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# *Yersinia pestis* kills *Caenorhabditis elegans* by a biofilm-independent process that involves novel virulence factors

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**It is known that *Yersinia pestis* kills *Caenorhabditis elegans* by a biofilm-dependent mechanism that is similar to the mechanism used by the pathogen to block food intake in the flea vector. Using *Y. pestis* KIM5, which lacks the genes that are required for biofilm formation, we show that *Y. pestis* can kill *C. elegans* by a biofilm-independent mechanism that correlates with the accumulation of the pathogen in the intestine. We used this novel *Y. pestis–C. elegans* pathogenesis system to show that previously known and unknown virulence-related genes are required for full virulence in *C. elegans*. Six *Y. pestis* mutants with insertions in genes that are not related to virulence before were isolated using *C. elegans*. One of the six mutants carried an insertion in a novel virulence gene and showed significantly reduced virulence in a mouse model of *Y. pestis* pathogenesis. Our results indicate that the *Y. pestis–C. elegans* pathogenesis system that is described here can be used to identify and study previously uncharacterized *Y. pestis* gene products required for virulence in mammalian systems.**

Keywords: innate immunity; forward genetics; virulence factors; plague; host–pathogen interactions

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## INTRODUCTION

The soil nematode *Caenorhabditis elegans* has been used as a model host to study the pathogenic mechanisms of several human pathogens, including Gram-positive and Gram-negative bacteria, as well as fungal species (reviewed by Aballay & Ausubel, 2002; Ewbank, 2002; Alegado *et al*, 2003; Nicholas & Hodgkin, 2004b; Mylonakis & Aballay, 2005; Sifri *et al*, 2005). Recently, it was reported that *Yersinia* spp kill *C. elegans* by a mechanism that correlates with the formation of a biofilm in the head of the animals. It was proposed that biofilm production, which requires the *hmsHFRS* operon, prevents food intake and causes death by starvation (Darby *et al*, 2002). To identify other virulence factors that are required for the biofilm-mediated killing of *Yersinia pseudotuberculosis*, the virulence phenotype of 39 transposon insertion strains was studied in *C. elegans*. Seven mutants in unknown genes and in genes that encode factors involved in haemin storage and lipopolysaccharide biosynthesis were identified (Joshua *et al*, 2003). A later report indicates that biofilm formation does not involve signalling between *Yersinia* and *C. elegans* and that it depends on a continuous exposure of the nematodes to *Yersinia* (Tan & Darby, 2004).

We sought to develop a *Yersinia pestis–C. elegans* pathogenesis model that aided identification and characterization of virulence factors that are not necessarily related to biofilm formation but are important for mammalian pathogenesis. Using the *Y. pestis* strain KIM5, which lacks the *hmsHFRS* genes, and standard conditions for *C. elegans* infection, we show here that *Y. pestis* is able to cause a persistent and lethal intestinal infection in nematodes. We also show that several genes that were previously associated with virulence in other bacterial pathogens, *ompT*, *y3857* and *yapH*, are required for the full virulence of *Y. pestis* in *C. elegans*. To identify previously undescribed virulence factors, a *Y. pestis* EZ::TN mutagenized library containing 984 clones was screened to identify insertion mutants that show reduced virulence in nematodes. Two out of the six mutants isolated on the basis of their reduced virulence in *C. elegans* also showed reduced virulence in a mouse model of *Y. pestis* pathogenesis. One of these mutants harbours an insertion in a gene that has not been related to virulence before, the product of which is similar to a putative exported protein of *Salmonella enterica* serovar Typhi. These experiments show that *C. elegans* can be used as an alternative model host to accelerate the identification and characterization of *Y. pestis* virulence factors that are required for pathogenesis in mammalian systems.

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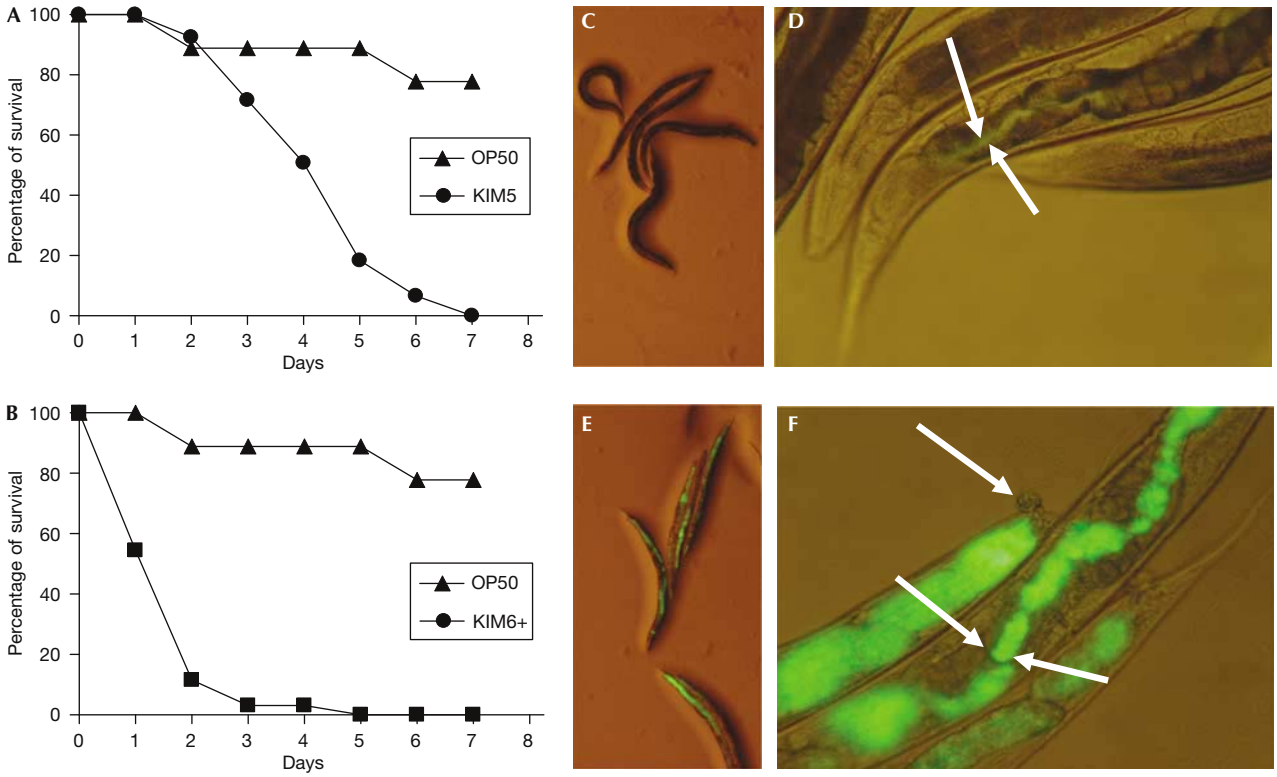


**Fig 1** | *Yersinia pestis* KIM5 accumulates in the *Caenorhabditis elegans* intestine and causes a lethal infection. (**A**) Groups of 40 *C. elegans* N2 (wild type) young adult animals were exposed to control *Escherichia coli* OP50 or *Y. pestis* KIM5 ($P<0.001$). (**B**) Groups of 40 *C. elegans* N2 young adult animals were exposed to control *E. coli* OP50 or *Y. pestis* KIM6+ ($P<0.001$). *C. elegans* N2 young adults were exposed to *E. coli* DH5α expressing green fluorescent protein (GFP) (**C,D**) or *Y. pestis* KIM5 expressing GFP (**E,F**) for 48 h, transferred to *E. coli* OP50 lawns and then visualized using a Leica MZ FLIII fluorescence stereomicroscope. The margins of the intestinal lumen of single nematodes are indicated with arrows. An arrow also points to the swollen tail of an animal infected with *Y. pestis*.

## RESULTS AND DISCUSSION

### *Y. pestis* kills *C. elegans* in a biofilm-independent manner

*Y. pestis* is the aetiological agent of bubonic plague, a zoonotic infection that is transmitted from natural animal reservoirs to humans, usually through the bite of an infected flea. In the infected flea, the digestive tract is blocked by a *Y. pestis* biofilm that is dependent on the same genes that are required for biofilm formation on the surface of *C. elegans* (Darby *et al*, 2002). We postulated that *Y. pestis* strains that are deficient in biofilm formation could enter the *C. elegans* intestine and eventually kill the animals, by a mechanism that is different from blocking food intake. As shown in Fig 1A, the nematodes died more quickly when fed on the commonly studied *Y. pestis* KIM5 strain than when fed on *Escherichia coli* OP50, the usual food source for growing *C. elegans* in the laboratory. Although *Y. pestis* KIM5 kills *C. elegans* more slowly than *Y. pestis* KIM6+ (Fig 1B), many other human pathogens, including *S. enterica*, *Enterococcus feacalis*, *Serratia marcescens* and *Cryptococcus neoformans*, also kill nematodes with similar kinetics (Aballay *et al*, 2000; Labrousse *et al*, 2000; Garsin *et al*, 2001; Mylonakis *et al*, 2002; Kurz *et al*, 2003). The time required for 50% of the nematodes to die (time to death 50; TD50) when fed on a *Y. pestis* KIM5 lawn at 25 °C was calculated in five independent experiments and was determined to be 3.63±0.27 days, whereas the TD50 was 9.54±1.67 days when fed on an *E. coli* OP50 lawn. The TD50 values of *C. elegans* pathogens range from 4 h to 8 days, and almost half of the reported TD50 values are as high as 3.63, or higher (reviewed by Sifri *et al*, 2005). When eggs were placed on *Y. pestis* lawns, the animals reached adulthood, which indicated that *Y. pestis*, in spite of its virulence, supports nematode development and that nematode death is not related to any deficiency in food intake (data not shown).

To test whether the premature death of the nematodes that were infected with *Y. pestis* KIM5 correlates with the presence of bacteria in the intestine, we infected the animals with *Y. pestis* KIM5 expressing the *Aequiorea victoria* green fluorescent protein (GFP). Fig 1 shows that KIM5 is not capable of forming a biofilm on the surface of the animals and that the intestinal lumen of the nematodes is distended and full of bacteria 48 h after initial exposure to the pathogen. These results show that *Y. pestis* lacking the *hmsHFRS* genes is still virulent in *C. elegans* and that virulence factors other than those involved in biofilm formation can be studied using the *Y. pestis–C. elegans* pathogenesis system described here.

### Characterization of the *Y. pestis* infection of *C. elegans*

Previous work has shown that certain human pathogens can resist the mechanical and humoral stresses of the *C. elegans* intestinal environment and persistently colonize the gut (Aballay *et al*, 2000; Labrousse *et al*, 2000; Garsin *et al*, 2001; Kurz *et al*, 2003).

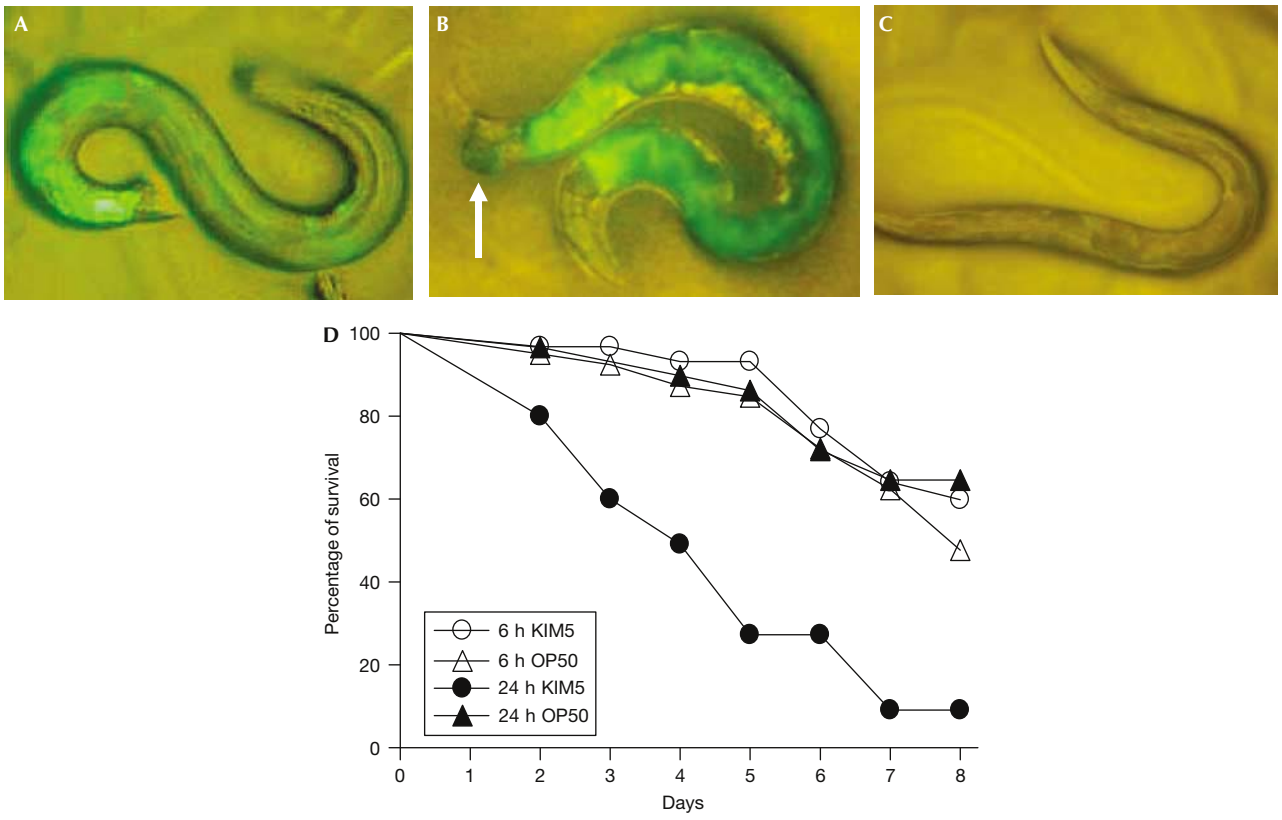


**Fig 2** | *Yersinia pestis* persistently colonizes the *Caenorhabditis elegans* intestine. *C. elegans* N2 young adult animals were exposed to *Y. pestis* KIM5/green fluorescent protein (GFP) (**A**,**B**) or *Escherichia coli* DH5α/GFP (**C**) for 24 h, transferred to *E. coli* OP50 for 24 h (**A**,**C**) or 48 h (**B**), and then visualized using a Leica MZ FLIII fluorescence stereomicroscope. (**D**) Groups of 40 *C. elegans* N2 young adult animals were exposed for 6 h to *Y. pestis* KIM5 ($P<0.90$) or *E. coli* OP50 ($P<0.32$), or for 24 h to *Y. pestis* KIM5 ($P<0.001$) or *E. coli* OP50, and then transferred to *E. coli* OP50. An arrow points to the swollen tail of an animal infected with *Y. pestis*.

To determine whether *Y. pestis* KIM5 was capable of persistently colonizing the *C. elegans* intestine, nematodes were fed on *Y. pestis* KIM5/GFP for 24 h and then transferred to nonlabelled *E. coli*. Fig 2 shows the presence of *Y. pestis*/GFP in representative animals 24 h (panel A) or 48 h (panel B) after the animals were transferred to *E. coli*, whereas *E. coli*/GFP was not observed in a representative animal 24 h after the animal was transferred to *E. coli* (panel C). We also tested whether the persistent colonization of the *C. elegans* intestine by *Y. pestis* correlated with the killing of the animals. Using the transfer experiment described above, we showed that nematodes exposed to *Y. pestis* KIM5 for 24 h died at the same rate as when they were continuously fed on *Y. pestis*, whereas those transferred after an infection time of only 6 h were rescued from a lethal infection (Fig 2D). These results indicate that an initial infection period of 24 h is sufficient for *Y. pestis* to establish a persistent colonization in *C. elegans*.

The presence of a swollen tail is a morphological phenotype characteristic of nematodes that are infected with *Y. pestis* (Figs 1F,2B). To our knowledge, the presence of a swollen tail has not been previously reported in *C. elegans* infected with other human pathogens, but it is very similar to that seen in animals that are infected with a specific *C. elegans* pathogen, *Microbacterium nematophilum*. These bacteria adhere to the anal region of the nematodes and induce localized swelling of the underlying hypodermal tissue (Hodgkin *et al*, 2000). After initial characterization of this infection, it was found that the extracellular signal-regulated kinase–mitogen-activated protein kinase (ERK–MAPK) signalling pathway mediates both tail swelling and a protective response against *M. nematophilum* attack (Nicholas & Hodgkin, 2004a). It will be interesting to use the large collection of mutants in the ERK–MAPK signalling pathway to determine whether a similar *C. elegans* defence response is required to fight *Y. pestis* infection.

## Known *Y. pestis* virulence factors in the killing of *C. elegans*

As many *Y. pestis* virulence determinants are encoded on virulence plasmids, we decided to study the role of plasmids pCD1 and pPCP1 on *Y. pestis* virulence in *C. elegans*. The pCD1 plasmid (also called pYV) encodes a type III secretion system (TTSS) required for full virulence in mammalian systems, although it is not sufficient to cause disease (Heesemann & Laufs, 1983; Heesemann *et al*, 1984). The pPCP1 plasmid encodes the plasminogen activator protease, which is important for dissemination of *Y. pestis* after subcutaneous injection into mammalian hosts (Wren, 2003). To determine the importance of these plasmids in *Y. pestis* virulence in *C. elegans*, we compared the susceptibility of the nematodes to strains cured of one or both plasmids. Fig 3A shows that strains lacking one or two virulence plasmids showed virulence that was comparable to that of

*Y. pestis* KIM5, which carries both virulence plasmids. These results show that virulence factors encoded in pCD1 and pPCP1 are not required for *Y. pestis* virulence in *C. elegans*.

In addition to plasmid-encoded virulence factors, several chromosomally encoded virulence factors have been identified in *Y. pestis*, including genes encoding a potential second TTSS (Revell & Miller, 2001). As shown in Fig 3B, at least three *Y. pestis* KIM5 deletion mutants in chromosomally encoded genes presumably related to virulence (*ompT*, *y3857* and *yapH*) that were available in our stock showed reduced virulence in nematodes. *ompT* encodes a member of the omptin family of outer-membrane proteases, which have been implicated in the virulence of *E. coli*, *S. enterica*, *Shigella flexneri* and *Y. pestis* virulence (Kukkonen & Korhonen, 2004). *y*3857 encodes a putative exported metalloprotease that shows significant amino-acid sequence similarity to virulence-related metalloproteases from *Yersinia ruckeri*, *Photorhabdus luminescens*, *S. marcescens* and *Proteus mirabilis* (Walker *et al*, 1999; Fernandez *et al*, 2002; Bowen *et al*, 2003). The *yapH* gene encodes a putative autotransporter adhesion protein; the importance of this family of proteins in bacterial attachment to the surface of host cells and protection from complement and antimicrobial peptides has been studied in several species (Roggenkamp *et al*, 2003; Rose *et al*, 2003; Wehrl *et al*, 2004).

## Identification of novel mammalian virulence factors

To identify novel *Y. pestis* virulence factors, an EZ::TN insertion library containing 984 *Y. pestis* clones was screened, as described previously (Tenor *et al*, 2004), for mutants that failed to kill or showed attenuated killing of *C. elegans*. Six out of 984 insertion mutants were identified on the basis of their slower killing of *C. elegans* than the killing by wild-type *Y. pestis* KIM5. The molecular characterization of the isolated strains indicates that all the mutations are in genes that have previously not been implicated in *Y. pestis* virulence. Three of the insertions, however, are in genes that are related to virulence in other species (Table I).

To determine whether *Y. pestis* virulence-related genes identified using *C. elegans* as a host have a role in mammalian pathogenesis, we used an intranasal mouse model of *Y. pestis* pathogenesis (supplementary information online) to study the virulence phenotypes of four isolated mutants. Using *Y. pestis* KIM5, we found that the medial lethal dose (MLD or $LD_{50}$) is about $10^5$ CFU. After intranasal inoculation of 10 $LD_{50}$, bacteria are present in the lungs at relatively low numbers for several days, but the lung infection is often cleared by days 4–6. Histology on days 3 and 4 after intranasal inoculation shows micro-abscesses in the spleen and liver. These abscesses enlarge, leading to the complete destruction of the spleen architecture in some mice by days 6–8 (Fig 4). Although the route of infection is the lungs, this model is more representative of septicemic than of human pneumonic plague. Fig 4 shows the virulence phenotypes of two mutants with reduced virulence both in the *C. elegans* model and in the mouse model of *Y. pestis* pathogenesis. The virulence phenotype

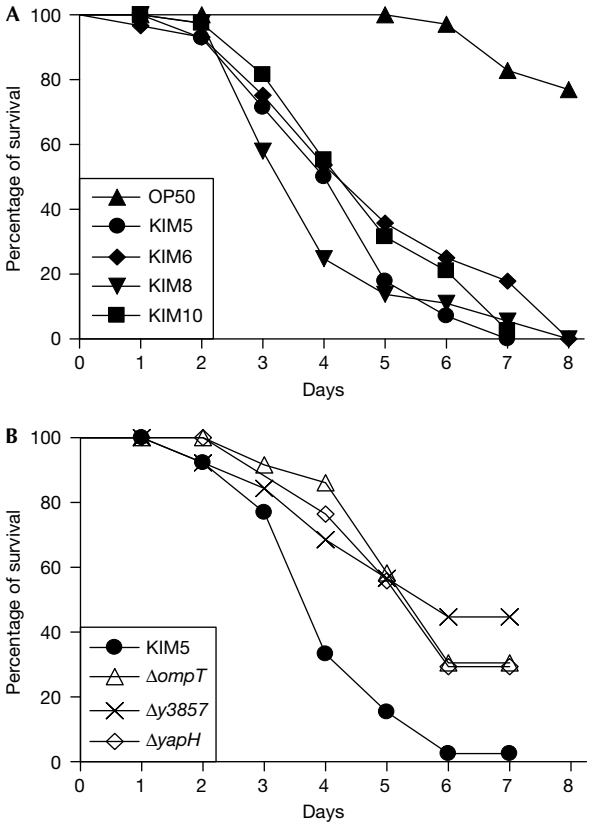


**Fig 3** | Role of known *Yersinia pestis* virulence factors in the killing of *Caenorhabditis elegans*. (**A**) Groups of 40 *C. elegans* N2 young adult animals were exposed to *Y. pestis* KIM5 (pCD1+, pPCP1+), *Y. pestis* KIM6 (pCD1−, pPCP1+; $P<0.0885$), *Y. pestis* KIM8 (pCD1+, pPCP1−; $P<0.5202$), *Y. pestis* KIM10 (pCD1−, pPCP1−; $P<0.1388$) or *E. coli* OP50 ($P<0.001$). (**B**) Groups of 40 *C. elegans* N2 young adult animals were exposed to *Y. pestis* KIM5 or *Y. pestis* KIM5 strains with deletion mutations in the genes *ompT* ($P<0.001$), *y3857* ($P<0.001$) and *yapH* ($P<0.001$).

**Table I** | *Yersinia pestis* KIM5 insertion mutants isolated based on attenuated *Caenorhabditis elegans* killing

| Strain | Gene | Gene product description | Virulence phenotype in mouse | Significance | *N* |
|---|---|---|---|---|---|
| 5B9 | *y0340* | Similar to putative exported protein of *Salmonella enterica* | Attenuated | $P=0.0004$ | 23 |
| 5G5 | *y1021*[a] | Cytochrome o ubiquinol oxidase subunit II | Attenuated | $P=0.0001$ | 23 |
| 5F1 | *y2663* | Putative membrane protein | Wild type | $P=1.000$ | 23 |
| 6B4 | *y3913*[a] | Putative RNase R exoribonuclease | ND | ND | ND |
| 8B7 | *y0941*[a] | Putative membrane protein; part of an adhesin system | ND | ND | ND |
| 9F11 | *y4018* | Unknown | Wild type | $P=0.3055$ | 8 |

ND, not determined; *N*, number of animals analysed.
[a]Genes related to virulence in other species.

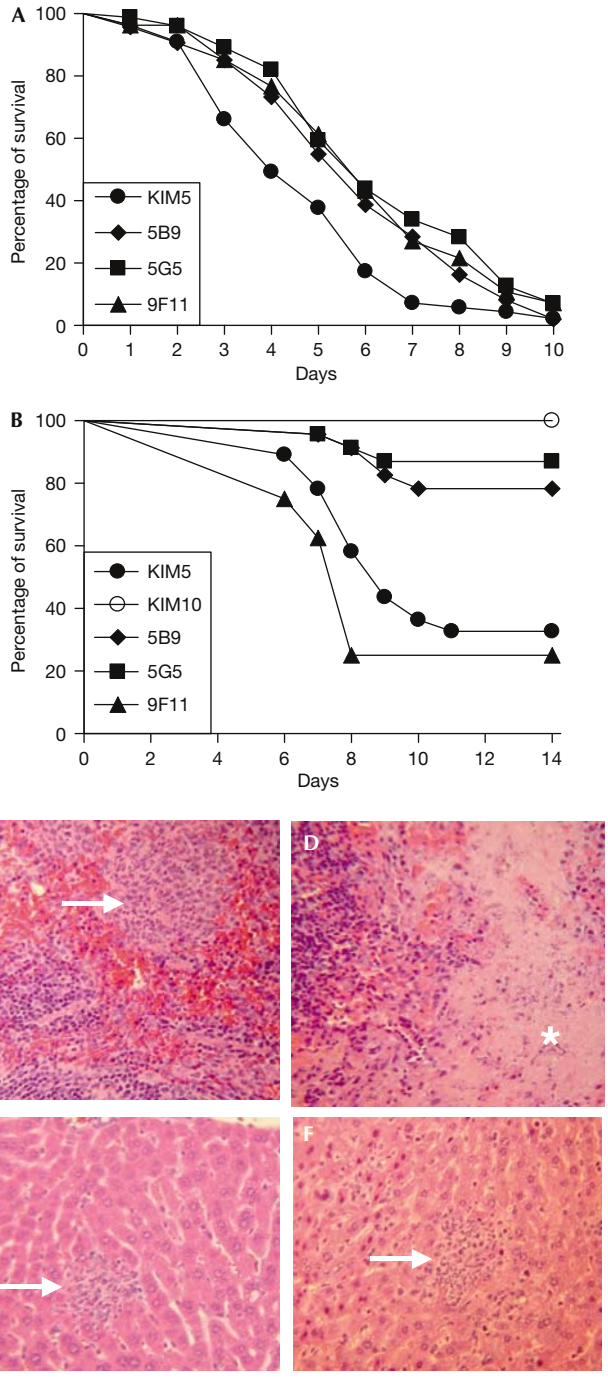


**Fig 4** | Genes identified in a *Caenorhabditis elegans*-based screen are also required for virulence in a mouse model of plague. (**A**) Groups of 80 *C. elegans* N2 young adult animals were exposed to *Yersinia pestis* KIM5, *Y. pestis* 5B9 ($P<0.0096$), *Y. pestis* 5G5 ($P<0.0001$) or *Y. pestis* 9F11 ($P<0.0006$). (**B**) Female BALB/c mice (12-week-old) were inoculated intranasally with *Y. pestis* KIM5 ($n=55$), *Y. pestis* KIM10 ($n=8$, $P<0.0001$), *Y. pestis* 5B9 ($n=23$; $P<0.0004$), *Y. pestis* 5G5 ($n=23$; $P<0.0001$) or *Y. pestis* 9F11 ($n=8$; $P<0.3055$) mutant strains ($10^6$ CFU/mouse). (**C–F**) Tissue sections after intranasal administration of *Y. pestis* KIM5. Murine spleen (**C,D**) and liver (**E,F**) are shown at day 3 (**C,E**) and at the time of killing for humane end points on day 6 (**D**) or day 8 (**F**). Circumscribed micro-abscesses (arrows) develop at early time points in the red pulp of the spleen (**C**) and the liver parenchyma (**E**), with preservation of the overall architecture and minimal or no surrounding inflammation. At the time of killing, the red pulp of the spleen (**D**) is largely replaced by necrotic tissue with nuclear debris (asterisk). Splenic rupture is common (not shown), and, in some cases, the spleen cannot be identified as a distinct organ at necropsy. In contrast, the liver (**F**) retains its normal architecture, with distinct micro-abscesses (arrows) and a general inflammatory response.

of *Y. pestis* 9F11 is provided as an example of a mutant in a gene that is probably required for full virulence in *C. elegans* but not in mammals. The reduced virulence of *Y. pestis* KIM10 indicates that the intranasal inoculation of bacteria alone does not cause lethality.

In summary, two out of the four mutants that were studied in the mouse model showed a statistically significant attenuation in virulence. The attenuated virulence shown by *Y. pestis 5G5* is not surprising, as *y1021* encodes a cytochrome subunit previously implicated in *S. enterica* virulence (Turner *et al*, 2003). The reduced virulence of *Y. pestis 5B9* is interesting because *y0340* has not previously been related to virulence and its product is a member of an uncharacterized family of putative exported proteins that are found exclusively in several pathogens in the family Enterobacteriaceae. *Y. pestis* KIM5 encodes seven individual orthologues of the *y0340* gene product in its chromosome. In fact, *y0340* is the last gene in an operon encoding three of the seven Y0340 orthologues. The gene downstream of *y0340* is predicted to be transcribed in the opposite direction of *y0340*, making the possibility of a polar effect due to the mutation highly unlikely. These results show that *C. elegans* can be used as an alternative model host to accelerate the identification and characterization of *Y. pestis* virulence factors required for pathogenesis in mammalian systems.

## Concluding remarks

One drawback of using *C. elegans* to model bacterial pathogenesis is the fact that the nematode is grown at temperatures below 25 °C. It is well established that exposure to temperatures of around 37 °C in the mammalian hosts results in the upregulation of a range of *Y. pestis* virulence factors. In contrast, other *Yersinia* virulence factors, such as invasin, and the *Y. enterocolitica* chromosomal YSA TTSS are preferentially expressed at 26 °C (Revell & Miller, 2001). In addition, *Y. pestis* virulence factors that are required for colonization of the flea are optimally expressed at lower temperatures. For example, the plasmid pMT1-encoded Ymt protein, which has phospholipase D activity, is preferentially expressed at 26 °C and has an important role in enabling infection of the flea (Hinnebusch *et al*, 2002).

Soon after being injected by the flea, *Y. pestis* has to deal with mammalian defences; therefore, the bacterium probably has to express proteins that are required for defence against many mammalian innate defences at 25 °C. For example, *Y. pestis* is constitutively serum resistant, whereas, in the enteropathogenic *Yersiniae*, serum resistance is expressed only at 37 °C. This could also be required owing the presence of serum-like products in the flea gut. Thus, although temperature is a limitation, we theorized that *C. elegans* could be useful for studying virulence factors that are

optimally expressed at 25 °C or constitutively expressed at both 25 and 37 °C. In spite of the long evolutionary distance and differences in growth temperatures between nematodes and mammals, the results reported here show that *C. elegans* can be used as an alternative host to study *Y. pestis* virulence factors that are required for pathogenesis in mammalian systems. These types of virulence factor expressed at low temperatures may be required to regulate innate immunity pathways conserved in nematodes, fleas and mammals.

## METHODS

**Bacterial strains and growth conditions.** *E. coli* OP50 (Brenner, 1974), *Y. pestis pgm*− strains KIM5, KIM6, KIM8 and KIM10 (Une & Brubaker, 1984) and *Y. pestis pgm*+ strain KIM6+ (Perry *et al*, 1990) have been described previously. *Y. pestis* KIM5Δ*ompT*, *Y. pestis* KIM5Δ*yapH* and *Y. pestis* KIM5Δ*y3857* were generated using the phage lambda Red recombinase method (Datsenko & Wanner, 2000), essentially as described previously (Jackson *et al*, 2004).

**Statistical analyses.** Animal survival was plotted using the PRISM (version 4.00) computer program. Survival curves are considered significantly different from the control when *P*-values are $<0.05$. Prism uses the product limit or the Kaplan–Meier method to calculate survival fractions and the log-rank test, which is equivalent to the Mantel–Heanszel test, to compare survival curves.

**Supplementary information** is available at *EMBO reports* online (http://www.emboreports.org).

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