Published in final edited form as:
Microb Pathog. 2009 November ; 47(5): 243–251. doi:10.1016/j.micpath.2009.08.005.

Evaluation of a *Yersinia pestis* Mutant Impaired in a Thermoregulated Type VI-Like Secretion System in Flea, Macrophage and Murine Models

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Abstract

Type VI secretion systems (T6SS) have been identified recently in several Gram-negative organisms and have been shown to be associated with virulence in some bacterial pathogens. A T6SS of *Yersinia pestis* CO92 (locus YPO0499-YPO0516) was deleted followed by investigation of the phenotype of this mutation. We observed that this T6SS locus of *Y. pestis* was preferentially expressed at 26° C in comparison to 37° C suggesting a possible role in the flea cycle. However, we found that the deletion of T6SS locus YPO0499-YPO0516 in *Y. pestis* CO92 had no effect on the ability of this strain to infect the oriental rat flea, *Xenopsylla cheopis*. Nevertheless, this mutant displayed increased intracellular numbers in macrophage-like J774.A1 cells after 20 hours postinfection for bacterial cells pre-grown at 26° C indicating that expression of this T6SS locus limited intracellular replication in macrophages. In addition, deletion of the YPO0499-YPO0516 locus reduced the uptake by macrophages of the *Y. pestis* mutant pre-grown at 37°C, suggesting that this T6SS locus has phagocytosis-promoting activity. Further study of the virulence of the T6SS mutant in murine bubonic and inhalation plague models revealed no attenuation in comparison with the parental CO92 strain.

Keywords

Yersinia pestis; Type VI Secretion System; Plague; Macrophage; Murine Model; *Xenopsylla cheopis*

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1. Introduction

Three species within the genus *Yersinia* are pathogenic for humans: *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica. Yersinia pestis*, the causative agent of plague, is the commonly accepted etiological agent of three historically significant pandemics [1]. This same bacterium is believed to be a clone derived from a serotype O:1b *Y. pseudotuberculosis* progenitor, which underwent certain genomic rearrangements and acquired additional plasmid-associated virulence and transmission elements that enabled it to be maintained in flea to rodent transmission cycles and spread over the globe [2,3]. Plague bacilli exist in nature at ambient temperature within the flea vector or in the mammalian host at approximately 37°C. Accordingly, global regulation of the expression of certain functions of *Y. pestis* in response to changes in temperature reflects adaptations to the mammalian host environment, which it encounters immediately upon being transmitted from the flea vector. Thus, differential monitoring of the cell functions during temperature transition and subsequent infection of hosts can be used to identify a useful list of putative virulence-associated genes as well as genes essential for the survival of *Y. pestis* in fleas and its transmission by these insects. Thermoregulation of the plague pathogen *in vitro* was studied on the whole-genome level by transcriptomic and proteomic approaches [4-9]. These approaches identified gene clusters YPO0499-YPO0516 and y3658-y3677 in *Y. pestis* CO92 and KIM10+, respectively, that were strongly affected by growth temperature and represented one of the five putative type VI secretion system (T6SS)-like gene clusters found within the genome of this bacterium.

Y. pestis CO92 contains at least five clusters of genes recognized to have features of T6SS [10] and gene expression in one of these clusters, YPO0499-YPO0516, is controlled by the global regulator RovA [11]. The type VI secretion systems have been described in numerous species of gram negative bacteria [12]. Many of these organisms have pathogenic or symbiotic associations with eukaryotic cells. However, other bacteria possessing T6SS gene homologs are free-living organisms [10]. A T6SS has been shown to be involved in virulence of pathogens *Vibrio cholerae* [13], *Pseudomonas aeruginosa* [14], enteroaggregative *Escherichia coli* (AggR regulated) [15], *Edwardsiella tarda* [16], *Burkholderia mallei* (VirA/G regulated) [17], *Francisella tularensis* [18], *Agrobacterium tumefaciens* [19], *Pectobacterium atrosepticum* (Quorum sensing regulated) [20], and *Aeromonas hydrophila* [21]. In *Salmonella enterica*, T6SS genes encoded on the Sci island and controlled by the SsrA/SsrB two-component regulatory system are involved in bacterial persistence, limiting the intracellular growth in macrophages at late stages, and attenuating lethality in murine hosts [22]. In *P. aeruginosa*, expression of a T6SS is controlled by the global virulence regulator proteins RetS and LadS, which control the formation of acute or chronic lung infections [14]. In *Rhizobium leguminosarum*, expression of T6SS proteins are involved in symbiosis, blocking the formation of effective, nitrogen-fixing nodules on legume species that are not natural hosts [23]. In general, T6SS appears to be tightly regulated, and usually expressed during interactions between bacteria and eukaryotic host cells.

In this study, we constructed an in-frame deletion mutant of the cluster YPO0499-YPO0516 in *Y. pestis* CO92 and investigated the phenotype of this mutation. We found that expression of this locus was strongly suppressed at 37°C in comparison to 26°C at both neutral and acidic pH values (7.2 and 5.5, respectively). The down-regulation of activity of a certain function at mammalian body temperature resembles a pattern that would be expected for transmission factors, such as murine toxin (Ymt) and the hemin storage locus (Hms), that are crucial for infection maintenance or biofilm-mediated transmission of *Y. pestis* by fleas and are preferentially expressed at ambient temperature [1,24-27]. Therefore, we examined whether the above mutation in this T6SS locus influenced the ability of *Y. pestis* to infect and survive in the midgut of the flea vector, *Xenopsylla cheopis*, and found no difference compared with the parental CO92 strain. Since it was shown for some pathogens that the T6SS is involved in

virulence, we tested the mutant in murine bubonic and inhalation plague models. These experiments revealed no attenuation in comparison with the parental CO92 strain. Nevertheless, we found differences between the parental and mutant strains when compared in a macrophage model. The deletion of YPO0499-YPO0516 region resulted in change of both the uptake and intracellular growth of *Y. pestis* in the macrophage-like cell line J774.A1.

2. Results

2.1. Expression of the T6SS locus YPO0499-YPO0516 in vitro at neutral and acidic pH conditions

It was shown by us [8] and recently by others [4,9] that the T6SS region YPO0499-YPO0516 is preferentially expressed at 26° C versus 37° C at neutral pH. However, this locus is controlled by the global transcriptional regulator RovA which directs the expression of the *Yersinia* invasin [11]. The expression of invasin gene *inv* of *Y. enterocolitica* is downregulated at 37° C at neutral, but not acidic pH [28]. Therefore, we tested a hypothesis that similar to the expression of the *inv* gene, the T6SS locus will be expressed at 37° C at low pH that *Y. pestis* may encounter during the intracellular cycle.

We compared the growth of the CO92_ΔYPO499-516 deletion mutant to the parental strain in both HIB and chemically defined BCS media buffered at pH 7.2 and 5.5 during experiments examining the temperature shift from 26° C to 37° C. We did not find significant differences in growth rate between the strains for up to 12-15 hours of growth post-temperature shift. However, at late time points (in the range of 24-30 hours post temperature shift) the T6SS mutant attained a higher cell density (up to a log of difference depending on medium used, as determined by OD_{600} measurements) than the parental strain in the media with low pH at 37° C (data not shown). To determine the conditions in which genes YPO0499-516 were expressed, total RNA was extracted and RT-PCR was performed using gene-specific primers. The expression of YPO0510, a hypothetical gene within the T6SS locus, was detected in the parental strain grown at 26° C at both pH 5.5 and pH 7.2 (Fig. 1 A, lanes 4 and 8), but not at 37° C at either pH value (Fig. 1 A, lanes 12 and 16). Additional RNA transcripts encoded by YPO0499, YPO0501, YPO0506, and YPO0515 were also detected in RNA extracts of the parental strain grown at 26° C but not at 37° C (data not shown). As expected, a control gene, *psaA*, encoding the adhesin subunit of the pH 6 antigen was expressed better at 26° C under acidic (Fig. 1 B, lanes 2 and 4) rather than neutral pH conditions (Fig. 1 B, lanes 6 and 8), and this pH-dependent effect was seen more clearly at 37° C (Fig. 1 B, lanes 10 and 12 versus lanes 14 and 16). Conversely, another control gene, *gyrB*, was expressed constitutively at both temperatures and pH values (Fig. 1 C). Thus, we showed for the first time that the expression of the T6SS locus was strongly downregulated at 37°C in comparison to 26°C not only at neutral pH, but also at acidic conditions.

2.2. Artificial infection of Xenopsylla cheopis

A preferential expression of this T6SS locus at 26° C in comparison to 37° C may suggest a possible role of this function in the flea cycle as shown for other plague transmission factors [1,24-27]. Therefore, we investigated whether a mutation in the T6SS locus would alter the behavior of *Y. pestis* in the flea midgut.

X. cheopis were fed defibrinated rat blood that was either uninfected or spiked with the following Lcr^- strains of *Y. pestis*: CO92 (Pgm^+) (3.2×10^9 CFU/ml), *Y. pestis* CO92 (Pgm^-) (3.7×10^9 CFU/ml), *Y. pestis* CO92_ΔYPO499-516 (Pgm^+) (4×10^9 CFU/ml), *Y. pestis* CO92 ΔYPO499-516 (Pgm^-) (6.1×10^9 CFU/ml), *Y. pestis* CO92_Δymt (Pgm^+) (1.1×10^{10} CFU/ml), or *Y. pestis* CO92_Δymt (Pgm^-) (5.1×10^9 CFU/ml). The infection status and bacterial loads of up to twenty fleas from each group were determined by plating or qPCR

on days 3, 7, 14, and 21 post-infection (Table 1). Over the course of the experiment there was no significant difference in the infection status of fleas infected with the parental strains CO92 (Pgm+) and CO92 (Pgm$^-$) and the YPO0499-YPO0516 deletion mutants.

The fleas infected with the YPO0499-YPO0516 mutant contained *Y. pestis* in their midguts for up to 21 days post-infectious feeding. As expected, the percent of fleas infected with the control CO92_Δymt strain with inactivated murine toxin rapidly declined [25]. Therefore, we detected no difference in the infectivity of T6SS mutant for fleas. Flea mortality and feeding were measured following maintenance feeds (Table 2). Overall, flea mortality was less than 50% in each group for all the time points. The number of fleas feeding in each group averaged over 80%, and the percentages of fleas feeding at each maintenance feeding did not differ significantly between fleas infected with the different *Y. pestis* strains. Also, the midgut contents predominantly consisted of similarly appearing mixed bloodmeals or old, undigested blood in both the infected fleas and the fleas fed uninfected blood (controls). Therefore, infection of *X. cheopis* with these strains of *Y. pestis* did not have a significant effect on their survivability, feeding behavior, or the appearance of partially digested blood meals in the flea midgut.

2.3. Infection of macrophages

Y. pestis is a facultative intracellular pathogen and the ability to survive and replicate in macrophages generally correlates with virulence of this type of microorganism. Therefore, we compared the uptake and intracellular proliferation of T6SS mutant with the parent CO92 strain in a macrophage model.

The murine macrophage-like cell line J774A.1 was infected with wild type *Y. pestis* CO92 (Lcr + Pgm+)(WT) and ΔYPO499-516 (Lcr+ Pgm+) grown at either 26° C or 37° C in HIB medium. The infectivity of both strains of bacteria harvested at the log-phase of growth at 37°C was considerably lower than those grown at 26° C. To achieve a similar number of bacteria within infected macrophage cells, a MOI of 50 was used for 37° C pre-grown *Y. pestis*, and a MOI of 1 was used for bacteria pre-grown at 26° C (Tables 3 and 4). We did not observe any substantial increase in the number of intracellular *Y. pestis* for either the parental or mutant strains. Both strains, however, showed a slow and not very pronounced tendency to decrease in the number of intracellular bacteria over 24 hours of observation time. In this respect, the number of viable intracellular bacteria of the mutant pre-grown at 26° C was significantly ($p < 0.001$) higher than that of the parental strain at late time points reaching more than a log difference at 24 hour post infection (Table 3). No significant death of eukaryotic cells was seen during the course of the experiment. When parental and mutant strains pre-grown at 37° C were used to infect macrophages, uptake of the WT strain was 10-fold higher than the T6SS mutant despite the addition of equal numbers of bacteria for both strains to J774A.1 cells. This result was repeatedly observed in a number of similar independent experiments. This effect can be seen in Table 4 at time = 0 hours, which shows the number of intracellular bacteria immediately after their initial uptake by the macrophages (4.8 compared to 3.8 log10 CFU).

2.4 Animal studies

Since we found that the mutation in the T6SS locus influenced the intracellular replication in macrophages, we used a murine plague model to test the effect of deletion of the YPO0499-YPO0516 cluster on the virulence of *Y. pestis* CO92.

In our pneumonic plague murine model (i.n. route of challenge), some mice received a sub-inhibitory dose of levofloxacin (0.3 to 0.5 mg/kg of body weight/day) for the first 5 days post-infection which prolonged survival time and was previously proven useful in revealing the degree of attenuation of *Y. pestis* mutants [29]. The mean survival times of mice infected with

WT treated with a curative dose of antibiotic, WT treated with a sub-inhibitory dose of antibiotic, T6SS mutant treated with a sub-inhibitory dose of antibiotic, WT with no antibiotic treatment and T6SS mutant with no antibiotic treatment were respectively 21, 14.4, 14.0, 7.0, and 5.2 days. Although, we saw no significant difference in the survival between i.n. infected mice with WT versus T6SS deletion mutant strains within differing antibiotic treatments, there was a significant difference in the survival distributions between the groups treated with the sub-inhibitory dose of antibiotic versus the mice cured with antibiotic ($p < 0.005$), and between mice which received no antibiotic treatment compared to both the mice cured with antibiotic ($p < 0.001$) and the mice treated with the sub-inhibitory dose of antibiotic ($p < 0.005$) (Fig. 2). Two doses (10 and 20 bacteria per mouse) of both parental and mutant strains were given in a s.c. challenge that mimics bubonic plague. A statistical comparison of the mean survival times for infected mice between these groups did not reveal a significant difference at $p < 0.05$ (data not shown) suggesting that T6SS mutation did not change virulence of *Y. pestis* for this route of inoculation.

3. Discussion

Type VI secretion systems (T6SS) have been identified recently in several Gram-negative organisms, and this number continues to grow [10,30,31]. The *Y. pestis* genome contains at least five putative T6SS loci each of which differs in gene number and arrangement [10]. Although it is unclear whether all of these loci are functional in the plague pathogen, the expression of one copy, located within a ~20 kb fragment on the chromosome (YPO0499-YPO0516 in *Y. pestis* CO92), was detectable. Analyzing the global transcriptional profile of *Y. pestis*, we were the first to show that expression of this cluster of 19 genes was strongly down-regulated within 1 hour after the cultivation temperature was shifted from 26° C to 37° C and that it remained repressed at the latter temperature for at least 10 additional hours [8]. This observation was confirmed by others who studied thermoregulation of the plague pathogen by transcriptomic and proteomic approaches using different strains of *Y. pestis*, media and growth conditions [4-7].

Thirteen putative T6SS proteins encoded by the *Y. pestis* KIM chromosomal locus y3658-y3677 (homologous to YPO0499-YPO0516 in CO92) were detected in association with the bacterial membrane of *Y. pestis* grown in static culture at 26° C but not at 37° C, an indication that *Y. pestis* likely assembles this large protein complex [9]. The preferred expression of T6SS at ambient rather than mammalian host temperature was also reported in the fish pathogen *Edwardsiella tarda* [16,32], and expression of T6SS orthologs in the leguminous plant symbiont *Rhizobium leguminosarum*, also appears to be thermoregulated [23] as well as in the close relative of *Y. pestis*, *Y. pseudotuberculosis* (Motin, V.L., unpublished results).

In this study, we investigated the phenotype of an in-frame deletion mutant of *Y. pestis* CO92_ΔYPO499-516 which had all 19 genes of the T6SS cluster inactivated. RT-PCR assays, using primers specific to several genes of this locus, revealed a severe reduction in the expression of this copy of T6SS in the parental *Y. pestis* CO92 strain after the temperature of cultivation was increased from 26° C to 37° C at pH 7.2. Interestingly, it was shown recently that this T6SS locus is regulated by the global transcriptional regulator RovA which controls the expression of a number of virulence factors in *Yersinia* species, including the *inv* gene encoding the invasion factor invasin [11]. The expression of *inv* of *Y. enterocolitica* is thermoregulated and reduced at 37°C at neutral pH, however, its expression was increased to the levels observed at 26° C when these bacteria were grown at 37° C at pH 5.5 [28]. Moreover, acidic conditions induced VirA/G-independent expression of a T6SS in *Agrobacterium tumefaciens* [33].

We tested the expression of the genes in the YPO0499-YPO0516 locus after bacteria were grown at pH 5.5 at both temperatures and did not find a difference between the expression at the neutral and low pH values. This might be an additional indication that RovA regulates this T6SS copy indirectly, as proposed previously [11]. Comparison of the growth curves of the mutant and parental strains at either pH did not show significant differences between them at either 26° C or 37° C for up to 15 hours after the temperature of cultivation was changed. To our surprise, we found at late time points (24-30 hours following temperature shift) that the culture of the T6SS mutant grown at 37° C in pH 5.5 media had a higher optical density than cultures of both parental and mutant strains grown at other conditions. This might be a further indication of the connection of T6SS expression in *Y. pestis* with the population density and stress response, as recently suggested [9]. A quorum sensing mechanism was shown to be involved in regulation of expression of T6SS in the plant pathogen *Pectobacterium atrosepticum* [20].

Since *Y. pestis* is considered a facultative intracellular pathogen, its virulence generally correlates with the ability to survive and replicate in macrophages [34-39]. In *V. cholerae* O: 37, T6SS genes were involved in cell-cell contact- dependent virulence towards *Dictyostelium ameobae*, as well as in cytotoxicity towards J774 murine macrophages [13]. Moreover, T6SS in Burkholderia pseudomallei, Salmonella enterica, Francisella tularensis, and *Edwardsiella tarda* are known to be expressed or to effect bacterial growth during infection of phagocytic cells [22,32,40,41]. Thus, we tested whether or not the mutational loss of the YPO0499-YPO0516 T6SS locus influenced the intracellular viability of *Y. pestis* by monitoring the fate of the mutant after phagocytosis by murine macrophage-like J774.A1 cells. We did not find a significant difference in the abilities of either the parental or YPO0499-YOP0516 mutant strains to maintain their viability within macrophages when pre-grown in liquid culture at 37° C prior to the infection. However, when the host cells were infected with *Y. pestis* pre-grown at 26° C, the T6SS mutant displayed a significantly greater survival rate at late time points (Table 3). This correlates with our *in vitro* observation that the mutant attains a higher cell density than the parental strain in stationary phase cultures at pH 5.5. Similarly, a Type VI secretion gene *sciS* in *Salmonella enterica* was activated in late stages of macrophage infection to control overgrowth, a possible mechanism for persistence of intracellular bacteria [22].

Overall, the expression of the YPO0499-YPO0516 T6SS in *Y. pestis* might influence the intracellular replication of the pathogen differently from the dispensable character of the functions encoded by the T3SS- bearing virulence plasmid pCad [42] and small plasmid pPCP [43]. We repeatedly observed that the uptake of the T6SS mutant pre-grown at 37°C was notably reduced in comparison to that of the parental strain (Note the log10 difference at time point 0 hours in Table 4). Although, the YPO0499-YPO0516 locus did not show a noticeable expression at 37°C as judged by RT-PCR (Fig. 1), our previous microarray data suggested that T6SS is weakly expressed at this temperature [8]. Thus, even this low expression level [6,9] was enough either to increase phagocytosis of *Y. pestis* bacteria by J774.A1 cells or to reduce the antiphagocytic functions of *Y. pestis*. However, this phagocytosis-promoting activity of the YPO0499-YPO0516 locus was not seen at 26° C (Table 3). At this temperature, the efficiency of bacterial uptake was very high and perhaps additional contribution of a T6SS is negligible in comparison with other phagocytosis-promoting determinants of *Y. pestis*.

Factors of *Y. pestis* crucial to this pathogen's adaptation to flea transmission, including the chromosomal Hms locus and the pMT plasmid-encoded *ymt* gene for murine toxin, are expressed preferentially at ambient temperature by the bacteria, indicating their importance for this life-stage [1,27]. Deletion of either of these transmission factors, reduces efficient maintenance and transmission of *Y. pestis* by the flea, since Ymt and Hms (Pgm+ phenotype) are crucial for *Y. pestis* survival and biofilm formation in the flea midgut [24,25,26,27]. Owing to their low temperature expression, we hypothesized that gene products of YPO0499-

YPO0516 could be involved in the ability of *Y. pestis* to infect, survive within, and/or be transmitted by flea vectors. *X. cheopis*, the oriental rat flea, has frequently been identified as one of the most efficient arthropod vectors of *Y. pestis* [44]. Using artificial feeding systems, we compared the infectivity of (Pgm+) and (Pgm$^-$) parental, YPO0499-516 T6SS deletion mutant, and Ymt deletion mutant strains of *Y. pestis* CO92 (Lcr$^-$) in laboratory-maintained *X. cheopis* fleas. Initially, fleas took equivalent amounts of bacteria with their infectious blood meals, and, as expected, the majority of fleas infected with the control Ymt deletion mutants cleared their infections over the duration of the experiment. However, we found no effect of the YPO0499-YPO0516 T6SS deletion on *Y. pestis* survival in similar experiments with *X. cheopis*. Not surprisingly, decreases in the fleas' bacterial loads occurred more prominently for Pgm$^-$ strains compared to Pgm+ strains. Furthermore, we monitored flea feeding behavior and mortality by counting the numbers of dead fleas found in tubes prior to each of the twice weekly maintenance feeding but failed to find any significant mortality differences between strains. The ability of fleas to ingest and digest blood also was examined but again, we saw no significant differences for these parameters between groups of fleas. Our study design, using low calcium response (Lcr) deficient strains for infection of fleas, did not allow us to test whether infected fleas could transmit these strains to susceptible mice or other animals. Whether T6SS plays a role in the early stages of the *Y. pestis* infection in mammals should be determined experimentally but was outside of the scope of our study.

Type VI secretion systems have been associated with increased virulence in mammalian models of infection for several Gram-negative bacterial pathogens including *A. hydrophila* [21], *P. aeruginosa* [45], *F. tularensis* [18,40] and *B. mallei* [17]. In contrast, a *sciS* mutant of *S. enterica* was hyper-virulent in mice when administered intragastrically, indicating that this gene product is involved in the attenuation of virulence in murine infection [22]. Accordingly, we tested the effect of YPO0499-516 T6SS deletion in murine models of bubonic and inhalational plague, but did not find any significant difference in virulence between the parental and mutant strains.

In summary, we observed that genes encoded by the ~20 kb chromosomal locus YPO0499-YPO0516 were expressed by *Y. pestis* at conditions likely to be encountered during infections of the flea vectors of this bacterium but not in mammalian hosts. However, we found no effect of this mutation in laboratory models of infected oriental rat flea *X. cheopis*, or in virulence using bubonic or pneumonic murine plague models. The only phenotype of *Y. pestis* CO92_ΔYPO499-516 mutant uncovered by us was a difference in the interaction of bacteria with macrophage-like cells. Removal of this copy of T6SS decreased the initial uptake of bacterial cells pre-grown at 37°C and increased intracellular survival of *Y. pestis* pre-grown at 26° C. As there are multiple putative type VI loci in the *Y. pestis* chromosome, with evidence of differential regulation [8,11], these gene products may play a role in *Y. pestis* virulence or in its maintenance in nature and should be examined further.

4. Materials and methods

4.1. Bacterial strains and media

Y. pestis CO92 (biovar Orientalis), which was isolated from a human pneumonic plague case [46], was the parent of all strains of *Y. pestis* used in this study, together with two derivatives of CO92, namely low calcium response negative (Lcr$^-$) and pigmentation-deficient (Pgm$^-$) variants, that lacked either the 70 kb virulence plasmid, pCad, or the chromosomal ~102-kb pigmentation locus, respectively. In preparation for growth in liquid culture, bacteria were plated from glycerol stocks maintained at -20° C on Heart Infusion Broth (HIB) agar plates and incubated at 26° C.

Various liquid media preparations were tested for their ability to grow *Y. pestis* at different pH and temperatures. Sterile HIB was buffered with either 0.05 M MOPS, pH 7.2 or 0.05 M MES, pH 5.5. Additionally, we adapted a previously described chemically defined medium BCS [47,48] that was buffered by the addition of MOPS or MES as described for HIB and eliminating HEPES from the stock salt solution. All other components making up the chemically defined BCS medium were the same as described in the original paper [48]. When indicated, 4.0 mM $CaCl_2$, 20 mM $MgCl_2$, and 0.1% galactose were added to the buffered medium.

4.2. Generation of deletion mutants

The knock-out mutants of *Y. pestis* CO92 were generated using the gene deletion protocol adapted from the Lambda Red recombinase mutagenesis system in which the target genes were first substituted with a marker of antibiotic resistance located between FRT sites and subsequently removed using FLP recombinase [49]. Two separate targets were used to obtain deletion mutants: the entire T6SS-like gene cluster, YPO0499-YPO0516, and the gene *ymt* which encodes murine toxin [50]. The mutagenesis was performed using primers containing a prolonged 85 bp homology region (underlined) to the target gene to facilitate recombination (YPO0499_lng_F:
CATCCTCTCTCAATTAGATGAGTCTGCGGACCCCCTTTCCCGCCCGCATGAACCC
CCTCAAATAAACTGGCACCATATCAGTGAGGTGTAGGCTGGAGCTGCTTCG;
YPO0516_lng_R:
CGAAGTCCTTCGGACGCTCCTCACTTTGCATTTTCTGGTGAGCATCAACATTGTC
CTGCAATCCTCCCAATATCTTATTCATATCATGGGAATTAGCCATGGTCCAT;
ymt_lng_F:
CGGCCAATCTCCAACAGTATTTATGAATGGGTTATCTGCTGCAACAAATGGCTC
CCCTGACTTTGTTGCTTTTAAATCAGAGTTAGTGTAGGCTGGAGCTGCTTCG;
ymt_lng_R:
TTGCAGTATAAGCGCTTTGTTCTAAATCAGGCCACTTGTAGGTTTCGCCTTCAAT
TGTATTTTCATCAGGTACTTTATCTGTAAAATGGGAATTAGCCATGGTCCAT). As a result, the mutant CO92_ΔYPO499-516 contained an in-frame deletion inactivating all 19 genes of the T6SS and another mutant CO92_Δymt had an internal deletion of 960 bp out of 1599 bp of the *ymt* gene. The size of the deletion was confirmed by PCR using primers flanking the removed region (YPO0499_F: TTGAATATGATCCGGCCTATGG; YPO0516_R: CCTCTGGGAAACCATTGGAGAG; ymt_F: GGCGAACTCAGCCAACTCAGTA; ymt_R: TAATACCTTGATGCGGGGGTTT), followed by sequencing of the corresponding fragments. All helper plasmids providing Lambda Red and FLP recombinases were cured from the constructed mutants as recommended elsewhere [49].

4.3. Measurement of in vitro growth rate and gene expression

Y. pestis cells were grown overnight in a shaking water bath at 26° C in HIB or in chemically defined BCS medium buffered with 0.05M MOPS, pH 7.2 or MES, pH 5.5 containing 0.1% galactose, 4 mM $CaCl_2$, and 20 mM $MgCl_2$. Overnight cultures were used to inoculate 8 flasks containing fresh growth medium to an OD_{600} = 0.1 and grown for an additional three hours at 26° C until reaching an OD_{600} ranging from 0.25-0.40. Four of flasks were shifted to 37° C, while the remaining four were maintained at 26° C. Following the temperature shift, OD_{600} measurements were taken every hour for 14 hours. Aliquots were removed from growth cultures and mixed in a 1:1 ratio with RNAlater (Ambion, Austin, TX) for RNA extraction and analysis of gene expression. RNA was extracted using the RNAqueous Kit (Ambion), treated with DNase I, and visualized using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). RNA was reverse transcribed to generate cDNA using the Retroscript Kit (Ambion). Control reactions were performed without the reverse transcriptase enzyme. PCR was performed using

previously described *Y. pestis* gene-specific primers [11]. The results of RT-PCR were evaluated by gel electrophoresis.

4.4. Artificial infection of Xenopsylla cheopis fleas

X. cheopis laboratory colonies were sifted to remove flea pupae. Prior to artificial infection, adult fleas were emerged from pupae and maintained for 5-6 days at 80% relative humidity in 50 ml conical tubes containing a strip of filter paper. *Y. pestis* (Lcr^-) cells were grown overnight with shaking at 26° C in HIB prior to addition to fresh, defibrinated rat blood, which was used as a potentially infectious blood meal for feeding fleas. For blood meal preparation, the overnight cultures were pelleted and resuspended in 1 ml of 0.85% NaCl and then diluted 1:10 into the rat blood. Blood meals were placed in artificial flea feeders made with mouse skin membranes as described elsewhere [51], and 150 fleas on each feeder were allowed to feed for one hour. To determine the bacterial load of the flea inocula, 10-fold serial dilutions of the blood meal were plated in duplicate on plates of blood agar containing 6% sheep blood. Following feeding, fleas were chilled on ice and observed under a dissecting microscope to determine whether they ingested the infectious blood meal. The fed infected fleas were maintained at 80% relative humidity in 50 ml conical tubes with filter paper for the duration of the experiment. Twice weekly, fleas were fed on uninfected Swiss Webster mice (maintenance feeding) for one hour. After each maintenance feeding, fleas were examined under the microscope to determine their feeding status and whether their guts contained old, partially digested blood from a previous blood meal. Up to twenty fleas were collected from each experimental group on days 3, 7, 14 and 21 post infection. These fleas were triturated in HIB containing 10% glycerol using a sterile pestle and frozen at -80° C. The bacterial loads of individual fleas were determined by plating triturates of individual fleas or by using a previously described quantitative, real-time PCR assay for the chromosomal *fur* gene [52]. For plating, colony forming units (CFU) of *Y. pestis* in an individual flea triturate were determined by making serial dilutions in HIB and then spreading these dilutions on the surfaces of agar plates made with a 1:1 mixture of CIN agar base (Remel, Lenexa, KS, 28.8 g/L (prepared by autoclaving and without the addition of supplemental antibiotics) and HIB (12.5 g/L HIB formulated with an additional 10 g/L of Bacto agar). CIN/HIB agar plates were more permissive for *Y. pestis* growth than commercially prepared CIN agar and were developed for this experiment to encourage complete recovery of *Y. pestis* cells, while restricting the growth of other organisms that might be present in flea triturates.

4.5. Growth in macrophages

The murine macrophage-like cell line J774A.1 (ATCC, Manassas, VA), was grown in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Grand Island, NY) supplemented with glutamine, 10% (v/v) FBS and sodium pyruvate at 37° C in the presence of 5% CO_2. After overnight incubation, the cells were washed twice in the medium without FBS and reconstituted with fresh culture medium supplemented with FBS (200-250 μl/well for the 24 well plates). *Y. pestis* CO92 wild type (Lcr+ Pgm+) and the mutant strain CO92_ΔYPO499-516 (Lcr+ Pgm +), were grown at 26° C or 37° C in HIB medium overnight. Overnight cultures were then diluted and the bacteria were grown to the exponential phase. At that point the bacteria were collected by centrifugation, washed once in PBS, and suspended in PBS before being used for infection of mammalian cells. To each well, a suspension of bacteria was added at different multiplicities of infection (MOI), and uptake was allowed for 30 minutes at 37° C. After bacterial uptake, the monolayer was washed twice with cell culture medium and 1 ml of fresh medium supplemented with 7 μg/ml gentamicin (Gibco BRL) was added and that was assigned as a time point 0 hours. Infected macrophages were incubated at 37° C in the presence of 5% CO_2 for various periods of time. For determination of the numbers of intracellular bacteria, the host cells were washed twice in PBS and lysed with 0.2 ml of 0.1% sodium deoxycholate

in PBS. After addition of 1.8 ml of PBS, serial dilutions were made, and 100 μl of each dilution was plated onto HIB agar plates in triplicate.

4.6. Murine model

Female 6- to 7-week-old Swiss-Webster mice were purchased from Charles River Laboratories (Wilmington, MA). *Y. pestis* (Lcr+ Pgm+) cells were grown on HIB agar plates for 36 hours at 26° C, suspended in cold phosphate buffer, and the cell concentration was adjusted by measuring optical density at 600 nm to calculate the challenge dose. The actual number of bacteria inoculated into the animals was determined by plating. Subcutaneous (s.c.) challenge was performed by injecting a 100 μl suspension of bacteria into the back hitch of the animal and intranasal (i.n.) challenge was performed by instilling 40 μl of the cell suspension into the nostrils of lightly anesthetized mice. As determined previously by us, the median lethal dose (MLD) of the wild-type *Y. pestis* CO92 used in our experiments was 5-15 CFU and 300-380 CFU for s.c. and i.n. challenge routes, respectively [29]. In some experiments, mice received a sub-inhibitory dose of levofloxacin (0.3 to 0.5 mg/kg of body weight/day) by the intraperitoneal (i.p.) route starting 24 hours post-infection for a period of five days. As shown by us recently, this scheme of treatment with the sub-inhibitory dose of levofloxacin prolonged time to death and increased the percent survival for mice after challenge with virulent plague organisms and was, therefore, useful for evaluating the degree of attenuation of the *Y. pestis* mutants [29]. To provide a complete cure from plague, one group of mice received a treatment dose of levofloxacin (50 mg/kg/day) using the same regime as the group that received the sub-inhibitory dose. The mice were observed for morbidity or mortality for the period of four to five weeks to evaluate their survival rate.

4.7. Statistical analysis

Mean and median survival times of infected mice were calculated and statistical analysis was performed using SAS package, version 9.2 (SAS Institute Inc., Cary, NC). For each comparison we used the log-rank, Wilcoxon, Peto, and modified Peto tests to statistically probe for possible significant differences between the survival distributions. A p value of ≤ 0.05 was considered significant. The macrophage assays were run in triplicate and repeated at least three times. Differences between experimental groups were analyzed using Student's t-test. All data were expressed as mean ± standard deviation.

Acknowledgments

This study was supported by the Sealy Center for Vaccine Development of the University of Texas Medical Branch, NIH/NIAID Western RCE grant U54 AI057156, and NIH/NIAID grant R01 AI064389. JBR was supported by the NIH T32 Biodefense Training grant AI060549. Likewise, SLA was supported in part by the T32 NIH training grant on Tropical and Emerging Infectious Diseases (AI07526) and Biodefense (AI060549). We thank Dr. A. Torres for thoughtful discussion and Dr. R. Pieper for sharing his manuscript on *Y. pestis* proteomic analysis prior to publication. We thank Eric Mandel for his help with statistical analysis.

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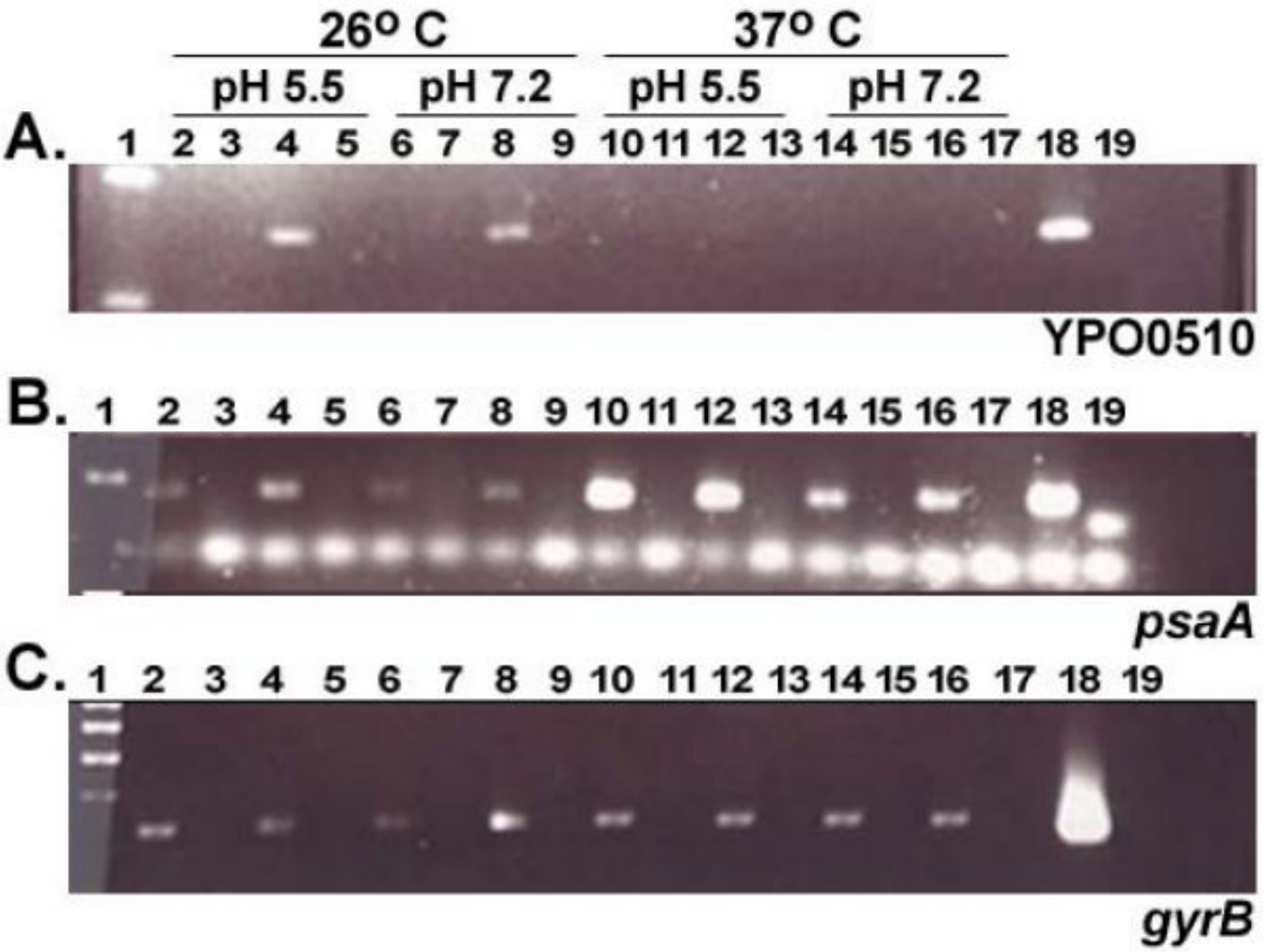

Figure 1.

RT-PCR detection of *Y. pestis* CO92 (Lcr$^-$ Pgm+) gene expression after growth in HIB media buffered with 50 mM MOPS, pH 7.2 or with 50 mM MES, pH 5.5, supplemented with 0.1% galactose, 4 mM $CaCl_2$, and 20 mM $MgCl_2$. RNA was isolated at 2 hours following a temperature shift from 26° C to 37° C. *Y. pestis* mutant strain ΔYPO499-516 (lanes 2, 3, 6, 7, 10, 11, 14, 15) and parental strain (lanes 4, 5, 8, 9, 12, 13, 16, 17). The even and odd lane numbers represent samples with and without reverse transcriptase added, respectively. Lanes 2-5 - 26° C, pH 5.5; lanes 6-9 - 26° C, pH 7.2; lanes 10-13 - 37° C, pH 5.5; lanes 14-17 - 37° C, pH 7.2; Lane 1- Standard DNA Ladder; Lane 18- positive PCR control, DNA of CO92; Lane 19- negative PCR control, no template. **A.** YPO0510, a hypothetical gene of the T6SS locus; Lane 1, ladder bands correspond to 200 and 300 bp. **B.** YPO1303, *psaA*, subunit of pH 6 antigen; Lane 1, ladder band corresponds to 100 bp. **C.** YPO4094, *gyrB*, DNA gyrase subunit B; Lane 1, ladder bands correspond to 200, 300 and 400 bp.

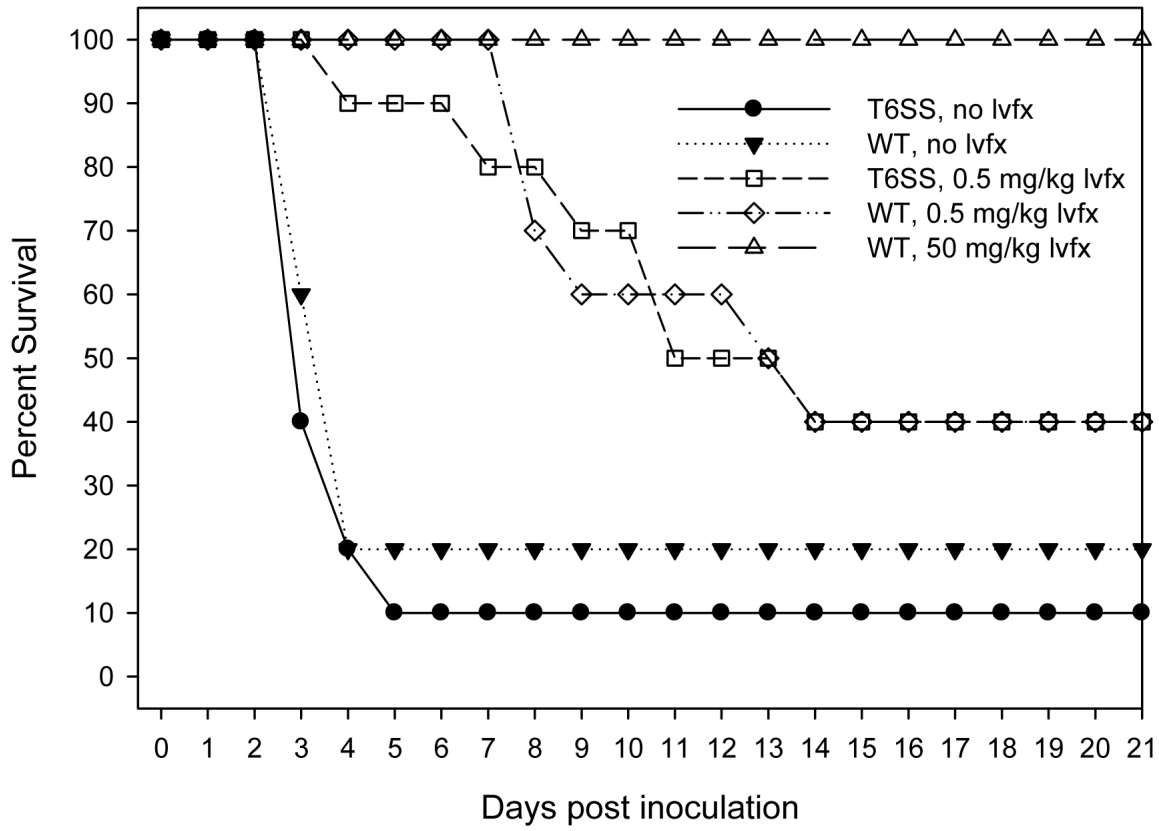

Figure 2.
Survival of Swiss-Webster mice (n = 20/group) after challenge via the i.n. route to mimic pneumonic plague. Mice were inoculated with 2×10^3 CFU of the wild type (WT) or the Lcr + Pgm+ mutant strain ΔYPO499-516 (T6SS) of *Y. pestis* CO92. At 24 hours of postinfection and for a total of 5 days, half of the mice were given a sub-inhibitory dose (0.3 to 0.5 mg/kg/day) of the antibiotic levofloxacin (lvfx). An additional group of mice (n = 10) received a treatment dose of 50 mg/kg/day of levofloxacin by the same schedule as for the treatment with the low dose of antibiotic.

Table 1

Infection status and bacterial load of *X. cheopis* fleas. Data are combined from fleas collected during two separate experiments. All strains were CO92 (Lcr^-)

Fleas infected with	Day p.i.	Fleas Collected (n)	Infected Fleas (%)	Median (range) CFU/Flea
Y. pestis CO92 Pgm+	0	10	100	1.5×10^4 (1.2×10^2-1.6×10^5)
	3	40	97.5	2.9×10^3 (0.0-4.9×10^4)
	7	22	100	4.7×10^3 (2.5×10^1-4.1×10^4)
	14	12	83.3	9.1×10^3 (0.0-3.9×10^4)
	21	-	-	-
Y. pestis CO92 Pgm^-	0	5	100	1.8×10^4 (7.9×10^3-8.4×10^4)
	3	20	85	1.1×10^2 (0.0-3.2×10^4)
	7	11	100	3.4×10^3 (6.3×10^2-4.9×10^4)
	14	-	-	-
	21	-	-	-
Y. pestis CO92_ΔYPO499-516 Pgm+	0	10	100	3.4×10^3 (2.6×10^2-1.8×10^5)
	3	40	100	1.0×10^3 (1.0-1.8×10^6)
	7	31	96.8	8.1×10^3 (0.0-8.0×10^4)
	14	5	100	1.7×10^3 (1.3×10^3-1.2×10^4)
	21	-	-	-
Y. pestis CO92_ΔYPO499-516 Pgm^-	0	10	100	2.7×10^3 (1.7×10^2-1.8×10^4)
	3	40	95	5.7×10^1 (0.0-1.8×10^4)
	7	20	100	1.2×10^3 (2.1×10^1-1.5×10^5)
	14	20	100	2.4×10^2 (1.0×10^1-2.2×10^4)
	21	10	100	9.7×10^2 (2.3×10^2-5.9×10^3)
Y. pestis CO92_Δymt Pgm+	0	10	100	1.4×10^3 (1.0×10^2-9.2×10^3)
	3	26	61.5	1.5×10^1 (0.0-2.6×10^4)
	7	20	75	4.5×10^2 (0.0-7.2×10^4)
	14	4	0	0.0 (0.0-0.0)
	21	-	-	-
Y. pestis CO92_Δymt Pgm^-	0	10	100	3.7×10^3 (6.5×10^2-1.8×10^5)
	3	40	30	0.0 (0.0-8.6×10^4)
	7	40	15	0.0 (0.0-5.6×10^1)
	14	40	2.5	0.0 (0.0-3.1×10^3)
	21	20	5	0.0 (0.0-1.4×10^2)
No bacteria	0	5	0	0.0 (0.0-0.0)
	3	40	0	0.0 (0.0-0.0)
	7	33	0	0.0 (0.0-0.0)
	14	40	0	0.0 (0.0-0.0)
	21	20	0	0.0 (0.0-0.0)

Table 2

Mortality and feeding behavior of fleas following artificial infection. For each time point, the percentages of total fed and unfed fleas are given. In addition, the fed fleas categorized by midguts containing only fresh blood and midguts containing a mixture of fresh and old blood, and unfed fleas were divided into empty (clear) midguts and those containing old blood from previous feeding. Results represent two separate experiments

Fleas infected with	Day p.i.	Fleas (n)	Flea Mortality (%)	Live Fleas					
				Fed (%)				Unfed (%)	
				Fresh	Mixed	Total	Clear	Old	Total
Y. pestis CO92 Pgm+	3	199	38.7	18.8	81.2	78.7	15.4	84.6	21.3
	7	136	4.4	4.8	95.2	80.8	4	96	19.2
	14	82	3.7	25.4	74.6	84.8	0	100	15.2
	21	-	-	-	-	-	-	-	-
Y. pestis CO92 Pgm$^-$	3	33	21.2	29.2	70.8	92.3	50	50	7.7
	7	5	0	25	75	80	0	100	20
	14	-	-	-	-	-	-	-	-
	21	-	-	-	-	-	-	-	-
Y. pestis CO92_ΔYPO499-516 Pgm+	3	179	43.6	23.4	76.6	76.2	29.2	70.8	23.8
	7	58	32.8	26.1	73.9	59	37.5	62.5	41
	14	6	16.7	33.3	66.7	60	0	100	40
	21	-	-	-	-	-	-	-	-
Y. pestis CO92_ΔYPO499-516 Pgm$^-$	3	214	34.1	23.1	76.9	85.8	10	90	14.2
	7	100	28	5.2	94.8	80.6	7.1	92.9	19.4
	14	33	6.1	6.9	93.1	93.6	0	100	6.4
	21	-	-	-	-	-	-	-	-
Y. pestis CO92_Δymt Pgm+	3	134	41.8	24.3	75.7	94.9	50	50	5.1
	7	51	43.1	15	85	69	0	100	31
	14	6	33.3	0	100	25	0	100	75
	21	-	-	-	-	-	-	-	-
Y. pestis CO92_Δymt Pgm$^-$	3	227	8.8	21.9	78.1	70.5	19.7	80.3	29.5
	7	162	21.6	13.4	86.6	88.2	26.7	73.3	11.8
	14	88	1.1	12	88	86.2	16.7	83.3	13.8
	21	38	10.5	9.1	90.9	32.4	8.7	91.3	67.6
No bacteria	3	275	1.1	12.1	87.9	91.5	17.4	82.6	8.5
	7	194	18	6.7	93.3	84.9	8.3	91.7	15.1
	14	159	6.9	25	75	78.4	25	75	21.6
	21	55	7.3	7.7	92.3	25.5	5.3	94.7	74.5

Table 3

Intracellular viability of 26° C pre-grown *Y. pestis* CO92 wild type (WT) or the Lcr+ Pgm+ mutant strain ΔYPO499-516 (T6SS) in murine macrophage like cell line J774.A1. Multiplicity of infection (MOI) = 1. Results represent three separate experiments

Number of bacteria (log10 CFU/well)							
MOI 1	**Time (hours)**						
Strain	0	4	8	12	16	20	24
WT	4.3±0.03	4.2±0.14	4.0±0.44	4.5±0.07	4.1±0.1	3.8±0.05*	2.9±0.24*
T6SS	4.7±0.05	4.6±0.24	4.7±0.09*	4.6±0.14	4.5±0.1*	4.2±0.19*	4.4±0.09*

* $p < 0.001$

Table 4

Intracellular viability of 37° C pre-grown *Y. pestis* CO92 wild type (WT) or the Lcr+ Pgm+ mutant strain ΔYPO499-516 (T6SS) in murine macrophage like cell line J774.A1. Multiplicity of infection (MOI) = 50. Results represent three separate experiments

	Number of bacteria (log10 CFU/well)				
MOI 50	**Time (hours)**				
Strain	0	6	18	24	30
WT	4.8±0.24*	4.7±0.17*	4.2±0.18*	4.3±0.05*	4.3±0.15*
T6SS	3.8±0.22*	3.7±0.12*	3.4±0.08*	2.9±0.2*	3.1±0.27*

* $p < 0.001$