

LPS modification promotes maintenance of *Yersinia pestis* in fleas

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Yersinia pestis, the causative agent of plague, can be transmitted by fleas by two different mechanisms: by early-phase transmission (EPT), which occurs shortly after flea infection, or by blocked fleas following long-term infection. Efficient flea-borne transmission is predicated upon the ability of *Y. pestis* to be maintained within the flea. Signature-tagged mutagenesis (STM) was used to identify genes required for *Y. pestis* maintenance in a genuine plague vector, *Xenopsylla cheopis*. The STM screen identified seven mutants that displayed markedly reduced fitness in fleas after 4 days, the time during which EPT occurs. Two of the mutants contained insertions in genes encoding glucose 1-phosphate uridylyltransferase (*galU*) and UDP-4-amino-4-deoxy-L-arabinose-oxoglutarate aminotransferase (*arnB*), which are involved in the modification of lipid A with 4-amino-4-deoxy-L-arabinose (Ara4N) and resistance to cationic antimicrobial peptides (CAMPs). These *Y. pestis* mutants were more susceptible to the CAMPs cecropin A and polymyxin B, and produced lipid A lacking Ara4N modifications. Surprisingly, an in-frame deletion of *arnB* retained modest levels of CAMP resistance and Ara4N modification, indicating the presence of compensatory factors. It was determined that WecE, an aminotransferase involved in biosynthesis of enterobacterial common antigen, plays a novel role in *Y. pestis* Ara4N modification by partially offsetting the loss of *arnB*. These results indicated that mechanisms of Ara4N modification of lipid A are more complex than previously thought, and these modifications, as well as several factors yet to be elucidated, play an important role in early survival and transmission of *Y. pestis* in the flea vector.

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INTRODUCTION

Yersinia pestis can undergo transmission from fleas to mammalian hosts by at least two different mechanisms: bacteria may be transmitted shortly after infection of a flea vector [early-phase transmission (EPT), 1–4 days post-infection] in a biofilm-independent manner (Eisen *et al.*, 2006; Kartman *et al.*, 1958; Vetter *et al.*, 2010) or after long-term infection, during which time they form a biofilm and block the feeding ability of the flea (Bacot & Martin,

1914; Burroughs, 1947; Chouikha & Hinnebusch, 2012). Currently, there is little known about the mechanisms by which EPT occurs, but many flea species are able to efficiently transmit *Y. pestis* in this manner (Eisen & Gage, 2012; Eisen *et al.*, 2007), which may play a significant role in the spread of plague.

Plague transmission via blocked fleas was described 100 years ago (Bacot & Martin, 1914) and, although there is still much that is unknown about the mechanisms involved, a small number of transmission factors required for survival and biofilm formation in fleas have been identified. These factors include the *ymt* (*Yersinia murine* toxin) gene, which encodes a product with phospholipase D activity. *Ymt* is important for maintaining *Y. pestis* infections in the flea vector, ostensibly by protecting the bacteria from unknown toxic byproducts generated during the digestion of the flea's blood meal (Hinnebusch *et al.*,

Abbreviations: Ara4N, 4-amino-4-deoxy-L-arabinose; CAMP, cationic antimicrobial peptide; ECA, enterobacterial common antigen; EPT, early-phase transmission; FRT, flippase recognition target; RT, reverse transcription; spPCR, single-primer PCR; STM, signature-tagged mutagenesis.

One supplementary table is available with the online Supplementary Material.

2000, 2002; Rudolph *et al.*, 1999). Additionally, the ability of *Y. pestis* to form stable biofilms is important in its ability to block fleas and thus be transmitted by the classic route. The *hms*, *gmhA* and *phoPQ* genes are necessary for flea blockage through their involvement in biofilm formation (Darby *et al.*, 2005; Hinnebusch *et al.*, 1996; Jarrett *et al.*, 2004; Rebeil *et al.*, 2013). Although these genes play important roles in the association of *Y. pestis* with fleas, they do not fully explain the organism's ability to survive in arthropods and undergo successful transmission (Eisen *et al.*, 2006; Hinnebusch *et al.*, 1996; Houhamdi & Raoult, 2008). Therefore, it is likely that there are other transmission factors yet to be identified that enable *Y. pestis* to utilize a flea vector and spread to vertebrate hosts.

Here, we describe the construction and screening of a *Y. pestis* signature-tagged mutagenesis (STM) library (Hensel *et al.*, 1995; Mazurkiewicz *et al.*, 2006) to identify genes that may play a role in survival within a well-recognized flea vector of plague, *Xenopsylla cheopis*. This approach identified seven mutants with significant survival or colonization defects in the flea vector, including at least two involved in lipid A modification, which is predicted to allow *Y. pestis* to resist the action of cationic antimicrobial peptides (CAMPs) produced by the insect. Furthermore, we identified a novel role for WecE, an enzyme normally involved in enterobacterial common antigen (ECA) biosynthesis, in modification of lipid A.

METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Y. pestis* strains were routinely grown in heart infusion (HI) broth (Difco; BD) or on HI agar at 28 or 37 °C, as indicated. *Escherichia coli* strains were grown in Luria-Bertani medium at 37 °C. Unless otherwise indicated, antibiotics were used at the following concentrations: 50 µg kanamycin ml⁻¹, 100 µg ampicillin ml⁻¹, 2 µg irgasan ml⁻¹, 15 µg tetracycline ml⁻¹ and 12.5 µg chloramphenicol ml⁻¹. The pUT-miniTn5Km2 (Ap^r, Km^r) STM plasmid library (a gift from D. Holden, Imperial College, London, UK) was used as the source of tagged transposons (Hensel *et al.*, 1995).

STM library generation. Tagged pUT-miniTn5Km2 plasmids were transformed into *E. coli* S17-1λpir and isolated colonies were picked into three 96-well plates, and screened for amplification and hybridization efficiency using a modified colony blot assay. Briefly, 50 µl overnight culture of each clone was transferred to a positively charged Nytran nylon membrane (Whatman) by vacuum filtration with a 96-well dot-blot apparatus (Bio-Rad). Bacterial cells were lysed and DNA was denatured with 1 N NaOH and washed with 2 × SSC (saline sodium citrate) prior to hybridization to labelled probes. Clones with the strongest hybridization signals were assembled into pools and screened for cross-hybridization as described by Hensel *et al.* (1995). Cross-reactive clones were removed prior to assembly of the final optimized set of 96 STM vectors.

Separate matings were performed between *Y. pestis* KIM6+ and each of the 96 *E. coli* S17-1λpir clones carrying uniquely tagged pUT-miniTn5Km2 using a 96-well broth mating strategy. Approximately equal numbers of cells from overnight cultures of *Y. pestis* and each *E. coli* clone were mixed, pelleted (10 000 g, 1 min) and allowed to mate

for 3 h at 28 °C. Cells were resuspended in 1 ml 0.9% saline and aliquots were plated onto HI agar containing kanamycin (300 µg ml⁻¹), ampicillin and irgasan. Individual colonies from each mating were picked to a series of 39 separate 96-well plates such that each sequence tag resided in the same position of each plate. Based on the Poisson distribution, we estimated that ~53% of *Y. pestis* genes were mutated in this library (Sambrook *et al.*, 1989). Genuine transposition events were confirmed by screening each clone for kanamycin resistance and ampicillin sensitivity.

In vitro infection of *X. cheopis* fleas. Adult *X. cheopis* fleas that had been starved for 7–10 days were fed through freshly prepared mouse skins attached to glass feeders containing defibrinated Sprague–Dawley rat blood (Bioreclamation) as described previously (Eisen *et al.*, 2006). Briefly, for STM screening, each pool of 96 clones was grown overnight and harvested by centrifugation. Cells were washed in 0.1 vol. 0.85% NaCl and resuspended in rat blood at a concentration of 1–4 × 10⁹ c.f.u. ml⁻¹. Mouse skins were recovered after euthanasia of animals used for other purposes under protocols approved by the Centers for Disease Control and Prevention, Division of Vector-Borne Diseases Animal Care and Use Committee (06-014, 08-022, 11-015). For *in vivo* competition assays, individual STM mutant and WT bacteria were mixed in rat blood and plated as described below to determine initial infection ratios. Up to 150 fleas per feeder were allowed to feed for 1 h and then scored by light microscopy for the presence or absence of a fresh blood meal. Unfed fleas were discarded. The remaining fed fleas were maintained for 4 days at 23 °C and 80–85% relative humidity, and then live fleas were transferred to vials and maintained at –80 °C until processed. To determine the concentration of bacteria in each feeder, the infected blood was serially diluted and plated in triplicate onto blood agar base containing 6% sheep blood supplemented with kanamycin where appropriate. Plates were incubated at 28 °C for 48 h prior to enumeration of colonies.

Identification of STM mutants with possible survival defects in the flea.

Viable *Y. pestis* mutants were recovered by culturing disrupted fleas (18 per pool) in HI supplemented with kanamycin (Eisen *et al.*, 2006). Genomic DNA was isolated from recovered mutants (output DNA) as well as viable *Y. pestis* isolated from infected blood meals (input DNA) using a Wizard Genomic DNA Purification kit (Promega). Probes for hybridization were generated essentially as described previously (Hensel *et al.*, 1995; Mei *et al.*, 1997). Briefly, transposon tags were PCR-amplified from genomic DNA samples using primers P2 and P4 (Table S1, available in the online Supplementary Material), and the resulting ~80 bp product was gel-purified. This pool of amplified tags was used as template for generation of DIG-dUTP-labelled probes (PCR DIG Probe Synthesis kit; Roche) using primers P2 and P4 (Mei *et al.*, 1997). Probes were digested with *Hind*III and the labelled tag sequences were excised from 4% agarose gels (Hensel *et al.*, 1995). The gel slices were melted and denatured in hybridization buffer (DIG Easy Hyb; Roche) and hybridized at 42 °C overnight to pairs of dot-blot membranes (Mei *et al.*, 1997) containing arrays of the 96 unique sequence-tagged plasmids that had been vacuum blotted, alkali denatured and UV cross-linked to Nytran nylon (Sambrook *et al.*, 1989). Following hybridization, membranes were washed under high stringency, incubated in DIG-blocking solution and blocking solution containing anti-DIG-alkaline phosphatase antibody conjugate for 30 min each, then washed three times with wash buffer according to the manufacturer's recommendations (DIG Wash and Block Buffer set; Roche). Membranes were incubated 5 min with detection buffer containing CSPD luminescent substrate (DIG Luminescent Detection kit; Roche), excess buffer was decanted and the membranes were incubated for an additional 10 min at 35 °C prior to exposing to autoradiography film. Mutants that displayed decreased hybridization signals in the output compared with the input DNA pools were

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics/usage	Reference/source
<i>Y. pestis</i> strains		
KIM6+	Parent strain; pCD ⁻ , pMT ⁺ , pPCP ⁺	Perry <i>et al.</i> (1990)
3C1	KIM6+ STM mutant with transposon insertion in <i>galU</i>	This study
3C1-C	3C1 complemented with pGEN-MCS plus <i>galU</i>	This study
3C1-EV	3C1 harbouring pGEN-MCS empty vector	This study
9H9	KIM6+ STM mutant with transposon insertion in <i>arnB</i>	This study
9H9-C	9H9 complemented with pCR2.1-TOPO plus <i>arnBCADTEF</i> operon	This study
9H9-EV	9H9 harbouring pCR2.1-TOPO empty vector	This study
KIM6+ Δ <i>arnB</i>	In-frame deletion of <i>arnB</i>	This study
KIM6+ Δ <i>arnB</i> -C	KIM6+ Δ <i>arnB</i> complemented with pCR2.1-TOPO plus <i>arnB</i>	This study
KIM6+ Δ <i>arnB</i> -EV	KIM6+ Δ <i>arnB</i> harbouring pCR2.1-TOPO empty vector	This study
KIM6+ Δ <i>wecE</i>	In-frame deletion of <i>wecE</i>	This study
KIM6+ Δ <i>wecE</i> -C	KIM6+ Δ <i>wecE</i> complemented with pCR2.1-TOPO plus <i>wecE</i>	This study
KIM6+ Δ <i>wecE</i> -EV	KIM6+ Δ <i>wecE</i> harbouring pCR2.1-TOPO empty vector	This study
KIM6+ Δ <i>arnB</i> Δ <i>wecE</i>	In-frame deletions of both <i>arnB</i> and <i>wecE</i>	This study
KIM6+ Δ <i>arnB</i> Δ <i>wecE</i> -Ca	KIM6+ Δ <i>arnB</i> Δ <i>wecE</i> complemented with pCR2.1-TOPO plus <i>arnB</i>	This study
KIM6+ Δ <i>arnB</i> Δ <i>wecE</i> -Cw	KIM6+ Δ <i>arnB</i> Δ <i>wecE</i> complemented with pCR2.1-TOPO plus <i>wecE</i>	This study
KIM6+ Δ <i>arnB</i> Δ <i>wecE</i> -Caw	KIM6+ Δ <i>arnB</i> Δ <i>wecE</i> complemented with pCR2.1-TOPO plus <i>arnB</i> , <i>wecE</i>	This study
KIM6+ Δ <i>arnB</i> Δ <i>wecE</i> -EV	KIM6+ Δ <i>arnB</i> Δ <i>wecE</i> harbouring pCR2.1-TOPO empty vector	This study
<i>E. coli</i> strains		
DH5 α	Cloning strain for complementation constructs	Invitrogen
S17-1 λ <i>pir</i>	Cloning strain for STM library generation	Simon <i>et al.</i> (1983)
Plasmids		
pKD46	λ Red recombinase expression plasmid; Ap ^r	Datsenko & Wanner (2000)
pCR2.1-TOPO	Complementation cloning plasmid; Ap ^r , Km ^r	Invitrogen
pFLP2	Source of Flp recombinase; Ap ^r	Hoang <i>et al.</i> (1998)
pFKM1	Kanamycin resistance cassette with flanking FRT sites; Km ^r	Choi <i>et al.</i> (2005)
pGEN-MCS	Complementation cloning plasmid; Ap ^r	Lane <i>et al.</i> (2007)
pUTmini-Tn5Km2	Pool of plasmids with uniquely tagged transposons; Km ^r	Hensel <i>et al.</i> (1995)

rescreened by flea infection in a different background of clones to confirm the loss of signal.

Insertion sites of STM transposons in mutants with possible survival defects were determined using the single-primer PCR (spPCR) technique as described by Karlyshev *et al.* (2000). Briefly, spPCR was performed on each STM mutant using the transposon-anchored, outward-facing primers, P6U and P7U (Table S1), which bind to sequences on opposite ends of the STM transposon. The resulting spPCR products were purified using ExoSAP-IT (USB), and sequenced on an ABI PRISM 3730 genetic analyser using the nested primers P6M and P7M (Table S1). Insertion sites were identified by performing sequence similarity searches using BLASTN, and verified by gene-specific PCR and sequencing.

Competition assays. STM mutants that showed repeated loss of signal in the pool-based screening were analysed for survival relative to WT bacteria both *in vivo* and *in vitro*. Mixtures of a single STM mutant and WT *Y. pestis* were used to infect fleas, and were also inoculated into HI broth. After 4 days, fed fleas were collected and stored at -80 °C until use. Fleas ($n=17-39$) were triturated in 250 μ l HI broth plus 10% glycerol, and then serial dilutions were plated on HI agar with and without kanamycin to calculate the output ratio of the total numbers of mutant (number of kanamycin-resistant colonies) to WT (total number of colonies minus number of kanamycin-resistant colonies) *Y. pestis*. Input blood meals and both time 0- and 4-day HI broth competition cultures were similarly diluted and plated to calculate input and output ratios. Competitive indices were calculated as the output ratio/input ratio for each competition.

RNA extraction and reverse transcription (RT)-PCR. RNA was extracted from *Y. pestis* strains using RiboZol RNA Extraction Reagent (Amresco) as directed. RT was performed using Moloney murine leukemia virus reverse transcriptase (Thermo Scientific) as directed, using 100 ng RNA and random hexamers for cDNA synthesis. Control reactions without the addition of reverse transcriptase were also performed. First-strand cDNA was amplified using *Taq* polymerase (Roche) and primers for each gene in the *arn* operon (y1917-y1923) as well as a reference gene, *rpoD* (Table S1). Products were detected by electrophoresis on a 4% agarose gel containing ethidium bromide.

Generation and complementation of mutants. Unmarked, in-frame deletions of the *arnB* and *wecE* genes were generated in KIM6+ using the λ Red recombineering approach (Datsenko & Wanner, 2000; Derbise *et al.*, 2003). Briefly, a kanamycin resistance gene flanked by FRT (flippase recognition target) sites was amplified from pFKM1 using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) and primers with 75 bp oligonucleotide tails homologous to the sequences up and downstream of the target gene (Table S1). The linear PCR product was transformed into KIM6+ electrocompetent cells harbouring the pKD46 plasmid (Datsenko & Wanner, 2000) that had been induced with 0.2% arabinose for 4-6 h. Recombinants were selected on HI plates with kanamycin and subsequently screened for loss of pKD46. Ampicillin-sensitive recombinants were transformed with pFLP2 (Hoang *et al.*, 1998) to remove the kanamycin cassette, leaving a 126 bp in-frame scar site that contained the start codon as well as the last 12 codons of the target gene. The STM and λ Red mutant

strains were complemented by amplifying the target gene, including its native promoter region, from KIM6+ using the Expand High Fidelity PCR System (Roche) and primers indicated in Table S1. The resulting amplicons were then cloned into pGEN-MCS (a gift from H. Mobley, University of Michigan, Ann Arbor, MI, USA) or pCR2.1-TOPO (Invitrogen) as indicated in Table 1, and mutant strains were transformed with complementation vectors by electroporation. Recombinants and complementation vectors were sequenced for verification prior to further experimentation.

CAMP susceptibility assays. CAMP susceptibility assays were performed as described by Felek *et al.* (2010), except as otherwise noted. Briefly, twofold serial dilutions of the CAMPs polymyxin B and cecropin A were prepared in HI broth in 96-well round-bottom plates. Bacterial strains were grown overnight at 28 or 37 °C and 10^5 cells were added to each well in triplicate. Plates were incubated overnight at 28 or 37 °C and the MIC for each strain was recorded as the lowest CAMP concentration at which bacterial growth was not observed. *E. coli* ATCC 25922 was used for quality control.

Lipid A extraction. LPS was extracted from overnight bacterial cultures grown at 28 °C using an LPS extraction kit (iNtRON Biotechnology) as directed, except 20 ml culture was initially harvested for use. The extracted LPS was subjected to mild acid hydrolysis as described by Kawasaki (2009). Briefly, the dried LPS pellet was suspended in 150 µl 100 mM sodium acetate buffer (pH 4.5) and incubated for 3 h at 95 °C. Then, 600 µl chloroform/methanol mixture (1:2), 200 µl chloroform and 100 µl PBS were added sequentially followed by centrifugation at 2000 r.p.m. for 5 min. The aqueous phase was discarded and 600 µl chloroform/methanol/0.1 M KCl mixture (3:47:48, by vol.) was added to the chloroform phase and mixed, followed by centrifugation at 2000 r.p.m. for 5 min. Finally, the aqueous phase was discarded and the remaining lipid A-containing chloroform phase was transferred to a new tube and dried under a stream of nitrogen. Lipid A samples were resuspended in 50 µl chloroform/methanol/water mixture (4:4:1, by vol.).

MS of lipid A. Lipid A preparations were analysed by MALDI-TOF MS in the negative-ion mode using a Bruker microflex LRF instrument (Bruker Daltonics). Lipid A samples were mixed with an equal volume of 5-chloro-2-mercaptobenzothiazole [20 mg ml^{-1} in chloroform/methanol/water (4:4:1, by vol.)] supplemented with 20 mM EDTA/ammonium hydroxide (Zhou *et al.*, 2010) and 0.5 µl aliquots were spotted onto the target plate. Spectra were acquired in the reflectron mode and analysed using flexAnalysis (Bruker Daltonics). External calibration was performed using diphosphoryl lipid A from *E. coli* F583 (Sigma-Aldrich). To identify relative differences in levels of 4-amino-4-deoxy-L-arabinose (Ara4N) modification, Ara4N-modified lipid A peaks were compared to their cognate, unmodified lipid A peaks [(sum of Ara4N-modified peaks at 1455, 1535, 1717 and 1953 *m/z*)/(sum of unmodified peaks at 1324, 1404, 1586 and 1822 *m/z*)] for the following *Y. pestis* strains: KIM6+ ($n=10$ biological replicates), 9H9 ($n=8$), 9H9-C ($n=5$), ΔarnB ($n=12$), ΔwecE ($n=10$), $\Delta\text{arnB}\Delta\text{wecE}$ ($n=5$), $\Delta\text{arnB}\Delta\text{wecE-Ca}$ ($n=5$), $\Delta\text{arnB}\Delta\text{wecE-Cw}$ ($n=5$) and $\Delta\text{arnB}\Delta\text{wecE-Caw}$ ($n=5$). Mean ratios (derived from 500 shots per biological replicate) \pm SD were plotted for each strain. Mann-Whitney tests were performed in GraphPad Prism to determine whether the mean proportions of Ara4N differed significantly between the mutant strains and WT.

RESULTS

STM mutant generation and negative selection

To identify genes involved in the survival or maintenance of *Y. pestis* in its flea vector, STM was used to randomly

generate ~3800 mutants with 96 sequence-tagged transposons. Pools of 96 mutants were used to infect fleas and were evaluated for loss of individual mutants after 4 days of infection. Mutants lost in the STM screen were re-pooled and reintroduced into fleas for verification. Those with reproducible STM results were subjected to *in vivo* and *in vitro* competition with WT *Y. pestis* KIM6+. Seven mutants showed dramatically reduced ability to compete with WT in the flea. These defects were not due to general growth deficiencies, as all mutants were able to compete effectively with WT *in vitro* over the same time period (Table 2).

Identification of genes disrupted in STM mutants

STM transposon insertion sites were identified using spPCR (Karlyshev *et al.*, 2000). Insertion sites were within ORFs for six of the seven STM mutants, whilst one was located in a promoter region (Table 2). Three mutants had insertions in genes encoding hypothetical proteins: one in a predicted permease and one in a predicted transposase. Two mutants had insertions in *galU* and *arnB*, which were predicted to encode proteins involved in LPS modification with Ara4N and resistance to CAMPs (Breazeale *et al.*, 2003; Fernández *et al.*, 2012; Klein *et al.*, 2012).

CAMP susceptibility

Modification of bacterial LPS with Ara4N confers resistance to CAMPs, some of which are produced by the insect host immune system (Bulet & Stöcklin, 2005; Ravi *et al.*, 2011; Vadyvaloo *et al.*, 2010). Both the 3C1 (*galU*) and 9H9 (*arnB*) mutants showed increased sensitivity to the CAMPs polymyxin B and cecropin A (Table 3). Complementation of the mutants with the *galU* gene or the complete *arn* operon *in trans* restored WT levels of CAMP resistance (Table 3). Complementation of 9H9 with only *arnB* did not restore resistance to CAMPs, indicating that the transposon insertion had polar effects on the *arn* operon. RT-PCR confirmed that the expression of the *arn* operon genes was essentially eliminated in the 9H9 mutant. To define the contribution of *arnB* to CAMP resistance, an in-frame deletion was generated (KIM6+ ΔarnB). Interestingly, this mutant displayed intermediate levels of resistance to CAMPs (Table 3), indicating that *Y. pestis* may have partial redundancy in the pathway for Ara4N modification of lipid A.

To further explore this pathway, in-frame deletions of a related sugar aminotransferase gene, *wecE* (KIM6+ ΔwecE), as well as a double mutant (KIM6+ $\Delta\text{arnB}\Delta\text{wecE}$) were generated. The double mutant was more susceptible to CAMPs than the *arnB* mutant, with MICs similar to the whole-operon mutant (9H9). Surprisingly, the *wecE* mutant was nearly as susceptible as the double mutant, indicating a significant role in CAMP resistance (Table 3). Complementation of the double mutant with both the *arnB* and *wecE* genes restored WT levels of CAMP resistance, whilst

Table 2. STM mutants with decreased competitive index in fleas

STM mutant	<i>Y. pestis</i> KIM10+ locus tag	Gene	Tn5 insertion site*	Product	Competitive index†	
					<i>In vivo</i>	<i>In vitro</i>
3C1	y2631	<i>galU</i>	2 895 235	UTP-glucose 1-phosphate uridylyltransferase	0.0037	5.23
3D9	y2283		2 519 947	Hypothetical	0.0383	2.07
5F7	y1748		1 929 492	Putative transposase	0.0024	1.67
9F7	y2422		2 689 958	Hypothetical	0.0306	1.20
9H9	y1917	<i>arnB</i>	2 110 322	UDP-4-amino-4-deoxy-L-arabinose-oxoglutarate aminotransferase	0.0222	1.10
10A3	y3386		3 734 381	Carbohydrate symporter permease	0.0259	1.32
17B4	y4035‡		4 474 082‡	Hypothetical	0.0331	3.28

*Base pair location of transposon insertion site according to KIM10+ genome.

†Competitive index of STM mutant to KIM6+ at 4 days post-infection/inoculation.

‡Transposon inserted into promoter region.

complementation with either *arnB* or *wecE* alone restored moderate levels of resistance, similar to those of the respective single mutants (Table 3).

MALDI-TOF MS of lipid A

As *galU* and *arnB* were previously implicated in CAMP resistance via Ara4N modification of lipid A (Gunn *et al.*, 1998; Jiang *et al.*, 2010; Klein *et al.*, 2012), the LPS of these mutants was examined. *Y. pestis* has rough LPS, lacking O-antigen (Skurnik *et al.*, 2000), and its structure varies depending on growth temperature, with Ara4N modifications

less prevalent at 37 °C than at lower temperatures (Anisimov *et al.*, 2005; Rebeil *et al.*, 2004). After growth at 28 °C, SDS-PAGE revealed no gross differences in LPS structure, so MALDI-TOF MS was performed on lipid A to identify more subtle alterations. The WT strain displayed peaks corresponding to tetra (1HPO₃)⁻, tetra (2HPO₃)⁻, penta- and hexa-acylated lipid A species at *m/z* 1324, 1404, 1586 and 1822, respectively, indicating unmodified lipid A, plus additional peaks corresponding to the Ara4N modification of each of these lipid A species (+131 *m/z*) (Fig. 1a). The lipid A spectrum of the 9H9 mutant displayed only the four unmodified lipid A peaks, with an absence of the

Table 3. Resistance of *Y. pestis* strains to cationic antimicrobial peptides

Strain	MIC (µg ml ⁻¹)			
	Polymyxin B		Cecropin A	
	28 °C	37 °C	28 °C	37 °C
KIM6+	≥256	2	≥80	20
3C1	0.25	0.125	5	5
3C1-C (pGEN- <i>galU</i>)	≥256	0.25	≥80	20
3C1-EV (pGEN-MCS)	0.125	0.125	5	2.5
9H9	0.25	0.125	5	5
9H9-C (pCR2.1- <i>arnBCADTEF</i>)	≥256	1	≥80	20
9H9-EV (pCR2.1-TOPO)	0.25	0.125	5	5
KIM6+Δ <i>arnB</i>	32	0.25	≥80	10
KIM6+Δ <i>arnB</i> -C (pCR2.1- <i>arnB</i>)	128	1	≥80	20
KIM6+Δ <i>arnB</i> -EV (pCR2.1-TOPO)	32	0.25	≥80	10
KIM6+Δ <i>wecE</i>	0.25	0.25	≥80	10
KIM6+Δ <i>wecE</i> -C (pCR2.1- <i>wecE</i>)	≥256	2	≥80	20
KIM6+Δ <i>wecE</i> -EV (pCR2.1-TOPO)	0.25	0.25	≥80	10
KIM6+Δ <i>arnB</i> Δ <i>wecE</i>	0.25	0.125	10	10
KIM6+Δ <i>arnB</i> Δ <i>wecE</i> -Ca (pCR2.1- <i>arnB</i>)	1	0.25	≥80	10
KIM6+Δ <i>arnB</i> Δ <i>wecE</i> -Cw (pCR2.1- <i>wecE</i>)	16	0.25	≥80	10
KIM6+Δ <i>arnB</i> Δ <i>wecE</i> -Caw (pCR2.1- <i>arnB</i> , <i>wecE</i>)	≥256	1	≥80	10

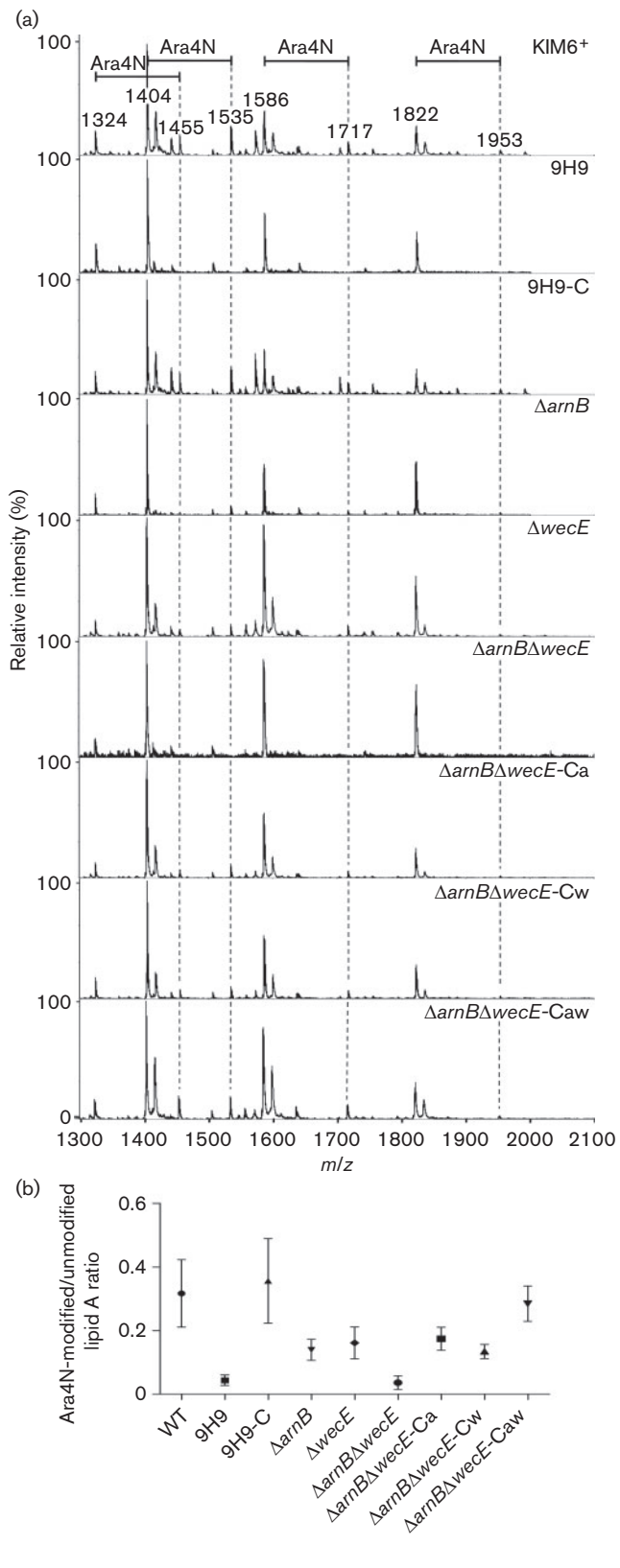


Fig. 1. MALDI-TOF MS analysis of lipid A from *Y. pestis*. (a) Representative spectra of *Y. pestis* strains. Four major lipid A species consisting of tetra (1HPO_3)-, tetra (2HPO_3)-, penta- and hexa-acylated forms are present in all strains (1324, 1404, 1586 and 1822 m/z , respectively). Peaks indicating the addition of an Ara4N group (+131 m/z) to each of these species are indicated by the dotted lines. (b) Mean ratios of Ara4N-modified (sum of peak intensities at 1455, 1535, 1717 and 1953 m/z) to unmodified (sum of peak intensities at 1324, 1404, 1586 and 1822 m/z) lipid A in *Y. pestis* strains. Bar, SD.

and 1822 m/z , respectively). Peaks indicating the addition of an Ara4N group (+131 m/z) to each of these species are indicated by the dotted lines. (b) Mean ratios of Ara4N-modified (sum of peak intensities at 1455, 1535, 1717 and 1953 m/z) to unmodified (sum of peak intensities at 1324, 1404, 1586 and 1822 m/z) lipid A in *Y. pestis* strains. Bar, SD.

peaks representing Ara4N modification (Fig. 1). Consistent with data from Klein *et al.* (2012), the 3C1 (*galU*) mutant showed a lipid A pattern nearly identical to the 9H9 mutant, indicating loss of Ara4N modifications (Fig. 2). Complementation of the 9H9 mutant with the full *arn* operon, but not *arnB* alone, resulted in a WT spectral pattern indicating restoration of Ara4N modification, which is consistent with the increased CAMP MICs (Fig. 1). As expected, the complemented 3C1 mutant displayed a WT lipid A profile, and the spectra for the 9H9-EV and 3C1-EV (empty vector control) strains appeared identical to those of their respective mutant strains. Taken together, these data confirmed that *galU* and the *arn* operon were involved in Ara4N modification of lipid A in *Y. pestis*.

In contrast to the 9H9 insertion mutant, which effectively lacked expression of the entire *arn* operon, the KIM6+ ΔarnB mutant displayed some Ara4N-modified lipid A, albeit at significantly lower levels than WT (Fig. 1b, $P=0.0001$), which was consistent with the intermediate CAMP susceptibility observed for this strain. Complementation with the *arnB* gene restored WT levels of Ara4N modification, whilst

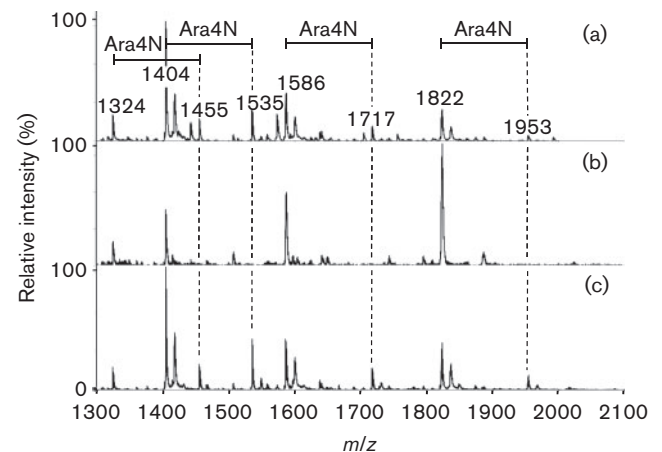


Fig. 2. MALDI-TOF MS analysis of lipid A from WT and 3C1 mutants. (a) Representative WT KIM6+ *Y. pestis* spectrum. Four major lipid A species consisting of tetra (1HPO_3)-, tetra (2HPO_3)-, penta- and hexa-acylated forms are present (1324, 1404, 1586 and 1822 m/z , respectively). Peaks indicating the addition of an Ara4N group (+131 m/z) to each of these species are indicated by the dotted lines. (b) Representative STM mutant 3C1 spectrum. Peaks representing Ara4N modifications are absent. (c) Representative STM mutant 3C1 complemented with *galU* (3C1-C) spectrum. Ara4N peaks are restored.

the empty vector alone did not alter the mutant's spectra. KIM6 + $\Delta wecE$ also showed significantly lower levels of Ara4N-modified lipid A than WT (Fig. 1b, $P=0.0040$), consistent with its susceptibility to CAMPs and complementation with *wecE* yielded spectra similar to WT. The KIM6 + $\Delta arnB\Delta wecE$ lipid A profile was similar to that of the 9H9 mutant, with no evidence of Ara4N modification (Fig. 1b). Complementation with both genes restored WT modification of lipid A ($P=0.8371$), whilst complementation with *arnB* or *wecE* individually restored partial Ara4N modification ($P=0.0159$ and $P=0.0028$, respectively, compared with double mutant), similar to that seen in the single mutants (Fig. 1b).

DISCUSSION

In this study, STM was used to generate an insertional mutant library to identify genes that contribute to the survival or maintenance of *Y. pestis* in its flea vector through the first 4 days of infection – the period of time during which *Y. pestis* can efficiently undergo EPT. STM is a powerful negative selection technique that allows for pool-based mutant analysis, enabling the identification of mutants unable to be effectively maintained in the flea (Hensel *et al.*, 1995; Mazurkiewicz *et al.*, 2006). A small number of other mutagenesis studies have aimed to dissect other parts of the *Y. pestis* infection cycle (Flashner *et al.*, 2004; Klein *et al.*, 2012) or used alternative model systems to approximate transmission dynamics (Darby *et al.*, 2005). In contrast, this study utilized a proven *Y. pestis* vector, *X. cheopis*, to identify genes that likely play a direct role in plague transmission. Few of the putative transmission factors identified in this screen have been extensively characterized in *Y. pestis*. However, the *galU* gene and the *arnBCADTEF* operon were shown in other organisms to be involved in Ara4N modification of lipid A (Gunn *et al.*, 1998, 2000). This modification plays an important role in other bacterial species by increasing their resistance to CAMPs produced by eukaryotic hosts (Gunn *et al.*, 1998; Raetz *et al.*, 2007). Recently, *galU* and the *arn* (or *pmr*) operon were shown to play an important role in allowing *Y. pestis* to survive within murine macrophages (Klein *et al.*, 2012). Therefore, the *galU* and *arnB* mutants identified from this STM screen were investigated to further examine the role of these genes in the flea vector. The 3C1 (*galU*) and 9H9 (*arn*) mutants both had dramatic survival or colonization defects in fleas at 4 days post-infection and showed decreased resistance to representatives of two major classes of CAMPs, cecropin A and polymyxin B. Cecropin A belongs to a well-studied class of linear, α -helical peptides often produced by insects, including *X. cheopis* (Vadyvaloo *et al.*, 2010), whilst polymyxin B belongs to a family of cyclic polypeptides and many insect-associated bacteria show resistance to this CAMP (Anisimov *et al.*, 2005; McCoy *et al.*, 2001). The production of CAMPs is a major defence mechanism against microorganisms for many insects and may play an important role

in protecting fleas from infectious agents, particularly within the midgut, as is seen in other blood-feeding insects (Lehane *et al.*, 1997; Vadyvaloo *et al.*, 2010). MS of these mutants confirmed that they were unable to modify their lipid A with Ara4N, rendering them susceptible to CAMPs. Together, these data confirm the role of *galU* and the *arn* operon in the modification of lipid A with Ara4N, as described in other organisms, and indicate that this modification of lipid A allows *Y. pestis* to be maintained early in the infection of fleas, facilitating transmission to other hosts.

The CAMP susceptibility of the 9H9 STM mutant, which contained a transposon insertion in the *arnB* gene, was not complemented with *arnB* alone. This indicated that the transposon had a polar effect on other genes in the operon, which was confirmed by RT-PCR. Consistent with this observation, complementation with the full *arn* operon restored WT levels of CAMP resistance and Ara4N-modified lipid A. Interestingly, an in-frame deletion of the *arnB* gene alone exhibited an intermediate phenotype. ArnB is an aminotransferase involved in the conversion of UDP-4-ketopentose to UDP-L-Ara4N during biosynthesis of Ara4N-modified lipid A (Raetz *et al.*, 2007). The decreased levels of Ara4N-modified lipid A in KIM6 + $\Delta arnB$, together with its intermediate level of CAMP resistance, suggested that at least one alternative transamination mechanism exists in *Y. pestis* in the absence of ArnB. This is in contrast to previously studied organisms that appear dependent on *arnB* for Ara4N modification and associated CAMP resistance (Gunn *et al.*, 2000). ArnB is a member of the DegT/Dnr//EryC1/StrS family of aminotransferases and orthologues of this gene have been found in bacteria that modify their lipid A with Ara4N (Breazeale *et al.*, 2003; Hwang *et al.*, 2004). The closest homologue to *arnB* in the *Y. pestis* genome is the sugar aminotransferase *wecE* (y0369; 30% amino acid identity, 48% similarity), which is part of an operon for ECA synthesis (Danese *et al.*, 1998). In *Y. pestis* and other organisms, these orthologues maintain similar active-site residues and form a similar overall structure (Hwang *et al.*, 2004; Noland *et al.*, 2002). The *wecE/arnB* double mutant showed greater CAMP sensitivity than KIM6 + $\Delta arnB$ and completely lacked Ara4N-modified lipid A, indicating that WecE may contribute to the Ara4N modification of lipid A. The *wecE* mutant was also more sensitive to polymyxin B and showed significantly less Ara4N-modified lipid A than WT ($P=0.0040$), yet significantly more than the *wecE/arnB* double mutant ($P=0.0159$, Fig. 1c). This is particularly interesting as the polymyxin B MIC for the *wecE* mutant is equivalent to that of mutants completely lacking Ara4N, even though this mutant maintains some Ara4N modification capability. The double mutant complemented with either the *arnB* or *wecE* gene alone restored low levels, and complementation with both genes restored WT levels of Ara4N modification. It is also interesting to note that complementation of this mutant with *wecE* alone yielded a 16-fold increase in polymyxin B resistance relative to complementation with *arnB*. Together, these data suggest that *Y. pestis* WecE plays not only a previously undescribed role in Ara4N modification of lipid A,

but perhaps additional Ara4N-independent roles in CAMP resistance.

ECA is a cell-surface carbohydrate found among members of the *Enterobacteriaceae* that is composed of $\epsilon\text{-}(1\rightarrow3)\text{-}\alpha\text{-D-Fucp4NAc-(1}\rightarrow4\text{)-}\beta\text{-D-ManpNAc-(1}\rightarrow4\text{)-}\alpha\text{-D-GlcpNAc-(1}\rightarrow\text{'}$ trisaccharide repeats of varying chain lengths that can exist in multiple forms (Mäkelä & Mayer, 1976; Rick & Silver, 1996). The predominant form of ECA, ECA_{PG}, is shared by all members of the *Enterobacteriaceae* and is linked via phosphodiester bonds to diacylglycerol in the outer membrane (Rick *et al.*, 1998). In some species, ECA can also be covalently linked to the LPS core (Goździewicz *et al.*, 2014; Muszyński *et al.*, 2013) or can exist in a free cyclic form in the periplasmic space (Rick & Silver, 1996; Vinogradov *et al.*, 1994). Cyclic ECA has been described from *Y. pestis* (Vinogradov *et al.*, 1994), but there is limited information on other forms it may produce. The role of ECA is not well understood, but it may be involved in virulence (Gilbreath *et al.*, 2012) or provide a protective function against environmental stress (Barua *et al.*, 2002). In *E. coli*, *wecE* mutants accumulate the lipid II intermediate of ECA, which results in structural disturbances in the outer membrane (Danese *et al.*, 1998). Although the *E. coli* *wecE* mutants do not show increased CAMP sensitivity (Nichols *et al.*, 2011), it is conceivable that similar membrane disturbances in the *Y. pestis* *wecE* mutant may affect CAMP sensitivity. Additionally, LPS-anchored ECA may alter CAMP affinity and susceptibility in *Y. pestis*. Taken together, our data point to a novel role for *WecE* in polymyxin B resistance and suggest that *Y. pestis* has evolved additional mechanisms of CAMP resistance that are distinct from those of other currently described systems.

Rebeil *et al.* (2013) recently demonstrated that *Y. pestis* mutants deficient in their ability to modify LPS with Ara4N were able to maintain long-term infections in fleas (4 weeks) and develop proventricular blockages. This initially appears to contrast with our data, which show that Ara4N modification is important for survival of *Y. pestis* in the early stages of flea infection. However, the methods used in these studies differ in important ways, which may explain this apparent difference. Rebeil *et al.* (2013) infected fleas using heparinized mouse blood rather than defibrinated rat blood as used in this study. Heparin is highly negatively charged, and is known to bind and inhibit the antibacterial activity of CAMPs (Barańska-Rybak *et al.*, 2006; Park *et al.*, 2001; Schmidtchen *et al.*, 2001). In addition, heparin was recently shown to be protective for *Y. pestis* mutants in blood by an unknown mechanism (Minato *et al.*, 2013). Furthermore, the difference in rat versus mouse blood could lead to alterations in host response to *Y. pestis*, infection prevalence and bacterial loads within the flea (Eisen *et al.*, 2008). Finally, the difference in time post-infection when the fleas were evaluated (4 days versus 4 weeks) could have a significant impact on the results. Early killing followed by recovery at later time points has been seen with the well-described *Y. pestis* transmission factor

ymt (Hinnebusch *et al.*, 2002). As *arn* mutants are still able to form biofilms (Rebeil *et al.*, 2013), cells that are not initially eliminated may be afforded an additional level of protection by the biofilm, allowing their numbers to increase over a 4-week infection such as that used by Rebeil *et al.* (2013) (Otto, 2006). Overall, these two studies imply that Ara4N modification, and CAMP resistance in general, is important for survival or colonization during the EPT period, but may not be essential for long-term colonization.

The *ymt* gene is important for survival in the flea midgut, and bacteria lacking this gene are deficient in flea colonization (Hinnebusch *et al.*, 2002); however, *ymt* mutants were not identified in this STM screen. This is not surprising as the STM mutants were pooled and any *ymt* mutants would be surrounded by bacteria producing functional Ymt, providing protection against the cytotoxic agents this protein is presumed to inactivate. In fact, Hinnebusch *et al.* (2002) showed that 81% of fleas maintained an infection with a *ymt* mutant strain (*ymtH188N*) in the proventriculus, the midgut or both when fed *ymtH188N* mixed with WT KIM6+. In the midgut, the mutant clustered within masses of WT bacteria, indicating it may not survive on its own, but could replicate when protected by Ymt-producing bacteria. Given the data, it is likely that in the pools of mutants, other strains were able to inactivate or serve as a sink for toxic compounds, minimizing their effects on *ymt* mutants. By extension, there may be additional genes required for survival in fleas that were not identified by this STM screening strategy due to protection the other pooled mutants may have provided. Other previously described transmission factors, *hms* and *gmhA*, were also not observed in the STM screen. This was expected as both are involved in biofilm formation, which is only required for late-phase transmission, and neither are required for survival in the flea (Darby *et al.*, 2005; Hinnebusch *et al.*, 1996). As this screen was designed to look for mutants that were unable to survive during the period required for EPT, mutants such as these would not be expected to be identified.

Investigation into the remaining STM mutants shows that the 3D9 and 9F7 mutants had transposon insertions in genes annotated as hypothetical in the KIM10+ genome (y2422 and y2283). There is little homology to known genes for y2422, whilst y2283 shows some similarity to a glycerophosphoryl diester phosphodiesterase (*ugpQ*). In *E. coli*, this enzyme is upregulated under limiting phosphate conditions and liberates phosphate from glycerophosphodiester (Ohshima *et al.*, 2008). The 10A3 mutation is in y3386, which is annotated in *Yersinia* spp. as a sodium:galactoside symporter family protein. Inactivation of this gene may result in a metabolic defect, leading to decreased survival in the flea or perhaps it is involved in uptake of a precursor of Ara4N that may be required for lipid modification. Further investigation may elucidate the substrate specificity of this transporter as well as the molecules it uses as co-transporters, providing insight into the possible role of this gene. The 17B4 mutation is located between y4035 (hypothetical) and y4036 (*proY*), and may

affect the promoter of *y4035*, interfering with gene expression. The role of *y4035* is currently unknown, but it is interesting to note that immediately downstream (*y4034*) is a phosphoethanolamine transferase gene. It is possible that expression of this transferase is also affected, which could interfere with LPS modification by phosphoethanolamine (Reynolds *et al.*, 2005). In other species, phosphoethanolamine modifications to the LPS core have been shown to be involved in maintaining outer membrane integrity and may increase resistance to some CAMPs (Cullen *et al.*, 2013; Reynolds *et al.*, 2005; Viau *et al.*, 2011). Lastly, the 5F7 mutant contains an insertion in a predicted transposase gene (*y1748*). It is unclear how this might affect bacterial survival in the flea; however, adjacent to this transposase is a small, unannotated ORF with similarity to *bssS*, which in *E. coli* regulates a wide variety of genes involved in biofilm formation, catabolite repression and stress response (Domka *et al.*, 2006). Altered expression of this gene may therefore disrupt survival in the flea. Further study is needed to understand exactly how these other mutants affect the ability of *Y. pestis* to colonize or survive in the flea vector.

There are likely other factors required by *Y. pestis* for flea survival that remain to be discovered, given that the mutant library was not fully saturated. However, even without reaching saturation, many studies in other systems have identified important survival or virulence factors, increasing our understanding of host–pathogen interactions (Flashner *et al.*, 2004; Merrell *et al.*, 2002). The approach of using STM to investigate *Y. pestis*–flea interactions revealed hitherto unidentified mechanisms by which this significant human pathogen can move between hosts to cause its devastating disease. Some of the mutants with maintenance defects in fleas have disruptions in hypothetical genes, and further investigation of these mutants may provide insight into novel factors that are important for arthropod infection and transmission.

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REFERENCES

Anisimov, A. P., Dentovskaya, S. V., Titareva, G. M., Bakhteeva, I. V., Shaikhutdinova, R. Z., Balakhonov, S. V., Lindner, B., Kocharova, N. A., Senchenkova, S. N. & other authors (2005). Intraspecies and temperature-dependent variations in susceptibility of *Yersinia pestis* to the bactericidal action of serum and to polymyxin B. *Infect Immun* **73**, 7324–7331.

Bacot, A. W. & Martin, C. J. (1914). LXVII. Observations on the mechanism of the transmission of plague by fleas. *J Hyg (Lond)* **13** (Suppl), 423–439.

Barańska-Rybak, W., Sonesson, A., Nowicki, R. & Schmidtchen, A. (2006). Glycosaminoglycans inhibit the antibacterial activity of LL-37 in biological fluids. *J Antimicrob Chemother* **57**, 260–265.

Barua, S., Yamashino, T., Hasegawa, T., Yokoyama, K., Torii, K. & Ohta, M. (2002). Involvement of surface polysaccharides in the organic acid resistance of Shiga Toxin-producing *Escherichia coli* O157:H7. *Mol Microbiol* **43**, 629–640.

Breazeale, S. D., Ribeiro, A. A. & Raetz, C. R. (2003). Origin of lipid A species modified with 4-amino-4-deoxy-L-arabinose in polymyxin-resistant mutants of *Escherichia coli*. An aminotransferase (ArnB) that generates UDP-4-deoxyl-L-arabinose. *J Biol Chem* **278**, 24731–24739.

Bulet, P. & Stöcklin, R. (2005). Insect antimicrobial peptides: structures, properties and gene regulation. *Protein Pept Lett* **12**, 3–11.

Burroughs, A. L. (1947). Sylvatic plague studies: the vector efficiency of nine species of fleas compared with *Xenopsylla cheopis*. *J Hyg (Lond)* **45**, 371–396.

Choi, K. H., Gaynor, J. B., White, K. G., Lopez, C., Bosio, C. M., Karkhoff-Schweizer, R. R. & Schweizer, H. P. (2005). A Tn7-based broad-range bacterial cloning and expression system. *Nat Methods* **2**, 443–448.

Chouikha, I. & Hinnebusch, B. J. (2012). *Yersinia*–flea interactions and the evolution of the arthropod-borne transmission route of plague. *Curr Opin Microbiol* **15**, 239–246.

Cullen, T. W., O'Brien, J. P., Hendrixson, D. R., Giles, D. K., Hobb, R. I., Thompson, S. A., Brodbelt, J. S. & Trent, M. S. (2013). EptC of *Campylobacter jejuni* mediates phenotypes involved in host interactions and virulence. *Infect Immun* **81**, 430–440.

Danese, P. N., Oliver, G. R., Barr, K., Bowman, G. D., Rick, P. D. & Silhavy, T. J. (1998). Accumulation of the enterobacterial common antigen lipid II biosynthetic intermediate stimulates *degP* transcription in *Escherichia coli*. *J Bacteriol* **180**, 5875–5884.

Darby, C., Ananth, S. L., Tan, L. & Hinnebusch, B. J. (2005). Identification of *gmhA*, a *Yersinia pestis* gene required for flea blockage, by using a *Caenorhabditis elegans* biofilm system. *Infect Immun* **73**, 7236–7242.

Datsenko, K. A. & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640–6645.

Derbise, A., Lesic, B., Dacheux, D., Ghigo, J. M. & Carniel, E. (2003). A rapid and simple method for inactivating chromosomal genes in *Yersinia*. *FEMS Immunol Med Microbiol* **38**, 113–116.

Domka, J., Lee, J. & Wood, T. K. (2006). YliH (BssR) and YceP (BssS) regulate *Escherichia coli* K-12 biofilm formation by influencing cell signaling. *Appl Environ Microbiol* **72**, 2449–2459.

Eisen, R. J. & Gage, K. L. (2012). Transmission of flea-borne zoonotic agents. *Annu Rev Entomol* **57**, 61–82.

Eisen, R. J., Bearden, S. W., Wilder, A. P., Monteneri, J. A., Antolin, M. F. & Gage, K. L. (2006). Early-phase transmission of *Yersinia pestis* by unblocked fleas as a mechanism explaining rapidly spreading plague epizootics. *Proc Natl Acad Sci U S A* **103**, 15380–15385.

Eisen, R. J., Lowell, J. L., Monteneri, J. A., Bearden, S. W. & Gage, K. L. (2007). Temporal dynamics of early-phase transmission of *Yersinia pestis* by unblocked fleas: secondary infectious feeds prolong efficient transmission by *Oropsylla montana* (Siphonaptera: Ceratophyllidae). *J Med Entomol* **44**, 672–677.

Eisen, R. J., Vetter, S. M., Holmes, J. L., Bearden, S. W., Monteneri, J. A. & Gage, K. L. (2008). Source of host blood affects prevalence of infection and bacterial loads of *Yersinia pestis* in fleas. *J Med Entomol* **45**, 933–938.

- Felek, S., Muszyński, A., Carlson, R. W., Tsang, T. M., Hinnebusch, B. J. & Krukoni, E. S. (2010).** Phosphoglucosyltransferase of *Yersinia pestis* is required for autoaggregation and polymyxin B resistance. *Infect Immun* **78**, 1163–1175.
- Fernández, L., Alvarez-Ortega, C., Wiegand, I., Olivares, J., Kocíncová, D., Lam, J. S., Martínez, J. L. & Hancock, R. E. (2013).** Characterization of the polymyxin B resistome of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **57**, 110–119.
- Flashner, Y., Mamroud, E., Tidhar, A., Ber, R., Aftalion, M., Gur, D., Lazar, S., Zvi, A., Bino, T. & other authors (2004).** Generation of *Yersinia pestis* attenuated strains by signature-tagged mutagenesis in search of novel vaccine candidates. *Infect Immun* **72**, 908–915.
- Gilbreath, J. J., Colvocoresses Dodds, J., Rick, P. D., Soloski, M. J., Merrell, D. S. & Metcalf, E. S. (2012).** Enterobacterial common antigen mutants of *Salmonella enterica* serovar Typhimurium establish a persistent infection and provide protection against subsequent lethal challenge. *Infect Immun* **80**, 441–450.
- Gozdziewicz, T. K., Lugowski, C. & Lukaszewicz, J. (2014).** First evidence for a covalent linkage between enterobacterial common antigen and lipopolysaccharide in *Shigella sonnei* phase II ECALPS. *J Biol Chem* **289**, 2745–2754.
- Gunn, J. S., Lim, K. B., Krueger, J., Kim, K., Guo, L., Hackett, M. & Miller, S. I. (1998).** PmrA–PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. *Mol Microbiol* **27**, 1171–1182.
- Gunn, J. S., Ryan, S. S., Van Velkinburgh, J. C., Ernst, R. K. & Miller, S. I. (2000).** Genetic and functional analysis of a PmrA–PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of *Salmonella enterica* serovar typhimurium. *Infect Immun* **68**, 6139–6146.
- Hensel, M., Shea, J. E., Gleeson, C., Jones, M. D., Dalton, E. & Holden, D. W. (1995).** Simultaneous identification of bacterial virulence genes by negative selection. *Science* **269**, 400–403.
- Hinnebusch, B. J., Perry, R. D. & Schwan, T. G. (1996).** Role of the *Yersinia pestis* hemin storage (*hms*) locus in the transmission of plague by fleas. *Science* **273**, 367–370.
- Hinnebusch, J., Cherepanov, P., Du, Y., Rudolph, A., Dixon, J. D., Schwan, T. & Forsberg, A. (2000).** Murine toxin of *Yersinia pestis* shows phospholipase D activity but is not required for virulence in mice. *Int J Med Microbiol* **290**, 483–487.
- Hinnebusch, B. J., Rudolph, A. E., Cherepanov, P., Dixon, J. E., Schwan, T. G. & Forsberg, A. (2002).** Role of *Yersinia* murine toxin in survival of *Yersinia pestis* in the midgut of the flea vector. *Science* **296**, 733–735.
- Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchna, A. J. & Schweizer, H. P. (1998).** A broad-host-range Flp–FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* **212**, 77–86.
- Houhamdi, L. & Raoult, D. (2008).** Different genes govern *Yersinia pestis* pathogenicity in *Caenorhabditis elegans* and human lice. *Microb Pathog* **44**, 435–437.
- Hwang, B. Y., Lee, H. J., Yang, Y. H., Joo, H. S. & Kim, B. G. (2004).** Characterization and investigation of substrate specificity of the sugar aminotransferase WecE from *E. coli* K12. *Chem Biol* **11**, 915–925.
- Jarrett, C. O., Deak, E., Isherwood, K. E., Oyston, P. C., Fischer, E. R., Whitney, A. R., Kobayashi, S. D., DeLeo, F. R. & Hinnebusch, B. J. (2004).** Transmission of *Yersinia pestis* from an infectious biofilm in the flea vector. *J Infect Dis* **190**, 783–792.
- Jiang, S. S., Lin, T. Y., Wang, W. B., Liu, M. C., Hsueh, P. R. & Liaw, S. J. (2010).** Characterization of UDP-glucose dehydrogenase and UDP-glucose pyrophosphorylase mutants of *Proteus mirabilis*: defectiveness in polymyxin B resistance, swarming, and virulence. *Antimicrob Agents Chemother* **54**, 2000–2009.
- Karlyshev, A. V., Pallen, M. J. & Wren, B. W. (2000).** Single-primer PCR procedure for rapid identification of transposon insertion sites. *Biotechniques* **28**, 1078–1082.
- Kartman, L., Prince, F. M., Quan, S. F. & Stark, H. E. (1958).** New knowledge on the ecology of sylvatic plague. *Ann N Y Acad Sci* **70**, 668–711.
- Kawasaki, K. (2009).** Alternative procedures for analysis of lipid A modification with phosphoethanolamine or aminoarabinose. *J Microbiol Methods* **76**, 313–315.
- Klein, K. A., Fukuto, H. S., Pelletier, M., Romanov, G., Grabenstein, J. P., Palmer, L. E., Ernst, R. & Bliska, J. B. (2012).** A transposon site hybridization screen identifies *galU* and *wecBC* as important for survival of *Yersinia pestis* in murine macrophages. *J Bacteriol* **194**, 653–662.
- Lane, M. C., Alteri, C. J., Smith, S. N. & Mobley, H. L. (2007).** Expression of flagella is coincident with uropathogenic *Escherichia coli* ascension to the upper urinary tract. *Proc Natl Acad Sci U S A* **104**, 16669–16674.
- Lehane, M. J., Wu, D. & Lehane, S. M. (1997).** Midgut-specific immune molecules are produced by the blood-sucking insect *Stomoxys calcitrans*. *Proc Natl Acad Sci U S A* **94**, 11502–11507.
- Mäkelä, P. H. & Mayer, H. (1976).** Enterobacterial common antigen. *Bacteriol Rev* **40**, 591–632.
- Mazurkiewicz, P., Tang, C. M., Boone, C. & Holden, D. W. (2006).** Signature-tagged mutagenesis: barcoding mutants for genome-wide screens. *Nat Rev Genet* **7**, 929–939.
- McCoy, A. J., Liu, H., Falla, T. J. & Gunn, J. S. (2001).** Identification of *Proteus mirabilis* mutants with increased sensitivity to antimicrobial peptides. *Antimicrob Agents Chemother* **45**, 2030–2037.
- Mei, J. M., Nourbakhsh, F., Ford, C. W. & Holden, D. W. (1997).** Identification of *Staphylococcus aureus* virulence genes in a murine model of bacteraemia using signature-tagged mutagenesis. *Mol Microbiol* **26**, 399–407.
- Merrell, D. S., Hava, D. L. & Camilli, A. (2002).** Identification of novel factors involved in colonization and acid tolerance of *Vibrio cholerae*. *Mol Microbiol* **43**, 1471–1491.
- Minato, Y., Ghosh, A., Faulkner, W. J., Lind, E. J., Schesser Bartra, S., Plano, G. V., Jarrett, C. O., Hinnebusch, B. J., Winogrodzki, J. & other authors (2013).** Na⁺/H⁺ antiport is essential for *Yersinia pestis* virulence. *Infect Immun* **81**, 3163–3172.
- Muszyński, A., Rabsztyn, K., Knapaska, K., Duda, K. A., Duda-Grychtol, K., Kasperkiewicz, K., Radziejewska-Lebrecht, J., Holst, O. & Skurnik, M. (2013).** Enterobacterial common antigen and O-specific polysaccharide coexist in the lipopolysaccharide of *Yersinia enterocolitica* serotype O:3. *Microbiology* **159**, 1782–1793.
- Nichols, R. J., Sen, S., Choo, Y. J., Beltrao, P., Zietek, M., Chaba, R., Lee, S., Kazmierczak, K. M., Lee, K. J. & other authors (2011).** Phenotypic landscape of a bacterial cell. *Cell* **144**, 143–156.
- Noland, B. W., Newman, J. M., Hendle, J., Badger, J., Christopher, J. A., Tresser, J., Buchanan, M. D., Wright, T. A., Rutter, M. E. & other authors (2002).** Structural studies of *Salmonella typhimurium* ArnB (PmrH) aminotransferase: a 4-amino-4-deoxy-L-arabinose lipopolysaccharide-modifying enzyme. *Structure* **10**, 1569–1580.
- Ohshima, N., Yamashita, S., Takahashi, N., Kuroishi, C., Shiro, Y. & Takio, K. (2008).** *Escherichia coli* cytosolic glycerophosphodiester phosphodiesterase (UgpQ) requires Mg²⁺, Co²⁺, or Mn²⁺ for its enzyme activity. *J Bacteriol* **190**, 1219–1223.

- Otto, M. (2006). Bacterial evasion of antimicrobial peptides by biofilm formation. In *Antimicrobial Peptides and Human Disease*, pp. 251–258. Edited by W. M. Shafer. Berlin: Springer.
- Park, P. W., Pier, G. B., Hinkes, M. T. & Bernfield, M. (2001). Exploitation of syndecan-1 shedding by *Pseudomonas aeruginosa* enhances virulence. *Nature* **411**, 98–102.
- Perry, R. D., Pendrak, M. L. & Schuetze, P. (1990). Identification and cloning of a hemin storage locus involved in the pigmentation phenotype of *Yersinia pestis*. *J Bacteriol* **172**, 5929–5937.
- Raetz, C. R., Reynolds, C. M., Trent, M. S. & Bishop, R. E. (2007). Lipid A modification systems in gram-negative bacteria. *Annu Rev Biochem* **76**, 295–329.
- Ravi, C., Jeyashree, A. & Renuka Devi, K. (2011). Antimicrobial peptides from insects: an overview. *Res Biotech* **2**, 1–7.
- Rebeil, R., Ernst, R. K., Gowen, B. B., Miller, S. I. & Hinnebusch, B. J. (2004). Variation in lipid A structure in the pathogenic yersiniae. *Mol Microbiol* **52**, 1363–1373.
- Rebeil, R., Jarrett, C. O., Driver, J. D., Ernst, R. K., Oyston, P. C. & Hinnebusch, B. J. (2013). Induction of the *Yersinia pestis* PhoP–PhoQ regulatory system in the flea and its role in producing a transmissible infection. *J Bacteriol* **195**, 1920–1930.
- Reynolds, C. M., Kalb, S. R., Cotter, R. J. & Raetz, C. R. (2005). A phosphoethanolamine transferase specific for the outer 3-deoxy-D-manno-octulosonic acid residue of *Escherichia coli* lipopolysaccharide. Identification of the *eptB* gene and Ca²⁺ hypersensitivity of an *eptB* deletion mutant. *J Biol Chem* **280**, 21202–21211.
- Rick, P. D. & Silver, R. P. (1996). Enterobacterial common antigen and capsular polysaccharides. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 104–122. Edited by F. C. Neidhardt. Washington, DC: American Society for Microbiology.
- Rick, P. D., Hubbard, G. L., Kitaoka, M., Nagaki, H., Kinoshita, T., Dowd, S., Simplaceanu, V. & Ho, C. (1998). Characterization of the lipid-carrier involved in the synthesis of enterobacterial common antigen (ECA) and identification of a novel phosphoglyceride in a mutant of *Salmonella typhimurium* defective in ECA synthesis. *Glycobiology* **8**, 557–567.
- Rudolph, A. E., Stuckey, J. A., Zhao, Y., Matthews, H. R., Patton, W. A., Moss, J. & Dixon, J. E. (1999). Expression, characterization, and mutagenesis of the *Yersinia pestis* murine toxin, a phospholipase D superfamily member. *J Biol Chem* **274**, 11824–11831.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schmidtchen, A., Frick, I. M. & Björck, L. (2001). Dermatan sulphate is released by proteinases of common pathogenic bacteria and inactivates antibacterial alpha-defensin. *Mol Microbiol* **39**, 708–713.
- Simon, R., Priefer, U. & Puhler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Nat Biotechnol* **1**, 784–791.
- Skurnik, M., Peippo, A. & Ervelä, E. (2000). Characterization of the O-antigen gene clusters of *Yersinia pseudotuberculosis* and the cryptic O-antigen gene cluster of *Yersinia pestis* shows that the plague bacillus is most closely related to and has evolved from *Y. pseudotuberculosis* serotype O:1b. *Mol Microbiol* **37**, 316–330.
- Vadyvaloo, V., Jarrett, C., Sturdevant, D. E., Sebbane, F. & Hinnebusch, B. J. (2010). Transit through the flea vector induces a pretransmission innate immunity resistance phenotype in *Yersinia pestis*. *PLoS Pathog* **6**, e1000783.
- Vetter, S. M., Eisen, R. J., Schotthoefer, A. M., Monteneri, J. A., Holmes, J. L., Bobrov, A. G., Bearden, S. W., Perry, R. D. & Gage, K. L. (2010). Biofilm formation is not required for early-phase transmission of *Yersinia pestis*. *Microbiology* **156**, 2216–2225.
- Viau, C., Le Sage, V., Ting, D. K., Gross, J. & Le Moual, H. (2011). Absence of PmrAB-mediated phosphoethanolamine modifications of *Citrobacter rodentium* lipopolysaccharide affects outer membrane integrity. *J Bacteriol* **193**, 2168–2176.
- Vinogradov, E. V., Knirel, Y. A., Thomas-Oates, J. E., Shashkov, A. S. & L'vov, V. L. (1994). The structure of the cyclic enterobacterial common antigen (ECA) from *Yersinia pestis*. *Carbohydr Res* **258**, 223–232.
- Zhou, P., Altman, E., Perry, M. B. & Li, J. (2010). Study of matrix additives for sensitive analysis of lipid A by matrix-assisted laser desorption ionization mass spectrometry. *Appl Environ Microbiol* **76**, 3437–3443.

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