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Directed Screen of *Francisella novicida* Virulence Determinants Using *Drosophila melanogaster*▽†

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***Francisella tularensis* is a highly virulent, facultative intracellular human pathogen whose virulence mechanisms are not well understood. Occasional outbreaks of tularemia and the potential use of *F. tularensis* as a bioterrorist agent warrant better knowledge about the pathogenicity of this bacterium. Thus far, genome-wide *in vivo* screens for virulence factors have been performed in mice, all however restricted by the necessity to apply competition-based, negative-selection assays. We wanted to individually evaluate putative virulence determinants suggested by such assays and performed directed screening of 249 *F. novicida* transposon insertion mutants by using survival of infected fruit flies as a measure of bacterial virulence. Some 20% of the genes tested were required for normal virulence in flies; most of these had not previously been investigated in detail *in vitro* or *in vivo*. We further characterized their involvement in bacterial proliferation and pathogenicity in flies and in mouse macrophages. Hierarchical cluster analysis of mutant phenotypes indicated a functional linkage between clustered genes. One cluster grouped all but four genes of the *Francisella* pathogenicity island and other loci required for intracellular survival. We also identified genes involved in adaptation to oxidative stress and genes which might induce host energy wasting. Several genes related to type IV pilus formation demonstrated hypervirulent mutant phenotypes. Collectively, the data demonstrate that the bacteria in part use similar virulence mechanisms in mammals as in *Drosophila melanogaster* but that a considerable proportion of the virulence factors active in mammals are dispensable for pathogenicity in the insect model.**

Francisella tularensis is the causative agent of tularemia, a zoonotic disease affecting a wide variety of small vertebrates as well as humans (49). The severity and the clinical manifestations of the disease are highly dependent on the infecting strain and the route of entry. If inhaled, as few as 10 bacteria can cause infection in humans, and if untreated, the mortality rate can reach 60% (45). To date, two subspecies that cause disease in humans, *F. tularensis* subspecies *holarctica* and *F. tularensis* subspecies *tularensis*, have been identified. A closely related species, *F. novicida*, is an environmental pathogen and appears not to affect healthy humans, since *F. novicida* infections have been reported almost exclusively in immunocompromised individuals (8, 19, 58), but it has been recognized as relevant when *Francisella* virulence in various mouse infection models is investigated. Genome comparisons revealed high sequence similarities between the *F. novicida* isolate U112 and the clinically important *F. tularensis* subspecies *tularensis* strain SCHU S4. The former represents the evolutionarily oldest and most complete *Francisella* genome, supporting good metabolic competence, while human-pathogenic strains, in adaptation to an intracellular niche, have lost many genes to genetic drift and are much more fastidious (26, 43).

The only tularemia treatment to date relies on antibiotics. To enable development of vaccines and new antimicrobial drugs, it is vital to understand the molecular mechanisms behind the interaction of this pathogen with humans. *F. tularensis* escapes from the host cell phagosome and propagates in the cytosol (16). Multiplication results in cell death and release of bacteria (25), allowing them to spread to regional lymph nodes and to colonize spleen, liver, and lung (52). A substantial proportion of the bacterial burden can persist extracellularly in the bloodstream (14, 59).

Despite knowledge about the *in vivo* life cycle, genome sequence data, and techniques for mutant generation, we still know little about specific virulence determinants of *F. tularensis*. Factors that are known to play a role are involved in lipopolysaccharide biosynthesis or intracellular survival. A focus of attention has been a genomic region called the *Francisella* pathogenicity island (FPI), which is required for escape from the phagosome and proliferation inside the cytosol and which encodes a putative type VI secretion system (reviewed in references 2, 13, and 35).

Lately, libraries of transposon insertion mutants of different *Francisella* reference strains were used to screen for virulence factors in various mammalian *in vitro* and *in vivo* infection models (23, 31, 38, 51, 53, 57). *In vivo* screens in mice applied a competition-based, negative-selection strategy to identify bacterial mutants that cannot survive in and/or colonize a target organ (23, 51, 57). Results from these studies suggested a large number of genes to be involved in *Francisella* pathogenicity. While this strategy is a sensitive and efficient way to screen the whole bacterial genome, it probably overestimates the number of virulence genes and their importance as well as

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providing limited information as to the specific roles of individual genes in pathogenesis.

Thus, far, the mouse has been the preferred model host for *in vivo* studies of *Francisella*. Recently, however, analysis of various human pathogens in model organisms like *Caenorhabditis elegans* or *Drosophila melanogaster* have demonstrated that bacteria to a large extent rely on the same virulence strategies in invertebrates as in humans (24). Because of their simplicity and the genetic tools available, nonmammalian models offer a unique opportunity to unravel the basis of host-pathogen interactions in great detail. Our previous work, in which we introduced *D. melanogaster* as a model host for *Francisella* infections, suggested that the fruit fly might be valuable for the identification and characterization of virulence determinants (56). In addition, blood-feeding arthropods, like ticks, mosquitoes, and biting flies, have long been acknowledged as vectors of tularemia (34, 37), implying that the bacterium has evolved strategies for persistence and replication in such organisms. *Drosophila melanogaster* might not represent a natural host for *Francisella tularensis*, but it is a highly relevant model for immune mechanisms and basic physiology in arthropods.

Here we individually analyzed nearly 250 genes that had previously been suggested as candidate virulence determinants by negative-selection screens in mice in order to confirm their importance for pathogenicity in a robust infection model. By using survival of infected flies as a measure of bacterial virulence, we identified 49 genes as being required for normal virulence in flies. These genes were further investigated for their role in bacterial proliferation in flies and in mouse macrophage-like cells. All mutant phenotypes were analyzed by hierarchical cluster analysis to provide new insights into functional relationships among the corresponding genes. Our collected data and comparison of bacterial mutants in two model systems also allowed for an evaluation of *D. melanogaster* as a screening model.

MATERIALS AND METHODS

Bacterial strains, fly strains, and culture media. Infection experiments with *D. melanogaster* Oregon R were performed using *F. novicida* U112 as the wild-type control and U112-derived transposon insertion mutants from the two-allele library provided by Colin Manoil's group (15). Mutants were grown initially directly from the 96-well plates containing the library and later from single-colony stocks. Other strains used were the live vaccine strain (LVS) of *F. tularensis* subspecies *holarctica*. Bacteria were cultured on modified GC agar plates (Difco GC medium base [Becton Dickinson] complemented with hemoglobin and Iso-Vitalex) or in supplemented TSB medium (Trypticase soy broth, 0.1% L-cysteine HCl, 0.2% dextrose) at 37°C and 5% CO_2. For transposon mutants, media contained 15 μg/ml kanamycin; for viable count analysis, agar plates were also supplemented with 50 μg/ml polymyxin B. For the *in vitro* proliferation test, bacteria were inoculated to an optical density at 600 nm (OD_{600}) of 0.05 from a standard overnight culture and incubated at 29°C and 200 rpm. Data points for growth curves were collected until the OD_{600} reached 2. Viable count analysis from infected flies was carried out as described previously (56), with sample preparation on days 1, 3, and 5 postinfection for attenuated mutants and on days 1, 2, 3, 4, and 5 postinfection for hypervirulent mutants. All experiments were repeated two or three times. Prior to infection, flies were reared on standard corn meal agar at room temperature.

Fly infection. In each test, 20 adult flies (10 females and 10 males, 2 to 8 days old) were infected by being pricked with a glass needle dipped in bacterial colonies. Flies were incubated at 29°C and transferred to fresh food every day. Living animals were counted once each day for 10 days, and the results were recorded as the percentage of the number of living animals in relation to the number of flies recorded 1 day postpricking. Bacterial mutants were tested in sets of 10 to 15 strains plus two wild-type replicates per set. Each mutant allele was examined in at least two independent replicates. The median life length (MLL) of each replicate was calculated by linear extrapolation between the two values surrounding 50% survival. In cases where 50% survival was not reached within the 10-day observation period, the data were censored and the MLL was set to be 10 days. Only sets of experiments in which the MLL values for the two wild-type controls differed by less than 1 day were accepted.

Intracellular growth assay. The mouse macrophage-like cell line J774A.1 was cultured in standard Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and kept at 37°C and 5% CO_2. Cells were infected as described previously (17), using 1.5×10^5 cells per well in 24-well plates and an approximate multiplicity of infection of 100. The release of lactate dehydrogenase as an indicator of cytotoxicity was measured using a commercial kit (Promega). All analyses were done twice, with duplicates in the second round.

Data analysis: fly survival. For each mutant and each replicate, we determined the difference in MLL compared to that for the corresponding U112 wild-type control and calculated the median for all replicates (mΔMLL). To identify mutant mΔMLL values significantly different from those for the wild type, we obtained cutoff criteria using a simulation approach that was based on the wild-type MLL values from all experiments. The difference between the two controls, ΔMLL_{wt}, was calculated for all experiments, and we assumed that these observations were normally distributed. The distribution parameters were estimated by the sample mean and standard deviation from the ΔMLL_{wt} data. The estimated normal distribution was used to simulate 10,000 pseudo-mΔMLL_{wt} observations, with four replicates per observation. The empirical distribution of the simulated data estimates the null distribution under which the mutants behave as the wild type. The 0.0025 and 0.9925 quantiles of the simulated data were selected as cutoff values (±1.5 days) and corresponded to a significance level of 0.5% ($P = 0.005$). Some mutants did not have four replicates, but by considering their numbers of replicates in the simulation procedure, we obtained their P values. All mutants outside the ±1.5-day interval were significant at a P value of 0.005, and no mutant inside the ±1.5-day interval was significant at this level. A mutant strain was called attenuated if the median ΔMLL was >1.5 days and hypervirulent if it was <1.5 days.

Bacterial growth *in vitro*. Based on values from the linear range of the growth curve ($0.5 < OD_{600} < 1.8$), the growth rate, ΔOD_{600}/h, was calculated for each mutant and each replicate. The mean growth rate of the U112 wild type was $0.510 \pm 0.057\ \Delta OD_{600}$/h ($n = 24$). The ratio between the mutant growth rate and that of the corresponding wild-type control was defined as the *in vitro* proliferation index.

Spearman's rank correlation (R_S), with a conservative significance level of $P < 0.001$, was used to determine whether results from the different assays were statistically significantly correlated with each other.

Gene information was gathered from http://www.francisella.org/.

RESULTS

Our group recently demonstrated that LVS kills *D. melanogaster* in a dose-dependent manner and that 50% of the flies infected with 3×10^5 bacteria succumb to the infection within 5 to 6 days (56). We also showed that mutants of single FPI genes were less virulent in flies.

For our analysis of putative virulence factors, we chose *F. novicida* transposon insertion mutants from a well-described two-allele library (15). This library contains two distinct strains for most of the 1,490 loci for which transposon insertions were verified and thereby provides internal controls, since the two alleles of each gene should result in similar phenotypes. We first established experimental conditions for infections with *F. novicida*. Advantageous for our screen, U112-infected flies died much more quickly than did LVS-infected flies (see Fig. S1A in the supplemental material), an observation that has also been made in mouse infection models (22). Infection by pricking reproducibly delivered an inoculum of $\sim 10^4$ CFU per fly, killing all flies within approximately 7 days. Furthermore, we ascertained that a U112 mutant of a known virulence determinant, MglA, had an attenuated phenotype similar in terms of fly survival to that previously demonstrated for an

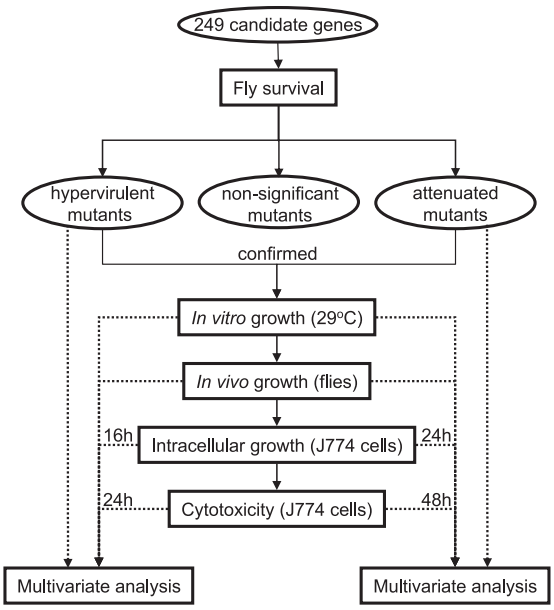

FIG. 1. Flow chart visualizing the sequence of experiments. The various assays and analysis methods are marked by rectangular boxes, and groups of bacterial mutants are marked by ovals. Assay readouts used for multivariate analysis are indicated by dotted arrows.

LVS mutant of the same gene (see Fig. S1B in the supplemental material and compare to reference 56).

Assessment of putative virulence determinants by fly survival. We then performed a directed screen of genes that had been implicated in pathogenicity in two recent negative-selection screens in mice. These two studies had identified either U112 transposon insertion mutants unable to spread to the spleen 48 h after intraperitoneal infection (57) or LVS mutants deficient in persistence in the lung 7 days after intranasal infection (51). A total of 240 mutants had been absent from the target organs in these two studies, and the corresponding genes were therefore considered candidate virulence factors in the mouse. Of these genes, 222 are represented in the U112 two-allele library; the residual loci are not covered by the library, since they belong to the large group of candidate essential genes that are required for bacterial survival in broth at 37°C (15). In addition to the 222 loci, we included a number of genes in our screen that had been suggested as putative virulence factors by other investigations.

Altogether, we individually tested mutants of 249 genes in flies by using the mutant allele with the transposon inserted in the 5′ half of the open reading frame (ORF) (see study outline in Fig. 1). For each mutant, we monitored survival of infected flies (see examples in Fig. 2A) and calculated the median difference in median life length compared to that for U112-infected flies (see Table S1 and Fig. S2 in the supplemental material). We then used a nonparametric statistical test to determine whether a mutant killed flies significantly more slowly or quickly than did U112 (see Materials and Methods). We identified 43 loci that were attenuated for killing *Drosophila*. These included most of the FPI genes, which grouped with an average median ΔMLL of 4.3 ± 0.7 days. Only mutants of four FPI genes, *FTN_1319* (*pdpC*), *FTN_1320* (*pdpE*), *FTN_1325* (*pdpD*), and *FTN_1326* (*anmK*), behaved like the wild-type control in flies. Surprisingly, we also found six mutants to be hypervirulent in flies (*FTN_0119*, *pilA*, *pckA*, *fimT*, *pilB*, and *pilM*).

For the 49 loci identified with significantly altered fly survival, we wanted to confirm the mutant phenotype by testing the sister alleles from the library. For 42 candidates, the library provided a second allele, which has the transposon inserted further downstream in the ORF. For all but one, we could confirm the effect of the bacterial gene knockout on fly survival (see Table S1 in the supplemental material). The correlation between the median ΔMLL of the first allele and that of the second allele was high ($R = 0.923$) (see Fig. S3 in the supplemental material), ensuring that our candidates were true "hits." In the case of *FTN_0672*, the second allele produced a different, nonattenuated phenotype. Sequencing confirmed that both alleles harbored the transposon at the expected position in the genome. However, in the nonattenuated allele the transposon is inserted only 9 bp from the end of the ORF and probably does not disrupt gene function. In the cases of *FTN_0119* and *greA*, for which we could not test a sister allele, mutants of the downstream genes *FTN_0118* and *fimT*, respectively, were demonstrated to have a phenotype different from that of the *FTN_0119* and *greA* mutants (see Table S1 in the supplemental material). We further checked the available information on insertion sites in all of the mutant strains but could not find indications of off-target effects on downstream genes in the same operon, so-called polar effects, with possible exceptions being insertions in *tig* and in *FTN_0772* (see Discussion). Since *tig* is the first of four genes in a putative operon (*tig-clpP-clpX-lon*), it is possible that a transposon insertion in *tig* disrupts expression of the entire operon and thereby creates an additive phenotype. With respect to fly survival, mutants of the other genes in the operon were mildly attenuated but did not differ significantly from the wild type, and SCHU S4 mutants of *tig* or *lon* were not attenuated in mice (21).

For the large number of mutant strains whose median ΔMLL differed by less than 1.5 days from that of the wild type and was not significant, it is possible that the transposon fails to disrupt gene function (see the example given above). However, bacterial strains in the two-allele library had originally been selected to carry transposon insertions at 5 to 70% ORF length to increase the chance of null alleles (15). Furthermore, mutants of 11 genes with a nonsignificant phenotype in flies were negatively selected in a mouse infection model, which used the same transposon library as we did (23). So, differences between bacterial mutant phenotypes in flies and in mice can be explained partly by the assumption that competitive selection in mice is a more sensitive assay than individual testing in flies and that this assay will detect even subtle phenotypes. In addition, the conservative cutoff criteria (>1.5-day difference, $P < 0.005$) we used to define attenuated and hypervirulent mutants have contributed to these differences (see Discussion). We decided on such conservative cutoff criteria to avoid a high percentage of false-positive hits. Consequently, the fly model becomes more robust, but false-negative hits may have occurred.

Importance of candidate genes for bacterial proliferation in flies. Next, we wanted to know whether the mutants' effect on fly survival was dependent on bacterial proliferation. Before

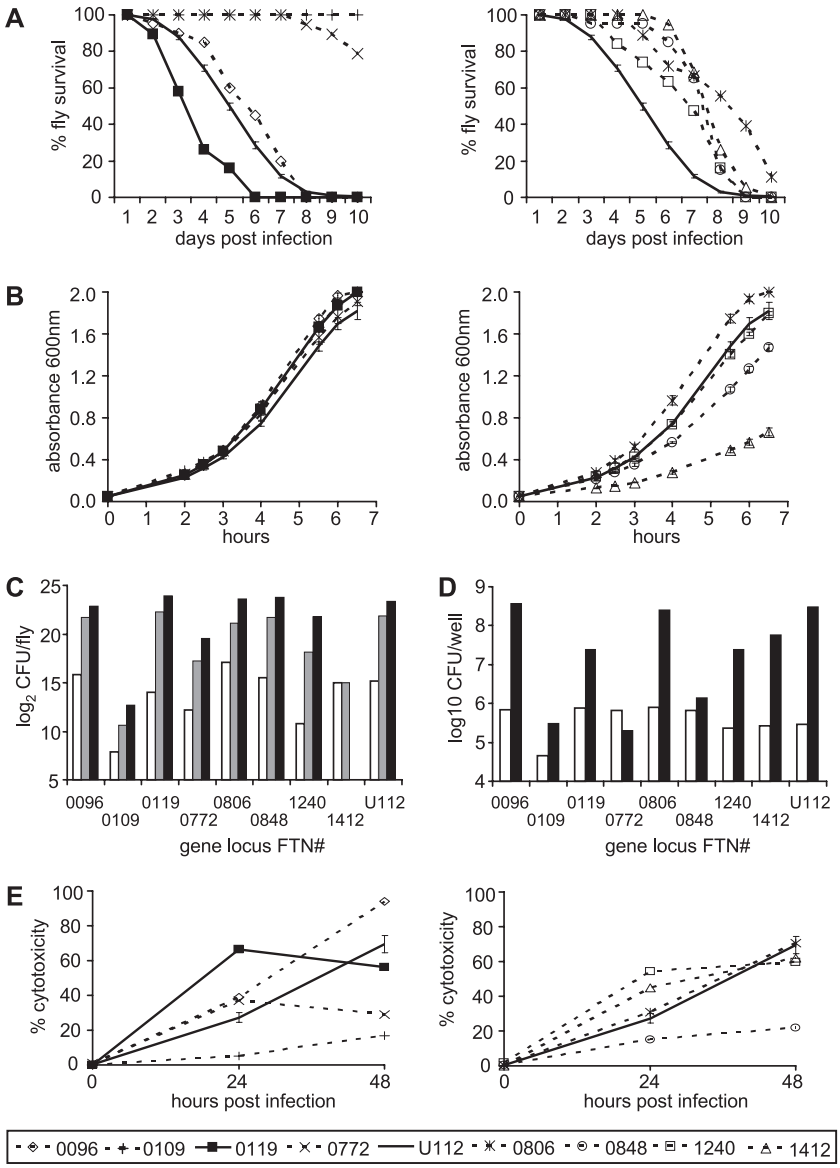

FIG. 2. Phenotypic features of attenuated and hypervirulent (*FTN_0119*) *F. novicida* transposon insertion mutants of nonannotated genes, as determined by various assays. If not otherwise noted, representative results from one experiment are shown for the mutants, and the averages are shown for U112. The legend at the bottom applies to panels A, B, and E, and numbers refer to FTN designations (e.g., 0096 represents the *FTN_0096* mutant). (A) Survival of infected flies. (B) Bacterial growth in liquid culture at 29°C. The averages and standard errors of the means (SEM) are shown for all mutants ($n = 3$). (C) Bacterial proliferation in adult flies on day 1 (white), day 3 (gray), and day 5 (black) postinfection. Day 5 values were not determined for the *FTN_1412* mutant. (D) Bacterial proliferation in J774 cells 45 to 60 min (white) and 24 h (black) after infection. Note that for the *FTN_0119* mutant, bacterial numbers were higher at 16 h (8.05 $\log_{10}$ CFU/well). (E) Bacterial cytotoxicity, as measured by the release of lactate dehydrogenase from J774 cells into the culture medium.

we carried out viable count determination with infected flies, we needed to ensure that attenuation in flies was not caused by a general growth defect of the mutants under our experimental conditions. As mentioned above, the two-allele library does not include mutants with a general growth defect at 37°C. Nevertheless, *FTN_0665*, *FTN_0821*, and *FTN_1661* mutant strains grew slowly on agar media at 37°C in our hands and were not investigated further. Since we carried out fly infection experiments at 29°C, the highest temperature at which *D. melanogaster* can be kept over an extended period, we monitored *in vitro* growth of the remaining 40 attenuated mutant strains and the six hypervirulent mutant strains at 29°C (see examples in Fig. 2B). For each mutant, we determined the *in vitro* proliferation index in relation to that for U112 (see Materials and Methods). Only a few mutants grew considerably more slowly than U112, i.e., the *glgB*, *sspA*, *manB*, *FTN_0848*, and *FTN_1412* mutants, confirming that in most cases the low temperature did not limit proliferation (Tables 1 and 2; also see Fig. S4A in the supplemental material).

We then followed bacterial proliferation in flies over the first 5 days of infection for all 46 strains (examples in Fig. 2C). The bacterial load on day 3 was chosen as the readout, since pro-

TABLE 1. Features of attenuated *F. novicida* transposon insertion mutants[f]

Gene name	Locus tag	Gene description	*In vitro* proliferation index ($n = 3$)	*D. melanogaster*		J774 cells	
				Fly survival[b] ($n = 2$–7)	Bacterial load[c] ($n = 2$ or 3)	Intracellular proliferation index[d] ($n = 2$)	Cytotoxicity index[e] ($n = 2$)
pyrB	*FTN_0019*	Aspartate carbamoyltransferase	1.03	3.57	20.35	0.12	1.02
carB	*FTN_0020*	Carbamoyl-phosphate synthase large chain	1.02	3.60	20.74	0.20	0.93
carA	*FTN_0021*	Carbamoyl-phosphate synthase small chain	1.01	2.18	20.21	0.13	0.93
pyrF	*FTN_0035*	Orotidine-5′-phosphate decarboxylase	0.94	2.68	20.31	0.22	1.01
pyrD	*FTN_0036*	Dihydroorotate oxidase	0.93	2.00	20.49	0.27	1.02
feoB	*FTN_0066*	Ferrous iron transport protein B	1.04	2.18	18.86	1.05	1.00
	FTN_0096	Conserved hypothetical membrane protein	1.11	1.59	20.39	0.54	1.12
	FTN_0109	Novel protein of unknown function	1.10	4.06	7.68	0.01	0.12
ribC	*FTN_0113*	Riboflavin synthase alpha chain	1.10	4.38	20.07	0.31	0.79
minD	*FTN_0330*	Septum formation inhibitor-activating ATPase	0.81	3.00	17.31	0.46	0.50
minC	*FTN_0331*	Septum formation inhibitor	0.87	2.60	16.82	0.38	0.99
slt	*FTN_0496*	Soluble lytic murein transglycosylase	0.89	3.63	14.49	0.04	0.84
glgB	*FTN_0513*	1,4-α-Glucan branching enzyme	0.75	4.44	8.26	0.24	0.97
sspA	*FTN_0549*	Stringent starvation protein A	0.44	3.43	17.93	1.76	0.69
secA	*FTN_0672*	Preprotein translocase, subunit A	0.92	2.06	16.19	0.10	1.16
	FTN_0772	Conserved protein of unknown function	0.95	4.16	17.06	0.01	0.41
	FTN_0806	Glycosyl hydrolase family 3	1.03	1.85	21.00	0.76	1.12
	FTN_0848	Amino acid antiporter	0.65	2.33	21.18	0.00	0.25
tig	*FTN_1058*	Trigger factor (TF) protein	0.87	1.95	20.58	0.40	1.11
	FTN_1240	BolA family protein	0.86	1.86	19.33	0.14	0.92
pdpA	*FTN_1309*	Protein of unknown function	1.08	4.01	18.85	0.00	0.04
icmF[a]	*FTN_1310*	Conserved protein of unknown function	1.04	5.50	19.07	0.00	0.03
iglE[a]	*FTN_1311*	Protein of unknown function	1.04	4.53	19.34	0.01	0.04
vgrG[a]	*FTN_1312*	Conserved hypothetical protein	1.03	3.03	20.22	0.01	0.03
iglF[a]	*FTN_1313*	Hypothetical protein	1.00	3.07	20.08	0.02	0.03
iglG[a]	*FTN_1314*	Conserved hypothetical protein	1.05	3.53	17.74	0.00	0.03
iglH[a]	*FTN_1315*	Protein of unknown function	0.99	4.25	18.13	0.01	0.03
dotU[a]	*FTN_1316*	Conserved protein of unknown function	0.95	4.71	17.31	0.00	0.03
iglI[a]	*FTN_1317*	Protein of unknown function	0.97	4.71	13.70	0.01	0.03
iglJ[a]	*FTN_1318*	Hypothetical protein	0.98	4.71	18.49	0.01	0.03
iglD	*FTN_1321*	Intracellular growth locus protein D	0.98	4.70	17.87	0.01	0.03
iglC	*FTN_1322*	Intracellular growth locus protein C	0.98	4.70	19.14	0.01	0.04
iglB	*FTN_1323*	Conserved protein of unknown function	1.00	4.56	20.15	0.01	0.04
iglA	*FTN_1324*	Conserved protein of unknown function	0.97	4.71	18.37	0.01	0.04
recB	*FTN_1357*	ATP-dependent exoDNase β subunit	0.97	1.49	21.88	0.85	1.16
feoA	*FTN_1368*	Fe^{2+} transport system protein A	1.05	1.98	19.05	0.85	1.10
	FTN_1412	DNA-directed RNA polymerase α subunit	0.42	3.78	14.52	0.24	0.97
manB	*FTN_1417*	Phosphomannomutase	0.73	2.19	19.88	0.00	0.72
dsbB	*FTN_1608*	Disulfide bond formation protein	1.01	4.43	15.90	0.00	0.12
clpB	*FTN_1743*	Chaperone ClpB	1.00	3.69	18.48	0.09	0.05

[a] Nomenclature according to reference 29.
[b] Fly survival is given as difference in median life length (ΔMLL) between mutant- and U112-infected flies (in days).
[c] Bacterial load at 3 days postinfection is given as $\log_2$ CFU/fly. The mean bacterial load in U112-infected flies was 21.7 ± 1.2 ($n = 18$).
[d] Intracellular proliferation index for 24 h postinfection.
[e] Cytotoxicity index for 48 h postinfection.
[f] Median values are given for all assays; *n*, number of experiments.

liferation had not reached the stationary phase at this time point (Tables 1 and 2; also see Fig. S4B in the supplemental material). Overall, there was a strong and significant negative correlation between bacterial load and fly survival (Table 3). Hypervirulent mutant strains grew mostly to numbers above the U112 average, and strongly attenuated mutants were much reduced in growth *in vivo*. However, among the attenuated mutants, quite a few strains grew to numbers similar to those for U112, indicating that *Francisella* virulence in flies might not depend only on the bacterial burden of the host.

Importance of candidate genes for infection of mouse macrophages. Only a few of the genes that had been suggested as putative virulence factors by the negative-selection screens in mice have been further investigated individually. Since intracellular survival of *F. tularensis* is a prerequisite for its virulence, we sought to establish whether our candidate genes were required for this virulence mechanism. We therefore tested the 46 mutant strains for intracellular growth and cytotoxicity in mouse macrophage-like J774 cells. The proliferation from time of uptake to 16 h and the percent cytotoxicity at 24 h were

TABLE 2. Features of hypervirulent *F. novicida* transposon insertion mutants[e]

Gene name	Locus tag	Gene description	*In vitro* proliferation index ($n = 3$)	*D. melanogaster*		J774 cells	
				Fly survival[a] ($n = 3$ or 4)	Bacterial load[b] ($n = 3$)	Intracellular proliferation index[c] ($n = 2$)	Cytotoxicity index[d] ($n = 2$)
	FTN_0119	Conserved outer membrane protein of unknown function	1.03	−1.88	22.71	0.24	3.66
pilA	*FTN_0415*	Type IV pili, pilus assembly protein	1.06	−2.66	22.68	0.03	3.36
pckA	*FTN_0540*	Phosphoenolpyruvate carboxykinase	0.94	−1.96	22.79	1.07	0.70
fimT	*FTN_0664*	Type IV pili, pilus assembly protein	0.97	−1.58	21.84	0.10	2.64
pilB	*FTN_1115*	Type IV pili ATPase	1.03	−1.98	23.38	0.15	2.77
pilM	*FTN_1141*	Type IV pili, pilus assembly protein	1.06	−2.16	20.22	0.06	3.52

[a] Fly survival is given as difference in median life length (ΔMLL) between mutant- and U112-infected flies (in days).
[b] Bacterial load at 3 days postinfection is given as $\log_2$ CFU/fly. The mean bacterial load in U112-infected flies was 21.7 ± 1.2 ($n = 18$).
[c] Intracellular proliferation index for 16 h postinfection.
[d] Cytotoxicity index for 24 h postinfection.
[e] Median values are given for all assays; *n*, number of experiments.

chosen as readouts for the hypervirulent strains; the proliferation from time of uptake to 24 h and the percent cytotoxicity at 48 h were chosen as readouts for the attenuated strains (examples in Fig. 2D and E). These time points demonstrated the best distinction between mutant and wild-type infections. Again, we related the mutants' values to those of the corresponding U112 control and expressed the results as indices (Tables 1 and 2). In concordance with negative selection in mice and reduced virulence in flies, most attenuated mutants had low intracellular proliferation indices, and many of these also demonstrated reduced cytotoxicity (see Fig. S4C and D in the supplemental material). Despite a strong and significant correlation between intracellular proliferation and cytotoxicity (Table 3), a few mutants had strongly reduced intracellular growth but were fully cytotoxic, i.e., the *pyrB*, *carA*, *secA*, and *FTN_1240* mutants. Similarly to the conclusion mentioned above, these results indicate that cytotoxicity might not depend on bacterial burden alone.

Five hypervirulent mutants had a 16-h proliferation index that was far below that of U112 (Table 2). Since these strains were highly cytotoxic already at 24 h postinfection, their reduced proliferation index was probably due to premature death of the host cells.

Identification of functionally linked genes by multivariate analysis. Finally, we asked whether the mutants' phenotypic features as determined by the different assays were correlated with one another. Apart from the correlation between bacterial load and virulence in both model systems, we found strong and significant correlations between fly survival and intracellular proliferation and between fly survival and cytotoxicity (Table 3). This correlation between readouts across the two model systems suggests that survival of infected flies is indeed a good indicator of bacterial virulence in mice. Encouraged by this result, we thought that a multivariate analysis of the mutants' phenotypic features might allow for conclusions regarding functional relationships between the corresponding genes. Based on all five readouts listed in Tables 1 and 2, we performed separate hierarchical cluster analyses on the attenuated and the hypervirulent mutants (Fig. 3). At an arbitrary height of 10, six clusters of loci with an attenuated mutant phenotype were clearly distinguishable. Cluster 1 mutants differed little from the U112 wild type, with the biggest deviation seen in intracellular proliferation. Candidate virulence genes in this group function primarily in basic metabolic processes, like energy metabolism (*ribC*) and arginine and pyrimidine biosynthesis (*carAB*, *pyrB*, and *pyrFD*), but they seem to be important for bacterial adaptation to oxidative stress. For instance, CarA, CarB, and PyrB are required for the suppression of the oxidative burst in neutrophils (46). *FTN_1240* has homology to BolA morphogens, which switch cell morphology from one type to another after being induced by carbon starvation or acidic or oxidative stress (44). Tig, also called trigger factor, might act in posttranslational protein modification in the periplasm; it was suggested as a putative virulence determinant by an extensive bioinformatic analysis of *Francisella* genomes (43). Interestingly, mutants of *FTN_0848*, a putative amino acid transporter, and *manB*, a phosphomannomutase, formed a subgroup within cluster 1; they did not proliferate well *in vitro* or in macrophage-like cells but grew normally in flies. Knowing that *Francisella* is able to grow extracellularly in the fly (56), we assume that the fly hemolymph, which is particularly rich in amino acids and in organic acids of the citric acid cycle (11), compensates for the deficiency in these two gene functions.

Phenotypic features of genes in cluster 2 were overall similar to those of the first group, but the mutants' growth in flies was more reduced. The functions of these genes are related to cell

TABLE 3. Spearman's rank correlations (R_S) among the five readouts discussed in the study

Group and readouts compared	R_S	*P* value
All mutants ($n = 46$)		
Fly survival/*in vitro* proliferation	−0.03	0.8257
Fly survival/bacterial load in flies	−0.65	0.0000
In vitro proliferation/bacterial load in flies	0.23	0.1280
Attenuated mutants ($n = 40$)		
Fly survival/intracellular proliferation	−0.59	0.0002
Fly survival/cytotoxicity	−0.71	0.0000
In vitro proliferation/intracellular proliferation	−0.08	0.5927
In vitro proliferation/cytotoxicity	−0.17	0.2770
Bacterial load in flies/intracellular proliferation	0.19	0.2458
Bacterial load in flies/cytotoxicity	0.30	0.0641
Intracellular proliferation/cytotoxicity	0.73	0.0000

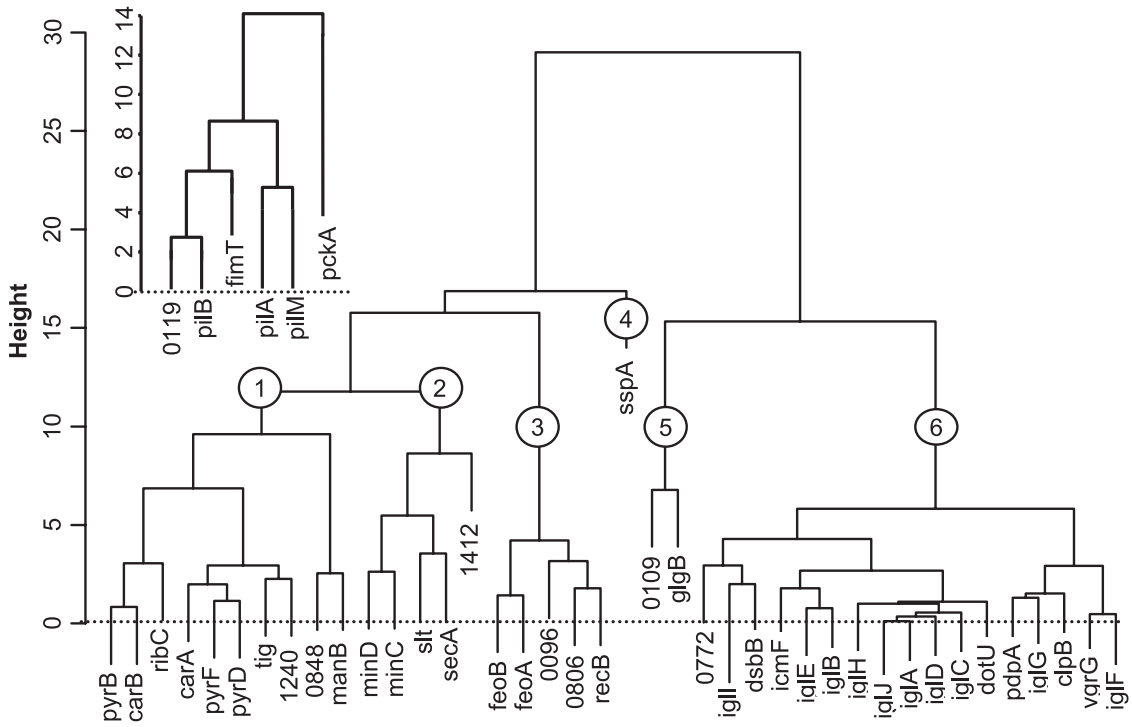

FIG. 3. Hierarchical cluster analysis of phenotypic features of attenuated and hypervirulent (inset) *F. novicida* mutants. Readout variables from all five analyses listed in Tables 1 and 2 were standardized. The Manhattan distance was used to calculate the distances between the mutants, and Ward's method was used to calculate the distances between clusters. Circled numbers identify clusters of phenotypically similar attenuated mutants, and numbers used to identify genes refer to FTN designations (e.g., 1240 represents *FTN_1240*). See the text for further details.

division and, again, to oxidative stress. MinC and MinD are homologous to proteins required for chromosome partitioning during cell division and to components of an oxyanion pump. Based on their findings that a *Francisella minD* mutant is sensitive to serum and to oxidative killing, Anthony et al. hypothesized that this locus either forms an ion pump to extrude toxic ions and radicals or is required to maintain cell wall integrity (1). Slt is a murein-degrading enzyme and is probably important during cytokinesis. Like Tig, Slt was listed as a putative virulence factor by Rohmer et al. (43). The gene product of *FTN_1412* is annotated as a DNA-dependent RNA polymerase. SecA takes part in a conserved protein secretion process across the inner membrane.

Cluster 3 mutants were weakly attenuated in flies but not attenuated in J774 cells, indicating that targeted genes are not important for intracellular survival. The *feoA* and *feoB* genes, which participate in iron transport, as well as *recB*, involved in DNA repair, are related to the defense against oxidative stress. Taken together, these observations suggest that cluster 3 genes play a role during the extracellular period of the *Francisella* life cycle. In the insect hemolymph, reactive oxygen and nitrogen species contribute to the antimicrobial defense.

The *sspA* mutant (cluster 4) separated from the other groups, due to its slow growth *in vitro* and its surprisingly high intracellular proliferation index. However, the absolute numbers of intracellular bacteria immediately after uptake and at 24 h were in fact only 10% of the levels for U112 (data not shown). SspA, stringent starvation protein A, is thought to be involved in intracellular survival; together with MglA, it directly regulates the expression of at least 30 genes, among those the genes of the FPI (6).

The two mutants in cluster 5 were extremely reduced in their growth in flies and strongly attenuated in killing flies, suggesting that they are particularly sensitive to the fly's immune defense. The *FTN_0109* mutant was also strongly affected in macrophage-like cells. FTN_0109 is a novel protein of unknown function and might be associated with the outer membrane; GlgB is annotated as a 1,4-α-glucan branching enzyme involved in glycogen biosynthesis.

The mutant strains in cluster 6 formed a very tight group, with strong effects on virulence in flies and in J774 cells. Most of the corresponding genes belong to the FPI. Six of these genes, *icmF*, *dotU*, *vgrG*, *iglA*, *iglB*, and *iglD*, have been suggested to function as components of a type VI secretion system (reviewed in references 3 and 13), and the *iglC* gene product depends on these components for proper localization to the outer membrane (29). Two additional cluster 6 loci, *clpB* and *dsbB*, are involved in protein modification and are important for proliferation in cells. ClpB belongs to the highly conserved ring-forming AAA^+ superfamily of ATPases, which is associated with virulence of several pathogens, including *F. tularensis* (7, 54, 60, 62). ClpB proteins disaggregate and reactivate strongly aggregated proteins and appear to be critically required for adaptation to various forms of stress. DsbB, together with DsbA and other components of the disulfide bond formation system, is required for efficient protein secretion into the periplasm in a number of bacterial pathogens (27, 33). Recent studies demonstrated their importance for *F. tularensis*

virulence in mice (39, 40, 50). We tested a mutant of the *F. novicida* homolog of *dsbA*, *FTN_0771*. This mutant was not attenuated in flies (see Table S1 in the supplemental material), which might be due to transposon insertion close to the 3′ end of the ORF. Instead, we found *FTN_0772* mutants to be attenuated and closely linked with the *dsbB* strain in the multivariate analysis. One possible explanation might be that the transposon insertion in the *FTN_0772* allele generated a polar effect on the downstream gene *FTN_0771*. Interestingly, the short *FTN_0772* ORF is 29% identical to that of *FTN_0771* and shows a homology to FK506 binding protein-like peptidyl-prolyl *cis-trans* isomerase 1 similar to that shown by DsbA. Therefore, it cannot be excluded that the phenotype of the two *FTN_0772* alleles truly reflects a deficiency in this gene function.

The hypervirulent mutants formed a relatively tight cluster characterized by increased virulence in flies and in J774 cells, with the *pckA* mutant as an outlier, due to its wild-type-like behavior in the cell culture assay (inset in Fig. 3). In concordance with these results, *pckA* was shown not to be important for virulence in mice (21). *pckA* is an outlier even in terms of gene function, since the other five loci all appear to contribute to type IV pili or to other surface structures of the bacteria (see Discussion).

In summary, the multivariate analysis provided ample examples of mutants with similar phenotypes, mutants that clustered tightly, and mutants for which the targeted genes are functionally linked.

DISCUSSION

We had previously established the fruit fly as a model organism for infections with *F. tularensis*, and here we employed this model to identify *F. novicida* virulence factors by individual testing of a large number of bacterial mutants. Combining fly experiments and mouse cell culture assays, we identified genes involved in virulence in two different host systems and genes necessary for intracellular survival and cytopathogenicity. A strong correlation between results from the two model systems evaluated *D. melanogaster* as a reliable model host.

Of 249 *Francisella* loci suggested as putative virulence determinants in mice, we found 49 to be required for normal virulence in flies. Six of these seem to act as virulence modifiers, since the corresponding transposon mutant strains were hypervirulent (see below). For a third of our 49 candidates, the recent literature provides confirmatory data from infections of mice with U112, LVS, or SCHU S4 mutants showing that these genes are required for normal virulence in the insect as well as in the mammalian model organism (see Table S1 in the supplemental material for references).

Of the 200 genes without a significant phenotype in flies, about 20% have been tested by individual mutant analysis in mice by other researchers (see Table S1 in the supplemental material for references). From these analyses, 26 genes were shown not to be important for virulence of U112, LVS, or SCHU S4 in mice. Thus, these genes can be considered false-positive hits from the original negative-selection screens, although for six of them, importance for virulence differed depending on genetic background. The residual 19 loci, again tested in various backgrounds, were confirmed to have an attenuated mutant phenotype in mice. There are many possible explanations for phenotypic differences of mutants in mice and in flies. We mentioned some of these in Results, and another might lie in the differences between the mutant strains that were tested (types of mutation and subspecies). Despite more than 97% sequence identity among U112, LVS, and SCHU S4, differences in the numbers of protein-encoding genes and in ORF lengths exist and affect the *in vivo* life cycle as well as the host immune response (12, 26, 43). For example, 16 of the 200 nonsignificant *F. novicida* genes are either strongly truncated in or are missing from the live vaccine strain (see Table S1 in the supplemental material).

However, differences in mutant phenotypes can also reflect physiological differences between the two model organisms compared here. For instance, it is noteworthy that genes involved in purine metabolism (*purM*, *purF*, *purL*, and *relA*) and other basic metabolic functions (*glpX*, *FTN_0720*, *aroG*, *ggt*, and *FTN_1213*) seem to be important in mice but not in flies (see Table S1 in the supplemental material). Assuming that intracellular conditions for *Francisella* are overall very similar in the two host models, the insect hemolymph probably represents the biggest difference. It is possible that, similarly to our observations regarding *FTN_0848* and *manB*, the corresponding deficiencies are compensated for by the rich composition of the *Drosophila* hemolymph (11). Another group of genes required for virulence in mice but not in flies encode enzymes of the lipopolysaccharide and capsule biosynthesis pathways (*capB*, *capC*, *capA*, and *wbtA*). Despite a high degree of conservation between components of the innate immune response in insects and mammals, differences between the two systems do exist and might contribute to the outcome of our screen. Pathogen recognition, for instance, is based on different pathogen moieties and different host receptors.

Induction of virulence factors upon a shift to mammalian body temperature is known for several pathogens, so one would assume that the lower temperature in the insect host might affect the hits that can be retrieved in this model. Indeed, mutations in 11 genes that were induced by a shift from 26° to 37°C did not generate a phenotype in flies (20). However, five other upregulated genes, *pyrB*, *carB*, *carA*, *clpB*, and *pilB*, were nevertheless required for normal virulence in flies. Similarly, five genes upregulated upon infection of mice (compared to expression during growth in broth at 37°C) did not have a mutant phenotype in flies, with the exception of *iglC* and *FTN_0119* (55).

Comparing the three *in vivo* screens in mice, it is evident that they were performed under widely varying experimental conditions (types of mutant library, routes of infection, etc.) and that they documented different readouts (target organs, time postinfection, and detection of mutants) (23, 51, 57). These differences explain the small overlap in candidate virulence factors obtained in the studies. The two reports on which we based our mutant selection had only 19 candidate genes in common, corresponding to 12% (57) and 20% (51) overlap. Compared to these numbers, the 19% overlap between our results and those from the original two mouse screens appears to be within an expected range. Mutations in *FTN_0848*, *iglA*, *iglB*, *iglC*, and *clpB* caused attenuation in the two mouse screens as well as in flies. A recent study by Kraemer et al., in which the *F. novicida* two-allele library was screened in an

aerosol infection model, identified nine genes that were also picked up by the two previous screens; two of these, *iglD* and *vgrG*, generated an attenuated phenotype in flies (23).

By analysis of bacterial growth *in vitro* and *in vivo* as well as of cytopathogenicity in cultured mouse macrophage-like cells, we further characterized the candidates with an attenuated or a hypervirulent phenotype in flies. Consistent with observations in mice, we found a significant correlation between bacterial proliferation and virulence in the *in vivo* fly model and in the cell culture model (47). More importantly, our experiments bridge the insect and mammalian models.

The strong correlation between mutant phenotypes in flies and in cultured mouse macrophages proves the survival of infected flies to be a reliable indicator of bacterial cytopathogenicity in the mouse model. It also demonstrates the importance of cytopathogenicity as a virulence mechanism in an insect host. This is consistent with our previous findings that LVS is able to replicate inside hemocytes, the phagocytes considered to be the functional equivalent of mammalian macrophages, and that flies in which phagocytosis was inhibited succumbed more quickly to infection (56).

By grouping the mutants into clusters of phenotypically similar genes, our multivariate analysis provides additional insight into gene function. Half of the candidate virulence factors are involved in cell division and basic metabolic processes, with a possible role in responses to oxidative stress. These genes almost exclusively formed the left branch of the cluster tree, while genes involved in intracellular survival segregated to the right branch. Within most clusters, we find examples of tightly linked genes that are known or are expected to act in the same cellular process. This linkage primes the hypothesis that unknown genes are involved in functions related to that of their better characterized nearby neighbor(s) in the cluster.

We found 14 of the 18 genes in the FPI to be required for full virulence in flies and for proliferation in J774 cells. For *iglE*, *iglF*, *iglG*, *iglH*, *dotU*, and *iglI*, such data have so far not been available, although these genes were believed to play a role in intracellular survival. There is also indirect evidence for the importance of FPI genes in a protozoan model, since mutants of *iglC* and the major regulator of FPI gene expression, *mglA*, do not proliferate well in *Acanthamoeba castellanii* (28). In our screen, the only FPI genes that did not affect virulence in flies were *pdpC*, *pdpE*, *pdpD*, and *anmK*. Consistent with our findings, Read et al. reported that *pdpC* and *pdpD* are not required for intracellular proliferation in an insect cell line (42). Similar results were obtained for *pdpD* in mouse macrophages, although in mice it seems that PdpD together with AnmK plays a role in virulence of *F. novicida* (29). Nevertheless, the requirement of FPI genes for *Francisella* survival in organisms as diverse as amoebae, flies, and mice indicates that this bacterium is adapted to sustain itself in a variety of host cells. This adaptation might be the key to the ability of *Francisella* to survive in the environment and provides an explanation for the occasional outbreaks of tularemia seen worldwide.

In addition to intracellular proliferation, interference with systemic physiological processes seems to play a role in *Francisella* pathogenicity. "Energy wasting" is a well-known effect of chronic inflammation in humans and has also been investigated in the fruit fly, for which infection-induced inhibition of the Akt kinase in the insulin receptor pathway has been described previously (9, 10, 48). In mammalian systems, *Francisella* has been demonstrated to interfere with Akt signaling (5, 41). We have previously speculated that energy wasting or similar disruptions of host physiology might contribute to fly death upon LVS infection (56). Despite an overall good correlation between bacterial load and virulence in flies, we here identified genes that appear to contribute to host death apart from a simple linkage to bacterial numbers, i.e., all loci in cluster 1; *FTN_0096*, *FTN_0806*, and *recB* in cluster 3; and *iglE*, *iglB*, *vgrG*, and *iglF* of the FPI. While mutants of these genes proliferated normally in flies, they were hampered in their proliferation in mouse macrophage-like cells. Nevertheless, many of them demonstrated nearly wild-type cytotoxicity in these cells. This discrimination between the cell culture system and the *in vivo* situation suggests that the targeted genes affect systemic host processes rather than a cell-autonomous response.

In addition to genes required for full virulence, we identified genes that could be considered "virulence modifiers," since they generated a hypervirulent mutant phenotype. Zogaj et al. put forward the idea of PilA being a virulence modifier after they had noted that the length of PilA in various *Francisella* species and strains is reciprocal to the virulence of the bacteria (61). As mentioned above, genes in the hypervirulent group are likely to contribute to the formation of type IV pili. The validity of our results is supported by the finding that *F. novicida pilA*, *pilB*, and *pilC* mutants exhibited increased virulence in mice (18). These authors demonstrated further that PilA and PilB are in fact required for the secretion of a number of substrates. Hager et al. hypothesized that type IV pilus-mediated secretion of the metalloprotease PepO inhibits spreading of the bacteria in the mammalian vascular system (18). PepO displays homology to the mammalian endothelin converting enzyme 1, which induces vasoconstriction (32). Type IV pilus-mediated secretion mutants could therefore more easily disseminate via the blood circulatory system. Although this theory may not explain the hypervirulent phenotype in the fly, it is interesting to note that *D. melanogaster* expresses several homologues of endothelin converting enzyme 1, the closest being neprilysin 3 (4, 30). Two other observations are interesting in this respect. First, expression of *pilB* is upregulated in the attenuated live vaccine strain upon exposure to 37°C (20), and second, the *FTN_0119* gene product is expressed at 2- to 16-fold-lower levels in SCHU S4 than in less virulent strains of *F. tularensis* subspecies *holarctica* (36). From their experiments, Zogaj et al. have also concluded that, while PilA and PilB form a secretion apparatus, the gene products of *FTN_0389* (*pilE4*) and *FTN_1622* (*pilT*) together with PilB form type IV pili (61). They could exclude a few other *pil* genes from being important for pilus formation. Information on the role of *fimT* and *pilM* is incomplete; they were, however, listed as candidate virulence factors by Rohmer et al. (43). From these observations, it seems that type IV pili somehow mask the true *Francisella* virulence and thereby slow down the infection process.

We have shown that *D. melanogaster* is a valuable host system for *in vivo* studies of *Francisella*. Our screening protocol reliably and efficiently identified attenuated and hypervirulent mutants on the basis of fly survival. Thanks to the availability of mapped transposon mutant libraries, a genome-wide screen to identify novel *Francisella* virulence determinants can be

performed using this protocol. From our observations, we can predict that not all determinants required for successful mammalian infections will be identified in such a screen. However, we have seen that important strategies, such as intracellular survival and adaptation to oxidative stress, are also active in an insect host. Hits from a screen in *D. melanogaster* will therefore most likely be universally relevant. In contrast to experiments in cell culture, the *in vivo* fly model offers the opportunity to discover bacterial interference with systemic host processes. It is also worth noting that our high-throughput screening procedure permits analysis of each mutant individually in a much more rapid and ethically and economically feasible way than in mammalian model systems. Thus, such an approach will surely aid the ongoing quest to identify new targets for drug and vaccine development needed for the treatment and prevention of *F. tularensis* infections.

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