324 **Bacterial strains and growth conditions.** The genotype and source for *Y. pestis* strains  
325 are presented in Table S1. Genotype information includes the codons removed by each  
326 in-frame effector deletion: for example, the notation  $yopH^{A3-467}$  denotes that the *yopH*  
327 gene harbors an in-frame deletion of codons 3-467 (inclusive). *In vivo* experiments  
328 (lethality, liver histology) were performed using strains made in the fully virulent  
329 KIM1001 background. *In vitro* experiments (macrophage cell death and bacterial survival  
330 in the presence of neutrophils) were performed in the JG150 background (equivalent to  
331 KIM5), which lacks the *pgm* locus required for iron acquisition, to permit  
332 experimentation under biosafety level 2 conditions.

333 *Y. pestis* was cultured in the rich medium TB, prepared to maximize plating  
334 efficiency as previously described (64), or in the defined Serum Nutritional Medium  
335 (SNM) (18). All *Y. pestis* cultures were supplemented with 2.5 mM CaCl<sub>2</sub> to suppress  
336 type III secretion. *E. coli* strains used in construction of *Y. pestis* mutants were cultured in  
337 Luria broth. Media were supplemented with 100 µg/ml ampicillin and/or 25 µg/ml

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

341 **Construction and complementation of nonpolar mutant strains of *Y. pestis*.** *Y. pestis*  
342 mutants were constructed via allelic exchange with the suicide vector pRE107 ((65), gift  
343 from D. Schifferli). Primers are listed in Table S2. Deletion mutants for each gene were  
344 constructed by amplifying flanking homology to the gene using primer pairs A+B and  
345 C+D, hybridizing the resulting fragments, and cloning this “stitched” product into the  
346 pRE107 plasmid before proceeding with allelic exchange as described (65). The *E. coli*  
347 donor strain  $\beta$ 2155 ((66), gift from B. Akerley) was used to propagate pRE107  
348 derivatives and to introduce them into *Y. pestis* by conjugation. Complementation of  
349 effector mutants was performed *in situ*, using pRE107-based allelic exchange to replace  
350 each deleted gene with a wild-type copy amplified using the primer pair A+D for each  
351 gene. Attenuated mutants for use in *in vitro* assays were generated by screening for  
352 spontaneous loss of the *pgm* locus on HIB agar with Congo Red, verified by PCR as  
353 described (21). Luminescent derivatives were constructed by electroporation of the  
354 pML001 plasmid containing the *lux* operon from *Photobacterium luminescens* (67)  
355 followed by selection of transformants on media supplemented with ampicillin.

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

361 containing 2.5 mM CaCl<sub>2</sub> for one overnight prior to infection. Bacterial cells were diluted  
362 in infection-grade phosphate-buffered saline (PBS) to the desired concentration. In every  
363 case, the number of viable bacteria present in each dose was verified by dilution plating  
364 of the inoculum. Mice were monitored every twelve hours for signs of illness such as  
365 ruffled fur, shallow breathing, limping, reluctance to move, and swollen lymph nodes.

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

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

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

389 **Survival of T3SS in co-culture with primary human neutrophils.** Viability assays  
390 were performed with bioluminescent *Y. pestis* strains using the plasmid pML001, which  
391 encodes the *lux* operon from *Photobacterium luminescens* (67). Luminescence from this  
392 system requires the reduced flavin mononucleotide FMNH<sub>2</sub> as a cofactor (68). As  
393 reduced FMNH<sub>2</sub> is rapidly depleted if metabolism or the cell membrane is disrupted,  
394 bioluminescence in this system serves as a proxy for determining viability of the bacterial  
395 population in real time. CFU plating confirmed that the decreased luminescence of a  
396 T3SS-deficient strain after 4 hours in the presence of neutrophils (Figure S2)  
397 corresponded to a 50-80% reduction in bacterial viability compared to the media-only (no  
398 neutrophil) condition.

399 Whole blood was collected from healthy adult human volunteers in compliance  
400 with protocols reviewed and approved by the University of Massachusetts Medical  
401 School Institutional Review Board (IRB). Neutrophils were isolated from whole blood on  
402 a gelatin gradient as described (69). *Y. pestis* viability assays were performed in opaque  
403 white flat-bottomed 96-well plates that were coated for 1 hour with 10 µg/mL fibrinogen  
404 in phosphate-buffered saline (PBS) and washed twice with PBS. 5x10<sup>5</sup> neutrophils were  
405 infected with luminescent strains of *Y. pestis* at MOI 0.1 in SNM supplemented with 2.5  
406 mM CaCl<sub>2</sub>, 100 µg/ml ampicillin, and 4% normal human serum. Plates were centrifuged

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

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421 role of T3SS effectors in potentiating host cell death.

422 **Table 1**

<b>Strain</b>	<b>Percent lethality (number of mice killed/total number of mice infected)</b>	<b>Median time to death (days)</b>	<b>Significance against KIM1001 (p value)</b>
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::+yopHEKMA	88% (14/16)	6	0.1305
KIM1001ΔT3SE::+yopHEKMT	87% (13/15)	6	0.1184
KIM1001ΔT3SE::+yopHEKMJ	85% (22/26)	5.5	0.1031
KIM1001ΔT3SE::+yopHEKMJT	100% (5/5)	7	0.1501
KIM1001ΔT3SE::+yopHEKMAJ	100% (5/5)	7	0.4756
KIM1001ΔT3SE::+yopHEKMAT	80% (4/5)	7	0.1206
KIM1001ΔT3SE::+yopHEKMAJT	100% (15/15)	5	0.3890
KIM1001	100% (12/12)	4.5	NA

423

424 **Virulence of *Y. pestis* strains expressing subsets of type III secretion effector**

425 **proteins.** All infection experiments were performed with a subcutaneous dose of  $1 \times 10^3$

426 CFU in C57BL/6 mice. Median time to death references only those mice within each

427 group that succumbed to infection; any mice that survived the duration of the experiment

428 were excluded from the calculation of this metric. Significance was calculated for each

429 curve compared to the KIM1001 curve by the Mantel-Cox test.

430

431 **Figure 1**

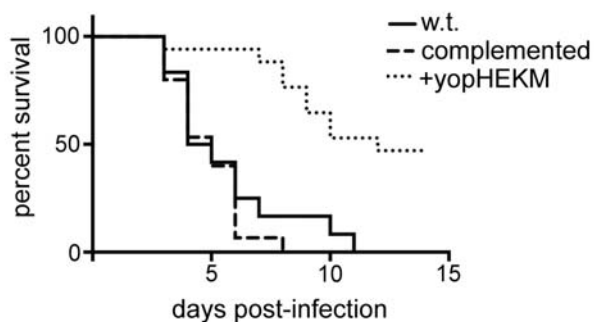
432

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434

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436



437 **YopH, YopE, YopK, and YopM are not sufficient for full virulence.** Subcutaneous

438 infection with 1000 CFU KIM1001 (w.t.) (n=12), KIM1001 $\Delta$ T3SE::+yopHEKMAJT

439 (complemented) (n=15), or KIM1001 $\Delta$ T3SE::+yopHEKM (+yopHEKM) (n=17). Mice

440 infected with KIM1001 or with the fully complemented strain rapidly succumbed to

441 infection, while KIM1001 $\Delta$ T3SE::+yopHEKM killed 9 out of 17 mice with delayed

442 kinetics.

443 **Figure 2**

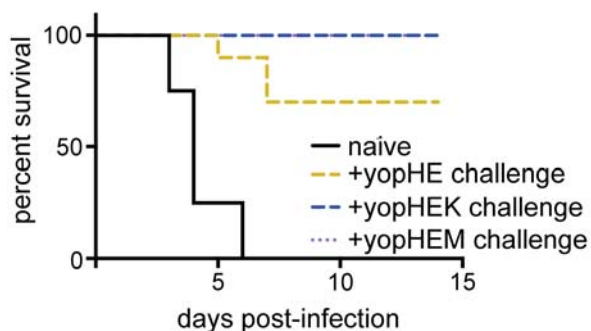
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448



449 **Protective immunity from exposure to *Y. pestis* mutants expressing subsets of T3SS**

450 **effectors.** When challenged with 1000 CFU KIM1001 28 days after initial infection,

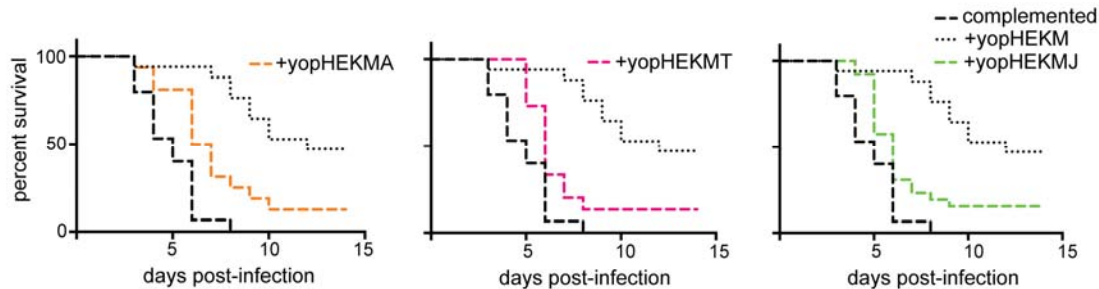
451 mice exposed to KIM1001 $\Delta$ T3SE::+yopHEM and KIM1001 $\Delta$ T3SE::+yopHEK were

452 fully protected (n=6 mice each), while exposure to KIM1001 $\Delta$ T3SE::+yopHE provided

453 partial protection (3 of 10 mice died). Naïve mice succumbed to infection within seven

454 days (n=8).

455 **Figure 3**



456

457 **Addition of YpkA, YopT, or YopJ enhances the virulence of a *Y. pestis* strain**

458 **expressing YopH, YopE, YopK, and YopM.** Survival following subcutaneous infection

459 with 1000 CFU KIM1001 $\Delta$ T3SE::+yopHEKMA (+yopHEKMA) (n=16),

460 KIM1001 $\Delta$ T3SE::+yopHEKMT (+yopHEKMT) (n=15), or

461 KIM1001 $\Delta$ T3SE::+yopHEKMJ (+yopHEKMJ) (n=26), compared to the survival curves

462 for KIM1001 $\Delta$ T3SE::+yopHEKMAJT (complemented) and

463 KIM1001 $\Delta$ T3SE::+yopHEKM (+yopHEKM) from Figure 1.

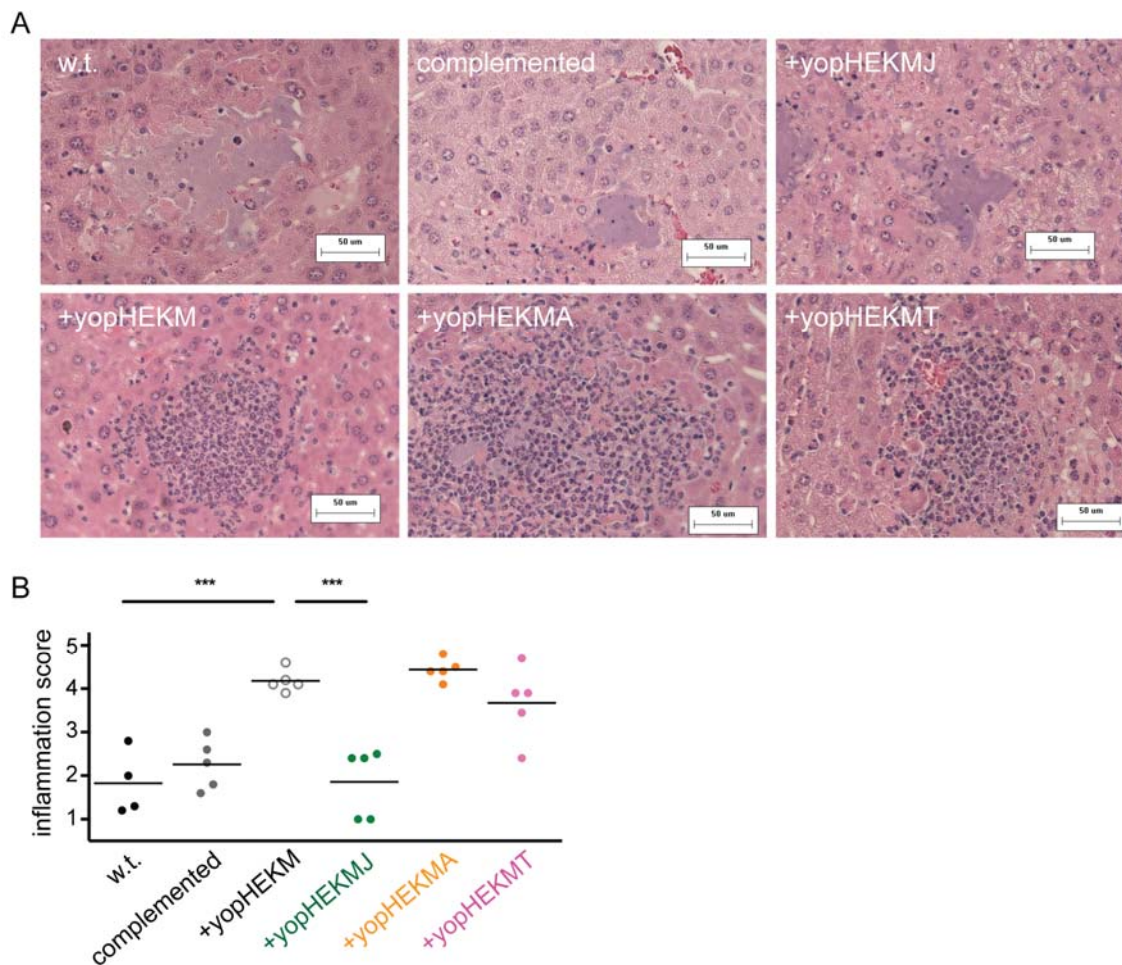
464 KIM1001 $\Delta$ T3SE::+yopHEKMA killed 14 out of 16 mice,

465 KIM1001 $\Delta$ T3SE::+yopHEKMT killed 13 out of 15 mice, and

466 KIM1001 $\Delta$ T3SE::+yopHEKMJ killed 22 out of 26 mice, all with kinetics similar to

467 KIM1001 $\Delta$ T3SE::+yopHEKMAJT.

468 **Figure 4**



469

470 **YopJ suppresses immune cell recruitment to foci of bacterial growth in liver tissue.**

471 (A) Representative liver sections stained with hematoxylin and eosin from mice 48 hours

472 after intravenous infection with  $10^3$  CFU KIM1001 (w.t.),

473 KIM1001 $\Delta$ T3SE::+yopHEKMAJT (complemented), KIM1001 $\Delta$ T3SE::+yopHEKMJ

474 (+yopHEKMJ), KIM1001 $\Delta$ T3SE::+yopHEKM (+yopHEKM),

475 KIM1001 $\Delta$ T3SE::+yopHEKMA (+yopHEKMA), or KIM1001 $\Delta$ T3SE::+yopHEKMT

476 (+yopHEKMT). Strains with a functional *yopJ* allele grow freely in liver tissue without

477 attracting inflammatory cells (top row), in contrast to strains deficient in *yopJ* (bottom

478 row). (B) Severity of inflammation was scored on an arbitrary scale (1 = free bacteria

479 with few or no inflammatory cells; 5 = abundant inflammatory cells with little or no

480 visible free bacteria). Each data point represents the average score for 10 lesions from a

481 single mouse. Representative of two independent blinded scorings.

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