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Francisella Is Sensitive to Insect Antimicrobial Peptides

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Key Words
Francisella tularensis · *Drosophila melanogaster* · Antimicrobial peptides · Lipopolysaccharide · Host-pathogen interactions

Abstract
Francisella tularensis causes the zoonotic disease tularemia. Arthropod vectors are important transmission routes for the disease, although it is not known how *Francisella* survives the efficient arthropod immune response. Here, we used *Drosophila melanogaster* as a model host for *Francisella* infections and investigated whether the bacteria are resistant to insect humoral immune responses, in particular to the antimicrobial peptides (AMPs) secreted into the insect hemolymph. Moreover, we asked to what extent such resistance might depend on lipopolysaccharide (LPS) structure and surface characteristics of the bacteria. We analyzed *Francisella novicida* mutant strains in genes, directly or indirectly involved in specific steps of LPS biosynthesis, for virulence in wild-type and *Relish*^{E20} immune-deficient flies, and tested selected mutants for sensitivity to AMPs in vitro. We demonstrate that *Francisella* is sensitive to specific fly AMPs, i.e. Attacin, Cecropin, Drosocin and Drosomycin. Furthermore, six bacterial genes, *kpsF, manB, lpxF, slt, tolA* and *pal*, were found to be required for resistance to *Relish*-dependent immune responses, illustrating the importance of structural details of *Francisella* lipid A and Kdo core for interactions with AMPs. Interestingly, a more negative surface charge and lack of O-antigen did not render mutant bacteria more sensitive to cationic AMPs and did not attenuate virulence in flies.

Introduction

Francisella tularensis is a gram-negative facultative intracellular bacterium and the causative agent of the zoonotic disease tularemia. Worldwide about 250 mammalian wildlife species such as rodents, rabbits, squirrels or deer are known carriers of tularemia agents. Humans contract infections through direct contact with carrier animals, inhalation or ingestion of contaminated dust or water, as well as through arthropod bites. Two subspecies of *F. tularensis*, subsp. *tularensis* (type A) and subsp. *holartica* (type B) are of clinical importance. Another spe-

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cies, *Francisella novicida*, is an environmental isolate that is not infectious for healthy humans, but causes a tularemia-like disease in wildlife. *F. novicida* is more than 97% genetically identical to *F. tularensis* [1] and is a widely used laboratory model for pathogenic *Francisella* species.

Hematophagous arthropods like hard ticks, horse flies or mosquitoes have been recognized as vectors of tularemia, and mosquito-borne infection has been linked to some of the largest epidemics of tularemia ever reported [2]. The bacteria have been detected in the midgut, salivary glands and in hemolymph of ticks [3, 4] and in midgut and Malpighian tubule cells of mosquitoes [5, 6], but it is not clear how *F. tularensis* survives in these hosts that are known to possess efficient cellular and humoral immune responses.

To date there is no laboratory model available using a natural arthropod vector of *Francisella*. We recently demonstrated that *Drosophila melanogaster* can be employed as an in vivo model of *Francisella* infections that enables analysis of bacterial survival and virulence mechanisms in an arthropod host [7]. Similar to *Francisella* infections of mammalian hosts, the bacteria are phagocytosed by the fly's macrophage-like hemocytes, and they proliferate in these cells relying on nearly the same set of virulence genes. In addition, *Francisella* propagates extracellularly in the open circulatory system (hemolymph) of infected flies and eventually kills them.

One of the immediate immune mechanisms induced by bacterial infections is the secretion of antimicrobial peptides (AMPs) into the extracellular space. Many of these mostly small and cationic peptides electrostatically interact with negative charges on the bacterial surface and integrate into the membrane by pore formation leading to disruption of the permeability barrier and the transmembrane potential. Moreover, certain peptides can also inhibit critical intracellular targets [8].

F. tularensis is known to circumvent important innate immune mechanisms in mammalian hosts. The O-antigen of *Francisella* lipopolysaccharide (LPS) does not activate the complement system; the lipid A has weak endotoxic activity and is not proinflammatory [9]. In contrast to the classical endotoxin of enterobacteriaceae, *Francisella* lipid A is not phosphorylated and carries only four acyl chains. Moreover, the majority of lipid A occurs in a free form without O-antigen. This free lipid A is monophosphorylated in the 1'-position with an adjacent galactosamine; in addition, it can be mannosylated or glycosylated in the 4'- or 6' position [10, 11]. Interestingly, these changes in the lipid A structure, more specifically the removal of a 3′-acyl chain and a 4′-phosphate, are also responsible for *Francisella's* natural resistance to the cationic AMP polymyxin B [12].

Here, we investigated whether *Francisella* is resistant to insect humoral immune responses, in particular to *D. melanogaster* AMPs, and asked to what extent such resistance might depend on the *Francisella* LPS structure and outer membrane composition.

Materials and Methods

Bacterial Cultures

The live vaccine strain (LVS) of *F. tularensis* subspecies *holarctica* was originally supplied by the US Army Medical Research Institute of Infectious Diseases (Fort Detrick, Frederick, Md., USA). The *F. novicida* wild-type strain U112 was obtained from C. Manoil together with the two-allele library of U112-derived transposon insertion strains from which most mutants originated [13]. Other strains used were an LVS-derived *wbtA* deletion mutant [14] and U112-derived deletion mutants of *lpxF* [12], *manB* and *manC* [15]. If not otherwise noted, bacteria were grown on modified GC agar (GC II agar base complemented with hemoglobin and ISO-VITALEX) supplemented with 50 μg/ml polymyxin B or 15 μg/ml kanamycin (for mutant strains) at 37°C and 5% CO_2 for 24 h. *Enterobacter cloacae* was obtained from Dan Hultmark's laboratory and grown on Luria broth agar plates.

Fly Strains

All fly strains were grown on standard potato meal agar at 25°C and 60% humidity. The *D. melanogaster* Oregon R strain served as wild type. For expression of single AMP genes in immune deficient background we crossed w^{1118}*; b pr imd; daughterless-GAL4 spz*^{rm7}*/TM6c* to w^{1118}*; UAS-AMP imd/CyO; UAS-AMP spz*^{rm7}*/TM6c Tb Sb* flies [16]. AMP stands for either *Attacin A (AttA), Diptericin (Dpt), Defensin (Def), Drosocin (Drc), Drosomycin (Drs)* or *Cecropin A (CecA)*. For expression of cecropin genes in wild-type background we crossed w^{1118}; +; *UAS-CecA* and w^{1118}; +; *UAS-CecB* (3 different lines; all generated by S. Ekengren/D. Hultmark, unpublished) to w^{1118}; +; *da-GAL4* flies. The *da>AMP* offspring used in experiments was incubated at 29°C for approximately 24 h prior to experiments to obtain a strong activity of the UAS-GAL4 system. Other fly strains used were *w*; +; *Relish*^{E20} [17].

Quantitative RT-PCR

Total RNA from 8 flies per sample was isolated using Trizol reagent (Invitrogen). cDNA synthesis was performed on DNase-I treated total RNA (2.5 μg) by oligo (dT) priming using the Superscript III preamplification system (Invitrogen). PCR was performed using a 7900 HT Fast Real-Time PCR. Primer sequences are listed in online supplementary table S1 (for all online suppl. material, see www.karger.com/doi/10.1159/000342468).

D. melanogaster *Infections*

Flies were infected either by injection of resuspended bacteria using a glass capillary (method A) or by pricking with plated bacteria using a glass needle (method B). For method A bacteria were resuspended in phosphate-buffered saline to $OD_{600\ nm}$ 1.0 for *F.*

tularensis LVS derivatives and to $OD_{600\,nm}$ 0.01 for *F. novicida* derivatives, corresponding to approximately 3×10^9 CFU/ml and 3×10^7 CFU/ml, respectively. For method B bacteria were grown on plates. Both methods were performed as previously described [7, 18]. Inhibition of phagocytosis with latex beads and viable count analysis was also performed as described previously [7].

Inhibition Zone Assay

This assay was adapted for *Francisella* from Hultmark [19]. Bacteria were grown to early log-phase ($OD_{600\,nm}$ 0.1, corresponding to approx. 3×10^8 CFU/ml) in trypticase soy broth supplemented with 0.1% L-cysteine and 0.2% dextrose. Thin-layer agarose plates were prepared from 10 μl bacterial suspension and 0.8% agarose in supplemented trypticase soy broth medium. Fine glass needles were loaded with 2 μl protease inhibitor solution (Roche) and then used to collect hemolymph from the heads of 5 flies per sample. Plates were incubated at 29°C until bacterial lawns and inhibition zones were clearly visible. For each combination of fly genotype and bacterial strain 3–18 hemolymph samples were tested. Protease inhibitor solution alone did not inhibit bacterial growth.

Data Analysis

We calculated differences in median life length (ΔMLL) between test and control flies to determine the effect of treatment on fly survival [7, 18]. For bacterial growth in flies original data from viable count analysis was logarithmically transformed and the absolute increase in bacterial load per fly from injection (day 0) to day 'X' after infection was used for comparisons between test and control (in vivo proliferation, proliferation index). To identify statistically significant differences we used Student's two-sample t test. Probability values below <0.05 were considered significant.

Results

F. tularensis *Is Sensitive to Drosophila Antimicrobial Peptides in vivo*

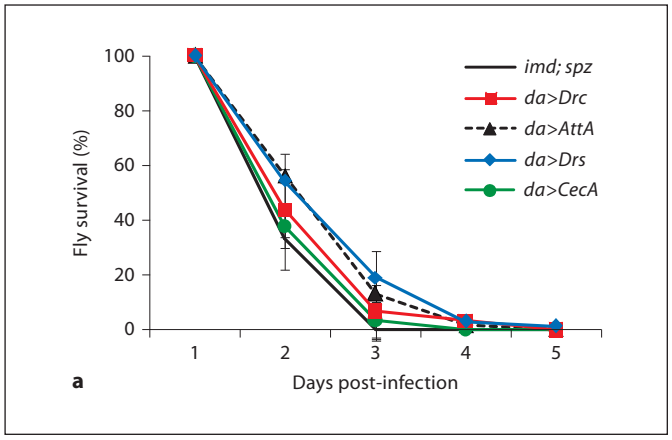
In the fruit fly induction of AMP genes upon bacterial infection is controlled by the NF-κB-dependent Toll and Imd/Relish signaling pathways. We previously demonstrated that in $Relish^{E20}$ mutant flies, which almost completely lack antimicrobial peptides, *Francisella* proliferates much faster than in wild-type flies [7]. Although Relish also regulates a number of other genes with less known or unknown relation to the immune system [20–22], the above-mentioned observation suggests that *Francisella* is sensitive to *Drosophila* antimicrobial peptides despite its overall virulence in flies. In order to test this hypothesis we utilized a genetic approach, the UAS/GAL4 system to ectopically express single *D. melanogaster* AMPs in immune deficient genetic background [16]. In contrast to commercially available synthetic AMPs, this approach offers in vivo testing and all putative post-translational modifications of the peptides that might be required for full antimicrobial activity. The fly strains used in these experiments were deficient in both the Toll (by mutation in the *spätzle* gene) and the Imd/Relish pathways, and are considered to completely lack AMP gene expression. Individual AMP genes representative of the major types of *D. melanogaster* AMPs were constitutively and ubiquitously expressed in this *imd; spätzle* double mutant background using the ubiquitously active *daughterless* promoter (*da>AMP* flies). We then injected *da>AMP* flies with LVS bacteria and monitored fly survival. LVS-infected *imd; spätzle* control flies died within 3 days, similar to infected $Relish^{E20}$ flies. Expression of *Attacin A*, *Drosocin* or *Drosomycin* significantly prolonged the survival of LVS-infected flies with 27, 8 and 37%, respectively (fig. 1a). We did not see a significant effect of *Cecropin A*, *Diptericin* or *Defensin* although overexpression of the respective AMP gene was confirmed using quantitative RT-PCR (table 1). However, the ectopic expression levels in *da>AMP* flies reached only about 20% of the levels induced by LVS bacteria in wild-type flies, except in the case of *Defensin*.

Upon microbial infection, very high steady-state concentrations of various AMPs can be detected in the hemolymph of wild-type flies. In addition, AMPs are thought to act in synergy with each other. Tzou et al. [16] have shown that combined expression of two AMP genes prolongs fly survival for longer than expression of only one AMP gene. We therefore tested whether overexpression of *Cecropin* genes in a wild-type background pro-

Table 1. Relative expression of AMP genes in noninfected *da>AMP* flies and in wild-type flies (OR) infected with *E. cloacae*, compared to LVS-infected OR

Gene	Fold expression		
	LVS-infected OR	*da>AMP* (mean ± SEM)	*E. cloacae*-infected OR (median)
AttA	1	0.23 ± 0.07	3.38
CecA	1	0.26 ± 0.04	1.94
Def	1	3.15	1.94
Dpt	1	0.51 ± 0.29	1.47
Drc	1	0.20	1.35
Drs	1	0.12 ± 0.08	0.70

The relative expression of AMP genes was determined by quantitative real-time PCR of whole flies. Infected flies were harvested 6–20 h after infection. Mean values of 2–3 independent experiments are shown.



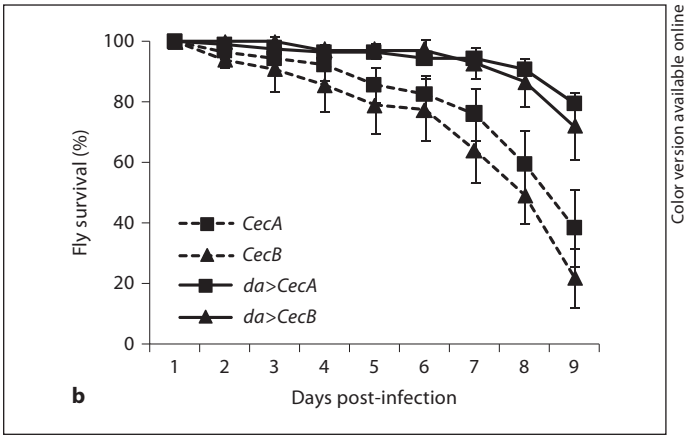


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Fig. 1. Protective effect of antimicrobial peptides on survival of flies infected with *F. tularensis* LVS. Antimicrobial peptides were ectopically expressed using the UAS/GAL4 system with the ubiquitously active *daughterless (da)* promoter indicated by '*da>*'. UAS-constructs were *Attacin A (AttA)*, *Cecropin A (CecA)*, *Cecropin B (CecB)*, *Drosocin (Drc)*, *Drosomycin (Drs)*. **a** Survival of LVS-injected *imd; spz* immune-deficient flies which overexpress single antimicrobial peptides. Control flies were w^{1118}*; b pr imd; da-GAL4 spz*^{rm7} with an MLL of 1.7 ± 0.1 days. The difference in MLL (ΔMLL) was 0.5 ± 0.2 days for *da>AttA*, 0.3 ± 0.1 days for *da>CecA*, 0.1 ± 0.0 days for *da>Drc* and 0.6 ± 0.1 days for *da>Drs*. **b** Survival of LVS-injected wild-type flies overexpressing *Cecropin A* or *B* with an MLL of 9.9 ± 0.5 and 10.3 ± 0.4 days, respectively, in comparison to 7.9 ± 0.4 days for LVS-infected genotype controls; results shown are from one fly strain transgenic for either *UAS-CecA* or *UAS-CecB*; for each UAS-construct similar results were obtained from two additional independent fly strains. Median values of three independent experiments with 20–80 flies per experiment are shown, error bars show standard error of mean.

vides flies with a greater survival advantage than if expressed in the immune-deficient background. Three different fly strains transgenic for expression of *Cecropin A* and three strains for *Cecropin B* were crossed and the survival of LVS-injected *da>CecA* and *da>CecB* offspring was monitored. Constitutive expression of either *Cecropin A* or *Cecropin B* in wild-type flies significantly prolonged fly survival with 23 or 30% of the lifespan of infected control flies, respectively ($p < 0.001$; fig. 1b). Taken together we found that overexpression of several antimicrobial peptides prolonged the survival of LVS-infected flies.

Modifications in Lipid A and an Intact Kdo Core Are Important for Virulence in D. melanogaster

We then wanted to investigate the importance of LPS and other membrane components for *Francisella* interaction with the fly humoral immune response. For this purpose we turned to *F. novicida* and made use of a well-characterized library of *F. novicida* U112 transposon insertion mutants [13] from which we chose roughly one hundred mutants in genes that had been annotated as being involved in 'cell wall/LPS/capsule', 'motility/attachment/secretion structure' or were simply 'unknown' [23], and that had been reported with attenuated mutant phenotypes in flies in at least one of three recent studies [18, 24, 25]. In addition, we chose a number of genes already known to be required for LPS biosynthesis in *F. novicida* or *F. tularensis* [10, 11, 15, 26, 27]: ten genes required for O-antigen synthesis, two genes reported to affect the Kdo core structure and five genes involved in modifying lipid A. In our experiments we also included *slt*, a gene encoding a putative soluble lytic murein transglycosylase. Mutations in this gene were expected to affect the structure of peptidoglycan rather than that of LPS.

First, we analyzed the LPS phenotype of the transposon insertion or deletion mutant strains and confirmed the absence of O-antigen in mutant strains for *FTN_1256*, the *wbt* and the *man* genes (online suppl. fig. S1, S2) [15, 27], as well as for *FTN_1222/kpsF*. To our surprise, even the *lpxF*, *flmK* and *slt* mutants appeared to have severely reduced O-antigen.

Second, we determined the surface charge (zeta potential) of the mutant strains, since this feature might correlate to their sensitivity to cationic antimicrobial peptides. The *F. novicida* U112 wild type had an almost neutral zeta potential of approximately –3 mV. Most mutant strains lacking O-antigen and/or the Kdo core demonstrated clearly more negative surface charges, while *slt*, *lpxF* and other mutant strains showed little but signifi-

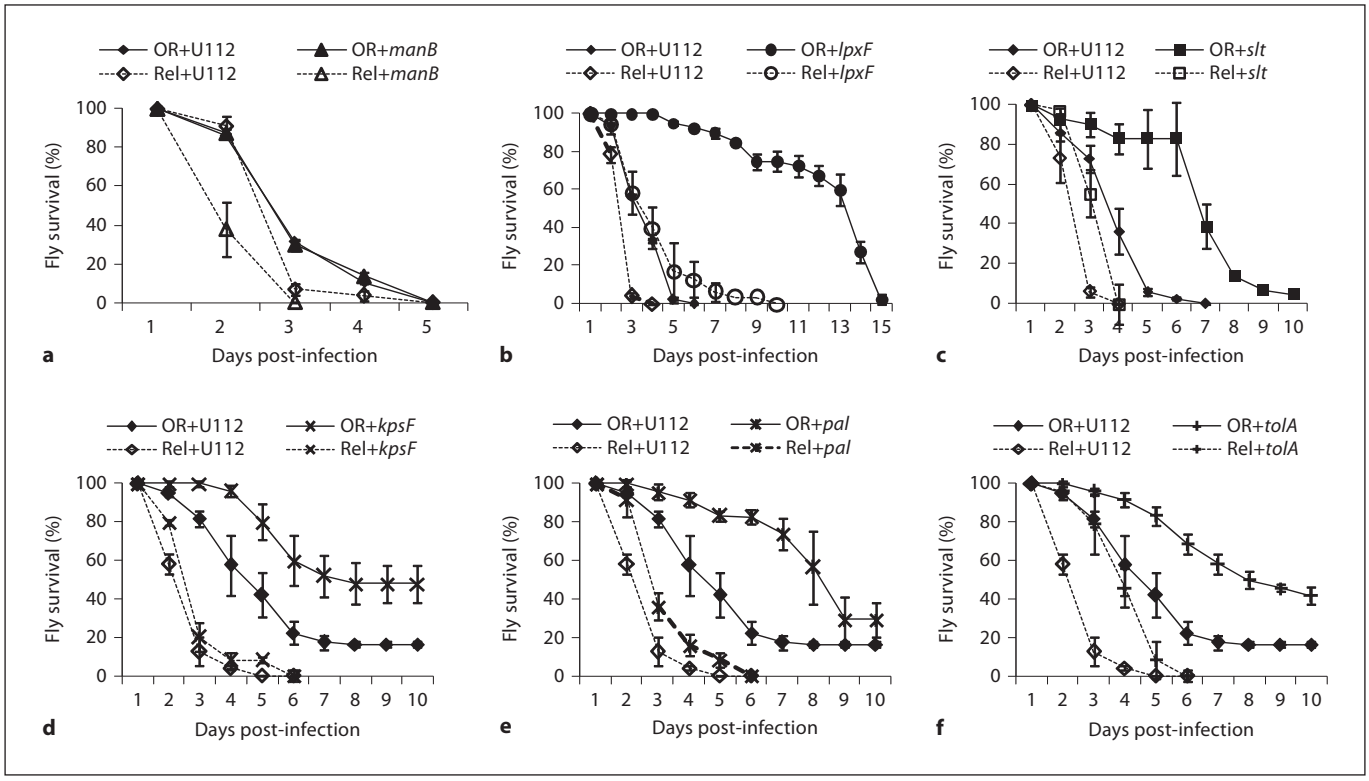


Fig. 2. Survival of wild-type (OR) and *Relish*^{E20} immune deficient (Rel) *D. melanogaster* flies infected with *F. novicida* U112 or U112-derived mutant strains as indicated. **a–c** 30–45 wild-type or 40–55 *Relish*^{E20} mutant flies per experiment were infected by injection. **d–f** 25 wild-type or *Relish*^{E20} mutant flies per experiment were infected by pricking. Median values of three independent experiments are shown, error bars show standard error of the mean. For statistical analysis of the data see online supplementary tables S2 and S3.

cant differences compared to the wild type (online suppl. tables S2, S3).

We then infected wild-type *D. melanogaster* flies either by pricking with mutants selected from the list of interesting candidates in online supplementary table S2 or by injection with mutants listed in online supplementary table S3. Fly survival and bacterial proliferation in flies was monitored (fig. 2, 3, online suppl. tables S2, S3). Among the mutations affecting O-antigen biosynthesis only transposon insertion in *FTN_1222/kpsF* led to significantly prolonged fly survival (fig. 2d, online suppl. table S2). In a previous study this mutant had shown a defect in intracellular proliferation in cultured *Drosophila* cells [24], but we did not observe reduced proliferation in flies (fig. 3d, online suppl. table S2). In *Escherichia coli*, *kpsF* encodes an arabinose-5-phosphate isomerase, which is involved in Kdo biosynthesis. *Neisseria meningitides* KpsF is required for the addition of core glycosyl residues like Kdo to lipid A and for the presence of a capsule [28]. Together, these findings suggest that KpsF has a similar role in *F. novicida* and that the *kpsF* mutant not only lacks O-antigen, but is also deficient in its core.

The other O-antigen mutants were not attenuated in flies, thus extending and confirming our previous results (online suppl. table S2) [18]. The sugar composition of O-antigen differs between *F. novicida* and *F. tularensis*, therefore we also tested an LVS *wbtA* deletion in flies. Similar to the *F. novicida wbtA* mutant, the LVS *wbtA* mutant was not significantly attenuated in the fly model (not shown).

ManB, a phosphomannomutase, and ManC, a guanylyltransferase, are consecutively required for the addition of mannose to the Kdo core; mutant bacteria in either of these genes contain a 'naked' Kdo moiety without O-antigen [15]. The *manB* mutant was as virulent as the U112 wild type with regard to fly survival, but proliferated less well in flies than U112 bacteria, which has also been observed by others (fig. 2a, 3a, online suppl. table S3) [25].

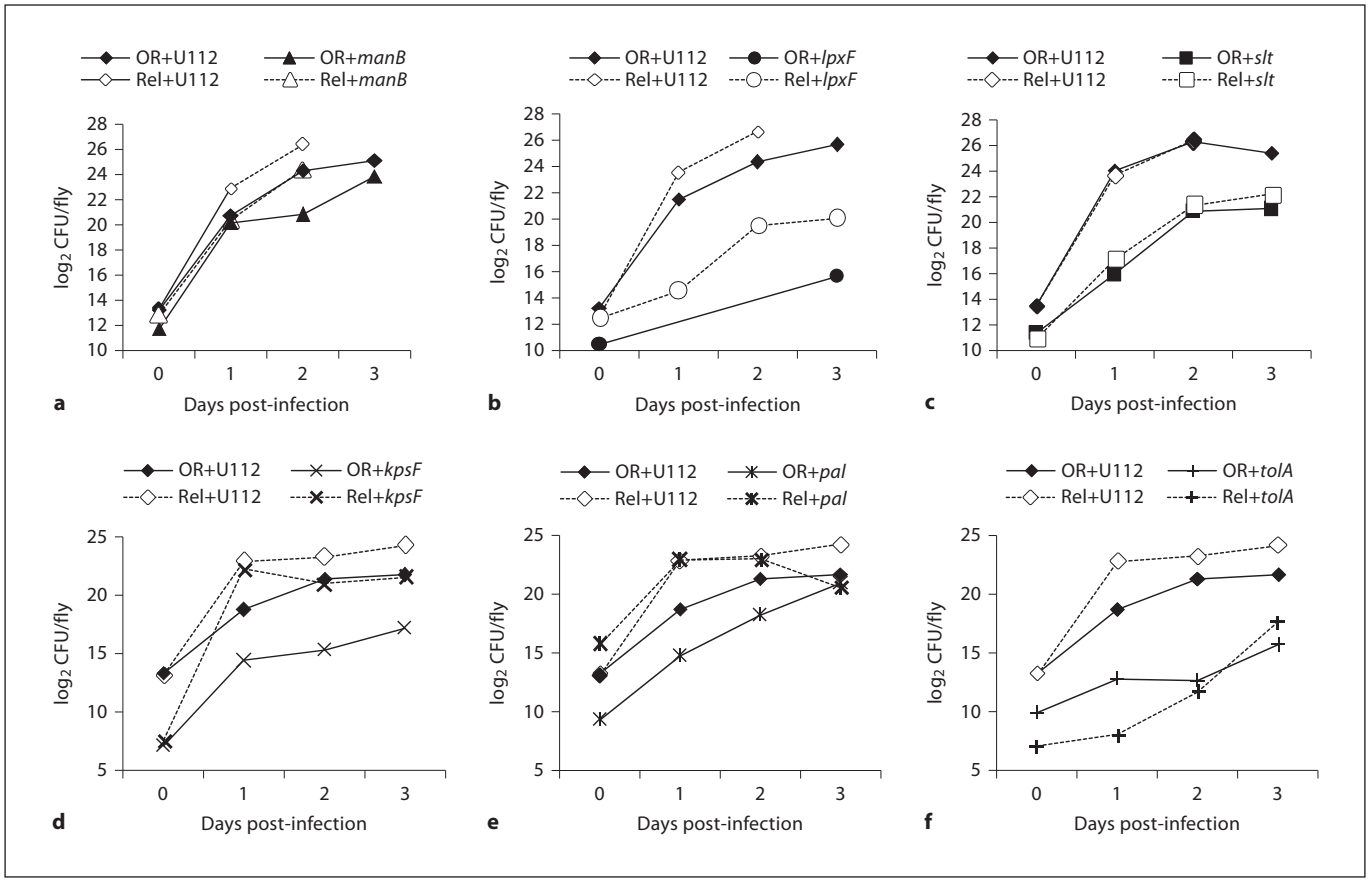


Fig. 3. In vivo proliferation of *F. novicida* U112 and U112-derived mutant strains in wild-type (OR) or in *Relish*^{E20} immune deficient (Rel) *D. melanogaster* flies. **a–c** Flies were infected by injection. **d–f** Flies were infected by pricking. Median values of three independent experiments are shown based on homogenates from 5 flies per sample and time point. For statistical analysis of the data see online supplementary tables S2 and S3.

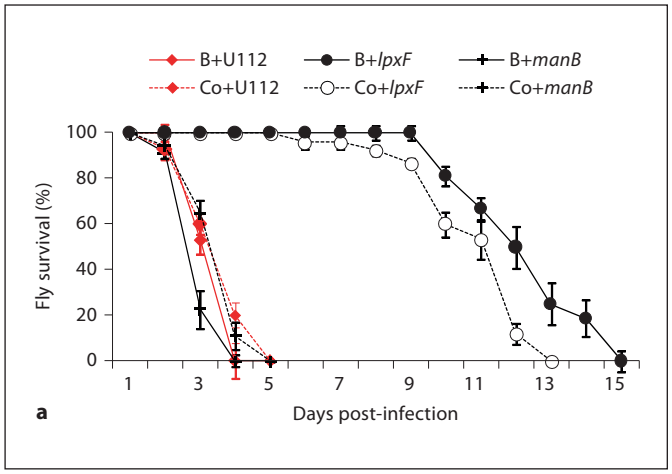
Surprisingly, neither the *manC* transposon insertion strain nor *manC* deletion strains demonstrated attenuation in flies (online suppl. table S3).

Among the mutations affecting lipid A only the *lpxF* mutant strain was significantly attenuated. For this mutant, which lacks the lipid A 4′-phosphatase and retains not only the 4′-phosphate but also a 3′-acyl chain, prolonged fly survival correlated with strongly reduced bacterial proliferation (fig. 2b, 3b, online suppl. table S3). However, it has to be noted that this mutant grows much slower in vitro than the U112 wild-type or other transposon insertion strains [12]. Other modifications of the lipid A component as caused by mutations in *lpxE* or in either of the *flm* genes did not affect bacterial virulence in flies. Similar to our previous observations, the *slt* mutant strain was clearly attenuated in both virulence and in vivo proliferation (fig. 2c, 3c, online suppl. table S3) [18].

Considering the zeta potential of the various mutant strains we tested in flies, there was no overall correlation between bacterial surface charge and attenuated mutant phenotypes in flies. Our results demonstrate that the LPS O-antigen of *Francisella* does not significantly contribute to bacterial survival and virulence in flies. The results rather suggest that small changes in outer membrane composition like the specific structure and charge distribution of the Kdo core and the lipid A component of *F. novicida* LPS are important for bacterial resistance to fly immunity.

tolA and pal Mutants Are Attenuated in D. melanogaster

In addition to the above-identified LPS genes, we also found *FTN_0354/tolA* and *FTN_0357/pal* (peptidoglycan-associated lipoprotein) to be required for virulence



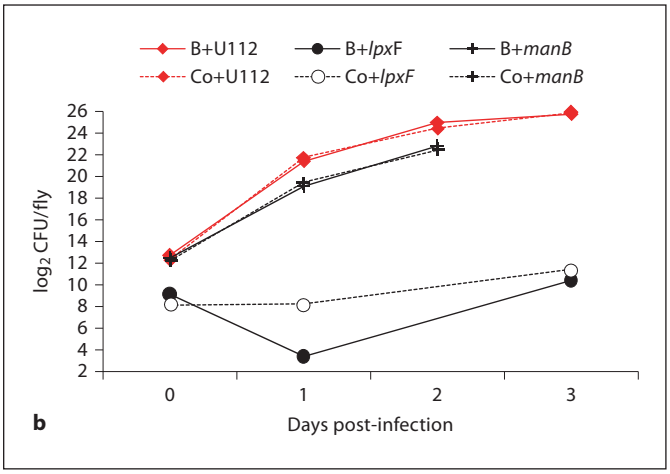


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Fig. 4. Blocking phagocytosis improved the survival of *lpxF* mutant-infected flies but not that of *manB* mutant-infected flies. **a** Survival of wild-type flies injected with either *F. novicida* U112 or U112-derived mutant strains as indicated. Prior to infection flies were injected with latex beads (B) to block phagocytosis of bacteria; control flies were injected with buffer (Co). **b** Growth of *Francisella* in wild-type flies. Flies were treated as described in **a**. Median values of three independent experiments with at least 35 flies per survival experiment and 4 flies per sample for viable count analysis are shown, error bars show standard error of the mean. For statistical analysis of the data see online supplementary table S4.

in flies, although only the *tolA* mutant was reduced in its in vivo proliferation (fig. 2e, f, 3e, f, online suppl. table S2). In *E. coli* TolA and Pal are part of a multiprotein complex, the Tol-Pal system, that bridges between the peptidoglycan and the outer membrane and that is important for proper structure and function of the outer membrane [reviewed in 29]. Mutations in *tol* and *pal* genes result in hypersensitivity to detergents and several antibiotics, leakage of periplasmic proteins, impaired motility and aberrant cell division [30]. Interestingly, TolA and Pal are necessary for correct surface polymerization of O-antigen chains that are assembled in a wzy (polymerase)-dependent manner. Although such a mechanism has been suggested for *Francisella* [31], we did not detect an O-antigen defect in *pal* or *tolA* mutants (online suppl. table S2).

F. novicida *LPS Genes Required for Resistance to the Fly's Humoral Immune Response*

We further asked whether the attenuated virulence and/or reduced growth of the various mutant strains were due to increased sensitivity to humoral immune responses in the hemolymph of *D. melanogaster*. We infected immune deficient *Relish*^{E20} mutant flies with mutant bacteria and determined whether the attenuated phenotype was reversed in such flies (phenotype 'rescue'). Loss of Relish function almost completely eliminates expression of the inducible AMP genes, but does not affect the phagocytic function of the hemocytes [17]. The *F. novicida* U112 wild-type strain killed *Relish*^{E20} flies more rapidly and proliferated faster than in wild-type flies, similarly as we have shown before for LVS, and others for U112 [7, 25]. The *manB* mutant also proliferated faster and killed *Relish*^{E20} flies more rapidly than wild-type flies (fig. 2a, 3a, online suppl. table S3). A comparison of proliferation and fly survival in *Relish*^{E20} versus wild-type flies revealed a small but significant rescue of the attenuated *manB* phenotype (not shown). The attenuation of the *lpxF, pal, tolA* and *kpsF* mutant strains was rescued in *Relish*^{E20} flies indicating that these mutants are indeed more sensitive to Relish-dependent defense mechanisms than the U112 wild type (fig. 2, 3, online suppl. tables S2, S3). Despite the severely reduced immune responses in these flies, *lpxF* mutant bacteria still grew more slowly than wild-type bacteria, which is probably due to this mutant's general growth defect. The *slt* mutant demonstrated some rescue of attenuation with regard to fly survival, but it's in vivo proliferation was similar in wild-type and in *Relish*^{E20} flies (fig. 2c, 3c, online suppl. table S3).

During *Francisella* infections of flies a growing proportion of the bacteria is localized in hemocytes, where they are protected from humoral immune responses in the hemolymph [7, 25]. We therefore forced the bacteria to remain in the extracellular compartment by blocking

Table 2. Growth inhibition of *F. novicida* U112 and U112-derived mutants by *D. melanogaster* hemolymph

Fly genotype	U112	*manB*	*lpxF*
imd; spz	–	–	+
da>Attacin A	–	–	+
da>Cecropin A	–	+	++
da>Cecropin B	+	+	++
da>Defensin	–	–	++
da>Diptericin	–	+	++
da>Drosocin	+	+	++
da>Drosomycin	+	–	+

Results are based on 3–18 (on average 9) independent replicates for each combination of fly genotype and bacteria.

phagocytosis prior to the infection. This treatment did not affect the virulence and proliferation of the U112 wild-type strain or of the *manB* mutant, but significantly prolonged fly survival after infection with the *lpxF* mutant and delayed proliferation of this mutant (fig. 4, online suppl. table S4). The results from these two approaches demonstrate that the LpxF, ManB, Pal and KpsF proteins contribute to *F. novicida* resistance against Relish-dependent immune responses, in particular against the humoral responses in the hemolymph. Such a function can also be concluded for TolA [this study, 25].

In vitro Sensitivity of Francisella *to AMPs*

To reduce the complexity of host-pathogen interactions and to test whether antimicrobial peptides directly inhibit *Francisella* growth, we utilized an inhibition zone assay in combination with pooled hemolymph samples of noninfected *da>AMP* flies. Overall, hemolymph from nonchallenged control flies (wild type or *imd; spz* double mutant) did not inhibit bacterial growth. However, occasionally hemolymph from *imd; spz* flies generated inhibition zones in lawns of the *lpxF* and *slt* mutants indicating that these strains are sensitive to humoral immune mechanisms, which might be dysregulated in the double mutant flies since hemolymph from $Relish^{E20}$ flies did not generate inhibition zones (table 2 and data not shown). Analysis of hemolymph samples enriched in single AMPs showed increasing inhibition of wild-type and mutant bacterial growth. Since neither the amount of hemolymph nor the concentration of AMPs could be measured exactly in these experiments, we refrained from a quantitative analysis. Nevertheless, the results show that hemolymph from *Drosocin*- or *Drosomycin*-expressing flies inhibited the growth of the U112 wild-type strain, and that *manB* or *lpxF* mutant bacteria were sensitive to additional *da>AMP* hemolymph samples. Attacin and Defensin samples generated larger or more consistent inhibition zones than did control samples (not shown). LVS bacteria were more sensitive than the U112 wild type, and the *slt* mutant did not demonstrate increased sensitivity to *da>AMP* hemolymph (not shown).

Discussion

We have demonstrated here that overexpression of AMPs like Attacin A, Cecropin, Drosocin and Drosomycin aid to protect *Drosophila* against the persistence of *Francisella* in vivo and/or inhibit bacterial growth in vitro. These antimicrobial peptides belong to three different structural groups: (i) linear α-helical peptides (cecropins, human LL-37), (ii) peptides forming disulphide bonds (Drosomycin, defensins) and (iii) peptides rich in particular amino acids (attacins, drosocins, pig PR-39, cow indolicidin) [32, 33]. It is likely that the various peptides interact differently with bacterial membranes, which is supported by our findings that wild-type *Francisella* was not sensitive to all peptides tested and that specific changes in the LPS core and lipid A extended sensitivity to additional AMPs. Specificity in the interactions between AMPs and bacteria was also demonstrated by Tzou et al. [16], who tested activity of various AMPs against a number of different microbes.

Cecropins probably act by binding to LPS and disrupting the bacterial membrane via pore formation, leading to release of cytoplasmic contents [34]. Drosocin and Attacin belong to the group of proline-/glycine-rich peptides. Like Cecropin, Drosocin is known to interact with LPS. In addition, it binds to cytoplasmic bacterial proteins like the heat shock protein DnaK and the chaperonin GroEL, both involved in protein folding [35]. In *E. coli* it has been shown that Attacin, which is a rather long peptide, partially integrates into the outer membrane via hydrophobic interactions with lipid A, and subsequently inhibits the synthesis of outer membrane proteins [36]. Rough *E. coli* strains are more sensitive to Attacin than strains carrying long O-antigen chains. In our experiments, the mere absence of O-antigen did not render *Francisella* mutants in *manB* or *lpxF* more sensitive to Attacin. But, with the 4′-phosphate and the 3′-acyl chain retained, the lipid A of the *lpxF* mutant is structurally similar to that of *E. coli*. This can explain this mutant's sensitivity to Attacin in contrast to the resistance of the

manB mutant. These structural alterations in *lpxF* mutant lipid A seem also to trigger TLR2 and TLR4 signaling, whilst wild-type *F. novicida* lipid A does not [37]. Surprisingly, we found Drosomycin, which is considered an antifungal peptide, to be effective against *Francisella*. However, Tzou et al. [16] showed that the combined overexpression of *Drosomycin* and *Drosocin* increased resistance to the gram-positive bacterium *Micrococcus luteus*. In line with these findings it is interesting to note that although Drosomycin contains an additional disulfide bond, it is structurally similar to insect defensins and to human β-defensins. The inducible human β-defensin hBD-3 exhibits antimicrobial activity against *F. tularensis* LVS, while hBD-1 and hBD-2 do not seem to affect the bacteria [38].

It is noteworthy that lack of O-antigen and higher negative surface charge did not per se render the bacteria attenuated in flies, and more sensitive to humoral immune responses. In mouse models of *Francisella* infection O-antigen mutant strains are attenuated. Such mutants are serum-sensitive since their LPS variant activates complement, which wild-type *Francisella* LPS does not [39]. In *D. melanogaster*, however, a homologue to the mammalian complement system has not been found.

Taken together, the unique structure of *Francisella* LPS, specifically the structure and charge distribution of the Kdo core and of lipid A appear to be essential for virulence and resistance to humoral immune responses in flies. Apart from genes directly involved in LPS biosynthesis, we also found components of the Tol-Pal outer membrane protein complex to be important, probably because of an overall effect on membrane integrity in the corresponding mutants. Interestingly, the Tol-Pal system also functions as receptor for certain AMPs like colicins. However, from our results it is not likely that the Tol-Pal system of *Francisella* is involved in the uptake of insect AMPs, since the *tolA* mutant was attenuated rather than more virulent in flies.

Arthropod-transmitted tularemia is a worldwide problem. Still not much is known about the interactions between *Francisella* and its arthropod vectors. Like *D. melanogaster*, mosquitoes express Attacins, Cecropins and Defensins, but also a fourth group of AMP genes, *gambicins,* which are only found in mosquitoes [40]. The small number of mosquito AMPs in contrast to *Drosophila* may reflect adaptations to their respective environment and the bacteria they encounter. Similarly, sequence variations in AMPs between mosquito species may account for the fact that only few wild-caught species tested positive for *Francisella* and that so far no competent mosquito vector has been established as a laboratory model [5, 6]. The ability of *Francisella* to survive in the presence of various insect AMPs indicates that the bacteria in turn are well adapted to survive in the hemolymph of an insect host. Even though we have demonstrated here that *Francisella* is sensitive to *Drosophila* AMPs, the peptides may only delay the outcome of the infection, death of the fly. In a competent arthropod vector of tularemia, however, such a delay might allow for *Francisella* transmission at the next blood meal.

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