A Transposon Site Hybridization Screen Identifies *galU* and *wecBC* as Important for Survival of *Yersinia pestis* in Murine Macrophages

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Yersinia pestis is able to survive and replicate within murine macrophages. However, the mechanism by which *Y. pestis* promotes its intracellular survival is not well understood. To identify genes that are important for *Y. pestis* survival in macrophages, a library comprised of ~31,500 *Y. pestis* KIM6+ transposon insertion mutants (input pool) was subjected to negative selection in primary murine macrophages. Genes underrepresented in the output pool of surviving bacteria were identified by transposon site hybridization to DNA oligonucleotide microarrays. The screen identified several genes known to be important for survival of *Y. pestis* in macrophages, including *phoPQ* and members of the PhoPQ regulon (e.g., *pmrF*). In addition, genes predicated to encode a glucose-1-phosphate uridylyltransferase (*galU*), a UDP-*N*-acetylglucosamine 2-epimerase (*wecB*) and a UDP-*N*-acetyl- α -mannosamine dehydrogenase (*wecC*) were identified in the screen. Viable-count assays demonstrated that a KIM6+ *galU* mutant and a KIM6+ *wecBC* mutant were defective for survival in murine macrophages. The *galU* mutant was studied further because of its strong phenotype. The KIM6+ *galU* mutant exhibited increased susceptibility to the antimicrobial peptides polymyxin B and cathelicidin-related antimicrobial peptide (CRAMP). Polyacrylamide gel electrophoresis demonstrated that the lipooligosaccharide (LOS) of the *galU* mutant migrated faster than the LOS of the parent KIM6+, suggesting the core was truncated. In addition, the analysis of LOS isolated from the *galU* mutant by mass spectrometry showed that aminoarabinose modification of lipid A is absent. Therefore, addition of aminoarabinose to lipid A and complete LOS core (*galU*), as well as enterobacterial common antigen (*wecB* and *wecC*), is important for survival of *Y. pestis* in macrophages.

Yersinia pestis is a Gram-negative bacterium and the causative agent of plague (41). Bubonic plague, the most common form of the disease, is transmitted via infected fleas and can disseminate throughout the host, giving rise to septicemia (41). Primary pneumonic plague is caused via inhalation of infectious respiratory droplets and shows particularly high morbidity and mortality compared to the bubonic and septicemic forms of plague. *Y. pestis* is classified as a category A select agent as a result of its potential to be used as a biological weapon (48).

Y. pestis is able to survive and replicate within murine macrophages (5, 56). However, the bacteria are more often found extracellularly within the tissues of an infected host (31), and *Y. pestis* is thus classified as a facultative intracellular pathogen. *Y. pestis* promotes its extracellular phase of growth by producing several types of antiphagocytic factors, which are upregulated at the host temperature of 37°C. These factors include several effector proteins known as Yops, which are secreted through the type III secretion system (T3SS), encoded on the virulence plasmid pCD1 (60). *Y. pestis* also produces a capsule composed of F1 protein that helps promote antiphagocytosis (10). Even though the bacteria have several antiphagocytic factors, evidence suggests that *Y. pestis* can initially survive within macrophages before being released to survive and replicate extracellularly in a host (34).

After a macrophage phagocytoses a bacterium into a vacuole termed a phagosome, the phagosome fuses with compartments of the endocytic pathway to mature into a phagolysosome (61). These fusion events can be detected by utilizing markers of late endosomes and lysosomes such as lysosome-associated membrane protein-1 (LAMP-1) and cathepsin D, as well as endocytic vesicle tracers such as Texas Red ovalbumin. As the phagosome matures, the bacterium is exposed to antimicrobial peptides as

well as reactive oxygen species (ROS). The environment of a phagolysosome is acidic (~pH 4.5), due to action of the vacuolar H⁺ ATPase (15). The acidity of the phagolysosome activates lysosomal proteases such as the cathepsins (27), which, together with antimicrobial peptides and ROS, contribute to the destruction of the bacterium.

Compared to other Gram-negative pathogens, such as *Salmonella enterica* serovar Typhimurium, it is poorly understood how *Y. pestis* promotes its survival within macrophages. Early studies suggested that *Y. pestis* survives within a phagolysosome (57), and further investigation of the trafficking of *Y. pestis*-containing vacuoles (YCVs) in macrophages indicated that the compartment colocalizes with cathepsin D and LAMP-1 (21), suggesting that YCVs undergo at least partial maturation by interacting with some components of endocytic pathway. It was recently shown that YCVs also colocalize with markers of autophagosomes (44). However, YCVs fail to acidify below a pH of 7, indicating that this vacuole does not fully mature to the stage of a phagolysosome (44). A chromosomal region referred to as the pigmentation

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TABLE 1 *Yersinia* strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
Strains		
KIM6+	<i>Y. pestis</i> biovar 2.MED, pCD1 [−] pMT1 ⁺ pPCP1 ⁺ <i>pgm</i> ⁺	19
KIM6+ <i>phoPΔ</i>	KIM6+ <i>phoPΔ</i> 127-429	21
KIM6+ <i>Δy0144</i>	KIM6+ <i>Δy0142</i> nt 61– <i>y0145</i> nt 724	This work
KIM6+ <i>Δy0157 (ptsN)</i>	KIM6+ <i>Δy0157</i> nt 22– <i>y0159</i> nt 151	This work
KIM6+ <i>Δy0887 (gshA)</i>	KIM6+ <i>gshAΔ</i> 40-1560	This work
KIM6+ <i>Δy0364-y0365 (wecBC)</i>	KIM6+ <i>ΔwecB</i> nt 46– <i>wecC</i> nt 1239	This work
KIM6+/pVector	KIM6+, pMMB67EH	This work
KIM6+ <i>galU</i> /pVector	KIM6+ <i>galU::magellan3</i> , pMMB67EH, Kn ^r Ap ^r	This work
KIM6+/pGalU	KIM6+ <i>galU::magellan3</i> , pGalU, Kn ^r Ap ^r	This work
KIM5	pCD1Ap, pMT1 pPCP1, <i>Δpgm</i> Ap ^r	33
KIM5 <i>galU</i>	KIM5 <i>galU::magellan3</i> Kn ^r	This work
IP2790c	<i>Y. pseudotuberculosis</i> O:1, pYV [−]	52
IP2790c <i>galU</i>	IP2790c <i>galU::magellan3</i> Kn ^r	20
Plasmids		
pMar2×T7	R6K replicon; contains <i>Himar1</i> element flanked by outward-reading T7 promoters, Ap ^r Gm ^r	32
pMar2×T7/TyK	Derived from pMar2×T7 by replacement of gentamicin resistance in <i>Himar1</i> with kanamycin resistance cassette, Ap ^r Kn ^r	This work
pSB890	Suicide vector with R6K replicon, Tc ^r	40
pMMB67EH	Expression vector with RSF1010 replicon, <i>tacP</i> Ap ^r	17
pGalU	pMMB67EH containing <i>galU</i> ORF from IP2790c, Ap ^r	This work

(*pgm*) locus contains an operon called *ripCBA* that promotes survival of the bacteria within activated macrophages (43). The products of the *ripCBA* operon appear to promote survival of *Y. pestis* by reducing the production of nitric oxide (43). PhoP, a transcriptional activator, as well as several PhoP-regulated genes, are important for the survival of *Y. pestis* in macrophages (21, 39). It has been suggested that products of PhoP-regulated genes (e.g., *pmrF* and *mgtC*) promote survival of *Y. pestis* in macrophages by facilitating resistance to antimicrobial peptides and the stress of low-Mg²⁺ conditions, respectively, encountered in YCVs (21). Global profiling of the transcriptome of *Y. pestis* inside macrophages showed that, in addition to *phoP* and genes in the *phoP* regulon, the expression of oxidative stress-response genes (e.g., *sodB*, *sodC*, and *katG*) and numerous genes involved in metabolism was induced intracellularly, suggesting the presence of various stresses and nutrient limitation inside YCVs (16).

The *Y. pestis* outer membrane contains a lipooligosaccharide (LOS) that plays a key role in resistance to antimicrobial peptides (24, 29). Products of the *pmr* genes promote antimicrobial peptide resistance in *Y. pestis* by the addition of the cationic sugar 4-amino-4-deoxy-L-arabinose (aminoarabinose) to lipid A (29, 46, 63). The inner heptose core of the LOS is also important for resistance of *Y. pestis* to antimicrobial peptides *in vitro* (2, 29).

Transposon site hybridization (TraSH) is a method that combines transposon mutagenesis with microarray technology to allow high-throughput screening of mutant libraries after negative selection (37). TraSH has been employed to identify mycobacterial genes required for growth under nutrient limitation conditions (50) as well as to identify genes contributing to the survival of *S. Typhimurium* within murine macrophages (6).

In this study, we performed a TraSH screen to identify genes that are important for the survival of *Y. pestis* in macrophages. Genes identified as potentially playing a role in the intracellular survival of *Y. pestis* include *wecB* and *wecC*, which are required for synthesis of enterobacterial common antigen. Another gene

identified, *galU*, is predicted to encode a glucose-1-phosphate uridylyltransferase, an enzyme required for the production of UDP-glucose. UDP-glucose is used as a substrate by a glucosyltransferase to add a single glucose residue to the LOS inner core of *Y. pestis*, as well as a metabolic precursor for aminoarabinose. The *wecBC* and *galU* genes were inactivated in *Y. pestis* in order to characterize their role in survival in macrophages.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *Y. pestis* strains and plasmids used in this study are listed in Table 1. KIM6+ is derived from KIM-10, a biovar 2.MED strain (1) that was isolated from a human case of pneumonic plague in Kurdistan Province, Iran, in 1961 (14). The *Escherichia coli* strain used for conjugation was S17-1λpir (51) and was grown at 37°C either on Luria-Bertani (LB) agar or in LB broth (36). *Y. pestis* strains were grown at 28°C on Difco heart infusion (HI) agar (Becton Dickinson Company), or in Difco HI broth (Becton Dickinson).

Macrophage cell culture. Bone marrow macrophages (BMMs) were isolated and cultured as previously described (42). BMMs were routinely prepared from the femurs of 6- to 8-week-old female C57BL/6J mice (Jackson Laboratories). Bone marrow cells from Camp^{−/−} (cathelicidin-related antimicrobial peptide [CRAMP]-deficient) mice on a C57BL/6 background (4) were obtained from R. L. Gallo (University of California, San Diego, San Diego, CA). The murine macrophage-like cell line J774A.1 was cultured as previously described (22).

Construction of a transposon library in *Y. pestis*. The transposon delivery vector pMar2xT7/TyK (Table 1), which carries a mariner-family transposon *Himar1* element flanked by outward-reading T7 promoters, was created by replacing the gentamicin resistance cassette in *Himar1* of pMar2xT7 (32) with a kanamycin resistance cassette. This replacement was achieved by PCR amplifying the TyK kanamycin resistance cassette from plasmid pFD1 (49) with primers Sfi_TyK_F1 and Nco_TyK_R1 (see Table S1 in the supplemental material) and inserting it into the Sfi/NcoI sites of pMar2xT7. *E. coli* S17-1λpir was transformed with pMar2xT7/TyK, and selection for the vector was achieved using 100 μg/ml ampicillin and 25 μg/ml kanamycin. *Y. pestis* KIM6+ was conjugated with S17-1λpir containing pMar2xT7/TyK on LB agar plates at 37°C for 5 h. Colonies of

TABLE 2 Negative selection screen results showing 11 genes with the highest negative change

Gene	Symbol	Change (fold)	P value	Predicted function
y1793	<i>phoQ</i>	-2.993	0.00009541	Sensor protein PhoQ
y2631	<i>galU</i>	-2.492	0.0005587	Glucose-1-phosphate uridylyltransferase
y1921	<i>pmrK</i>	-2.322	0.03572	Hypothetical protein
y1794	<i>phoP</i>	-2.233	0.004432	Transcriptional regulatory protein
y1918	<i>pmrF</i>	-2.134	0.00003637	Putative sugar transferase
y1919	<i>pmrI</i>	-2.126	0.0004919	Hypothetical protein
y1920	<i>pmrJ</i>	-2.115	0.00006396	Hypothetical protein
y0364	<i>wecB</i>	-2.034	0.00007054	UDP-N-acetylglucosamine-2-epimerase
y0157	<i>ptsN</i>	-1.84	0.00007054	Phosphotransferase system enzyme IIA
y0365	<i>wecC</i>	-1.789	0.0001052	UDP-N-acetyl-D-mannosaminuronic acid dehydrogenase
y0887	<i>gshA</i>	-1.76	0.0001309	Glutamate-cysteine ligase

KIM6+ transconjugants were selected on plates containing *Yersinia*-selective medium (YSM) (Oxoid) and 100 μ g/ml kanamycin. Approximately 31,500 transconjugant colonies were pooled together in LB with 50% glycerol and frozen at -80°C .

Negative selection of *Y. pestis* mutants in bone marrow macrophages. The negative selection screen was performed similarly to those in previously published studies (6, 50). This screen differed from previous screens (6, 50) in that the PCR amplification step utilizing a linker sequence was omitted, and the RNA was not reverse transcribed to cDNA but was hybridized directly to microarray slides. This decreased the number of steps required to produce the target for hybridization to the arrays. Briefly, a sample of the KIM6+ transposon library from the frozen stock was grown in HI broth culture at 28°C overnight with shaking. A portion of the bacterial culture was harvested for use as an unselected input control. Another portion of the culture was used to infect approximately 10^7 BMMs at a multiplicity of infection (MOI) of 2 for 15 min. At this MOI, approximately 50% of the BMMs were infected, and the majority of those cells contained only one bacterium. This low MOI was used to mitigate any possible effects from the presence of multiple bacteria within a BMM. The infected BMMs were then washed with phosphate-buffered saline (PBS), and incubated in tissue culture medium containing 8 μ g/ml gentamicin to kill extracellular bacteria. The gentamicin concentration was reduced to 2 μ g/ml after 1 h. At 8 h postinfection, the BMMs were washed with PBS and lysed with H_2O containing 0.1% Triton X-100. Bacteria were harvested by centrifugation and resuspended in HI, and an outgrowth step was performed overnight at 28°C with shaking. The bacteria were then collected as the selected output condition. Four independent infection experiments were performed.

Genomic-DNA processing, and *in vitro* transcription. Genomic DNA (gDNA) was isolated from the input and output pools of KIM6+ using Genomic-tip 100/G reagents per the manufacturer's protocol (Qiagen). Twenty micrograms of purified gDNA was sheared by sonication, precipitated with ammonium acetate and ethanol, and resuspended in H_2O containing diethyl pyrocarbonate (DEPC). Two micrograms of sheared purified gDNA was used for transcription with the amino-allyl MEGAscript kit (Ambion) and amino allyl UTP. The transcription reaction was allowed to proceed for 14 h at 37°C to make amino allyl-modified RNA. The allyl-modified RNA was purified using the Qiagen nucleotide removal kit (Qiagen) and subsequently coupled with cyanine 3 (Cy3) or Cy5 (GE Healthcare). The indirect labeling of RNA through incorporation of amino allyl UTP followed by coupling of amino allyl-modified RNA to Cy dyes has been found to be more efficient and yield stronger signal than the direct incorporation of Cy-UTPs (58).

For two experiments, the input RNA was labeled with Cy3 and the output RNA was labeled with Cy5, and the reciprocal was done with the remaining two experiments. Thus, four hybridization experiments were performed, with two experiments per dye swap to correct for dye bias (8).

Microarray hybridization and processing. The 70-mer DNA oligonucleotide microarrays representing *Y. pestis* open reading frames (ORFs)

(version 2) were obtained from the Pathogen Functional Genomics Resource Center (PFGRC) at the J. Craig Venter Institute (formerly The Institute for Genomic Research). The microarray slides were prehybridized, washed, and dried immediately before hybridization by using the recommended protocol (<http://pfgrc.jcvi.org/index.php/microarray/protocols.html>). For hybridization, RNA labeled with Cy3 or Cy5 was dye matched to 200 to 250 pmol and resuspended in 130 μ l of hybridization solution containing 25% (vol/vol) formamide, $5\times$ SSC, 0.1% sodium dodecyl sulfate (SDS), and 300 μ g/ml of sheared salmon sperm DNA ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Samples were heated at 95°C for 10 min prior to hybridization. Hybridization was performed on a Tecan HS4800 Pro hybridization station as per the manufacturer's instructions. Hybridized arrays were sequentially washed with $5\times$ SSC-0.1% SDS, $2\times$ SSC-0.1% SDS, $0.1\times$ SSC-0.1% SDS, 0.1% SDS, 95% ethanol, and deionized water. Arrays were directly scanned after the last wash using an Agilent G2505B DNA microarray scanner controlled by Agilent scan control software, with a pixel size of 5 μm on a single-pass sequential line. The laser power was set to 100% for both red and green channels, and photomultiplier tube (PMT) gains were automatically adjusted by the scanner. Slide spots were gridded using GenePix Pro 6 software. Lossless image files were stored for later analysis.

Microarray statistical analysis. Statistical analysis on the microarray data was done as described previously (21). Briefly, the microarray data were analyzed using the Limma module of the Bioconductor package for the R statistical environment (55). The microarray data for each gene were fitted to a linear model, and statistics were generated using the lmFit and eBayes functions. The *P* values displayed were adjusted for multiple testing using the Benjamini and Hochberg method within Limma. The log base 2 of the ratio of output and input signals [$\log(\text{FC})$] was first calculated for each gene, and then the fold change (FC) was calculated using the following formula (where $^{\wedge}$ indicates "to the power of"): if $\log(\text{FC}) \geq 0$, then $\text{FC} = 2^{\wedge} [\log(\text{FC})]$; if $\log(\text{FC}) < 0$, then $\text{FC} = -2^{\wedge} [-\log(\text{FC})]$. The resulting fold change value is a positive output/input ratio if the output signal is greater than the input and a negative input/output ratio if the input signal is greater than the output. Genes with *P* values of <0.05 and a negative log fold change were considered negatively selected (Tables 2 and 3; also, see Table S1 in the supplemental material). Annotations for microarray data were derived from the annotation files in a GenePix array list (GAL) format provided by PFGRC.

Inactivation of negatively selected genes in *Y. pestis*. Several negatively selected genes (Table 3) were inactivated in KIM6+ using an allelic exchange protocol (40) and the suicide plasmid pSB890 (Table 1). Antibiotic selection for pSB890 in S17-1 λ pir or KIM6+ was achieved with 20 μ g/ml tetracycline. Constructs for introducing gene deletions were generated using splicing by overlap extension by PCR (25). Sequences of the PCR primers used to construct deletions are listed in Table S1 in the supplemental material. PCR products containing gene deletions were inserted into pSB890. The resulting suicide plasmids were conjugated from S17-1 λ pir into KIM6+, and following allelic exchange, the presence of the

TABLE 3 *Y. pestis* genes selected for inactivation and analysis of mutants for intracellular survival in macrophages^a

Position in screen	Gene	Symbol	Change (fold)	Predicted function	Bacterial intracellular survival ^b
2	y2631	<i>galU</i>	−2.492	Glucose-1-phosphate uridylyltransferase	—
8	y0364	<i>wecB</i>	−2.034	UDP- <i>N</i> -acetylglucosamine-2-epimerase	— ^c
10	y0265	<i>wecC</i>	−1.789	UDP- <i>N</i> -acetyl-D-mannosaminuronic acid dehydrogenase	— ^c
9	y0157	<i>ptsN</i>	−1.84	Phosphotransferase system enzyme IIA	+
11	y0887	<i>gshA</i>	−1.76	Glutamate-cysteine ligase	+
21	y0144		−1.631	Hypothetical protein	+

^a Genes identified in the negative selection screen that were chosen for inactivation are listed according to each gene's position in the screen results from Table 2.

^b A plus sign indicates ability to survive in macrophages similar to wild-type bacteria, while a minus sign indicates that the mutant was defective for survival in macrophages.

^c The KIM6+ Δ *wecBC* double mutant was tested.

deletion (Table 1) was verified by PCR. To inactivate *galU*, a mutant allele of this gene was obtained from the *Yersinia pseudotuberculosis* strain IP2790c (Table 1) (20). The predicted amino acid sequence of *Y. pseudotuberculosis* IP2790c *galU* is identical to that of *Y. pestis* KIM6+ *galU* (data not shown). IP2790c *galU* contains the *mariner*-based minitransposon *magellan3* (Kn^r) inserted in *galU* (20, 49). PCR was used to amplify from IP2790c *galU::magellan3* chromosomal DNA to make a product consisting of the *galU::magellan3* allele and flanking sequences. The product was inserted into pSB890, and the resulting suicide vector was used to inactivate *galU* in KIM6+ and in KIM5 (Table 1) by allelic exchange. Inactivation of *galU* in KIM6+ and in KIM5 was verified by PCR and selection for Kn^r with 25 μ g/ml kanamycin. A plasmid for complementation of the *galU* mutation in KIM6+ was created using pMMB67EH (Table 1). PCR was used to amplify the *galU* ORF from IP2790c. The PCR product was inserted into pMMB67EH, placing *galU* under the control of the *tac* promoter (20). The resulting plasmid, pGalU (Table 1), or the empty vector was introduced into KIM6+ *galU* via conjugation with S17-1 λ pir. The empty vector pMMB67EH was introduced into KIM6+ by the same method. Selection for pMMB67EH-derived plasmids was achieved using 25 μ g/ml ampicillin for KIM6+ strains and 100 μ g/ml ampicillin for S17-1 λ pir.

Assays for determination of bacterial survival in macrophages. The CFU assay was performed as previously described (22) with the following modifications. BMMs from C57BL/6 mice or Camp^{−/−} (CRAMP-deficient) C57BL/6 mice were seeded in 24-well tissue culture plates at 1.5×10^5 cells/well. Cells were infected with *Y. pestis* at an MOI of 10, and 0.05 mM isopropyl- β -D-thiogalactopyranoside (IPTG) was added to the medium throughout the course of infection to induce expression of the *tac* promoter-driven gene on the bacterial plasmids. Infected BMMs were lysed in 0.1% Triton X-100 in double-distilled H₂O. After incubation for 10 min at 37°C, lysates of the infected BMMs were serially diluted and plated onto HI agar plates supplemented with 25 μ g/ml ampicillin for determination of CFU. The KIM6+ *galU* mutant was examined for increased sensitivity to detergent by incubating the bacteria in 0.1% Triton X-100 for 10 min at 37°C, followed by CFU assay. Statistical analysis of CFU data was performed using one-way analysis of variance and Dunnett's posttest or paired *t* test (GraphPad Prism version 4.0c for Macintosh; GraphPad Software, San Diego, CA). The procedures for detecting intracellular bacteria by a GFP induction assay have been described previously (22).

Polymyxin B and CRAMP MIC assay. The MICs of polymyxin B and CRAMP for *Y. pestis* were determined as follows. Polymyxin B (Sigma) was diluted 2-fold into HI broth supplemented with 0.05 mM IPTG at concentrations from 0.094 to 192 μ M. Murine CRAMP obtained from BACHEM (catalog no. H-6526) or Phoenix Pharmaceuticals, Inc. (catalog no. 072-15), was dissolved in water and diluted 2-fold in HI broth with 0.05 mM IPTG to yield concentrations ranging from 0.31 to 80 μ M. One hundred microliters of each dilution was placed into a well of a 96-well plate. Overnight cultures of *Y. pestis* were diluted in HI broth, and 100 μ l of the dilution was added to the wells at a concentration of $\sim 1 \times 10^6$ CFU/ml. Plates were placed at 28°C or 37°C for 20 h with shaking in a C24

shaker (New Brunswick Scientific) at 200 rpm and then examined for growth and compared to a control grown in HI broth with no polymyxin B or CRAMP. The MIC was estimated as the lowest concentration of polymyxin B or CRAMP at which there was no visible growth of *Y. pestis*.

Purification and analysis of *Y. pestis* LOS by deoxycholate polyacrylamide gel electrophoresis (DOC-PAGE) and silver staining. *Y. pestis* LOS was isolated as previously described (53), with the following modifications. Overnight cultures of *Y. pestis* were grown in HI broth at 28°C with shaking in the presence of the appropriate antibiotics and 100 μ M IPTG. Bacterial cells in 1 ml of culture were harvested by centrifugation. The isolated LOS samples were analyzed by DOC-PAGE (13% gel) as described previously (30). Commercially available LPS from *E. coli* (Sigma) and LPS isolated from IP2790c as above were analyzed in parallel as mobility controls. Silver staining of the gel was performed using a protocol modified from that of Tsai and Frasch (59). The gel was fixed in FIX1 solution (50% methanol, 10% acetic acid) for 30 min and in FIX2 solution (7.5% methanol, 5% acetic acid) for 1 h to overnight, then oxidized in FIX2 containing 0.7% periodic acid for 5 to 15 min, and washed in water 4 times for 10 min each. The gel was then stained in 0.02 M NaOH, $\sim 0.4\%$ NH₄OH and 0.7% silver nitrate for 15 min, washed in water 3 times for 15 min each, and developed in 0.005% citric acid and $\sim 0.019\%$ formaldehyde until strong bands appeared. The developing reaction was stopped in 1% acetic acid. The gel was washed with water and 5% glycerol and photographed. Two independent LOS *Y. pestis* samples were isolated and analyzed.

Analysis of *Y. pestis* lipid A structure by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. The *Y. pestis* LOS that was analyzed by MALDI-TOF mass spectrometry was prepared using a published isolation method (11). Briefly, approximately 10 mg of lyophilized material derived from an overnight culture of each strain was resuspended in 400 μ l of isobutyric acid and 1 M ammonium hydroxide (5:3 vol/vol) and incubated at 100°C for 1 h. After cooling, individual samples were centrifuged for 15 min at $2,000 \times g$, and supernatants were collected and diluted 1:1 (vol/vol) with endotoxin-free water. The samples were subsequently frozen and lyophilized overnight. The resultant powdered material was then washed twice with 1 ml of methanol, and the insoluble lipid A was extracted in 200 μ l of a mixture of chloroform, methanol, and water (3:1:0.25 [vol/vol/vol]). One microliter of this extract was then spotted onto a MALDI plate followed by 1 μ l of norharmane matrix and air-dried. Samples were analyzed on a Bruker AutoFlex Speed (Bruker Daltonics, Billerica, MA), which was calibrated using Agilent Tuning mix (Agilent Technologies, Foster City, CA).

Lysotracker assay. Colocalization of YCVs containing *Y. pestis* with the acidic tracer Lysotracker Red DND-99 (Invitrogen) was determined using J774A.1 cells as previously described (44).

Mouse virulence assay. All experiments were carried out with the approval of the Stony Brook University Institutional Animal Care and Use Committee (IACUC). Female 6- to 8-week-old BALB/c mice were purchased from Taconic Laboratories. The mice were injected via the lateral tail vein with 100 μ l of *Y. pestis* KIM5 or KIM5 *galU* (Table 1) suspended in PBS. The dose was $\sim 1,000$ CFU or ~ 100 times the 50% lethal dose

[LD₅₀] of the KIM5 strain. The mice were monitored three times a day for severe signs of disease. Mice exhibiting severe signs of disease (hunched posture, ruffled fur, and immobility upon stimulation) were considered moribund and were humanely euthanized.

Microarray data accession number. All microarray data described in this study have been submitted to Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/projects/geo/>) under accession no. GSE33982.

RESULTS

Use of a negative selection approach to identify genes important for survival of *Y. pestis* in macrophages. The transposon site hybridization (TraSH) technique (50) was utilized to identify genes important for the survival of *Y. pestis* within murine macrophages. A transposon library was created with ~31,500 *Y. pestis* KIM6+ insertion mutants, which is equal to approximately 7.3× coverage of the genome. A portion of the *Y. pestis* transposon insertion mutant library was used to infect BMMs, and the surviving bacteria (output pool) were recovered (see Materials and Methods). TraSH was used to compare the output pool to a portion of the library that was not subjected to selection (input pool) in order to identify *Y. pestis* genes important for survival in macrophages. The basis of the technique is that each end of the transposon used for mutagenesis contains an outward-reading T7 RNA polymerase promoter. RNAs transcribed from the T7 promoters are complementary to the chromosomal DNA flanking each transposon in the library, and therefore the RNAs can be used as “targets” to identify the approximate position of each transposon insertion in the mutant pool. Differentially labeled targets generated from the output and input pools are competitively hybridized to DNA microarrays containing oligonucleotide probes representing all *Y. pestis* ORFs. Genes important for survival of *Y. pestis* in macrophages are identified by determining the ratio of the signal intensities for the output and input targets hybridizing to a given probe. The values reported for the change (fold) in Table 2 and 3 represent an output/input ratio if the signal intensity for the output is greater than or equal to that of the input and a negative input/output ratio if the input is greater than the output. For example, a negative 3-fold change means that the input/output ratio is 1/3. Therefore, the negative value for the change indicates that the gene represented by the probe was important for survival of *Y. pestis* in macrophages.

The combined results of four TraSH screens using a single library in which BMMs were infected with the *Y. pestis* KIM6+ transposon mutants at an MOI of 2 are presented here. Statistical analysis of the results indicated that the screen identified 525 “negatively selected genes” (negative change; $P < 0.05$) (see Table S2 in the supplemental material). The 11 genes with the largest negative changes are shown in Table 2. The *phoP* gene, as well as *pmrF*, a member of the PhoP-regulated *pmr* operon, had large negative changes. As these genes had been previously characterized as being important for the survival of *Y. pestis* in macrophages (21), these results confirmed the validity of the screen.

Construction and analysis of *Y. pestis* mutants to assess importance of negatively selected genes for survival in macrophages. Several of the top negatively selected genes were chosen to validate their importance for survival of *Y. pestis* in macrophages. As shown in Table 2, three of the top negatively selected genes (*pmrK*, *pmrL*, and *pmrF*) were predicted to be in the *pmr* operon along with *pmrF*. These genes were not chosen for further study, since the phenotypes of the mutants would likely be similar to that

FIG 1 Role of *wecBC* for survival of *Y. pestis* in macrophages, as determined by CFU assay. BMMs were infected with the indicated KIM6+ strains at an MOI of 10. After 15 min of infection, the macrophages were washed to remove nonadherent bacteria and then incubated in medium containing gentamicin to kill extracellular bacteria. At the indicated time points, washed BMMs were lysed with 0.1% Triton X-100, and serial dilutions were plated to enumerate CFU. The data are averaged from three independent experiments, and the error bars indicate standard errors of the means (SEM). For statistical analysis, CFU data were transformed to log₁₀ achieve Gaussian distribution. The asterisk indicates a significant difference between the wild type and Δ *wecBC* mutant at the indicated time point ($P < 0.05$), as determined by paired *t* test.

of a *pmrF* mutant. Three other top genes, y2631 (*galU*), y0364 (*wecB*), and y0365 (*wecC*), were chosen for further study. These genes are predicted to be involved in biosynthesis of LOS (*galU*) and enterobacterial common antigen (ECA) (*wecB* and *wecC*) (Table 2). From the genes in Table 2, two additional genes were selected for study on the basis that they were predicted to be involved in metabolism (y0157; *ptsN*) or resistance to oxidative stress (y0887; *gshA*). The *galU* gene was disrupted by insertion of a transposon (Table 1; also, see Materials and Methods). The *wecB* and *wecC* genes were removed by a single large deletion. The *ptsN* gene (y0157) along with a gene encoding a hypothetical protein (y0158) and *ptsO* (y0159) (see Table S2 in the supplemental material) were also removed by a single large deletion under the assumption that they were contained in an operon (Table 1). An additional gene, y0144 (position 21, −1.631-fold change; see Table S2 in the supplemental material) was chosen for inactivation on the basis that it encodes a hypothetical protein with similarities to an ABC transporter component. A *Y. pestis* mutant lacking y0144 was constructed by deleting a region of the chromosome encoding y0142 to y0145 (Table 1).

A summary of the genes inactivated in KIM6+ and the phenotypes of the corresponding mutants is shown in Table 3. When tested for survival in BMMs by CFU assay and/or by visual inspection of GFP-expressing bacteria using fluorescence microscopy (see Materials and Methods), the *galU* mutant and the *wecBC* mutant exhibited a defect compared to the wild-type KIM6+ control (Table 3; Fig. 1 and 2). The defect of the *wecBC* mutant was mild, only showing a significant decrease in the CFU after 24 h postinfection (Fig. 1). In contrast, the *galU* mutant exhibited much more severe defect in intracellular survival, showing a decrease in CFU as early as 1.5 h postinfection and an approximately 2-log decrease after 24 h (Fig. 2). The KIM6+ *galU* mutant exhibited normal growth rates in HI medium at 28°C and 37°C, indicating that it was specifically defective for survival within macrophage phagosomes. The KIM6+ *galU* mutant did not show increased sensitivity to the detergent conditions (0.1% Triton X-100 in H₂O) used to lyse the BMMs for the CFU assay (data not

FIG 2 Role of *galU* for survival of *Y. pestis* in macrophages, as determined by CFU assay. BMMs were infected with the indicated KIM6+ strains (Table 1) at an MOI of 10 in the presence of IPTG. After 15 min of infection, the macrophages were washed to remove nonadherent bacteria and then incubated in medium containing gentamicin to kill extracellular bacteria. At the indicated time points, washed BMMs were lysed with 0.1% Triton X-100, and serial dilutions were plated to enumerate CFU. The data are averaged from three independent experiments, and the error bars indicate standard errors of the means (SEM). Asterisks indicate a significant difference compared to the data for the 15-min time point for each strain (*, $P < 0.05$; **, $P < 0.01$), as determined by one-way analysis of variance with Dunnett's posttest.

shown). In addition, the MIC of gentamicin for KIM6+ *galU* was identical to that for the parental KIM6+ strain (0.5 μ g/ml) (data not shown), indicating that the mutant was not more sensitive to the antibiotic used in the CFU assay.

Complementation of the *Y. pestis galU* mutant for survival in macrophages. The *galU* gene is predicted to encode a glucose-1-phosphate uridylyltransferase (accession number NP_669934), an enzyme required for the production of UDP-glucose. Genomic sequence analysis indicated that in *Y. pestis galU* is the first gene in a two-gene operon (*galUF*). The product of the *galF* gene (accession number NP_669933) does not appear to have enzymatic activity and may form a complex with GalU to stabilize the reaction balance toward accumulation of UDP-glucose (35).

To demonstrate that the inability of KIM6+ *galU* to survive in BMMs is specifically due to loss of GalU activity, the mutant was complemented with a wild-type copy of *galU* present on an expression vector (pGalU) (Table 1). As shown in Fig. 2, introduction of pGalU into KIM6+ *galU* restored the ability to survive in BMMs, while introduction of the empty vector (pVector) (Table 1) did not. Thus, GalU is required for the survival of *Y. pestis* in murine macrophages.

A *Y. pestis galU* mutant shows reduced resistance to polymyxin B and CRAMP. It was previously reported that inactivation of *galU* in *Vibrio cholerae* (38) or *Proteus mirabilis* (28) causes an increase in susceptibility to antimicrobial peptides. In addition, *Yersinia enterocolitica* serotype O:3 and O:9 mutants lacking the LPS outer core have defects in resistance to the cationic antimicrobial peptide polymyxin B (53, 54), and *Y. pestis* mutants defective for the biosynthesis of the inner heptose core of LOS are sensitive to polymyxin B (2, 29). To determine if *galU* is important for resistance of *Y. pestis* to antimicrobial peptides, the MICs of polymyxin B and the murine cathelicidin CRAMP for KIM6+ and KIM6+ *galU* were determined (see Materials and Methods). KIM6+ *galU*/pVector showed an ~520-fold-increased susceptibility to polymyxin B compared to KIM6+/pVector at both growth temperatures (Table 4). Resistance to polymyxin B was

partially (28°C) or fully (37°C) restored in the complemented strain (KIM6+/pGalU) (Table 4). KIM6+ *galU*/pVector was also more sensitive to CRAMP than KIM6+/pVector, especially at 37°C, and this phenotype could be fully complemented (Table 4).

CRAMP is expressed within murine macrophages and has been shown to play a role in restricting survival of *phoP* mutant *S. Typhimurium* in these cells (47). To determine if CRAMP is important for decreased intracellular survival of a *Y. pestis galU* mutant, BMMs from *Camp*^{-/-} (CRAMP-deficient) C57BL/6 mice were used in the CFU assay. KIM6+ *galU* showed the same magnitude of decrease in intracellular CFU in *Camp*^{-/-} BMMs as in wild-type BMMs (data not shown), indicating that CRAMP is not required for BMMs to kill intracellular *Y. pestis*.

Loss of GalU is associated with an altered LOS and virulence reduction in *Y. pestis*. It has been shown for several Gram-negative bacteria, such as *Vibrio cholerae* (38) and *Klebsiella pneumoniae* (7), that inactivation of *galU* results in an attenuation of virulence. It has been suggested that the attenuation in virulence is directly related to the inability of *galU* mutants to synthesize a complete LPS, since UDP-glucose is an important substrate used in the synthesis of LPS. LPS isolated from *galU* mutants of *V. cholerae*, *P. aeruginosa*, and *K. pneumoniae* migrates faster on SDS-polyacrylamide gels than the LPS isolated from the respective wild-type bacteria (7, 9, 38). To examine if the *galU* mutant of *Y. pestis* has an altered LOS structure, DOC-PAGE and silver staining were used to compare the LOS extracted from KIM6+/pVector, KIM6+ *galU*/pVector, and KIM6+ *galU*/pGalU. Samples of LPS from *Y. pseudotuberculosis* (IP2790c) or *E. coli* (complete core) were analyzed in parallel as mobility controls. The LOS of KIM6+ *galU*/pVector showed slightly increased mobility compared to the LOS of KIM6+/pVector and KIM6+ *galU*/pGalU (Fig. 3, compare lanes 2, 3, and 4). These results suggest that the *Y. pestis galU* mutant has a truncated LOS core.

In *Aeromonas hydrophila*, inactivation of *galU* results in lack of addition of aminoarabinose to lipid A as well as defects in biosynthesis of O antigen or core of LPS (62). To investigate the role of *galU* in addition of aminoarabinose to *Y. pestis* lipid A, the structure of lipid A isolated from the KIM6+ *galU* mutant growing at 26°C or 37°C was analyzed using MALDI-TOF mass spectrometry and compared with that of the parental KIM6+ strain. Analysis of the lipid A portion of LOS showed that the peaks corresponding to the aminoarabinose-containing lipid A species were present in the parental strain but absent in the *galU* mutant at both temperatures (see Fig. S1 and Table S3 in the supplemental material). These

TABLE 4 *Y. pestis galU* is important for resistance to antimicrobial peptides^a

Strain	MIC (μ M) of:			
	Polymyxin B		CRAMP	
	28°C	37°C	28°C	37°C
KIM6+/pVector	96	96	>80	>80
KIM6+ <i>galU</i> /pVector	0.184	0.184	80	10
KIM6+/pGalU	48	96	>80	>80

^a Bacteria were grown for 20 h in HI broth containing either polymyxin B (0.094 to 192 μ M) or murine CRAMP (0.31 to 80 μ M) at the indicated temperatures, and the MIC of each compound was determined as the lowest concentration at which bacterial growth was not seen. Three independent experiments were performed and the results of one representative experiment are shown.

FIG 3 The *Y. pestis galU* mutant has altered LOS. Samples of LOS extracted from KIM6+/pVector, KIM6+ *galU*/pVector and KIM6+ *galU*/pGalU (lanes 2 to 4, respectively) grown in HI broth in the presence of IPTG at 28°C were analyzed by DOC-PAGE and silver staining. Samples of LPS from *Y. pseudotuberculosis* IP2790c (lane 1) and *E. coli* (complete core; lane 5) were analyzed in parallel for comparison.

results demonstrated that the addition of aminoarabinose to lipid A does not take place in LOS of the *Y. pestis galU* mutant.

To determine if *galU* is important for virulence in *Y. pestis*, BALB/c mice were infected with conditionally virulent KIM5 or the isogenic KIM5 *galU* mutant (Table 1) by the intravenous route to initiate septicemic disease. Approximately 1,000 CFU or ~100 times the 50% lethal dose [LD_{50}] of the KIM5 strain was injected into each mouse. Five mice infected with KIM5 succumbed to the disease by day 7, while 5 mice infected with KIM5 *galU* survived to the end of the experiment (day 21) without signs of illness. Therefore, the *Y. pestis galU* mutant is substantially attenuated for virulence in this infection model.

DISCUSSION

The aim of this study was to identify genes that contribute to the intracellular survival of *Y. pestis* in murine macrophages. A transposon library of 31,500 KIM6+ mutants was created, and negative selection was performed using primary murine macrophages. Genes previously shown to be required for survival of *Y. pestis* in macrophages, including *phoP* and *pmrF* (21), were identified in the TraSH screen. In addition, new genes important for survival of *Y. pestis* in macrophages, *galU* and *wecBC*, were identified in the screen (Tables 2 and 3; Fig. 2). Together, these results indicate that the library and the procedures used for negative selection and comparison of input and output pools were sufficient to detect the desired class of mutants. However, not all genes required for survival in macrophages were identified in the screen. For example, the *ugd* and *mgtC* genes, which are required for survival of *Y. pestis* in macrophages (21), were not identified. A likely explanation for the failure to detect such genes is that the 70-mer probes representing these ORFs were not optimally configured to allow reproducible detection of transposon-generated targets. For example, if a gene is inactivated by transposon insertion near the 5' end of the gene but the probe is located at the 3' end of the gene, it is possible that the targets generated from sequences next to the site of insertion will not be long enough to hybridize to the probe. It is also

possible that these genes were not inactivated by transposon insertions in the library. Our library had $7.3\times$ coverage of the genome, but it is possible that the insertion may not have happened at a location appropriate for inactivation of the gene.

Several genes that appeared to be negatively selected in the screen were not required for survival of *Y. pestis* in macrophages when the corresponding KIM6+ mutants were tested by CFU assay (Tables 2 and 3). TraSH reproducibly detects transposon mutants that are 10-fold less abundant in an output pool compared to an input pool (37). Inspection of the results in Table 2 show changes of -2 - to -3 -fold for genes such as *phoP*, *pmrF*, and *galU*. The finding that the screen yielded a number of false positives, combined with the relatively small negative changes for known intracellular survival genes, suggests that the negative selection conditions used could have been more stringent. It is possible that serial rounds of selection (6) would have decreased the detection of false positives.

Two of the mutants constructed, the *galU* mutant and the *wecBC* mutant, showed defects in survival inside macrophages when tested by CFU assay (Table 3; Fig. 1 and 2). The *wecB* and *wecC* genes encode UDP-*N*-acetylglucosamine 2-epimerase (accession number NP_667702.1) and UDP-*N*-acetyl-D-mannosamine dehydrogenase (NP_667703.1), respectively. These enzymes are part of the biosynthetic pathway for ECA. ECA is a glycolipid produced by all *Enterobacteriaceae*, and it consists of trisaccharide repeats attached to a phospholipid anchor or an LPS core. ECA has been reported to play a role in resistance to bile salt as well as in virulence in *Salmonella* (45), organic acid resistance in *E. coli* O157:H7 (3), and swarming motility in *E. coli* K-12 (26). However, the role of ECA in the survival of bacteria inside mammalian host cells has been unclear. Our results suggest a role of ECA in the survival of *Y. pestis* inside macrophages, although its role may be minor, since the phenotype of the *wecBC* was mild. The exact role of ECA in intracellular survival remains to be elucidated.

The *galU* mutant exhibited a severe intracellular survival phenotype. The *galU* mutant was studied further to determine if GalU is important for production of the LOS and antimicrobial peptide resistance in *Y. pestis*. The LOS from the KIM6+ *galU* mutant had slightly increased mobility on DOC-PAGE compared to the wild type KIM6+ LOS (Fig. 3), and the mutant also showed increased susceptibility to polymyxin B and CRAMP (Table 4). The analysis of lipid A isolated from the *galU* mutant by mass spectrometry showed that aminoarabinose modification of lipid A that normally occurs in the wild-type strain is absent in the *galU* mutant (see Fig. S1 and Table S3 in the supplemental material).

The *galU* gene is predicted to encode a glucose-1-phosphate uridylyltransferase. This enzyme catalyzes the addition of UMP to glucose-1-phosphate yielding UDP-glucose and pyrophosphate. UDP-glucose is used as a substrate by a glucosyltransferase to add a single glucose residue to the LOS inner core of *Y. pestis*. The LOS core of *Y. pestis* contains a single glucose (Glc) attached laterally to heptose I (Hep-I) (24, 29). The absence of UDP-glucose in the *galU* mutant would prevent the addition of this Glc on Hep-I. Anisimov et al. have shown that a *Y. pestis* mutant (*waaE*) defective for the glucosyltransferase that is responsible for addition of this Glc produces a "deep rough" LOS that terminates with Hep-II (2). Therefore, the *galU* mutant and the *waaE* mutant could have the same form of truncated LOS, although it is possible that WaaE could use another NDP-sugar as a substrate to add to this site. We

also cannot rule out the possibility that the LOS core of the *galU* mutant does not contain a significant change compared to wild type. Disruption of another pathway for synthesizing UDP-glucose (*pgmA*, phosphoglucosyltransferase) in *Y. pestis* did not lead to alterations in the LOS core even though the *pgmA* mutant had dramatically increased sensitivity to polymyxin B (13). Further analyses are necessary to understand the composition and the structure of the LOS core in the *galU* mutant.

In addition to its role in LOS core synthesis, UDP-glucose is required for the synthesis of UDP-glucuronic acid, which serves as a precursor for aminoarabinose, which in turn modifies lipid A of LOS. It is thus expected that aminoarabinose synthesis is reduced or absent in the *galU* mutant. Consistent with this idea, we observed a lack of aminoarabinose modification in lipid A isolated from the *galU* mutant (see Fig. S1 in the supplemental material). Therefore, the effects of the *galU* mutant are likely pleiotropic, resulting in both truncated LOS core and lack of lipid A modification by aminoarabinose. Similar pleiotropic effects of *galU* inactivation have been reported previously for *Aeromonas hydrophila* (62).

The complete LOS core is important for resistance of *Y. pestis* to antimicrobial peptides, since the inactivation of *waaE* in *Y. pestis* is associated with increased susceptibility to polymyxin B (2). Aminoarabinose modification of lipid A also increases resistance to cationic antimicrobial peptides. We showed previously that *ugd* (which encodes a UDP glucose dehydrogenase that converts UDP-glucose to UDP-glucuronic acid [12]) and genes in the *pmr* operon (the products of which are responsible for the aminoarabinose modification of LOS [12]) are important for survival of *Y. pestis* in macrophages (21). Taking all these data together, we believe that the lack of a complete LOS core and the absence of aminoarabinose modification in lipid A both contribute to the decreased resistance of the *Y. pestis galU* mutant to antimicrobial peptides found in phagosomes of macrophages. In the *galU* mutants of other pathogenic bacteria, including *V. cholerae*, *P. aeruginosa*, and *K. pneumoniae*, evidence for changes in LPS core structure has been shown, but the possibility that lipid A in these mutants lacks aminoarabinose has not been discussed (7, 9, 38).

It is interesting that *galU* has been reported to be essential for growth of *Y. enterocolitica* serotype O:9 in laboratory media (53). We found that a *galU* mutant of *Y. pestis* grew normally in laboratory media, and similar results (data not shown) were obtained for a *Y. pseudotuberculosis galU* mutant (IP2790c *galU*) (Table 1). The *Y. pseudotuberculosis galU* mutant was also defective for survival in macrophages (data not shown). The reason for the difference is unclear, but there may be a distinct role for *galU* in *Y. enterocolitica*.

Previous studies reported that resistance of *Y. pestis* KIM6+ to antimicrobial peptides is temperature dependent, with increased resistance to polymyxin B occurring with bacteria grown at 21°C compared to 37°C due to increased acylation of lipid A at 21°C compared to 37°C (46). In our experiments, KIM6+ cells tested at 28°C and those tested at 37°C showed similar levels of resistance to polymyxin B (Table 4). This may be because the temperature-dependent acylation that leads to increased resistance to polymyxin B takes place only partially at 28°C. Alternatively, since the bacteria used in our experiments were grown overnight at 28°C prior to the MIC test, even the bacteria tested at 37°C may have had lipid A characteristics of 28°C initially, leading to similar levels of polymyxin B resistance at both temperatures.

Recently, it was shown that the Pla protease can proteolytically cleave cationic α -helical peptides such as LL-37, but only under conditions in which the F1 capsule is not expressed in *Y. pestis* (18). This raises the possibility that the Pla protease contributes to antimicrobial resistance in KIM6+, particularly when the bacteria are grown at 28°C, a condition that would allow Pla expression but not lead to high production of F1. Moreover, the possibility exists that the modified LOS in the *galU* mutant might reduce Pla activity, resulting in increased sensitivity to antimicrobial peptides. Anisimov et al. have shown that Pla activity is reduced, but not abolished, in a *waaE* mutant (2); thus, it is conceivable that reduced Pla activity contributes to increased sensitivity of the mutant to cationic α -helical peptide CRAMP at 28°C (Table 4). However, the *galU* mutant also shows increased sensitivity to CRAMP at 37°C (Table 4), a condition that should allow F1 to inhibit Pla, and therefore these results argue that the LOS core and the aminoarabinose-modified lipid A itself are important for resistance to CRAMP. Furthermore, because Pla does not affect resistance to polymyxin B, possibly due to the cyclic structure of polymyxin B preventing proteolysis (18), the increased sensitivity of the mutant to this antibiotic at either temperature can be taken as evidence that the LOS protects *Y. pestis* from this antibiotic.

It is interesting that while the growth of the *Y. pestis galU* mutant to polymyxin B was inhibited at a concentration as low as 0.184 μ M (~500-fold increase compared to wild type), the sensitivity of the *galU* mutant to CRAMP did not seem to increase to the same extent. Similar results were reported recently by Guo et al., who showed that the induced expression of the *pgbP* (also known as *pmr*) operon in *Y. pestis* conferred resistance to polymyxin B but not to human cathelicidin LL-37 (23). The reason for this phenotype is unclear, but it appears that the effects of changes in LOS and/or bacterial surface characteristics on *Y. pestis* sensitivity to antimicrobial peptides are different for cyclic (polymyxin B) versus α -helical (cathelicidin) peptides.

Although CRAMP effectively killed the *Y. pestis galU* mutant *in vitro*, we were not able to establish an important role for CRAMP in killing of the mutant in phagosomes. BMMs from Camp^{-/-} mice killed the *galU* mutant as effectively as BMMs from wild-type mice. It is likely that other antimicrobial peptides expressed in macrophages (e.g., defensins) function to kill the *galU* mutant in the absence of CRAMP.

Because a neutral to slightly alkaline pH is optimal for the activity of antimicrobial peptides such as defensins, the lack of YCV acidification imposes on intracellular *Y. pestis* a requirement for highly effective mechanisms of resistance to antimicrobial peptides. As shown by the Lysotracker assay, the *galU* mutant was able to inhibit phagosome acidification for the short period of time that it remained viable in phagosomes (data not shown). This lack of YCV acidification may act synergistically with antimicrobial peptides to effectively kill the intracellular *galU* mutant.

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