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# Tn5AraOut mutagenesis for the identification of *Yersinia pestis* genes involved in resistance towards cationic antimicrobial peptides

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ABSTRACT

Bacterial pathogens display a variety of protection mechanisms against the inhibitory and lethal effects of host cationic antimicrobial peptides (CAMPs). To identify *Yersinia pestis* genes involved in CAMP resistance, libraries of DSY101 (KIM6 *caf1 pla psa*) minitransposon Tn5AraOut mutants were selected at 37 °C for resistance to the model CAMPs polymyxin B or protamine. This approach targeted genes that needed to be repressed (null mutations) or induced (upstream $P_{BAD}$ insertions) for the detection of CAMP resistance, and predictably for improved pathogen fitness in mammalian hosts. Ten mutants demonstrated increased resistance to polymyxin B or protamine, with the mapped mutations pointing towards genes suspected to participate in modifying membrane components, genes encoding transport proteins or enzymes, or the regulator of a ferrous iron uptake system (*feoC*). Not all the mutants were resistant to both CAMPs used for selection. None of the polymyxin B- and only some protamine-resistant mutants, including the *feoC* mutant, showed increased resistance to rat bronchoalveolar lavage fluid (rBALF) known to contain cathelicidin and β-defensin 1. Thus, findings on bacterial resistance to polymyxin B or protamine don't always apply to CAMPs of the mammalian innate immune system, such as the ones in rBALF.

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## 1. Introduction

*Yersinia pestis* is best known as the causative agent of bubonic plague, a disease transmitted by the bite of infected fleas. However, if inhaled, this pathogen also produces a severe primary pneumonia known as pneumonic plague, which is contagious and most often lethal. This bacterial agent uses an arsenal of virulence factors that render its entrance into the host as surreptitious as possible. These tools are thought to be most important during the first few hours following infection to avoid alarming the innate immune system and to avoid phagocytosis. We and others have previously shown that in addition to being antiphagocytic, the surface proteins F1 and Psa inhibit bacterial uptake by respiratory tract epithelial cells or macrophages [1–3]. The antiphagocytic mechanism of Psa was suggested to be due to Psa binding to host receptors that don't direct internalization [4]. *Y. pestis* injects several antiphagocytic proteins directly into host cells through its type III secretion system (T3SS) [5,6], some of which have strong anti-inflammatory properties [6–11]. In addition, *Y. pestis* also makes a non-inflammatory LPS at mammalian body temperature, thereby escaping the typical LPS-induced stimulation of TLR4 [12,13]. Although the anti-inflammatory activities of *Y. pestis* also affect DCs and their migratory properties [14,15], *Y. pestis* delivered through a fleabite still spreads to the local lymph node, causing lymphadenitis (bubo). Further spreading steps leading to bacteremia or septicemia are not infrequent, particularly when bubonic plague remains untreated. Plague lethality is generally assumed to be due to sepsis. Dissemination is facilitated by core LPS, the Psa fimbriae, the outer membrane adhesin Ail and the plasminogen activator outer membrane protein Pla [16–20]. Pla acts as a protease that cleaves plasminogen to plasmin and mediates bacterial binding to extracellular matrix proteins [21,22]. It is essential for bubonic plague, (but not for septicemic plague) after flea-mediated transmission [18,19,23,24]. Pla is also essential for the development of (but not for the dissemination from) primary pneumonic plague [25].

*Y. pestis* has not only evolved to survive but also to thrive in a hostile host environment that includes the antimicrobial peptides

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of the innate immune system, as best exemplified by successful bacterial replication in local lymph nodes or lungs, leading to bubonic or primary pneumonic plague, respectively. Although the anti-inflammatory molecules of *Y. pestis* might down-regulate the expression of host cationic antimicrobial peptides (CAMPs) [26,27], it is likely that this bacterium also expresses a battery of tools aimed at inactivating CAMPs in vivo. Accordingly, we recently observed that Pla, through its proteolytic activities, increased bacterial resistance to CAMPs at 37 °C in vitro [48]. Curiously, this activity was counteracted in vitro by the F1 protein, an in-vivo expressed protein, suggesting that *Y. pestis* might use additional mechanisms of bacterial resistance to CAMPs. In addition to antimicrobial peptide degradation by proteases, other bacteria have been reported to trap CAMPs extracellularly, alter their surface, particularly their surface charge, pump CAMPs out, or modulate host cell expression or degradation of CAMPs [28,29]. The aim of this study was to identify new *Y. pestis* genes involved in any of these survival strategies. For this purpose, a minitransposon with an outward-oriented inducible promoter was constructed and used to identify genes that either increase CAMP resistance by being repressed (null mutants) or that demonstrate resistance by being activated (inducible gene expression).

## 2. Results

### 2.1. Isolation of CAMP-resistant minitransposon mutants

Tn*5*AraOut mutants of strain DSY101 that demonstrated increased resistance to polymyxin B or protamine were isolated as described in Materials and Methods. Briefly, *Y. pestis* strain DSY101 was transformed with plasmid pTn*5*AraOut and bacteria were selected on kanamycin-containing media at 37 °C containing L-arabinose and supplemented with polymyxin B or protamine at concentrations previously shown to inhibit strain DSY101 growth. Selection was done in the presence of arabinose to simultaneously obtain both arabinose-dependent and -independent CAMP-resistant mutants. Consistency in the phenotypes of clone-purified mutants was confirmed for five polymyxin-resistant and five protamine-resistant isolates on selective agar plates. No spontaneous resistant bacteria could be isolated in parallel control experiments with mock ($H_2O$)-transformed bacteria, strongly suggesting that the isolated polymyxin- and protamine-resistant clones were due to the minitransposon insertions. These mutants were further characterized by determining the arabinose-dependency of their resistance and by a quantitative analysis of their CAMP resistance levels. For this, each strain was grown in broth to log phase and further multiplication or killing was studied in the presence or absence of CAMPs. Bacterial growth was monitored in the presence of 0.2% arabinose, to activate the inducible promoter on the minitransposon, or 0.2% glucose as a repressor of this promoter (negative control). All our studies were done with bacteria grown at 37 °C, since our aim was to evaluate CAMP effects on *Y. pestis* at mammalian body temperature. In addition to the determination of MICs, growth was analyzed more precisely by determining absorbance values ($A_{600}$). That all the mutants and the parental strain grew somewhat better in the presence of arabinose than in the presence of glucose was probably related to a previously described negative effect of glucose on *Y. pestis* growth [30]. However, this effect did not interfere with the interpretation of the results concerning antimicrobial resistance, since bacterial growth of the mutants was compared with growth of the parental strain studying both media with or without polymyxin B. Fig. 1A shows that polymyxin B was clearly bactericidal on parental strain DSY101 at 1.25 μg/ml or more, the lower dose of 0.625 μg/ml being still able to inhibit bacterial growth, as compared to the bacteria in polymyxin B-free broth. In contrast, four of the five mutants grew well at the significantly higher dose of 5 μg/ml polymyxin B, growth of three of these resistant mutants (PB7-23, PB8-2 and PB8-4) being clearly arabinose-dependent, since their growth was inhibited by polymyxin B only in the absence of arabinose. Mutant PB8-8 demonstrated arabinose-independent polymyxin B resistance, its growth being not inhibited by polymyxin B, irrespective of the presence or absence of arabinose (Fig. 1A). Similarly, mutant PB7-10 still grew in the presence of twice the concentrations of polymyxin B that was killing the parental strain in the presence of arabinose

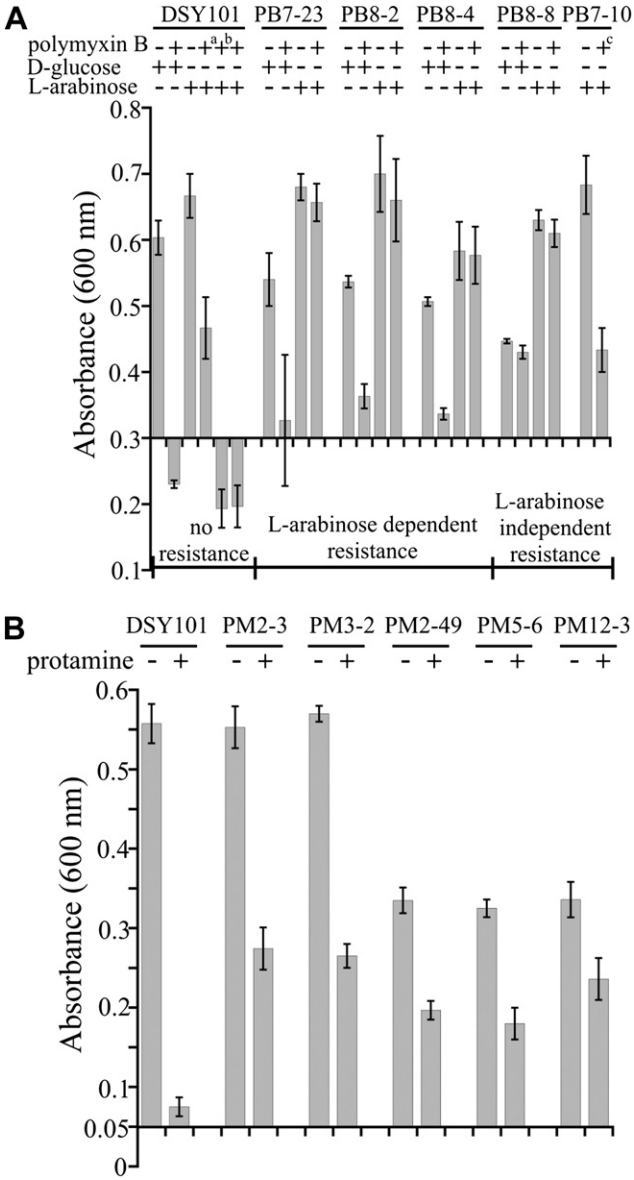



**Fig. 1.** Bacterial growth with the CAMPs used to select the mutants. (A) Bacteria were grown in BHI with or without glucose or arabinose to an $A_{600}$ of 0.3 at 37 °C, polymyxin B was added to a concentration of 5 μg/ml (or [a]0.625 μg/ml, [b]1.25 μg/ml, and [c]2.5 μg/ml), the cultures were further incubated for 18 h before measuring growth ($A_{600}$). MICs were determined in microtiter plates and were 1 μg/ml for DSY101 and 8 mg/ml for all the mutants (except PB7-10, 4 μg/ml). (B) Bacteria were grown in BHI to an $A_{600}$ of 0.3 at 37 °C, diluted to an $A_{600}$ of 0.05 (to use concentrations of protamine that do not interfere significantly with $A_{600}$ reading), protamine was added to a concentration of 0.4 mg/ml and the cultures were further incubated for 18 h before measuring growth ($A_{600}$). MICs were determined in microtiter plates and were 0.4 mg/ml for DSY101 and 0.8 mg/ml for all the mutants. All the data are means of at least three independent experiments with standard errors.

(Fig. 1A) or glucose (data not shown), indicating also arabinose-independent polymyxin B resistance. The five mutants selected on protamine agar were able to grow significantly in the presence of a protamine-concentration that blocked multiplication of the parental strain DSY101 (Fig. 1B). Interestingly, protamine resistance was arabinose-independent for all the five mutants (data not shown). Although three of the mutants grew slower than the parental strain in the absence of protamine, mutants PM2-3 and PM3-2 grew as well, indicating that protamine resistance wasn't necessarily linked to some effect on bacterial multiplication under the used growth conditions. Taken together, the quantitative analysis of bacterial growth in broth clearly confirmed the polymyxin B and protamine resistance phenotypes of the mutants.

### 2.2. Dissociation of polymyxin B and protamine resistance

To determine whether the two CAMPs target different bacterial components or possibly share their mode of actions, the polymyxin B-resistant mutants were tested for protamine resistance, and vice versa, using the same experimental conditions described above, including the inducer arabinose in all the assays. Although four polymyxin B-resistant mutants were also resistant to protamine, strain PB8-8 didn't multiply significantly with protamine (Fig. 2A). Conversely, mutant strains PM2-3 and PM3-2 that showed resistance to protamine were killed by polymyxin B, whereas the other three protamine-resistant mutants grew in the presence of polymyxin B (Fig. 2B). That some mutants showed a dissociation of resistance properties between polymyxin B and protamine highlighted the existence of different cationic peptide-specific mechanisms of CAMP resistance.

### 2.3. Susceptibility of selected mutants towards rBALF antimicrobials and LL-37

Since the polymyxin B- and protamine-resistance profiles were only partially overlapping, we wondered how the mutants would respond to constitutively expressed respiratory tract antimicrobial peptides. Again, the assays were undertaken as described above, in the presence of arabinose. By first testing bacterial growth in rBALF, which contains rCRAMP [4], the rat ortholog of the human cathelicidin LL-37, it was observed that all the mutants selected for polymyxin B resistance did not multiply (Fig. 3A). In contrast and with the exception of strain PM2-3 and PM3-2, the three other mutants selected for protamine resistance were able to grow in the presence of rBALF. To test these results more specifically, we investigated growth of these mutants in the presence of the purified mammalian CAMP, LL-37. Confirming the data obtained with rBALF, all the rBALF-susceptible mutants were also sensitive to LL-37, as exemplified with mutant PB7-23 (Fig. 3B). The other rBALF-susceptible mutants showed comparable LL-37 concentration-dependent killing effects, with $\geq 10^3$ reductions of CFUs for 160 μg/ml LL-37. Conversely, the three rBALF-resistant mutants were also resistant to LL-37 (Fig. 3B). To summarize, only two of the polymyxin B-susceptible mutants (PM2-3 and PM3-2) were resistant to protamine and rBALF/LL-37 and all polymyxin B-resistant mutants (most being also protamine-resistant) were susceptible to rBALF/LL-37. These results indicated that polymyxin B- or protamine resistance is not necessarily representative of resistance towards mammalian antimicrobial peptides, such as the ones of the respiratory tract.

### 2.4. Mapping Tn5AraOut in the CAMP-resistant mutants

The mutants were further characterized by mapping their Tn5AraOut insertion sites and orientations as described in Materials and Methods (Table 1). Briefly, DNA including Tn5AraOut for each of the ten resistant mutants was cloned by using different restriction enzymes. For each mutant-restriction enzyme pair, plasmid clones of similar sizes were obtained and DNA flanking the minitransposon inserts of these plasmids was sequenced. Sequencing plasmids from each individual mutant always yielded the same flanking sequences, strongly suggesting that each mutant had only one Tn5AraOut copy in its genome. This was confirmed by Southern blot analysis using genomic DNA restricted with either one of two different enzymes and a probe lacking the corresponding sites, each mutant showing only one major hybridizing fragment (Supplemental Fig. 1). Moreover, since spontaneous resistant mutants were not detected with mock

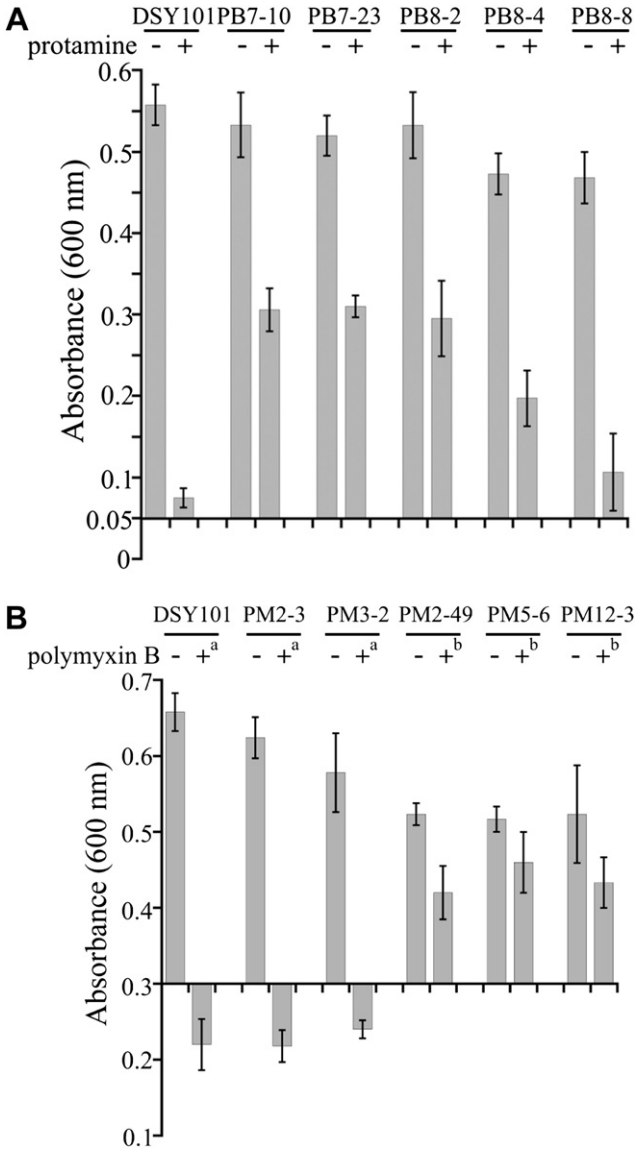



**Fig. 2.** Bacterial growth with CAMPs different from the ones used to select the mutants. (A) The polymyxin B-resistant mutants and the parental strain DSY101 were grown in BHI to an $A_{600}$ of 0.3 at 37 °C, diluted to an $A_{600}$ of 0.05, protamine was added to a concentration of 0.4 mg/ml and the cultures were further incubated for 18 h before measuring growth ($A_{600}$). MICs were determined in microtiter plates and were 0.8 mg/ml for all the mutants. (B) The protamine-resistant mutants were grown in BHI to an $A_{600}$ of 0.3 at 37 °C, polymyxin B was added to a concentration of [a]1.25 μg/ml or [b]5 μg/ml, the cultures were further incubated for 18 h before measuring growth ($A_{600}$). MICs were determined in microtiter plates and were 1 μg/ml (PM2-3, PM3-2) or 16 μg/ml (PM2-49, PM5-6 and PM12-3). All the data are means of at least three independent experiments with standard errors.

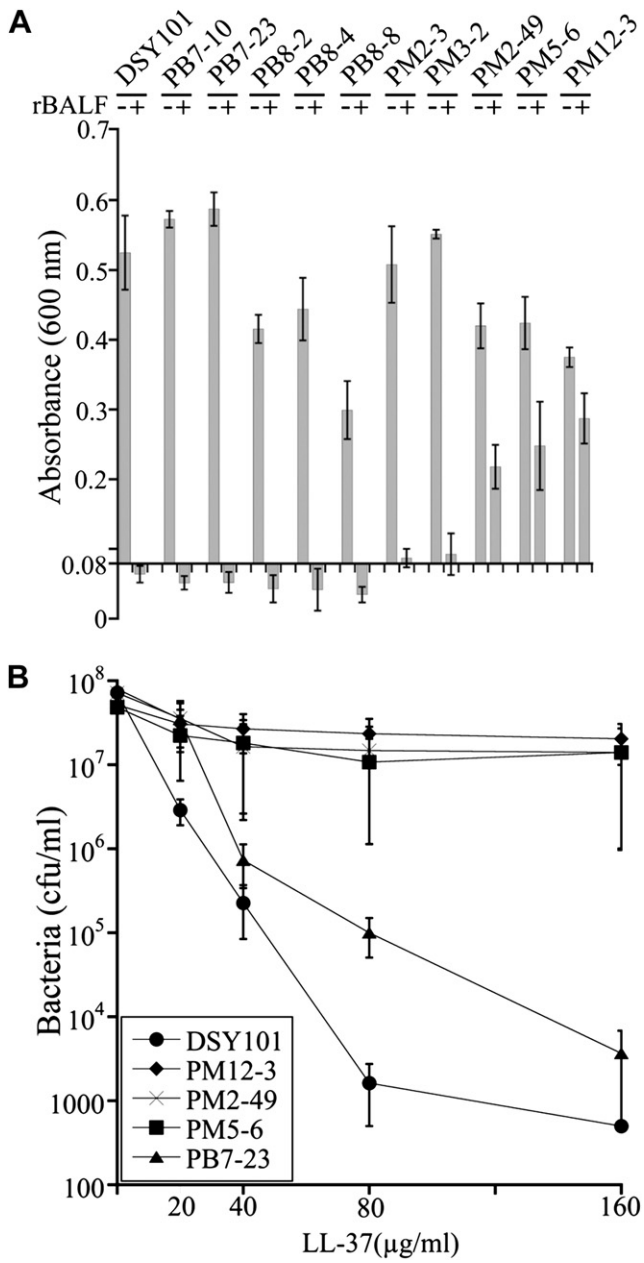



**Fig. 3.** Bacterial growth of all the mutants in the presence of rBALF or LL-37. (A) Bacteria were grown in BHI to an $A_{600}$ of 0.3 at 37 °C, diluted to an $A_{600}$ of 0.08, rBALF was added to a concentration of 60 μg/ml protein and the cultures were further incubated for 18 h before measuring growth ($A_{600}$). MICs were determined in microtiter plates and were 60 μg/ml protein for DSY101, all the polymyxin B mutants, PM2-3 and PM3-2, and 120 μg/ml protein for PM2-49, PM5-6 and PM12-3. (B) Bacteria were grown in BHI to an $A_{600}$ of 0.3 at 37 °C, diluted to an $A_{600}$ of 0.08, LL-37 was added to 20, 40, 80 or 160 μg/ml protein and the cultures were further incubated for 18 h before determining bacterial survival (CFUs). All the data are means of at least three independent experiments with standard errors.

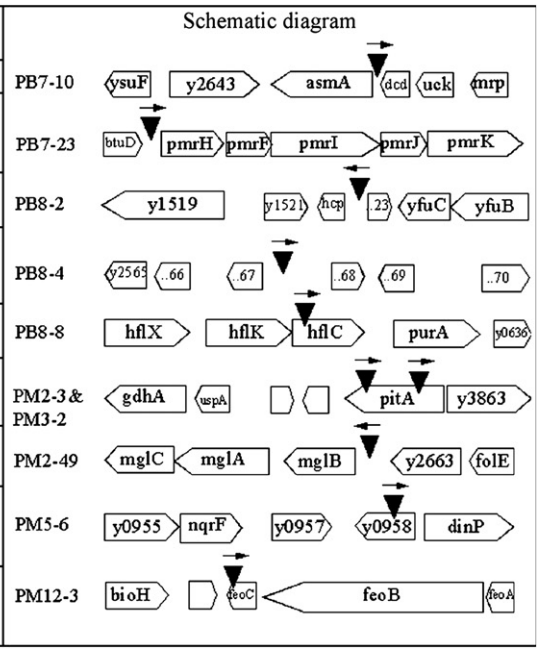
transformed bacteria, the mapped minitransposon inserts were most likely responsible for the described resistance phenotypes. Further supportive evidence on single transposon insertions responsible for resistance phenotypes in individual mutants were derived from detailed characterization of mutants of interest, as described below. Three of the CAMP-resistant mutants selected with polymyxin B had an arabinose-dependent resistance phenotype that was presumably linked to the activation of downstream genes (Table 1 and Fig. 1A). For example, mutant PB7-23 had the arabinose-inducible promoter upstream of the *pbgP* operons, which includes 7 cotranscribed genes (*pmrHFIJKLM* or *arnBCADTEF*) required for the synthesis of 4-amino-arabinose that is added to the phosphates of lipid A in *Y. pestis* LPS [13,31,32]. Arabinose-dependent induction of *pmrH* transcription was confirmed in this mutant by qRT-PCR (mean ± standard error = 15.0 ± 4.5 fold increase with the inducer, $n = 4$, versus 1.4 ± 0.7 without inducer, $n = 3$, $P = 0.037$). The detection of such a mutant in our screen supported our experimental method, since it had been previously shown that 4-aminoarabinose-decorated LPS renders *Y. pestis* more resistant to polymyxin B. The two other mutants with arabinose-inducible resistance to polymyxin B (PB8-4 and PB8-2) had minitransposon insertions upstream of two single or monocistronic genes, y2568 which encodes a hypothetical protein, and y1522 which encodes an Hcp-like protein that is not linked to any of the five predicted type 6 secretion systems (T6SS) of *Y. pestis* [33]. In contrast, two arabinose-independent polymyxin B-resistant mutants (PB7-10 and PB8-8) had minitransposon insertions in the promoter region of *asmA* and *hflC* orthologs of *Escherichia coli*. AsmA modulates porins, glycerophosphates and LPS levels in the outer membrane [34–36], whereas *hflC* encodes a repressor of the proteolytic activity of the integral inner membrane protein FtsH [37] which can regulate LPS concentrations through proteolysis of LpxC [38,39]. Thus, apart from the targeted hypothetical protein, the mutants selected for increased resistance to polymyxin B had a Tn*5*AraOut insertion in gene loci for bacterial envelope components or potentially secreted molecules. Of the five additional CAMP-resistant mutants that were selected with protamine, four had mutations in orthologs of *E. coli* or *Salmonella* transport systems, including two independent minitransposon insertions (PM2-3 and PM3-2) in the gene for the low affinity metal phosphate transporter PitA [40,41]. The PM2-49 mutant had its transposon insertion mapped 42 bp upstream of a predicted operator site for the GalR repressor of the galactose import system MglBAC [42]. Polymyxin B resistance of this mutant was arabinose-independent. This was consistent with *mglB* transcription in PM2-49, which was turned on whether it was induced (mean ± standard error = 6.9 ± 4.8 fold increased transcript levels, $n = 4$, as compared to the parental strain) or not (9.2 ± 6.1 fold increased transcript levels, $n = 3$; induction effect $P = 0.394$), as detected by qRT-PCR. Thus, the minitransposon insert of PM2-49 is suggested to have inactivated GalR-mediated repression of the *mglBAC* operon [43]. The protamine-resistant mutant PM5-6 had an insertion in y0958 that encodes a putative glycerophosphoryldiester diesterase, designated GlpQ-like. That protamine resistance of this mutant was linked to the inactivation of this gene and not to a potential activation of downstram *dinP* transcription was confirmed by *dinP* qRT-PCR (comparison with the parental strain, $P = 0.99$). The GlpQ-like orf predicts a significantly different primary structure than those of the *E. coli* (or *Y. pestis*) GlpQ and UgpQ enzymes, which are involved in the intracellular uptake and production of *sn*-glycerol-3-phosphate, a carbon and energy source and a precursor for novel phospholipid synthesis and recycling of membrane phospholipids [44]. The last protamine-resistant mutant (PM12-3) mapped in the predicted inhibitor of the ferrous iron importer system designated Feo, as discussed later [45].

### 2.5. CAMP resistance and bacterial surface modifications

Most of the mutants detected in this study had Tn*5*AraOut insertions that were located in or upstream of genes encoding proteins involved in membrane components or molecular transport through membranes. LPS, a major component of the outer membrane, is known to interact with CAMPs. Accordingly, one Tn*5*AraOut insertion located upstream of the *pbgP* operon involved in LPS modification (Table 1, PB7-23) showed arabinose-dependent resistance to CAMPs. Thus, we wondered whether the increased

**Table 1**
Mapped Tn5AraOut insertions in CAMPs-resistant mutants.

| Mutants | Locus tag | Gene | Protein | Arabinose dependency |
|---|---|---|---|---|
| PB7-10 | y2644 | *asmA* | putative assembly protein | - |
| PB7-23 | y1917-y1923 | *pbgP* operon | UDP-4-amino-4-deoxy-L-arabinose—oxoglutarate aminotransferase,etc. | + |
| PB8-2 | y1522 | *hcp*-like | DUF796, T6SS effector hcp like | + |
| PB8-4 | y2568 | unknown | hypothetical protein | + |
| PB8-8 | y0634 | *hflC* | FtsH protease regulator HflC | - |
| PM2-3& PM3-2 | y3862 | *pitA* | phosphate ABC transporter permease | - |
| PM2-49 | y2662 | *mglB* | galactose-binding protein | - |
| PM5-6 | y0958 | *glpQ*-like | glycerophosphoryl diester phosphodiesterase family protein | - |
| PM12-3 | y3910 | *feoC* | ferrous iron transport protein C,transcriptional regulator | - |




CAMP resistance of some of the other mutants could be related to an indirect effect on LPS. We first analyzed LPS levels by SDS-PAGE and found no significant differences between the mutants and the parental strain (data not shown). Subsequently, we evaluated whether the mutations affected bacterial surface properties resulting in a reduction of CAMP binding by labeling the bacteria grown at 37 °C with dansyl-conjugated polymyxin B. Preliminary microscopic evaluation suggested decreased binding of polymyxin B for several mutants (not shown). To evaluate this effect in a quantitative manner, the level of binding was determined by fluorometry. Five polymyxin B-resistant mutants (PB7-10, PB7-23, PM2-49, PM5-6 and PM12-3) showed a significant decrease in polymyxin B binding (Fig. 4), whereas polymyxin-B susceptible mutant PM2-3 bound polymyxin B as well as the parental strain. These data supported the assumption that the polymyxin B-resistant mutants with the corresponding mapped mutations (*asmA*, *pbgP*, *mglB*, *glpQ*-like and *feoC*) are involved in modifying the molecules or molecular composition of the bacterial outer membrane. Other mechanisms might explain the phenotypes of mutants PB8-4 (hypothetical gene) and PB8-2 (*hcp*-like gene). Arabinose-induced expression of the latter gene (y1522) in PB8-2 slightly increased polymyxin B binding, albeit not significantly. Since Hcp proteins have been reported in other bacteria to be secreted in the medium, spent broth of PB8-2 was tested by western blot analysis. Although the protein was present in the bacteria, it could not be detected in spent broth of the mini-transposon mutant (not shown). A mutant lacking the corresponding gene and complemented with a plasmid expressing the Hcp-like protein from an ITPG-inducible plasmid gave the same result (not shown). Increased polymyxin B resistance was only detected in the complemented strain when production of the plasmid-encoded protein was activated, confirming the arabinose-dependency of the minitransposon mutant phenotype. Moreover, deletion of the neighboring y1523 gene, which could have been affected in its promoter region (Table 1), did not increase polymyxin B resistance, indicating that this gene was not involved in the PB8-2 phenotype. The Hcp-like protein could not be detected in membrane fractions or on the surface of PB8-2 (not shown), possibly because of the insufficient basal expression of a complementary type 6 export machinery. That the observed phenotype

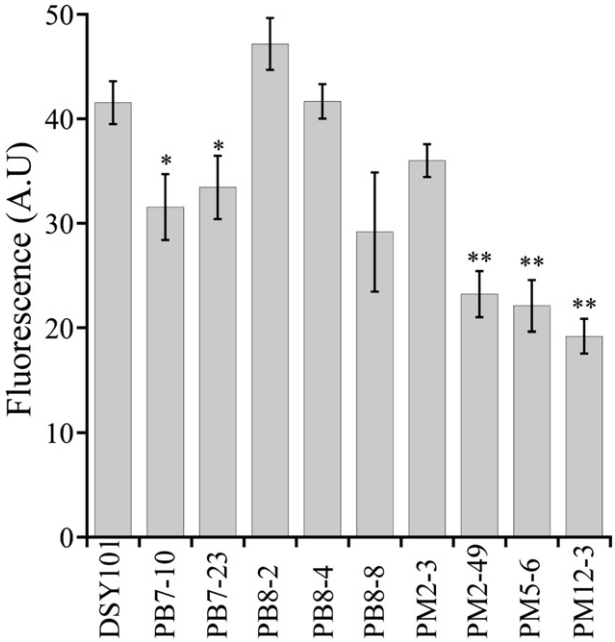



**Fig. 4.** Binding of polymyxin B to bacterial surfaces. Standardized concentrations of bacteria were incubated with dansyl-polymyxin B for fluorometric analysis and determination of arbitrary units (A.U.) of fluorescence, as described in Material and Methods. All the data are means of at least three independent experiments with standard errors. Unpaired *t*-tests comparing each mutant to the parental strain DSY101 showed statistical significant differences for several comparisons ($^{*}P < 0.05$, $^{**}P < 0.001$).

was due to an indirect effect resulting from the accumulation of the y1522 gene product in the cytoplasm was unlikely, since *E. coli* or *Salmonella enterica* strains expressing the y1522 in trans were not polymyxin B resistant (not shown).

### 2.6. CAMP resistance and inactivation of feoC

As expected, polymyxin B resistance of the *feoC*::Tn*5*AraOut strain and a *feoC* deletion were comparably increased (Figs. 2B and 5). Since the *feoC* gene has been suggested to be a repressor of the *feoAB* genes [45], polymyxin B resistance was thought to be associated with *feoAB* derepression. Accordingly, a *feoBC* mutant (and a *feoB* mutant, not shown) was as sensitive to polymyxin B as the parental strain DSY101 (Fig. 5). Moreover, when the *feoAB* genes were expressed from an inducible vector, they overrode *feoC* repression in DSY101, confirming the proposed model (Fig. 5). These results suggested that *feoC* represses the *feoAB* genes in vitro and that the *feoAB* genes are involved in mediating polymyxin B resistance. Increased FeoAB-mediated ferrous iron uptake is anticipated to cause oxidative stress by the Fenton reaction, resulting in induction of the Fur regulon. In addition to repressing iron uptake systems, Fur up-regulates the expression of a variety of genes, including the ones for several surface proteins and catalase (or hydroperoxidase II) [46]. Accordingly, the *feoC*::Tn*5*AraOut mutant was four times as much resistant to peroxide as the parental strain (MIC of 16 mM versus 4 mM, respectively), suggesting increased catalase expression as a response to the activation of the FeoAB-mediated ferrous iron import. Since the *feoC*::Tn*5*AraOut mutant was also more resistant to protamine and rBALF, as compared to the parental strain, we wondered whether the *feoC* gene was repressed in vivo, possibly increasing bacterial resistance to CAMPs, and thus improving bacterial survival, growth and dissemination in an infected host. To test this possibility, the parental strain KIM5 and an isogenic *feoB* mutant were administered together by the s.c. route to C57BL/6 mice and dissemination of the two bacteria was studied over time. Interestingly, the parental strain was always found in higher numbers than the *feoB* mutant when bacteria were isolated from the livers and spleens (Fig. 6). Bacteria could not be detected in the organs of many mice, indicating that *Y. pestis* KIM5 administered s.c. at the dose used had been rapidly eliminated in the host. Although daily differences were statistically significant in the liver only at day 4 ($P < 0.005$), differences of the aggregated data for the four studied days were highly significant ($P < 0.002$ for the livers and $P < 0.0004$ for the spleens). These results suggested that *feoB* is expressed in vivo and improves bacterial dissemination, possibly by increasing CAMP resistance and/or purveying iron to the multiplying bacteria.

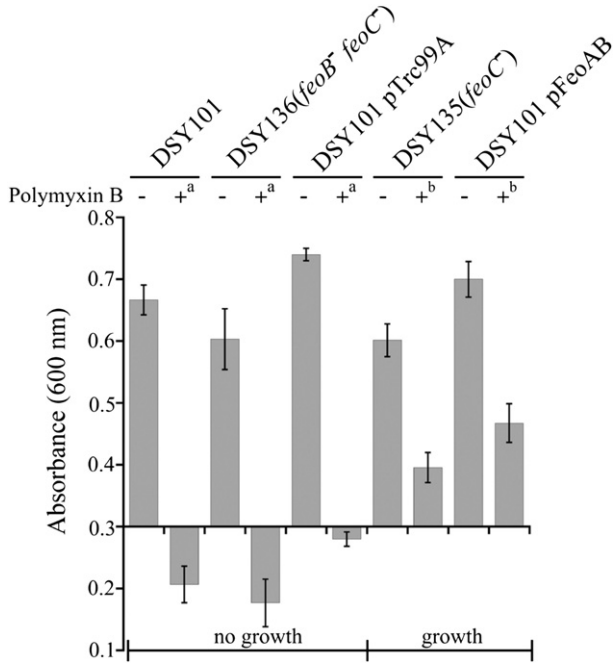



**Fig. 5.** Growth of *feo* mutants and complemented strains in the presence of polymyxin B. (A) Bacteria were grown in BHI to an $A_{600}$ of 0.3 at 37 °C, polymyxin B was added to a concentration of [a]1.25 μg/ml or [b]5 μg/ml, the cultures were further incubated for 18 h before measuring growth ($A_{600}$). All the data are means of at least three independent experiments with standard errors.

## 3. Discussion

*Y. pestis* encounters constitutively expressed CAMPs such as the cathelicidin LL-37 when it accesses the lower respiratory tract or

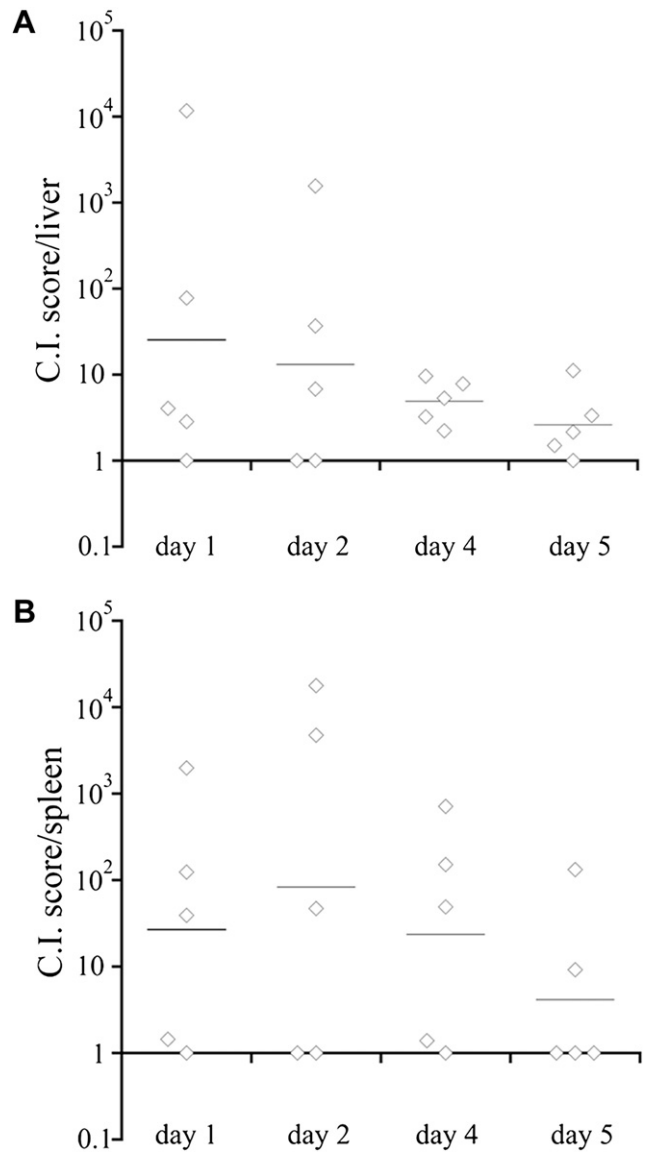



**Fig. 6.** *Y. pestis* KIM5 and DSY137 (KIM5 *feoB*) competition experiment in mice. C57BL/6 mice were challenged s.c. with a 1:1 mixture of KIM5 and DSY137 ($3 \times 10^7$ CFU each). Five mice were sacrificed on days 1, 2, 4 and 5 and CFUs were determined in livers (A) and spleens (B) to calculate competitive index (C.I.) scores (KIM5 CFUs divided by the DSY137 CFUs). C.I. scores for each mouse are shown as diamonds and bars represent geometric means.

the skin after a fleabite [47]. We have previously shown that cathelicidins and β-defensins have bactericidal activities towards *Y. pestis* and that Pla inactivates these CAMPs [48]. Since the F1 protein inhibits the CAMP-inactivating effect of Pla and both proteins are expressed in vivo, it is likely that *Y. pestis* utilizes other mechanisms to resist CAMPs in host tissues. The goal of this study was to identify new *Y. pestis* genes involved in CAMP resistance. Traditionally, such genes have been detected in various pathogens by screening for CAMP sensitive mutants out of a library of knockout mutants [49–51]. This approach implies that the "resistance" genes are expressed in vitro. Here, we used a minitransposon engineered with an inducible promoter to identify genes that need to be repressed or activated for detection of CAMP resistance. By selecting mutants for increased resistance towards CAMPs, we focused on genes that could be beneficial to bacterial survival in vivo whether repressed or activated, as determined with null mutations or inducible promoter insertions, respectively.

The used protocol identified several genes encoding proteins that were previously described in other bacteria to be involved directly or indirectly in the organization of the outer membrane. For example, a mutation resulting in an inducible polymyxin B resistance was related to the activation of the *pbgP* operon which decorates the phosphates of lipid A with 4-aminoarabinose, an LPS modification reported to increase polymyxin B resistance in various bacteria [31]. Other genes involved in polymyxin B resistance detected in this study, such as *asmA* [34–36] and *hflC* [37,38] have been previously shown to have indirect effect on LPS levels in the outer membrane. Consistent with a reduction in LPS levels, we observed that polymyxin B binding to the surface of the corresponding mutants was reduced. Whether import machineries for phosphate (PitA, GlpQ-like protein) and galactose (MglBAC) have indirect effects on species structures and compositions of membrane lipids that can explain CAMP resistance remains to be studied.

The y1522 and y3910 genes were investigated in more details since their gene products were potentially involved in modulating new mechanisms of CAMP resistance. Expression of the Hcp-like protein encoded by the y1522 gene determined CAMP resistance, irrespective of whether induced expression was from the *ara* promoter of the minitransposon or from the *trc* promoter of a complementing plasmid in a y1522 mutant. Hcp proteins are type 6 secreted proteins that form hexameric rings suggested to assemble in a tubular system for effector molecule delivery to eukaryotic cells [52,53]. Similar to some *hcp* genes of other bacteria, y1522 is an orphan *hcp* gene (i.e. unlinked to any of the T6SS gene clusters). Hcp proteins are either secreted or tightly associated to bacterial surfaces [33,52,54]. Interestingly, the y1522 gene product was not secreted or detected in bacterial membranes under the growth conditions used. Thus, it remains possible that the y1522 product is only secreted under specific conditions that were not duplicated here. Alternatively, artificial expression of the Hcp-like product of y1522 without activated expression of its corresponding T6SS might not result in secretion. Finally, expression of the Hcp-like product of y1522 might result in activation of an intracellular response that indirectly leads to CAMP resistance independent of type 6 secretion. Although a *Y. pestis* mutant lacking one of its Hcp molecule was not attenuated in models of bubonic or pneumonic plague, replication of this mutant in J774.A1 macrophages was limited, suggesting some nonessential role of a T6SS in *Y. pestis* pathogenesis [55].

CAMP resistance of the *feoC*::Tn*5*AraOut mutant was duplicated with a *feoC* (y3910) deletion mutant, confirming that the phenotype was due to the disruption of *feoC.* FeoC has been proposed to be a repressor of the *feoAB* operon [45], which encodes one of the two ferrous iron uptake systems of *Y. pestis*, the other one being encoded by the *yfeABCD* genes [56]. Consistent with the regulatory role of FeoC, FeoC-independent expression of FeoA and FeoB mimicked the CAMP-resistant phenotype obtained with the *feoC* mutant. Moreover, a *feoBC* deletion mutant was as sensitive to CAMP as the parental non-mutated strain. Taken together, these data indicated that induction of FeoAB expression increased CAMP resistance and supported a regulatory role for FeoC. How activation of ferrous iron uptake into the bacterial cytoplasm results by itself in CAMP resistance is not clear. One potential mechanism is that this resistance was directed by the *Y. pestis* Fur protein, which differentially regulates a large number of proteins, including membrane proteins [46]. This would be consistent with our finding that *feoC*⁻-mediated resistance to all tested CAMPs was linked to decreased polymyxin B-binding, suggesting some membrane modifications. Moreover, intracellular iron acts as a coregulator of the Fur protein to down-regulate iron uptake systems, protecting the bacterial cell against the toxic effects of the Fenton reaction [57]. In addition, Fur activates the expression of various scavenging proteins of oxidative intermediates, including catalase [46,58]. Consistent with the activation of the Fur pathway, the *feoC* mutant was also more resistant to peroxide than the parental strain. A *feoB* mutant of *Y. pestis* was attenuated in a competition assay with the parental strain after s.c. administration to mice, highlighting the bacterial use of its Feo system in vivo to augment virulence. Similarly, *E. coli* or *Salmonella feoA* or *feoB* mutants demonstrated reduced virulence properties in mice models of infections [45]. Interestingly, *Y. pestis* has two ferrous uptake systems (Yfe and Feo) and both systems were shown to be needed but redundant for optimal *Y. pestis* growth in a murine macrophage cell line [56]. A *yfe* mutant had a 100-fold increased LD50 after s.c. infection of mice [59]. In vivo studies with a *feo yfe* double mutant might show a more drastic effect on virulence and bring further evidence to the current data that supports a role for intra-macrophage survival of *Y. pestis* in the early stages of plague [60]. Additional studies will be needed to differentiate the contributions of the *feo* genes in iron replenishment and CAMP resistance for the intracellular survival and growth of *Y. pestis*. Interestingly, another study has recently linked iron uptake with polymyxin B resistance in *Yersinia pseudotuberculosis,* a coregulator of the *yfe* and *pbgP* genes being responsible for both phenotypes [61].

Finally, this study illustrates a clear dissociation between the resistance profiles of *Y. pestis* mutants towards polymyxin B, protamine and rBALF/LL-37. Increased resistance of the *hflC* or *pitA* mutants was specific for polymyxin B or for protamine, respectively. Moreover, only three of the protamine-resistant and none of the polymyxin B-resistant mutants showed increased resistance towards rBALF, known to contain at least rCRAMP and rat β-defensin 1 [48]. The three rBALF-resistant mutants were also resistant to human cathelicidin LL-37, whereas growth of the polymyxin B-resistant mutant PB7-23 with an activated *pbgP* operon was significantly affected by LL-37. The latter result was surprising since LL-37 was described to activate the PhoPQ two-component system in *Salmonella* [62], which itself activates the *pbgP* operon in *Salmonella* and *Y. pestis* [31]. Whether mammalian CAMPs are not good activators of the *Y. pestis* PhoPQ system, aminoarabinose decoration of LPS doesn't protect *Y. pestis* significantly from LL-37 or the experimental conditions used were suboptimal to detect an effect remains to be determined. Previously detected differences between polymyxin B, cecropin A and/or defensin A resistance of *Y. pestis phoP* or *lpxM lpxP* or *Y. pseudotuberculosis pbgP* operon mutants remained essentially unexplained [13,63,64]. Nevertheless, accumulating evidence indicates that CAMP resistance is CAMP-type specific and that this specificity is determined by a variety of bacterial genes, particularly those capable of modulating directly or indirectly the bacterial surface architecture,

including the composition and content of its protein and lipid species.

## 4. Materials and methods

### 4.1. Bacterial strains, growth conditions and reagents

Bacterial strains and plasmids used in this study are listed in Table 2. Media and media components were from Difco (BD Diagnostics, Sparks, MD). *Y. pestis* strains were grown overnight in brain heart infusion (BHI) broth at 26 °C, diluted to an $A_{600}$ of 0.1 in BHI and further grown at 37 °C. For colony selection, *Y. pestis* was grown on BHI or TB agar (10 g tryptose, 2.5 g NaCl, 3.0 g Bacto beef extract paste and 1.5% Bacto-agar per liter) for 24–48 h at 26 °C for colony counts or at 37 °C for mutant selection. For quantitative RT-PCR, bacteria grown at 26 °C in heart Infusion (HI) were sub-cultured in HI for 4 h at 37 °C. *E. coli* strains were grown at 37 °C in LB broth or agar. When required, the media were supplemented with kanamycin (Km, 45 mg/l), ampicillin (Ap, 200 mg/l) or chloramphenicol (Cm, 20 mg/l). When appropriate, the arabinose and lactose promoters were induced with 0.2% L-arabinose and 1 mM IPTG, respectively, using X-Gal (40 mg/l) as chromogenic substrate with the latter. Reagents, including polymyxin B sulfate salt and salmon protamine sulfate salt were from Sigma–Aldrich Corp. (St Louis, MO), and LL-37 was from AnaSpec (Fremont, CA). Rat bronchoalveolar lavage fluid (rBALF) was generously supplied by Sandra R. Bates-Kenney, and was shown to contain the cathelicidin rCRAMP and rat β-defensin 1 [48].

**Table 2**
Bacterial strains and plasmids.

| Strain or plasmid | Description | Source |
|---|---|---|
| *E. coli* | | |
| CC118λpir | Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA thi-1 rpsE rpoB argE*(Am) *recA1*, lysogenized with λ*pir* phage | [74] |
| DH5α | *fhuA2* Δ(*argF-lacZ*)*U169 phoA glnV44* *Φ80* Δ(*lacZ*)*M15 gyrA96 recA1 relA1* *endA1 thi-1 hsdR17* | Invitrogen Corp., Carlsbad, CA |
| BL21(DE3) | *E. coli* B (DE3)[F− *dcm ompT hsdSB* ($rB^-$ $mB^-$)] | Novagen, EMD Biosc., Madison, WI |
| *Y. pestis* | | |
| KIM5 | *Y. pestis* KIM *pgm* | [75] |
| KIM6 | KIM5 $pCD1^-$ ($LCR^-$) | [75] |
| DSY88 | KIM6 Δ*caf* Δ*psa* pPCP1 Ω*aphA* Δ*pla* | [48] |
| DSY101 | DSY88 PCP1Δ*pla* | This study |
| DSY131 | DSY101 Δy1522::*aphA* | This study |
| DSY132 | DSY101 Δy1523::*aphA* | This study |
| DSY133 | DSY131 pTrc99A | This study |
| DSY134 | DSY131 pHcpy1522 | This study |
| DSY135 | DSY101 Δy3910(*feoC*)::*aphA* | This study |
| DSY136 | DSY101 Δy3910-y3911 (*feoBC*)::*aphA* | This study |
| DSY137 | KIM5 Δy3911(*feoB*)::*aphA* | This study |
| Plasmids | | |
| pNJ17 | TnAraOut delivery vehicle; $Km^r$ | [65] |
| pRL27 | Plasmid with miniTn*5* and *ori*R6K; $Km^r$ | [66] |
| pTn5AraOut | pRL27 with *araC*-PBAD from pNJ17; $Km^r$ | This study |
| pRS415 | *lacZ* transcriptional fusion vector; $Ap^r$ | [76] |
| pSIM9 | pRK2 *gam exo bet*; $Cm^r$ | [77] |
| pKD4 | Plasmid with the $Km^r$ template for PCR | [72] |
| pET22b(+) | Expression vector; His6 affinity tag; $Ap^r$ | Novagen |
| pETHcp | pET22b(+)-y1522; $Ap^r$ | This study |
| pTrc99A | Expression vector with *trc* (*trp-lac*) promoter; $Ap^r$ | [78] |
| pHcpy1522 | pTrc99A-y1522 | This study |
| pFeoAB | pTrc99A-y3911-y3912 | This study |

### 4.2. Construction and testing of plasmid pTn5AraOut

Preliminary studies showed that the minitransposon miniTn*5* of pRL27 transposed efficiently in KIM6. Plasmid pTn*5*AraOut was engineered by inserting the *Eco*RI fragment of pNJ17 that contains the *araC* gene and $P_{BAD}$ promoter [65] into the *Swa*I site of pRL27, which carries a miniTn*5* delivery vector [66]. The *araC*-$P_{BAD}$ DNA fragment was blunted by filling in its protruding ends with the Klenow fragment of DNA polymerase before the ligation reaction. A successful construct carrying the miniTn*5* with $P_{BAD}$ facing out was identified by restriction analysis and renamed pTn*5*AraOut. Preliminary studies showed that the minitransposon of pRL27 transposed efficiently in KIM6 and the transposition efficiency of Tn*5*AraOut was only marginally lower than that of pRL27. To test the function of the arabinose-dependent promoter in Tn*5*AraOut, *E. coli* DH5α harboring the promoter-trap plasmid pRS415 was transformed with pTn*5*AraOut and the bacteria were screened for β-galactosidase activity on LB agar plates containing Ap, Km, X-gal and L-arabinose. Bacteria isolated from $Lac^+$ (blue) colonies were shown by restriction analysis to harbor Tn*5*AraOut upstream of the *lacZ* gene in pRS415. Arabinose-dependent induction was further confirmed on MacConkey agar plates.

### 4.3. Minitransposon mutagenesis, mutant selection and screening

*Y. pestis* strain DSY101 was randomly mutagenized with the minitransposon Tn*5*AraOut. For this, plasmid pTn*5*AraOut was introduced into strain DSY101 by 20 independent electroporations, using in parallel water as negative controls. After 3 h recovery at 26 °C, the bacteria were incubated in kanamycin-containing BHI broth at 37 °C, overnight, in a roller drum. Mutants resistant to antimicrobial peptides were then selected on TB-kanamycin agar plates containing 0.2% L-arabinose and supplemented with polymyxin B (4 mg/l, 4 × MIC) or protamine (1 g/l, 1 × MIC on agar plates) after incubation at 37 °C for 2–3 days. Colonies were isolated and resistance to the corresponding cationic antimicrobials was verified by growing the bacteria to an $A_{600}$ of 0.6 in BHI-kanamycin broth with 0.2% L-arabinose at 37 °C and plating in duplicate 0.01 ml onto corresponding cationic antimicrobial selection agar plates. Growth was inspected after incubation at 37 °C for 1–3 days. Because of the intrinsic property of *Y. pestis* to aggregate [67,68], most consistent absorbance data ($A_{600}$) were obtained by growing the bacteria in glass tubes and dissociating the aggregated bacteria by strong vortex mixing just before absorbance readings. Bacteria were grown at 37 °C to an $A_{600}$ of 0.3 in 3 ml BHI-kanamycin broth, with 0.2% L-arabinose or 0.2% D-glucose when appropriate to differentiate arabinose-dependent from arabinose-independent resistance. Serial two-fold dilutions of polymyxin B (1.25–5 mg/l) were directly added to such cultures. In contrast, dilutions of protamine (0.2–0.8 g/l), rat bronchoalveolar lavage fluid (rBALF, 30–240 μg protein/ml), LL-37 (20–160 μg/ml) or $H_2O_2$ (1–32 mM) were added to BHI cultures, prepared as described above and diluted 10-fold. The latter dilutions were needed to use concentrations of inhibitors that did not interfere significantly with $A_{600}$ readings. The experiments with rBALF were done with 0.5 ml cultures and the ones with LL-37 were done with 0.1 ml cultures in smaller tubes. The bacterial cultures were further incubated in

a roller drum or agitated at 37 °C for 18 , when final $A_{600}$ values were checked, or CFUs were determined for the LL-37 experiment.

### 4.4. Determination of MICs

*Y. pestis* DSY101 and its derived mutants being unable to grow significantly at 37 °C in small volumes of cation-adjusted Mueller-Hinton broth (100 μl/well, 96-well microtiter plate; according to the NCCLS, now CLSI, guidelines), MICs were evaluated by the following method. Briefly, bacteria were grown to an $A_{600}$ of 0.08–0.1 in BHI broth at 37 °C, diluted 10 fold in BHI (approximately $10^6$ CFU/ml), and distributed into round-bottom wells of 96-well microtiter plates ($10^5$ CFU/well). Serial two-fold dilutions of the various antimicrobials were prepared in triplicates and MICs were determined after overnight incubation on a rotary shaker at 37 °C. MICs were defined as the lowest concentration of antimicrobials that inhibited visible growth.

### 4.5. Southern blotting, subcloning and sequencing of minitransposon insertion sites

To check for single insertions by Southern blotting, genomic DNA was extracted from parental strain Y101 or minitransposon mutants (DNA purification kit, Gentra, USA) and digested with *Sty*I or *Hpa*I and separated on a 0.8% Seakem LE agarose gel. DNA was then transferred to Nytran SPC nylon membranes (GE Healthcare BioSciences Corp., Piscataway, NJ) by the capillary transfer method. A 575-bp coding region of the kanamycin resistance gene that lacks *Sty*I and *Hpa*I sites was amplified from pTn5AraOut with primers PrMK150 and PrMK151 (Table 3), labeled with the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Indianapolis, IN) and used for hybridization as described by the supplier. To clone minitransposon insertion sites, genomic DNA of each mutant was digested with either one of a variety of restriction enzymes. Subsequently, the digested DNA was treated with T4 DNA ligase and used to transform *E. coli* CC118λpir by electroporation. Circularized DNA fragments containing the Tn*5*AraOut replicated as plasmids, most clones originating from genomic DNA originally restricted with *Bcl*I or *Bam*HI. The plasmids were isolated and served as template for DNA sequencing, using a pair of outward-facing primers (pTn*5*AraOutUP and pTn*5*AraOutLOW, Table 3) that anneal to the *ori*R6 K or araC-$P_{BAD}$ ends of the minitransposon. The *Y. pestis* KIM genome [69] was analyzed by BLAST [70] with the obtained sequences to determine the minitransposon insertion sites.

### 4.6. Quantitative RT-PCR

HI broth cultures of bacteria grown to log-phase at 37 °C were split in two and grown further in the presence of 0.2% arabinose or glucose for 20 . The cells were pelleted at 6000 × g for 10 min at 4 °C and RNA was extracted using Trizol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol. Residual genomic DNA contamination in 10 μg RNA samples was removed by treating with the Turbo DNA-free Kit (Ambion, Austin, TX). RNA concentration was determined with the ND1000 spectrophotometer (NanoDrop products, Wilmington, DE) and RNA integrity was verified by agarose gel electrophoresis. Four hundred nanogram of RNA was used in reverse transcription reactions using random hexamer primers and Superscript RTIII Reverse Transcriptase (Invitrogen Corp.) according to manufacturer's protocol. Quantitative Real-Time RT-PCR was carried out using cDNA corresponding to 20 ng RNA, gene-specific primers (Table 3) and the Power SYBR Green PCR Master Mix with the ABI Fast 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Expression levels were normalized to the transcription levels for the house keeping gene *rpo*B [20]. Statistical significance of compared results between the parental strain and mutants or arabinose-induced and non-induced conditions was determined by the unpaired *t*-test ($P < 0.05$).

**Table 3**
Primers.

| Name | Sequence 5′–3′ |
|---|---|
| Tn5AraOutUP | tgccatagcatttttatccat |
| Tn5AraOutLOW | aacaagccagggatgtaacg |
| *dinP*-PrMK140 | ggtaagtttggtcgcgtattgtgg |
| *dinP*-PrMK141 | atcctcggccagtgttttctcc |
| *pmrH*-PrMK142 | cgagatcaatggattggtgagcag |
| *pmrH*-PrMK143 | gaacttaagccgacggactctgg |
| *mglB*-PrMK144 | gtattgctggctaagggcgtgaaag |
| *mglB*-PrMK145 | gtagctatccaatgccttgcgagaag |
| 16SrDNA-PrMK146 | gtgtgaagaaggccttcgggttg |
| 16SrDNA-PrMK147 | ttagccggtgcttcttctgcgag |
| *rpoB*-PrMK148 | tgctgtacaacgcacgtatcatccc |
| *rpoB*-PrMK149 | caggcaatttacggcgacggtc |
| PrMK150 | tcgggcaatcaggtgcgacaatctat |
| PrMK151 | gtctgcgattccgactcgtccaacat |
| y1522KOUP | aataatagggatggtgtaggccatcc<br>ccgaataaagtgtaggctggagctgcttc |
| y1522KOLOW | ccctgcatcttgaaggcgacgggtatata<br>aggacatcatatgaatatcctccttag |
| y1522VFUP | tcaccccctgaggatatagat |
| y1522VFLOW | ggatgagcaagggcaactaac |
| y1523KOUP | gaagctgtcctcagtatgaccacgtaggaaac<br>caggtgcggtgtaggctggagctgcttc |
| y1523KOLOW | tcgccataaccagagcggagcctcagttcttg<br>gaacccgtcatatgaatatcctccttag |
| y1523VFUP | caggcgagcggttattctatc |
| y1523VFLOW | ctccatgtcggcttaactgtt |
| *feoC*KOUP | attgatcacccattagccagccgtaaaaagag<br>ggggaacggtgtaggctggagctgcttc |
| *feoC*KOLOW | atacggaatcaacatattgagatgcccatat<br>gggcatctccatatgaatatcctccttag |
| *feoC*VFUP | tcacccattagccagccgtaa |
| *feoC*VFLOW | gctgacgccgaggtttat |
| *feoB*KOUP | cgcgcagtttgattttaatttattgatatcacgt<br>tggatcgtgtaggctggagctgcttc |
| *feoB*KOLOW | cgttgagggcaatcgcatcacgtag<br>ttgcagtaggctggccatatgaatatcctccttag |
| *feoB*VFUP | ttctatctgcgctacggtttg |
| *feoB*VFLOW | gctgatggccttcgggaca |
| y1522CMUP | gactcccgggttaatacacgcgatcatcccat |
| y1522CMLOW | gactcgtctcccatgagaatggctaacatgatt |
| *feoAB*CMUP | gactcgtctcccatgcatcttatcccccaacgatcctacaaa |
| *feoAB*CMLOW | gactcccgggtttttacggctggctaatgggtga |
| pETHCP-UP | gactcatatgagaatggctaacatgatt |
| pETHCP-LOW | gactctcgagatacacgcgatcatcccagatgctataaccact |

### 4.7. Preparation and analysis of LPS

LPS was prepared and resolved by SDS-PAGE as described elsewhere [71]. Briefly, overnight bacterial cultures were diluted to an $A_{600}$ of 0.5, and the cell pellets from 1 ml aliquots were suspended in 50 μl of lysis buffer (2% SDS, 4% 2-mercaptoethanol, 10% glycerol, 1 M Tris–HCl [pH 6.8], 0.002% bromophenol blue). The samples were heated at 100 °C for 10 min, cooled to room temperature, and incubated with 25 μg of proteinase K (Roche Diagnostics, Mannheim, Germany) at 60 °C for 1 h. The sample preparations were resolved by electrophoresis on a 12% polyacrylamide gel at 150 V, and the gels were silver stained (Silver Stain Kit; Bio-Rad, Hercules, CA).

### 4.8. Dansyl-polymyxin B binding test

Bacteria were grown at 37 °C to an $A_{600}$ of approximately 0.3–0.4 in 3 ml BHI broth. Bacteria were twice centrifuged and

resuspended in PBS, adjusted to an $A_{600}$ of 0.3 and 3 ml aliquots were concentrated by centrifugation and resuspension in 400 μl PBS. Half of this bacterial suspension was supplemented with dansyl-polymyxin B (Molecular Probes, Eugene, OR) to a final concentration of 2 μg/ml, whereas the other half was used as negative control to evaluate background fluorescence. After incubation at 37 °C for 5 min, the bacteria were centrifuged and washed twice in PBS, resuspended in 400 μl PBS and used for fluorescence microscopy or fluorometry. For the latter analysis, the bacterial suspension was transferred to transparent flat-bottom 96-well microtiter plates in triplicate. The $A_{600}$ and fluorescence values were read with a BioTek's Synergy™ HT Microplate Reader. The following calculations were done: Standardized fluorescence (arbitrary units or A.U.) value = (fluorescence (A.U.)/$A_{600}$)$_{test}$ - (fluorescence (A.U.)/$A_{600}$)$_{control}$. Data were presented as means ± SD of three separate experiments and statistical significance was determined by the unpaired *t*-test.

### 4.9. Construction of deletion mutants and plasmids, and complementation assays

Some of the genes targeted by Tn*5*AraOut were deleted from strain DSY101 by using the lambda red recombination system [72]. Briefly, primers flanking the corresponding genes were designed and used with pKD4 (Table 3) as template to prepare amplicons for the replacement of the target genes with a kanamycin cassette. Strain DSY101 carrying the lambda red plasmid pSIM9 (Table 2) was grown to log phase at 26 °C, the red recombinase genes were induced for 15 min at 42 °C, and the bacteria were transformed by electroporation with the gel-purified amplicons. Kanamycin-resistant recombinants were selected on BHI agar and verified by colony PCR, using primers that hybridize to flanking sequences of the target genes. Mutants cured of pSIM9 were selected by bacterial growth at 37 °C. Using this approach, deletion mutants were engineered for genes y1522(*hcp*-like), y1523, y3910(*feoC*) and y3910-3911(*feoCB*) in strain DSY101, and for gene y3911 in strain KIM5. The resulting strains were designated DSY131, DSY132, DSY135, DSY136 and DSY137, respectively (Table 2). Moreover, genes y1522, or y3911 and y3912(*feoA*) together, were cloned by PCR into the *Nco*I-*Xma*I sites of the expression plasmid pTrc99A for complementation studies. The generated plasmids were designated pHcpy1522 and pFeoAB, respectively (Table 2). Gene y1522 was also cloned by PCR into the *Nde*I-*Xho*I restriction sites of pET22b(+) to generate plasmid pETHcp (Table 2). Mutants complemented with plasmid constructs or with the empty vector were grown in BHI broth overnight at 26 °C, sub-cultured in BHI with IPTG (0.1 mM) at 37 °C and grown for 2–3 h to an $A_{600}$ of 0.3. Polymyxin B was added (1.25–5 mg/l) and further growth was monitored ($A_{600}$) after 18 h at 37 °C.

### 4.10. Expression and purification of the Hcp-like protein

The Hcp-like protein encoded by y1522 was expressed and purified from *E. coli* BL21(DE3)pETHcp following the standard protocol (Qiagen, Valencia, CA). The protein antigen was used to prepare polyclonal rabbit antisera (Cocalico Biologicals, Inc.). To determine whether the protein was secreted, bacteria and spent media of a 10 ml culture (BHI, 37 °C for 18 h) were studied by SDS-PAGE and Western blot. For this, spent media were first filtered (Millex, MF-millipore™, 0.22 μm, Millipore, Billerica, MA), concentrated to 1 ml (Amicon Ultra-15, 10 kDa, Millipore), precipitated in 10% TCA and the pellet was rinsed twice in 0.5 ml cold acetone before being resuspended in 100 μl sample buffer and split in two for SDS-PAGE and Western blot analysis. The gels were stained with Coomassie Blue and the blots were probed with the specific antibody, HRP-conjugated secondary antibody and enhanced chemiluminescence substrate [73].

### 4.11. Infection of mice

Six to eight week old female C57BL/6 mice were purchased from the Jackson Laboratories. The mice were provided food and fresh water *ad libitum* during the experiments, which were performed following the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee. Bacteria were grown at 26 °C, washed two times by centrifugation and suspension steps in sterile PBS. Numbers of colony-forming units (CFU) inoculated were determined by plating serial dilutions onto TB agar plates [48]. Mice were anesthetized (ketamine/xylazin 100/10 mg/kg body weight intra-peritoneally, i.p.) and infected subcutaneously (s.c.) with $3 \times 10^7$ CFU each of KIM5 and DSY137 (KIM5 *feoB*) in 200 μl PBS. At days 1, 2, 4 and 5, livers and spleens were surgically removed and homogenized in 5 ml sterile PBS by using a Stomacher Lab Blender (Seward Medical Limited). CFU/organ were determined by plating serial dilutions onto TB agar plates containing or lacking kanamycin. CFUs for KIM5 were calculated by subtracting the DSY137 CFUs (Km plates) from the total CFUs (no antibiotic). Competitive index (C.I.) scores were calculated as the ratio of the KIM5 CFUs to the DSY137 CFUs. Statistical significance was determined by the paired *t*-test.

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## Appendix. Supplementary data

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