Mus spretus SEG/Pas mice resist virulent Yersinia pestis, under multigenic control

Charlène Blanchet, Jean Jaubert, Elisabeth Carniel, Corinne Fayolle, Geneviève Milon, Marek Szatanik, Jean-Jacques Panthier, Xavier Montagutelli

ORIGINAL ARTICLE

Mus spretus SEG/Pas mice resist virulent *Yersinia pestis*, under multigenic control.

Running title : *Mus spretus* mice resistant to virulent *Yersinia pestis*

Charlène Blanchet[1,2], Jean Jaubert[1,2], Elisabeth Carniel[3], Corinne Fayolle[3], Geneviève Milon[4], Marek Szatanik[5], Jean-Jacques Panthier[1,2] and Xavier Montagutelli[1,2]

[1] Institut Pasteur, Mouse Functional Genetics Unit, F-75015 Paris, France

[2] CNRS, URA2578, F-75015 Paris, France

[3] Institut Pasteur, Yersinia Unit, F-75015 Paris, France

[4] Institut Pasteur, Immunophysiology and Intracellular Parasitism Unit, F-75015 Paris, France

[5] Institut Pasteur, Invasive Bacterial Infections Unit, F-75015 Paris, France

Corresponding author :

Xavier MONTAGUTELLI

25 rue du Docteur Roux, 75724 Paris cedex 15, France

Tel: (33) 1 45 68 85 54 - Fax: (33) 1 45 68 86 34

email: xmonta@pasteur.fr

ABSTRACT

Laboratory mice are well known to be highly susceptible to virulent strains of *Yersinia pestis* in experimental models of bubonic plague. We have found that *Mus spretus* derived SEG/Pas (SEG) mice are exceptionally resistant to virulent CO92 and 6/69 wild-type strains. Upon subcutaneous injection of 10^2 CFU, 90% of females and 68% of males survived, compared with only an 8% survival rate for both male and female C57BL/6 mice. Furthermore, half of the SEG mice survived a challenge of up to 10^7 CFU. The time required for mortality was similar between B6 and SEG, suggesting that survival is dependent on early rather than late processes. The analysis of 322 backcross mice identified three significant QTLs on chromosomes 3, 4 and 6, with dominant SEG protective alleles. Each QTL increased the survival rate by approximately 20% and act additively, thereby accounting for 67% of the difference between the parental phenotypes. Mice heterozygous for the three QTLs were just as resistant as SEG mice to *Y. pestis* challenge. The SEG strain therefore offers an invaluable opportunity to unravel mechanisms and underlying genetic factors of resistance against *Y. pestis* infection.

Keywords : Plague, mouse, resistance, *Yersinia pestis*, *Mus spretus*

Introduction

Yersinia pestis, a Gram-negative bacterium, is the etiological agent of human bubonic and pneumonic plague. Starting from the bite of a *Y. pestis*-hosting flea, bubonic plague is characterized by rapid multiplication of the bacteria in the bite site-draining lymph node, and is fatal in at least 50% of untreated cases, due to sepsis and multiple organ failure. Pneumonic plague results from the colonization of the lung by bacteria via infectious respiratory droplets. It is the most contagious and deadliest manifestation of the disease, with a 100% mortality rate in the absence of effective antibiotherapy [1].

Although the current worldwide incidence of plague is low compared to historical records, the steady increase in reported human cases during the past 15 years coupled with its re-emergence in areas otherwise declared plague-free for several decades has led to classify plague as a re-emerging disease [2], a threat exacerbated by the recent identification of a antibiotic resistant strain [3]. Moreover, rapid disease progression with a high mortality rate, the absence of efficient, safe vaccines, and the possibility of aerosol dispersion make *Y. pestis* a potential major agent for bioterrorism [4].

Historical records of plague pandemics indicate that a fraction of the individuals who developed bubonic plague were able to survive, suggesting that genetic factors of the mammalian host may also influence the outcome of the infection. It is likely that plague, which killed one third of the European population during the 1347-1351 Black Death, has exerted a rapid but strong selective pressure on the human genome. In animals, not all species are equally susceptible to plague. While most rodents, cats, camels and monkeys are susceptible, dogs and cows are usually resistant to *Y. pestis* [5]. These differences in susceptibility could help understand how *Y. pestis* overcomes the human immune system, identify which individuals are at risk, and form a basis for the development of new strategies for therapeutic and vaccine intervention.

Using forward genetic approaches to decipher the genetic control of natural variation in host resistance to viral, parasitic or bacterial infections has already demonstrated its power to unravel the molecular mechanisms engaged in host-microbes interactions [6]. The strength of this approach resides in its ability to identify previously unknown mechanisms, and characterize their hierarchy, interactions and individual contributions to the pathogenesis of infectious agents. However, its power remains very limited in humans, in particular because *Y. pestis*-infected patients undergo antibiotic treatment, thereby masking their intrinsic ability to control bacterial burden, the associated tissue damage and systemic inflammatory processes.

The availability of animal models relevant to human infectious diseases makes possible the identification of susceptibility genes under controlled experimental conditions in which strain, virulence, dose and route of *Y. pestis* inoculation are standardized. Among rodents, mice are of special interest since they are naturally susceptible to plague [7], and mouse offers powerful genetics tools to identify susceptibility and resistance genes even when the trait is under complex genetic control. Genetic and phenotypic dissection, which is critical in untangling complex sequences of events with many participating tissues and cell types, can be achieved using congenic strains, in which specific genomic regions harboring a small group of genes are transferred between susceptible and resistant backgrounds.

Differences in susceptibility to *Y. pestis* have been recently reported between laboratory inbred strains of mice [8], including the mapping of two resistance alleles, close to the interleukin-10 gene (chromosome 1) [9] and histocompatibility complex (chromosome 17) [10]. These results were obtained upon intravenous injection of the *Y. pestis* mutant strain KIM5, which lacks the chromosomal *pgm* locus. Since this strain is highly attenuated, it has to be given intravenously to cause a lethal infection. Although direct penetration of *Y. pestis* into

the blood stream may occur during a flea bite, it is a very uncommon mode of transmission. Furthermore, the relevance of these results to natural infection in animals and humans is limited, since the same inbred strains of mice are susceptible to virulent *Y. pestis* strains [8].

All classical laboratory strains (such as C57BL/6 (B6)) are susceptible to subcutaneous (s.c.) injection of fully virulent *Y pestis* strain C092, an experimental setting designed to mimic many features of natural exposure [11]. In this study, we report that the SEG/Pas (SEG) inbred strain, which was established from *Mus spretus* progenitors, exhibits a high level of resistance under the same conditions. The genetic analysis of a large backcross cohort between SEG and B6 identified three dominant, additively acting loci that account for most of the difference in susceptibility observed between the two strains.

Results

SEG mice resist virulent *Yersinia pestis*. Several inbred strains of mice were tested for their ability to survive an infection with the fully virulent *Y. pestis* strain CO92. Inoculation by s.c. injection of 100 colony-forming units (CFU) in the ventral region was lethal within 4-12 days for almost 100% B6 mice. This dose was used to challenge females of three additional laboratory inbred strains (FVB/N, BALB/cByJ and SJL/J), as well as one *Mus musculus castaneus*-derived strain (CAST/Ei) and the *Mus spretus*-derived SEG strain (Figure1). B6 and SEG mice were tested in several experiments and since the results were consistent throughout, the data were pooled.

SEG female mice were exceptionally resistant (90% survival; 62/69) compared to 23% (FVB; 5/22) or less in other strains. Only 6.3% (7/111) of B6 mice survived the infection (Figure 1). Interestingly, the few SEG mice that did succumb to the infection did so at the same average timepoint as B6 mice (Figure 2). SEG survivors developed symptoms characterized by a mild hypotonia and reduced activity between days 4 to 6 post-inoculation, before recovering. SEG mice that were susceptible progressed towards marked depression and prostration, and finally died. In B6 mice, death often occurred rapidly after the appearance of the first symptoms.

To further evaluate the level of resistance of SEG mice to the fully virulent CO92 strain, groups of females were inoculated s.c. with ten-fold dilutions ranging from 10^3 to 10^7 CFU of CO92. Remarkably, even the highest doses were unable to cause death in more than 50% of the mice (Supplementary figure 1A). The mortality timepoint of susceptible SEG mice varied across groups, but did not correlate with the number of bacteria inoculated. We therefore concluded that the SEG strain exhibits an exceptional level of resistance to s.c. inoculation with fully virulent *Y. pestis*. Such a resistance has never been reported for any other mouse strain.

Female SEG mice are more resistant than males. B6 males and females inoculated s.c. with 10^2 CFU of strain CO92 exhibited the same high level of susceptibility (Supplementary figure 1B). By contrast, SEG males were significantly less resistant (68.5% survival; 37/54) than females (89.9% survival; 62/69, p=0.005). Since the difference of susceptibility between SEG and B6 strains was higher in females than in males, further studies, including genetic mapping of QTLs, were carried out with females.

The exceptional resistance of SEG mice to *Y. pestis* is not CO92 strain specific. In order to assess whether the resistant phenotype of SEG mice was specific to *Y. pestis* strain CO92, we challenged B6 and SEG female mice with 100 CFU of another wild-type virulent strain, denoted as strain 6/69 [12]. Survival curves obtained with the two bacterial strains were similar (Supplementary figure 2), demonstrating that SEG mice resistance is not *Y. pestis* strain specific.

Multigenic control of resistance to *Y. pestis* in SEG mice. When a group of (B6 × SEG)F1 females (n=16) were challenged by s.c. inoculation of 10^2 CFU of strain CO92, 75% (12/16) of them survived, a resistance level not significantly lower than that of SEG mice (p=0.21). This suggests a dominant mode of inheritance for SEG-derived resistance alleles. An interspecific backcross was produced to characterize the genetic regions that account for the resistance of SEG mice. F1 females, born from the cross of B6 females and SEG males, were mated with B6 males to yield BSB progeny, out of which 322 females were challenged s.c. with 10^2 CFU of *Y. pestis* CO92. One hundred and ninety females survived the infection, giving a survival rate of 59%. This proportion was significantly lower than that of SEG mice ($p=3.2x10^{-7}$), but significantly higher (p=0.0015) than the 50% expected if resistance was inherited as a simple Mendelian trait. From these data, we conclude that resistance of SEG mice to *Y. pestis* is under the control of multiple genes, likely with SEG alleles acting in a dominant fashion.

The mortality timepoint of susceptible BSB females was similar to that of B6 and SEG mice in both mean value and standard deviation (Figure 2), suggesting that this cross did not segregate genes controlling this trait. In fact, we did not find any significant QTL controlling mortality time. The QTL search therefore focused on survival, analyzed as a binary trait.

SNP genotyping was performed using 721 polymorphic markers covering the entire genome of BSB females. QTL analysis using R/qtl software, revealed the presence of three chromosomal regions significantly associated with survival on mouse chromosomes 3, 4 and 6 (Figure 3). In all cases, the LOD curve reached the 5% genome-wide significance threshold computed by data permutation. These loci were named *Yprl1* (*Yersinia pestis* resistance locus-1), *Yprl2* and *Yprl3*, respectively. Their putative location was taken as the position of the marker with the highest LOD score value. *Yprl1*, *Yprl2* and *Yprl3* were positioned at 116, 62 and 95 Mb on chromosomes 3, 4 and 6, respectively. Their 95% confidence intervals were 50, 84 and 81 Mb long, respectively (Table 1).

The individual effects of each QTL were assessed by comparison of survival curves of BSB females depending on their genotype at the peak SNP marker. The SEG allele at the *Yprl1* locus was associated with a highly significant increase in resistance (Figure 4A), rising from 48.5% in mice homozygous for the B6 allele, to 68.1% in the B/S heterozygous mice. The effects of the *Yprl2* and *Yprl3* loci were similar in direction and magnitude (Figure 4B and C, Table 1).

Interactions between pairs of loci were investigated by comparing survival rates of the four genotype combinations in two-locus effect plots. For example, the difference of survival between $Yprl2^{B/B}$ and $Yprl2^{B/S}$ mice was 21.1 % within the group of $Yprl1^{B/B}$ mice, while it was 18.0% within the $Yprl1^{B/S}$ mice (Supplementary figure 3). Since these two values were not significantly different (p=0.97), we concluded that the effect of *Yprl2* was independent of the genotype of the mice at the *Yprl1* locus, and reciprocally. Similar observations were made

with *Yprl1*-*Yprl3* and *Yprl2*-*Yprl3* pairs of QTLs, thus suggesting that the three QTLs add up their individual effects without epistasis.

We also searched for additional QTLs which could have remained undetected using the one-QTL search, because of epistatic interactions. The "scantwo" feature of R/qtl did not detect any additional locus significantly associated with survival.

***Yprl1, 2* and *3* act additively to confer survival in BSB mice.** As the three QTLs appear to act independently of one another, we investigated the level of resistance of BSB mice for each of the eight possible genotype combinations at the three loci (Figure 5). This analysis was made possible by the large size of the cross which resulted in group sizes of at least 33 individuals. Groups were sorted by increasing proportion of SEG alleles at the three QTLs. The distribution of survival rates across groups was compared to that of B6 and SEG. Mice heterozygous for the SEG allele at the three QTLs show a level of resistance similar to that of SEG (82.7% and 89.9%, respectively). Conversely, mice homozygous for the B6 allele at the three QTLs had the lowest level of resistance (27.0%), but were significantly less susceptible than B6 mice ($p=0.0017$). The other groups of BSB mice showed intermediate levels of resistance, and groups of mice heterozygous for only one QTL were globally more susceptible than those heterozygous for two QTLs (48.5% and 68.0% survival, respectively; $p=0.0043$).

We draw four main conclusions from this analysis. First, SEG resistance alleles are dominant over B6 susceptibility allele. Second, the three QTLs act additively and together they account for 67% of the difference in survival to *Y. pestis* challenge between the two parental strains. Third, these QTLs provide BSB mice with the same level of resistance as parental SEG mice. Fourth, there remain undetected QTLs that contribute to significant resistance even in the absence of the three *Yprl* loci.

Discussion

Although plague is no longer a major threat to the human population, the recent re-emergence of the disease in several countries [2], the isolation of antibiotic resistant strains [3], and the exceptional pathogenicity of *Y. pestis* fully justify studies aimed at unravelling the mechanisms by which this bacterium is able to induce so efficiently and rapidly a deadly infection of its host. The mouse is a relevant animal model to explore the pathophysiology of plague owing to the fact that it is a natural and susceptible host for *Y. pestis*. Most previous studies have explored the bubonic form of the disease, although a model for primary pneumonic plague has been recently described and investigated [13].

The majority of *in vivo* genetic studies previously published on mouse models have focused on the role of genetic determinant in *Y. pestis* on bacterial load and histological damages in different host tissues [14, 15]. However, two recent reports identified regions of the mouse genome associated with the variable susceptibility of classical inbred mouse strains to *Y. pestis*. The first study showed that the previously reported resistant phenotype of *Il10* knock-out mice was partially due to a closely linked resistance allele inherited from the 129/P2J genetic background of the ES cells used for gene targeting [9]. The second study described the resistant phenotype of the BALB/cJ strain, compared to susceptible BALB/c substrains, and C57BL/6J. Using a combination of backcrossing and intercrossing, a QTL was mapped in the *H2* region, on proximal chromosome 17 [10]. However, both studies were performed using the attenuated *Y. pestis* strain KIM5 which lacks the *pgm* locus, an important virulence factor [16]. This deletion requires that inoculation of *Y. pestis* be performed intravenously, a route that ensures rapid dissemination of the bacteria, but one that also bypasses several important processes, in particular those deployed in the skin and the draining lymph node.

In this study, we aimed to identify host genetic determinants of resistance to plague, under experimental conditions that more closely resemble natural infection, i.e. using a wild-type, fully virulent *Y. pestis* strain and a s.c. route of transmission. We took advantage of our collection of wild-derived strains maintained at the Institut Pasteur to explore mouse genetic diversity. This strategy has been previously successful in our hands, with the identification of *Oas1b* gene as a major determinant of resistance to West Nile virus [17]. Indeed, while almost all laboratory inbred strains are highly susceptible to this virus due to a point mutation in the *Oas1b* gene, all wild-derived strains are highly resistant and carry a functional allele.

In the present study, all tested strains (including *Mus m. castaneus* CAST/EiJ) were found to be susceptible to plague, with the exception of the *Mus spretus* derived SEG strain, which exhibited remarkable resistance. Even inoculation of very high bacterial titres did not lead to mortality in more than 50% of the mice. One possible explanation for the high dose resistance of SEG mice could be genetic heterogeneity in the breeding colony, with a mutation conferring complete resistance to high doses segregating in a low dose-resistant inbred genetic background. However, careful analysis of the pedigree of the SEG mice used in this experiment ruled out this possibility. Another explanation might be a dose-dependent stimulation of the SEG innate immune response, with an enhanced, unsaturated capacity to clear the bacteria.

We have identified for the first time a mouse strain that displays high level resistance to inoculation of fully virulent *Y. pestis* strains, opening a double perspective to better understand the interactions between this highly pathogenic bacterium and its host. First, having both highly resistant and very susceptible mouse strains is an invaluable resource to identify the mechanisms, especially during the early phases following *Y. pestis* inoculation, which are triggered or disengaged by *Y. pestis*, and influence the outcome of host-pathogen interactions. Second, genes controlling this difference can be identified by QTL mapping in

two generations, although interspecific crosses between *Mus spretus* and laboratory strains invariably resulted in sterile F1 males, thereby preventing the production of F2 progeny. This forward genetic strategy has already been proven to be successful for the identification of key genes for the control of bacterial, viral or parasitic diseases [6].

An interspecific backcross using SEG and B6 as parental strains was therefore produced. We analyzed more than 300 females since we suspected that SEG resistance was not controlled by a single gene, and that scoring the phenotype as a binary (death or survival) trait, resulting from a complex series of host-bacteria interactions, would result in reduced power of QTL detection. In fact, this large number of animals is required to identify a QTL controlling 20% difference in survival, with a 0.05 genome-wide significance threshold. We identified three significant QTLs, each of which was able, in the context of the segregating background of BSB mice, to confer a 20% increase in survival, from 48 to 68% on average. Moreover, pair-wise analysis of QTL effects revealed an additive mode of action. Subsequently, the survival rate of BSB mice increased as a function of the SEG alleles present at the three QTLs, and mice heterozygous at *Yprl1*, *2* and *3* displayed the same level of resistance as SEG mice (Figure 5). However, since mice homozygous for the B6 allele at the three QTLs were significantly more resistant than B6 mice, there remain other resistance genes to be identified, which may be too numerous, or may have too weak effects to be detectable in a cross of this size.

Taken together, these data suggest a model for resistance to *Y. pestis* virulent strains in SEG mice (Figure 6). The B6 strain has a very weak ability to resist *Y. pestis*, which results most frequently in rapid mortality. One or more yet unidentified loci increase the resistance to a level where approximately 30% of mice eventually survive the infection. The three *Yprl* loci mapped in our study significantly improve resistance to *Y. pestis* and together recapitulate the resistance of SEG mice. We cannot predict the contribution the three *Yprl* loci provide to

resistance in the absence of the unidentified loci. It is possible that these unknown loci play a critical role and create a genomic context favourable to the action of the *Yprl* loci, so that strains congenic for only one of any of the *Yprl* loci may not show a significant increase in survival rate compared with B6.

The survival curves of the mice that died of the infection showed exactly the same decrease over time, across the B6, SEG and BSB populations (Figure 2). This observation suggests that the disease course in the mice which finally succumb to the *Y. pestis* challenge is very similar in these three different genetic backgrounds. Thus, the final outcome of infection would depend on events taking place during the first two or three days, before the first mice die.

The *Yprl1*, *2* and *3* regions overlap several QTLs associated with susceptibility to other bacteria, to viruses or parasites (Supplementary table 1). Regarding bacterial diseases, *Tbs1* is involved in control of both body weight loss and survival time following challenge with virulent *Mycobacterium tuberculosis*, which are rather late phenotypes. *Msts2* influences acute inflammatory reaction which correlates with survival following *Salmonella typhimurium* s.c. injection. This QTL could be relevant to our phenotype and maps close to the *Yprl3* peak location. Three other QTLs, associated with susceptibility to *Chlamydia trachomatis* and *Salmonella enteritidis* have been mapped at the boundaries of the *Yprl* confidence intervals and are hence less attractive.

Although the confidence intervals of the *Yprl1*, *2* and *3* loci encompass large chromosomal segments (between 50 and 84 Mb), it is also possible to consider potential candidate genes in these regions that may, because of their known function in host immune or inflammatory responses, contribute to the observed phenotype. The *Yprl1* confidence interval contains the *Pglyrp3* and *Pglyrp4* genes which are anti-microbial peptides secreted by skin, eyes, oral cavity and gastrointestinal tract in response to pathogen recognition. They play an

important role against pathogenic bacteria such as *Listeria monocytogenes* and *Staphylococcus aureus*, but seem to be less efficient against Gram-negative bacteria [18]. Interleukin 6 receptor α chain (*Il6ra*) also maps to *Yprl1*. IL-6 is a pleiotropic cytokine expressed by antigen presenting cells (APCs) such as dendritic cells, macrophages and B cells but also by a variety of non-hematopoietic cells [19]. It acts synergistically with a number of growth factors and cytokines to sustain normal proliferation and maturation of macrophages, T and B cells. It has been shown to play a role in resistance to bacteria (such as *Listeria monocytogenes* [20]) or viruses [21].

The main candidate in the *Yprl2* region is the Toll-like receptor 4 gene (*Tlr4*), located on the QTL peak. It is involved in the recognition of lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria [22]. Several mouse strains are hyporesponsive to LPS due to *Tlr4* mutations and show increased susceptibility to Gram-negative *S. typhimurium*, while transgenic mice possessing more than two extra-copies of the gene survived longer and in a greater percentage to *Salmonella* infection [23]. *Y. pestis* also produces LPS, however the *Tlr4* stimulating activity of its LPS-lipid A is reduced when the bacteria is at mammalian body temperature [24]. It would therefore be interesting to compare the *Tlr4* responses of B6 and SEG mice after exposure to *Y. pestis* LPS. Other genes of interest in the *Yprl2* region are 14 genes of the type I interferon and interferon receptor families, although studies have emphasized the role of type II gamma interferon in the control of *Y. pestis* multiplication in the host [25, 26].

Yprl3 overlaps several potential candidate genes. The nucleotide-binding oligomerization domain containing 1 (*Nod1*) gene is an intracellular pattern recognition receptor that mediates innate and acquired immunity by recognizing diaminopimelate-containing muropeptides from Gram-negative bacteria peptidoglycan, which activates the NF-κB pathway [27]. *Nod1* deficiency results in increased susceptibility to various Gram-negative

bacteria, including *Helicobacter pylori* [28], *Pseudomonas aeruginosa* [29] and *S. typhimurium* [30]. The interleukin-17 pathway is another interesting lead. There are six IL-17 family members and five receptors. Specifically, IL-17RA and IL-17RC subunits of IL-17 receptor, which map to the *Yprl3* interval (Supplementary table 1), interact with IL-17 and IL-17F to induce production of other proinflammatory cytokines, chemokines and growth factors [31], leading to an accumulation of neutrophils at the sites of infection and inflammation [32]. In fact, several studies have reported that IL-17R deficiency results in significant delay in neutrophil recruitment and impaired host defense against bacteria including *L. monocytogenes* [33], *Bacillus subtilis* [34] and *Klebsiella pneumoniae* [35]. Finally, *Yprl3* also contains IL-23 receptor which has been shown to regulate the function of specific IL-17–producing T-cells [36].

Future work will aim to narrow down the list of candidate genes by reducing the confidence intervals using congenic strains, and by searching for variations in the primary sequence or expression level of specific genes. Whatever the genes involved, it is likely that the SEG resistance alleles are gain-of-function variants, due to their dominant mode of inheritance.

The *Mus spretus* species diverged from *Mus musculus* more than 1 million years ago [37]. The genetic divergence between them at the nucleotide level is estimated to be 1 to 1.5%, the same order of magnitude seen between human and chimpanzee. Our observation of the exceptional resistance of SEG mice to *Y. pestis* illustrates the usefulness of *Mus spretus* derived strains as a source of genetic and phenotypic polymorphism [38]. The fact that the differences in susceptibility to plague between B6 and SEG strains is under the control of multiple genes with additive effects stands in contrast with other infectious diseases in which single gene mutations with major effects were identified. In the case of *Oas1b*, a nonsense mutation was probably present in the very early founders of laboratory strains and became fixed in the absence of any selective pressure in the vast majority of these strains [17]. This

mutation has not yet been found in inbred strains derived from progenitors of different *Mus musculus* subspecies. In the case of plague, the highly resistant phenotype of the SEG strain results from the accumulation of several gain-of-function genetic variations, each of which provides a moderate but significant advantage in the control of *Y. pestis* proliferation and systemic dissemination. The acquisition of this phenotype may have resulted from selective pressure exerted by endemic exposure to *Y. pestis*-hosting fleas in wild rodents.

The identification of genes controlling the resistant phenotype of SEG mice will involve the creation and characterization of congenic mouse strains carrying the three genomic regions identified in this study. This will be followed by the genetic dissection of each genomic region to prioritize positional candidates for DNA sequencing and functional evaluation using loss of function alleles at these genes. At the same time, construction and characterization of bi- or tricongenic strains will aim to recapitulate the resistant phenotype observed in the *Mus spretus* derived SEG strain.

Materials and methods

Mice and crosses. SEG/Pas was first bred as a closed colony by François Bonhomme in Montpellier, France, from *Mus spretus* progenitors trapped in Spain, near Granada [37]. It was later established as an inbred strain and maintained since 1995 at the Institut Pasteur. C57BL/6 (B6), FVB/NCrl, BALB/cByJ and SJL/JOrlCrl mice were purchased at 6 weeks of age from Charles River Laboratory (L'Arbresle, France). They were maintained for at least three weeks in the Institut Pasteur animal facilities prior to inoculation. CAST/Ei mice were bred at the Institut Pasteur from progenitors purchased from The Jackson Laboratory (Bar Harbor, ME). To produce B6×(SEG×B6)F1 backcross mice (BSB), SEG males were mated with B6 females and the resulting F1 females were mated with B6 males. All animal

experiments were approved and conducted in accordance with the Institut Pasteur Biosafety Committee, and in compliance with French and European regulation on protection of animals used for scientific purposes. Mice were kept in isolators after inoculation. They were given food and water ad libitum and were kept at 22±2°C with alternating 12h periods of light and dark.

Culture of *Yersinia pestis* strains and experimental infections of mice. Experimental infections were performed using the virulent *Y. pestis* strains CO92 [11] and 6/69 [12]. Cultures were carried out at 28°C for 48h on LB agar medium supplemented with 0.2% (W/v) hemin (LBH). Bacteria were collected and suspended in saline. Bacterial concentration was estimated by spectrometry at 600 nm (1 O.D. ~10^9 CFU/ml) and confirmed by colony-forming units (CFU) counts on LBH agar plates. Mice were injected s.c. between 8 and 12 weeks of age, in the ventral region, with a 100 µl inoculum, and were then monitored daily for 14 days.

Genotyping. Tail biopsies were collected at weaning from BSB mice and high-quality DNA was prepared by standard phenol-chloroform extraction. For BSB genotyping, 1,536 Single Nucleotide Polymorphisms (SNPs) were genotyped at the *Centre National de Génotypage* (Evry, France) using the Illumina GoldenGate platform. A total of 721 markers covering the entire mouse genome were polymorphic and gave reliable genotypes.

Statistical and QTL analysis. Survival curves were compared by a logrank (Mantel-Cox) test using StatView 5.0 (SAS Institute Inc, Cary NC). Survival rates were compared by Fisher's exact test. QTL analysis was performed by using the R/qtl software version 1.07-12 running under R 2.6.0 [39]. The survival rate was analyzed as a binary trait. Significance thresholds of LOD scores were estimated by 1 000 permutations of experimental data, and were 2.77 and 2.43 for 5% and 10% thresholds, respectively. Ninety-five percent confidence

intervals were estimated using the bayesint() function of R/qtl. QTL analysis of mortality time in susceptible BC mice was also performed with R/qtl using a non-parametric model.

Acknowledgements

CB is a recipient of a DGA (*Délégation Générale pour l'Armement*) fellowship. This work was supported by a grant from Aventis Pharma (Sanofi-Aventis group) and Bayer Pharma as part of a multi-organism call for proposal. The Mouse functional Genetics Unit is supported by Merck Serono. We thank Jean-Louis Guénet for his constant interest and challenging discussions. We are grateful to Colin Crist for critical reading and suggestions on the paper, to Isabelle Lanctin for careful breeding of the SEG/Pas colony, and to the CNG (*Centre National de Génotypage*) for the SNP genotyping of BC mice.

Supplementary information is available at Genes and Immunity's website.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

1. Titball RW, Williamson ED. Vaccination against bubonic and pneumonic plague. *Vaccine* 2001; **19**(30)**:** 4175-4184.
2. Schrag SJ, Wiener P. Emerging infectious diseases: what are the relative roles of ecology and evolution? *Trends Evol. Ecol. (TREE)* 1995; **10:** 319-324.
3. Galimand M, Guiyoule A, Gerbaud G, Rasoamanana B, Chanteau S, Carniel E *et al.* Multidrug resistance in Yersinia pestis mediated by a transferable plasmid. *N Engl J Med* 1997; **337**(10)**:** 677-80.
4. Inglesby TV, Dennis DT, Henderson DA, Bartlett JG, Ascher MS, Eitzen E *et al.* Plague as a biological weapon: medical and public health management. Working Group on Civilian Biodefense. *Jama* 2000; **283**(17)**:** 2281-90.
5. Perry RD, Fetherston JD. Yersinia pestis--etiologic agent of plague. *Clin Microbiol Rev* 1997; **10**(1)**:** 35-66.
6. Vidal SM, Malo D, Marquis JF, Gros P. Forward genetic dissection of immunity to infection in the mouse. *Annu Rev Immunol* 2008; **26:** 81-132.
7. Pollitzer R. Plague. *W.H.O. Monograph Series 22. World Health Organization, Geneva, Switzerland.* 1954.
8. Congleton YH, Wulff CR, Kerschen EJ, Straley SC. Mice naturally resistant to Yersinia pestis Delta pgm strains commonly used in pathogenicity studies. *Infect Immun* 2006; **74**(11)**:** 6501-4.
9. Turner JK, Xu JL, Tapping RI. Substrains of 129 mice are resistant to Yersinia pestis KIM5: implications for interleukin-10-deficient mice. *Infect Immun* 2009; **77**(1)**:** 367-73.
10. Turner JK, McAllister MM, Xu JL, Tapping RI. Resistance of BALB/cJ mice to Yersinia pestis maps to the major histocompatibility complex of chromosome 17. *Infect Immun* 2008.
11. Doll JM, Zeitz PS, Ettestad P, Bucholtz AL, Davis T, Gage K. Cat-transmitted fatal pneumonic plague in a person who traveled from Colorado to Arizona. *Am. J. Trop. Med. Hyg.* 1994; **51**(1)**:** 109-114.
12. Buchrieser C, Prentice M, Carniel E. The 102-Kilobase Unstable Region Of *Yersinia Pestis* Comprises a High-Pathogenicity Island Linked to a Pigmentation Segment Which Undergoes Internal Rearrangement. *J. Bacteriol.* 1998; **180**(9)**:** 2321-2329.
13. Lathem WW, Crosby SD, Miller VL, Goldman WE. Progression of primary pneumonic plague: a mouse model of infection, pathology, and bacterial transcriptional activity. *Proc Natl Acad Sci U S A* 2005; **102**(49)**:** 17786-91.
14. Leary SEC, Griffin KF, Galyov EE, Hewer J, Williamson ED, Holmstrom A *et al.* *Yersinia* outer proteins (YOPS) E, K and N are antigenic but non-protective compared to V antigen, in a murine model of bubonic plague. *Microb. Pathog.* 1999; **26**(3)**:** 159-169.
15. Lindler LE, Klempner MS, Straley SC. *Yersinia pestis* pH-6 Antigen - Genetic, Biochemical, and Virulence Characterization of a Protein Involved in the Pathogenesis of Bubonic Plague. *Infect. Immun.* 1990; **58**(8)**:** 2569-2577.
16. Leal-Balbino TC, Leal NC, do Nascimento MGM, de Oliveira MBM, Balbin VDQ, de Almeida AMP. The pgm locus and pigmentation phenotype in *Yersinia pestis*. *Genetics Mol. Biol.* 2006; **29**(1)**:** 126-131.
17. Mashimo T, Lucas M, Simon-Chazottes D, Frenkiel MP, Montagutelli X, Ceccaldi PE *et al.* A nonsense mutation in the gene encoding 2'-5'-oligoadenylate synthetase/L1 isoform is associated with West Nile virus susceptibility in laboratory mice. *Proc Natl Acad Sci U S A* 2002; **99**(17)**:** 11311-6.

18. Kapetanovic R, Cavaillon JM. Early events in innate immunity in the recognition of microbial pathogens. *Expert Opin Biol Ther* 2007; **7**(6)**:** 907-18.
19. Dienz O, Rincon M. The effects of IL-6 on CD4 T cell responses. *Clin Immunol* 2009; **130**(1)**:** 27-33.
20. Dalrymple SA, Lucian LA, Slattery R, McNeil T, Aud DM, Fuchino S *et al.* Interleukin-6-deficient mice are highly susceptible to Listeria monocytogenes infection: correlation with inefficient neutrophilia. *Infect Immun* 1995; **63**(6)**:** 2262-8.
21. LeBlanc RA, Pesnicak L, Cabral ES, Godleski M, Straus SE. Lack of interleukin-6 (IL-6) enhances susceptibility to infection but does not alter latency or reactivation of herpes simplex virus type 1 in IL-6 knockout mice. *J Virol* 1999; **73**(10)**:** 8145-51.
22. da Silva Correia J, Soldau K, Christen U, Tobias PS, Ulevitch RJ. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. transfer from CD14 to TLR4 and MD-2. *J Biol Chem* 2001; **276**(24)**:** 21129-35.
23. Bihl F, Lariviere L, Qureshi ST, Flaherty L, Malo D. LPS-hyporesponsiveness of mnd mice is associated with a mutation in Toll-like receptor 4. *Genes Immun* 2001; **2**(1)**:** 56-9.
24. Montminy SW, Khan N, McGrath S, Walkowicz MJ, Sharp F, Conlon JE *et al.* Virulence factors of Yersinia pestis are overcome by a strong lipopolysaccharide response. *Nat Immunol* 2006; **7**(10)**:** 1066-73.
25. Kummer LW, Szaba FM, Parent MA, Adamovicz JJ, Hill J, Johnson LL *et al.* Antibodies and cytokines independently protect against pneumonic plague. *Vaccine* 2008; **26**(52)**:** 6901-7.
26. Nakajima R, Brubaker RR. Association between virulence of Yersinia pestis and suppression of gamma interferon and tumor necrosis factor alpha. *Infect Immun* 1993; **61**(1)**:** 23-31.
27. Girardin SE, Boneca IG, Carneiro LA, Antignac A, Jehanno M, Viala J *et al.* Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* 2003; **300**(5625)**:** 1584-7.
28. Viala J, Chaput C, Boneca IG, Cardona A, Girardin SE, Moran AP *et al.* Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. *Nat Immunol* 2004; **5**(11)**:** 1166-74.
29. Travassos LH, Carneiro LA, Girardin SE, Boneca IG, Lemos R, Bozza MT *et al.* Nod1 participates in the innate immune response to Pseudomonas aeruginosa. *J Biol Chem* 2005; **280**(44)**:** 36714-8.
30. Le Bourhis L, Magalhaes JG, Selvanantham T, Travassos LH, Geddes K, Fritz JH *et al.* Role of Nod1 in mucosal dendritic cells during Salmonella pathogenicity island 1-independent Salmonella enterica serovar Typhimurium infection. *Infect Immun* 2009; **77**(10)**:** 4480-6.
31. Zhou X, Chen Q, Moore J, Kolls JK, Halperin S, Wang J. Critical role of the interleukin-17/interleukin-17 receptor axis in regulating host susceptibility to respiratory infection with Chlamydia species. *Infect Immun* 2009; **77**(11)**:** 5059-70.
32. Kolls JK, Linden A. Interleukin-17 family members and inflammation. *Immunity* 2004; **21**(4)**:** 467-76.
33. Sieve AN, Meeks KD, Bodhankar S, Lee S, Kolls JK, Simecka JW *et al.* A novel IL-17-dependent mechanism of cross protection: respiratory infection with mycoplasma protects against a secondary listeria infection. *Eur J Immunol* 2009; **39**(2)**:** 426-38.
34. Simonian PL, Roark CL, Wehrmann F, Lanham AM, Born WK, O'Brien RL *et al.* IL-17A-expressing T cells are essential for bacterial clearance in a murine model of hypersensitivity pneumonitis. *J Immunol* 2009; **182**(10)**:** 6540-9.

35. Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P *et al.* Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med* 2001; **194**(4)**:** 519-27.
36. Riol-Blanco L, Lazarevic V, Awasthi A, Mitsdoerffer M, Wilson BS, Croxford A *et al.* IL-23 receptor regulates unconventional IL-17-producing T cells that control bacterial infections. *J Immunol*; **184**(4)**:** 1710-20.
37. Guénet JL, Bonhomme F. Wild mice: an ever-increasing contribution to a popular mammalian model. *Trends Genet* 2003; **19**(1)**:** 24-31.
38. Dejager L, Libert C, Montagutelli X. Thirty years of Mus spretus: a promising future. *Trends Genet* 2009; **25**(5)**:** 234-41.
39. Broman KW, Wu H, Sen S, Churchill GA. R/qtl: QTL mapping in experimental crosses. *Bioinformatics* 2003; **19**(7)**:** 889-90.

FIGURE LEGENDS

Figure 1 Survival, over a 14-day period, of various mouse inbred strains following s.c. inoculation with virulent 10^2 CFU of strain CO92 *Y. pestis* : SEG (N=69), FVB/N (N=22), BALB/cByJ (N=21), CAST/Ei (N=10), B6 (N=111) and SJL/J (N=9) females.

Figure 2 Survival curves of the B6 (N=104), SEG (N=7) and BSB (N=132) female mice that died of the *Y. pestis* infection. Mortality time did not differ, either in mean, or in variability, across the three groups.

Figure 3 QTL mapping of survival to a *Y. pestis* challenge in 322 BSB females, showing three significant QTLs on chromosomes 3 (*Yprl1*), 4 (*Yprl2*) and 6 (*Yprl3*). Dashed lines denote thresholds of significance (p = 0.1 and p = 0.05, permutation test).

Figure 4 Survival curves of BSB females showing the effect of the genotype at the marker at the peak LOD score of each QTL. (A) *Yprl1* : Chromosome 3 at 116Mb; B/B : N = 139; B/S : N = 182. (B) *Yprl2* : Chromosome 4 at 62Mb; B/B : N = 159; B/S : N = 160. (C) *Yprl3* : Chromosome 6 at 95Mb; B/B : N = 148; B/S : N = 174. Significance values were calculated from Fisher's exact test on survival rates.

Figure 5 Survival rate of B6, BSB and SEG females according to their haplotype at the *Yprl1*, *Yprl2* and *Yprl3* QTLs (same markers as in Figure 4), and their genetic background. The top table gives the genotype of each group at the three QTLs and the genetic background. White box : B/B; hatched box : B/S; black box : S/S. Split boxes depict loci for which fully genotyped BSB animals were either B/B or B/S. The graph shows the survival rate (with error bars indicating one standard deviation) of each group. The difference between the two leftmost groups reflects the effect of unidentified QTLs. Mice heterozygous for *Yprl1*, *Yprl2* and *Yprl3* show the same survival rate as the SEG strain.

Figure 6 A model illustrating the additive effects of *Yprl1*, *Yprl2* and *Yprl3* on survival to a *Y. pestis* challenge. Unidentified QTLs provide some resistance mechanisms which result in ~19% increase in survival rate compared to B6J. Each of the *Yprl1*, *Yprl2* and *Yprl3* loci provide, even in B/S heterozygotes, additional ability to survive the infection, up to the survival rate observed in SEG mice.

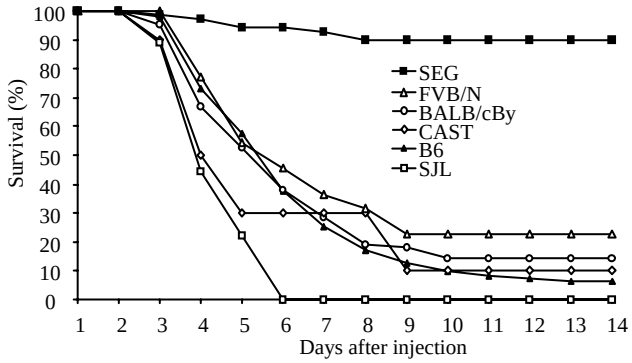

Survival (%)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days after injection
SEG
FVB/N
BALB/cBy
CAST
B6
SJL

Figure 1

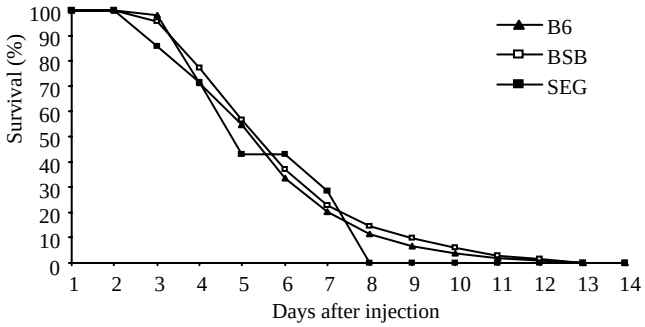

Survival (%)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days after injection
B6
BSB
SEG

Figure 2

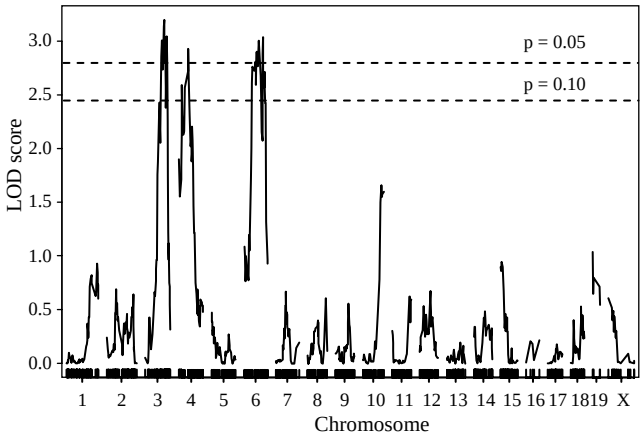

p = 0.05
p = 0.10
LOD score
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 X
Chromosome

Figure 3

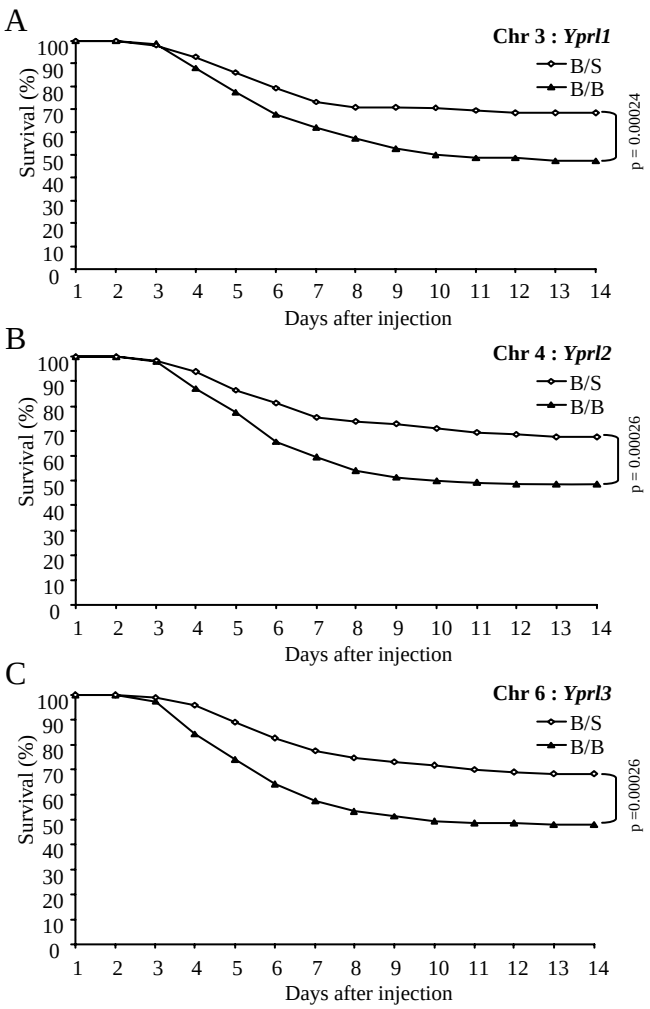

A
Chr 3 : Yprl1
B/S
B/B
p = 0.00024
Survival (%)
Days after injection
B
Chr 4 : Yprl2
B/S
B/B
p = 0.00026
Survival (%)
Days after injection
C
Chr 6 : Yprl3
B/S
B/B
p =0.00026
Survival (%)
Days after injection

Figure 4

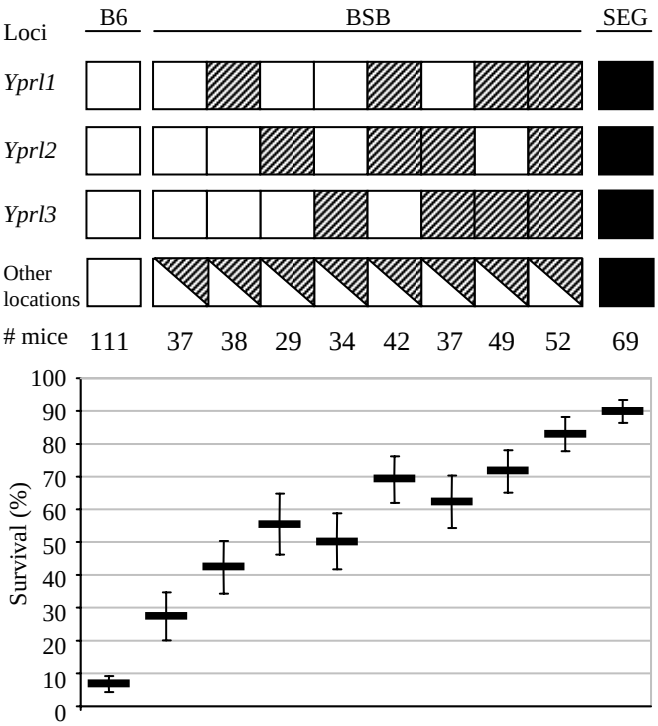

Loci
B6
BSB
SEG
Yprl1
Yprl2
Yprl3
Other locations
mice
111
37
38
29
34
42
37
49
52
69
Survival (%)
100
90
80
70
60
50
40
30
20
10
0

Figure 5

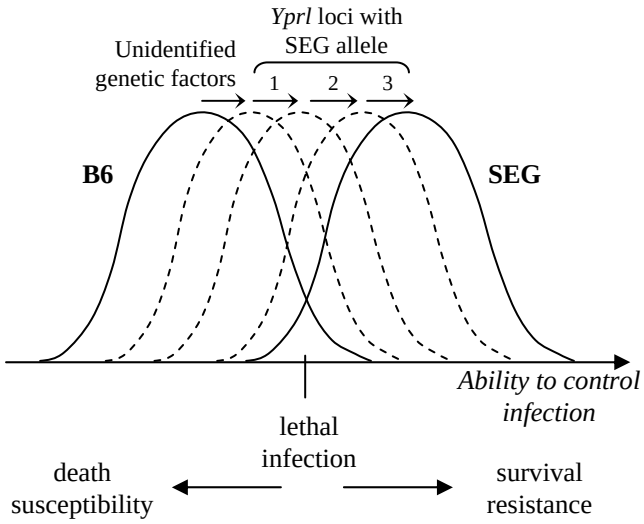

Yprl loci with
SEG allele
Unidentified
genetic factors
1
2
3
B6
SEG
Ability to control infection
lethal
infection
death
susceptibility
survival
resistance

Figure 6

Table 1 QTL summary

QTL	Chr	Peak position (Mb)	95% C.I. (Mb)	Effect on survival	Nb of genes in the interval[(1)]
Yprl1	3	116	89 - 139	20.6 %	469
Yprl2	4	62	6 - 90	20.3 %	371
Yprl3	6	95	53 - 134	20.4 %	644

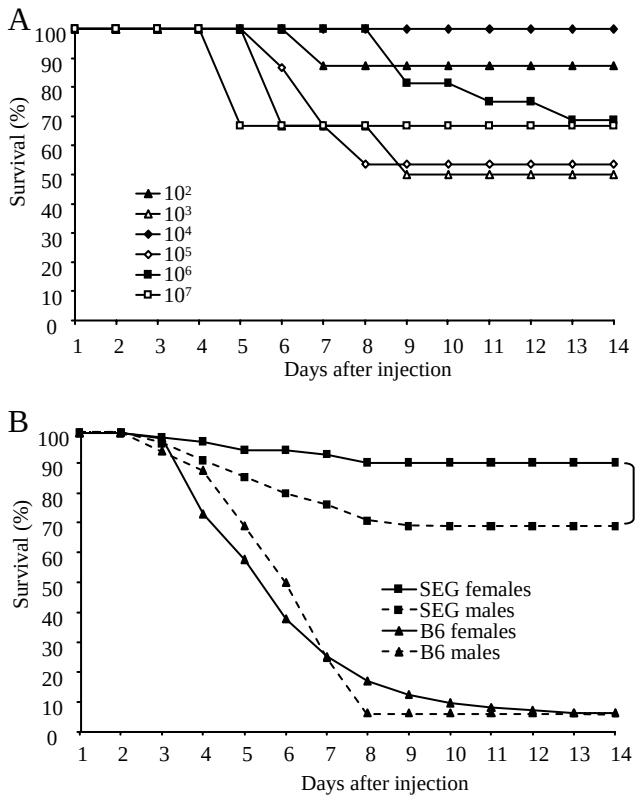
[(1)] excluding anonymous cDNA sequences and predicted genes

LEGENDS TO SUPPLEMENTARY FIGURES

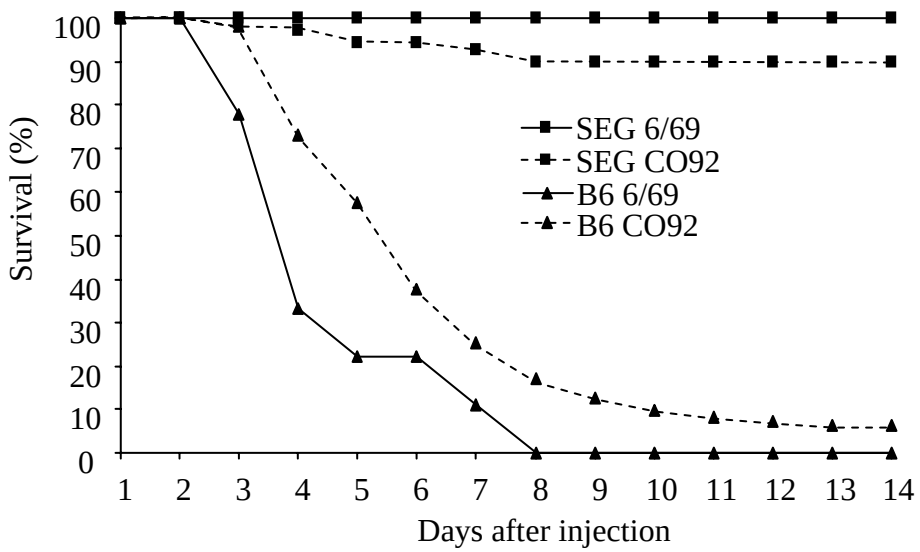
Supplementary figure 1 Survival, over a 14-day period, of various mouse inbred strains following s.c. inoculation with virulent *Y. pestis* strains CO92 or 6/69. (A) Groups of SEG females were inoculated with 10^2 (N=8), 10^3 (N=6), 10^4 (N=11), 10^5 (N=15), 10^6 (N=16) and 10^7 (N=6) CFU of strain CO92. (B) Comparison of susceptibility between male and female in B6 (N=16 and N=111, respectively) and SEG (N=54 and N=69, respectively) mice following inoculation with 10^2 CFU of strain CO92. SEG females were more resistant than males (shown in brackets; 62/69 and 37/54, respectively; p=0.005). Large groups (N>25) are the result of pooling several experiments.

Supplementary figure 2 Comparison of susceptibility of B6 and SEG females to 10^2 CFU of strain CO92 (N=111 and N=69, respectively) and of strain 6/69 (N=9 and N=16, respectively). Large groups (N>25) are the result from pooling several experiments.

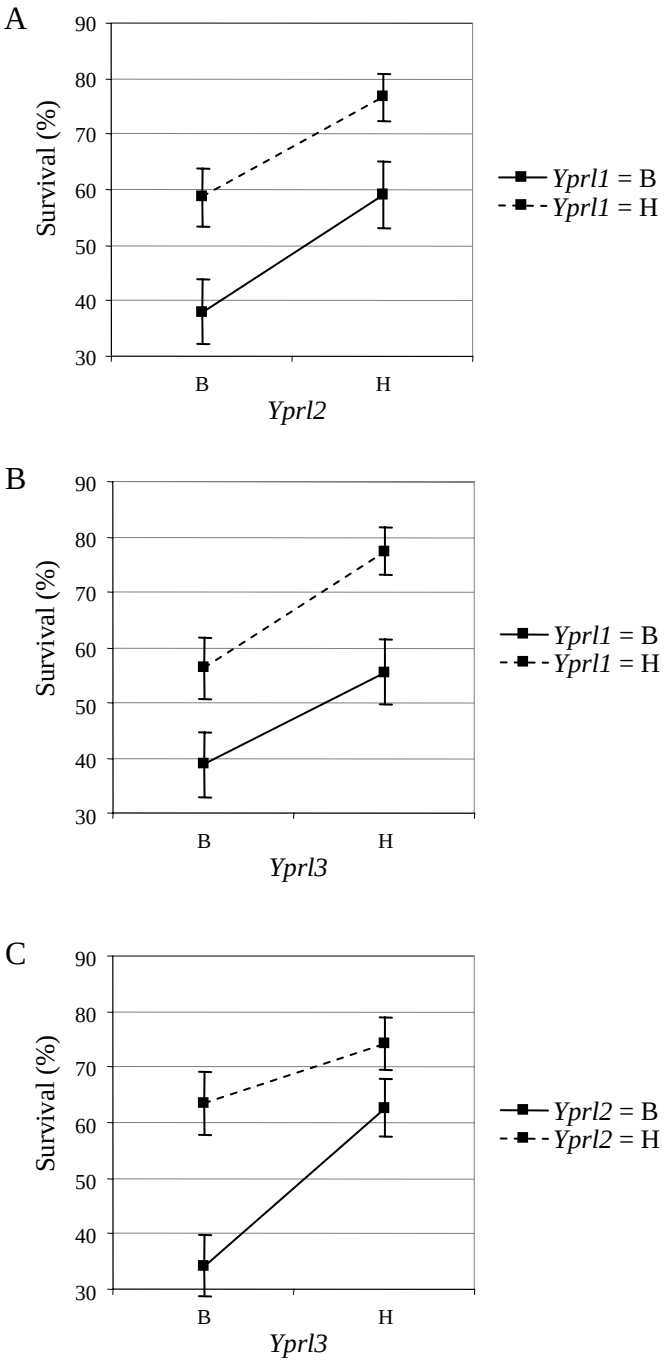
Supplementary figure 3 Two-locus effect plots showing the effect of one QTL depending on the genotype at the other QTL. For each combination of genotypes, survival rate is given with error bars indicating one standard deviation. The interaction between the two loci was tested by two-way ANOVA after arcsine transformation of the survival rates. (A) *Yprl1* and *Yprl2*, p(interaction) = 0.97. (B) *Yprl1* and *Yprl3*, p(interaction) = 0.38. (C) *Yprl2* and *Yprl3*, p(interaction) = 0.11.



Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3