JOURNAL OF BACTERIOLOGY, Feb. 2006, p. 1381–1388 Vol. 188, No. 4
0021-9193/06/$08.00+0 doi:10.1128/JB.188.4.1381–1388.2006

Characterization of Late Acyltransferase Genes of *Yersinia pestis* and Their Role in Temperature-Dependent Lipid A Variation

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Received 14 October 2005/Accepted 27 November 2005

Yersinia pestis **is an important human pathogen that is maintained in flea-rodent enzootic cycles in many parts of the world. During its life cycle, *Y. pestis* senses host-specific environmental cues such as temperature and regulates gene expression appropriately to adapt to the insect or mammalian host. For example, *Y. pestis* synthesizes different forms of lipid A when grown at temperatures corresponding to the in vivo environments of the mammalian host and the flea vector. At 37°C, tetra-acylated lipid A is the major form; but at 26°C or below, hexa-acylated lipid A predominates. In this study, we show that the *Y. pestis msbB* (*lpxM*) and *lpxP* homologs encode the acyltransferases that add C_{12} and $C_{16:1}$ groups, respectively, to lipid IV_A to generate the hexa-acylated form, and that their expression is upregulated at 21°C in vitro and in the flea midgut. A *Y. pestis* Δ*msbB* Δ*lpxP* double mutant that did not produce hexa-acylated lipid A was more sensitive to cecropin A, but not to polymyxin B. This mutant was able to infect and block fleas as well as the parental wild-type strain, indicating that the low-temperature-dependent change to hexa-acylated lipid A synthesis is not required for survival in the flea gut.**

The cell envelope of gram-negative bacteria includes two lipid bilayers, an inner membrane composed primarily of phospholipids, and an outer membrane containing primarily phospholipids in the inner leaflet and lipopolysaccharide (LPS) in the outer leaflet. Tight stacking of the long-chain fatty acids of lipid A, the hydrophobic anchor of LPS, creates a permeability barrier against toxic compounds encountered in the environment and the host (25). In *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, the final steps of lipid A synthesis occur in the inner membrane, where two acyl groups are added to the tetra-acylated Kdo-lipid IV_A before the mature hexa-acylated lipid A is exported to the outer membrane. At normal growth temperatures, the late acyltransferases HtrB (LpxL) and MsbB (LpxM) consecutively add lauroyl (C_{12}) and myristoyl (C_{14}) groups to the tetra-acylated intermediate (7, 8, 31). At 12°C, however, the cold-temperature-specific late acyltransferase LpxP acts instead of LpxL to add palmitoleate ($C_{16:1}$) (4). Mutation of *htrB* and *msbB* leads to growth defects and hypersensitivity to rifampin and vancomycin in *E. coli*, and to decreased virulence and resistance to macrophage killing in *E. coli* and *S. enterica* (37).

Yersinia pestis, the zoonotic agent of bubonic and pneumonic plague in humans, is primarily a pathogen of rodents that is transmitted by fleas (27). Upon transmission to a mammalian host and an increase in temperature to 37°C, *Y. pestis* upregulates the expression of virulence factors that aid colonization and invasion. Conversely, after *Y. pestis* is taken up in a blood meal by a flea vector, the decrease in temperature results in downregulation of virulence factors and activation of genes and gene products important for survival in the flea midgut and transmission to a new mammalian host (5, 11, 17, 19, 24a).

One example of a temperature-dependent phenotype in *Y. pestis* is lipid A structural variation (23, 24, 28). At the mammalian host temperature of 37°C, *Y. pestis* produces and exports primarily tetra-acylated lipid A to the outer membrane. However, at a temperature (21°C) typical of the flea vector, *Y. pestis* generates primarily hexa-acylated lipid A modified with C_{12} and $C_{16:1}$, a form that resembles lipid A produced by *E. coli* at 12°C (4). The increased acylation of lipid A at 21°C correlates with resistance of *Y. pestis* to cationic antimicrobial peptides (CAMPs) (28). These observations led us to hypothesize that expression of the *Y. pestis* late acyltransferases is temperature controlled, and that the shift from tetra-acylated lipid A at 37°C to hexa-acylated lipid A at 21°C is required for CAMP resistance and for the ability to survive in the flea digestive tract.

To test these hypotheses, we identified *Y. pestis* homologs of the *msbB* and *lpxP* acyltransferase genes and characterized their role in the temperature-dependent change from tetra-acylated to hexa-acylated lipid A. We also examined the role of these late acyltransferases and lipid A variation on outer membrane permeability, resistance to CAMPs, and the ability to colonize and produce a transmissible infection in fleas.

MATERIALS AND METHODS

Protein sequences and phylogenetic analysis. Predicted *Y. pestis* homologs of the *E. coli* and *Salmonella* MsbB, LpxP, and HtrB acyltransferases were identified in the *Y. pestis* genome sequence by means of a TBlastN (protein versus. translated DNA) search of the *Y. pestis* CO92 genome (http://www.sanger.ac.uk/Projects/Y_pestis/). The open reading frame numbers of the *Y. pestis msbB* and *lpxP* homologs are YPO2063 and YPO3632, respectively (26). Phylogenetic anal-

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TABLE 1. Bacteria and plasmids

Strain or plasmid	Description	Source or reference
Strains		
Y. pestis KIM6+	pYV-negative derivative of strain KIM	35
Y. pestis Δ*msbB*	KIM6+ with a 391-bp deletion in *msbB*	This study
Y. pestis Δ*lpxP*	KIM6+ with a 276-bp deletion in *lpxP*	This study
Y. pestis Δ*msbB* Δ*lpxP*	KIM6+ double deletion mutant	This study
Y. pestis Δ*msbB*(pLG*msbB*)	Complemented *msbB* mutant	This study
Y. pestis Δ*msbB* Δ*lpxP*(pLG*lpxP*)	Double mutant complemented with *lpxP*	This study
E. coli TOP-10	Plasmid host strain	Invitrogen
E. coli S17-1	*recA* λ*pir*; host for suicide vector pCVD442	29
Plasmids		
pCR 2.1-TOPO	Cloning vector	Invitrogen
pLG338	Low-copy-number vector used for complementation	32
pCVD442	Suicide vector used for allelic exchange	10
TOPO-*msbB*	1.6-kb *msbB* locus cloned into pCR 2.1-TOPO	This study
TOPO-*lpxP*	1.2-kb *lpxP* locus cloned into pCR 2.1-TOPO	This study
TOPO-Δ*msbB*	1.2-kb *msbB* deletion allele cloned in pCR 2.1-TOPO	This study
TOPO-Δ*lpxP*	1.0-kb *lpxP* deletion allele cloned into pCR 2.1-TOPO	This study
pCVD442-Δ*msbB*	1.2-kb *msbB* deletion allele cloned into pCVD442	This study
pCVD442-Δ*lpxP*	1.0-kb *lpxP* deletion allele cloned into pCVD442	This study
pLG*msbB*	1.4-kb wild-type *msbB* locus cloned into pLG338	This study
pLG*lpxP*	1.7-kb wild-type *lpxP* locus cloned into pLG338	This study

ysis was performed using the neighbor-joining and bootstrap programs of MacVector version 7.2.3 (Accelrys, San Diego, CA).

Bacterial strains and mutagenesis. The bacterial strains and plasmids used are listed in Table 1. To create the acyltransferase mutants, the predicted *Y. pestis msbB* and *lpxP* genes were amplified by PCR, gel purified, and cloned into the pCR-2.1 TOPO vector (Invitrogen, Carlsbad, CA). The resulting recombinant plasmids were used to transform *E. coli* TOP-10 (Invitrogen) by electroporation. Internal 391-bp and 276-bp deletions in the *msbB* and *lpxP* homologs were generated by inverse PCR of the recombinant TOPO 2.1 plasmids (38), followed by blunt-end ligation to yield a TOPO 2.1-Δ*msbB or* -Δ*lpxP* product. The mutated alleles were subcloned into the suicide vector pCVD442 and electroporated into *E. coli* S-17. The plasmids were then introduced into *Y. pestis* KIM6+ by conjugation, and clones in which allelic exchange had occurred were selected (10).

A *Y. pestis* Δ*msbB* Δ*lpxP* double mutant was generated by allelic exchange of the Δ*lpxP* allele into the *Y. pestis* Δ*msbB* strain. DNA sequencing confirmed the deletions. Complementation plasmids were generated by PCR amplification of the *msbB* and *lpxP* open reading frames and promoter-operator regions using primers flanked with KpnI and EcoRI sites. The complementation vector pLG338 and the *Y. pestis msbB* and *lpxP* PCR fragments were digested with KpnI and EcoRI, and the products of the reactions were gel-purified and ligated to generate pLG*msbB* and pLG*lpxP*, which were introduced by electroporation into the *Y. pestis* Δ*msbB* and Δ*msbB* Δ*lpxP* mutant strains, respectively. Primer sequences used for cloning, inverse PCR, complementation, and sequencing are listed in Table 2.

Lipid A isolation and structural analysis. *Y. pestis* strains were grown in Luria broth (LB), pH 7.4, at 21°C without aeration and harvested in late exponential phase (optical density at 600 nm [OD_{600}] ~1.0) and analyzed as described (28). LPS was purified by Mg^{2+}-ethanol precipitation (9). Lipid A was isolated by mild acid hydrolysis of LPS in 1% sodium dodecyl sulfate at pH 4.5 (3). Negative-ion spectra were acquired from a delayed extraction matrix-assisted laser desorption ionization-time-of-flight (DE-MALDI-TOF) mass spectrometer (Biflex III, Bruker Daltonics; Billerica, MA) (12). Briefly, lipid A was dissolved in 10 μl of a mixture of 5-chloro-2-mercaptobenzothiazole (20 mg/ml) in chloroform/methanol 1:1 (vol/vol), and 1-μl samples were analyzed by MALDI-TOF MS. Acyl groups from LPS samples were derivatized to fatty methyl esters with 2 M methanolic HCl at 90°C for 18 h and were identified and quantified by gas chromatography (GC) using an HP 5890 series II with a 7673 autoinjector (Hewlett Packard; Palo Alto, CA) (31). MS and GC analyses were performed on a minimum of two independent samples for each bacterial strain.

RNA isolation. For RNA extraction, bacteria were grown in LB, pH 7.4, at 21°C with aeration and harvested at an OD_{600} of 0.6. RNA for real-time PCR analysis was obtained using the RNeasy minikit (QIAGEN; Valencia, CA). Bacteria in 2 ml of culture were harvested by centrifugation for 1 min at 16,000 × *g*, resuspended in 1.0 ml ice-cold RLT buffer (QIAGEN), flash-frozen in liquid nitrogen, and stored at −80°C. The suspensions were transferred to chilled tubes containing lysing matrix B (Q-BIOgene; Carlsbad, CA) and the bacteria were disrupted by agitation for 30 seconds at speed 6.0 in a FastPrep device (Q-BIOgene). Lysates were then mixed with 0.4 ml of 100% ethanol, and the total RNA was isolated by means of RNeasy minicolumns (QIAGEN). Contaminating DNA in RNA samples was removed by using the DNA-free DNase kit (Ambion, Austin, TX).

Total RNA was also extracted from samples of 50 infected fleas that had been placed in lysing matrix B tubes (Q-BIOgene), flash frozen in liquid nitrogen, and stored at −80°C; 1 ml of ice-cold RLT buffer mix was added to each tube containing the frozen, infected fleas. Release of bacteria from the flea midguts and cell disruption was accomplished by two consecutive 30 sec FastPrep cycles at speed 6.0; tubes were cooled on ice for 1 min after each agitation cycle. Lysates were passed through a QIAshredder column (QIAGEN) to remove particulate matter and RNA was isolated and DNase-treated as described above.

Real-time quantitative reverse transcription-PCR (TaqMan). Total RNA samples obtained from three biological replicates for each condition tested were diluted to 1 to 10 μg/ml after DNase treatment and A_{260} quantitation. Reverse transcription and TaqMan PCR analysis of *Y. pestis* transcript levels were performed in triplicate for sample as previously described (6) using an ABI 7700 thermocycler (Applied Biosystems; Foster City, CA) and primer-probe sets listed in Table 2. The relative quantities of *msbB*, *lpxP*, and *ymt* mRNA were normalized to the amount of *proS* mRNA present in the samples.

Antimicrobial susceptibility assays. Susceptibility to polymyxin B (ICN Biochemicals, Aurora, OH), deoxycholate, rifampin, vancomycin, and cecropin A (Sigma-Aldrich, St. Louis, MO) was determined by bactericidal assays. *Y. pestis* KIM6+, Δ*msbB*, Δ*lpxP*, and Δ*msbB*Δ*lpxP* strains were grown in LB supplemented with 1 mM $MgCl_2$ at 21°C. Overnight cultures were diluted in Mueller Hinton broth to ~5×10^5 CFU/ml. Antimicrobial agents were dissolved in 0.1% bovine serum albumin and 0.01% acetic acid, and serial dilutions were prepared. In a 96-well plate, 11 μl of each dilution was added to 100 μl of bacterial suspension and incubated at 21°C for 2 h. Samples were then serially diluted, plated on LB agar plates, and CFU were counted after 48 h incubation at 37°C. Percent survival was calculated relative to CFU counts from wells with no added antimicrobial agent. All assays were performed in triplicate.

Flea infections. *Xenopsylla cheopis* fleas were infected with *Y. pestis* KIM6+ or *Y. pestis* KIM6+ Δ*msbB* Δ*lpxP* by allowing them to feed on heparinized mouse blood containing approximately 5.0×10^8 bacteria per ml using a membrane feeder apparatus (16). Fleas that took the infected blood meal were maintained at 21°C and 75% relative humidity, and fed twice weekly on uninfected mice. At three different times after infection, samples of 50 fleas were collected for RNA extraction and reverse transcription (RT)-PCR analysis. Additional samples of 50 female and 50 male fleas were monitored over a 4-week period for proventricular blockage, indicated by the presence of fresh blood in the esophagus and absence of fresh blood in the midgut after feeding (16). At 1 h and 1, 7, and 24

TABLE 2. Sequences of primers and probes used in this study

Target gene	Use	Sequence (5′ to 3′)
msbB	Cloning	TTTGGATGAACCAGCAAGCG
		GATAGGCAGAGGAGTAAAGCGTCC
	Deletion	GGATCACGGAATTTGGGTGGAATATAAGCGAGCGCCGC
		GCTTTACATTTCGGTGGTCGGATCCATGCCCGCG
	Complementation	GCGCGGTACCCGCAGTTACAACTGGATTAGCAGTG
		GCGCGAATTCCTATCACTAGCGGGCCTTTTACC
	Taq Man primers	ACCCAAATTCCGTGATCCTTTA
		ACGGGCGCTTTTAGCAAAT
	TaqMan probe[a]	CTGGCATAGGGCGTCTCGCTGG
	Sequencing	AGAAAACCTTCCTCCATCC
lpxP	Cloning	TGCGTCAATTTTCGTCCTCAC
		TGGCGGGTATCAGTAATGCTAAG
	Deletion	CCGGAGACGGAAAACCAGCGCTGTATCCGGGTATCGGACC
		CCCGCGCGGCAGCGTTTTTGCACCACTGTTCGCCG
	Complementation	GCGCGGTACCGTTTTCTTTCAGGTAACGGAACGG
		GCGCGAATTCTGTGATTCCTACGACCCCAACG
	TaqMan primers	CACCACTGTTCGCCGTAGAA
		AACGGGCGAGCATAAAGGT
	TaqMan probe	ATGCGGCAACCACCAGCGG
	Sequencing	CTTGAGCAGACTATCATCGG
ymt	TaqMan primers	CACCAATCAACGATACAAGAATGAC
		TGTCCACCAACAAGAGCTTCAG
	TaqMan probe	TGGAATCACACAAAAATAATGGCCTCAGATG
proS	TaqMan primers	ACGCGCACCGGCTACA
		CTCGGCGATGGTTTTTGC
	TaqMan probe	AGAGCTGCGAATCGTTGACACCCC

[a] TaqMan probes contain 5′-6-carboxyfluorescein reporter and 3′-6-carboxy-tetramethyl-rhodamine quencher.

days after infection, samples of 20 female fleas were collected and stored at −70°C. The infection rate and bacterial load in these fleas were determined by CFU count (16).

RESULTS

Identification of lipid A late acyltransferases in *Y. pestis*. Two potential homologs of lipid A late acyltransferases were identified in the *Y. pestis* genome (26). The predicted product of one (YPO2063) has 65% amino acid identity and 79% similarity to *E. coli* MsbB (LpxM), and the second (YPO3632) has 67% identity and 80% similarity to *E. coli* LpxP (Fig. 1). No obvious *Y. pestis* HtrB (LpxL) homolog was detected, although the predicted product of YPO3632 also shared 59% identity and 78% similarity, to the *E. coli* HtrB (Fig. 1). No other *Y. pestis* open reading frames were similar to the three *E. coli* late acyltransferase genes. *Y. pestis* mutant strains deleted of the *msbB* and *lpxP* homologs were used to determine whether these genes were responsible for the temperature-dependent change from tetra-acylated to hexa-acylated lipid A.

Role of the *Y. pestis* MsbB and LpxP homologs in temperature-dependent lipid A variation. Lipid A produced by the *Y. pestis* KIM6+, Δ*msbB*, and Δ*msbB* Δ*lpxP* strains grown at 21°C was purified for structural analysis by MALDI-TOF MS and gas chromatography. As previously reported (28), lipid A from *Y. pestis* KIM6+ grown at 21°C was predominantly hexa-acylated (*m/z* 1824), containing four 3-OH-C_{14}, one C_{12} and one $C_{16:1}$ (Fig. 2A). Other identifiable minor lipid A species produced at 21°C included previously described tetra-acylated

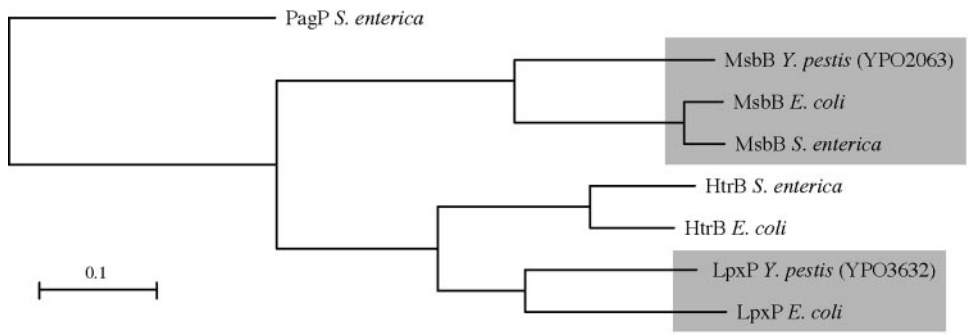

FIG. 1. Cladogram comparing the two *Y. pestis* late acyltransferases to HtrB, MsbB, and LpxP of *E. coli* and *S. enterica*. *Salmonella* PagP, an unrelated lipid A acyltransferase, was used to root the tree. The scale bar indicates the calculated evolutionary distances.

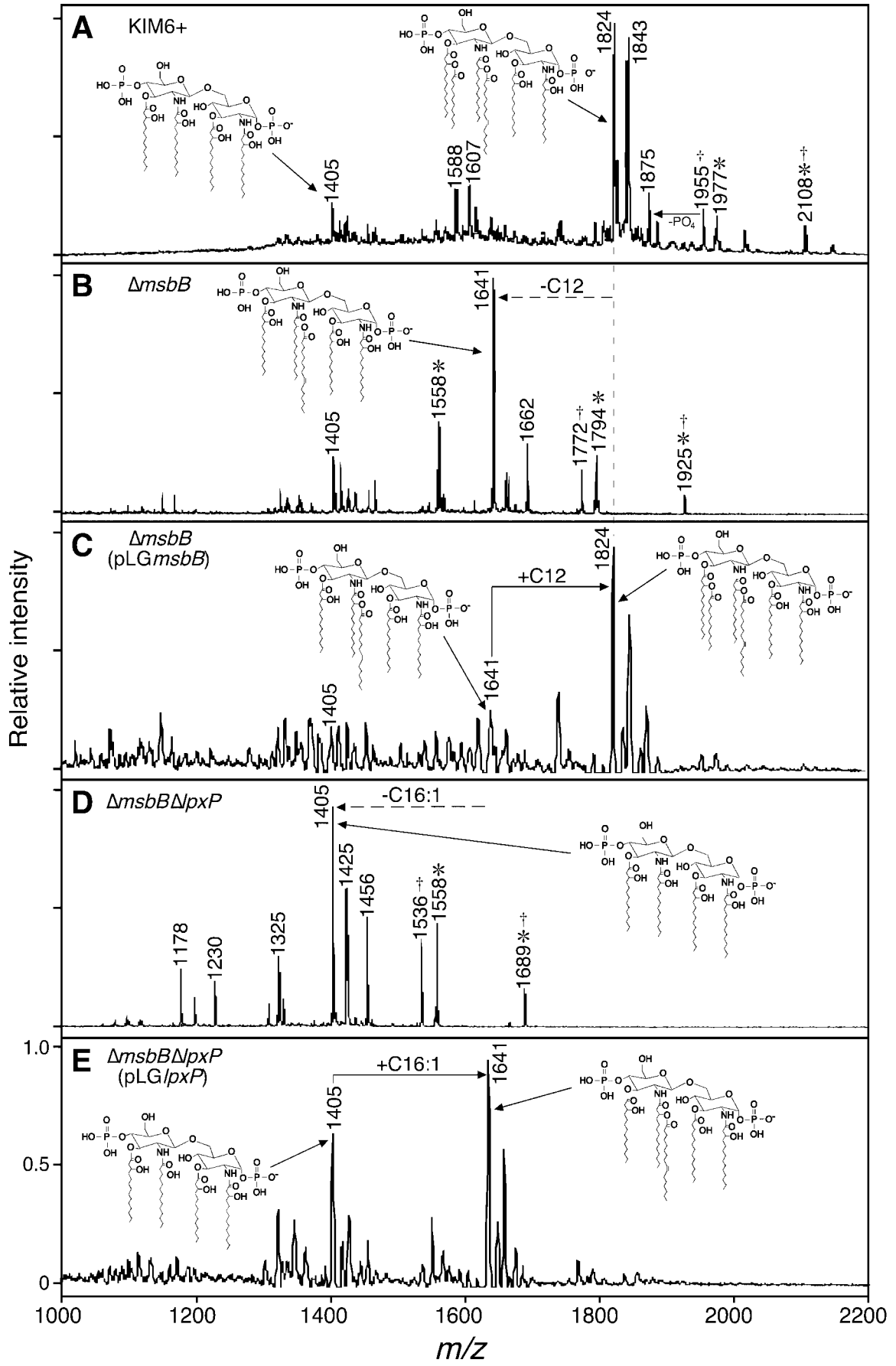
A
KIM6+
1824
1843
1405
1588
1607
1875
1955 -†
1977*
2108*-†
-PO4
B
ΔmsbB
1641
-C12
1558*
1405
1662
1772-†
1794*
1925*-†
C
ΔmsbB
(pLGmsbB)
+C12
1824
1641
1405
D
ΔmsbBΔlpxP
1405
-C16:1
1425
1456
1536-†
1558*
1178
1230
1325
1689*-†
E
ΔmsbBΔlpxP
(pLGlpxP)
+C16:1
1641
1405
Relative intensity
1.0
0.5
0
1000
1200
1400
1600
1800
2000
2200
m/z

TABLE 3. Relative amount of different acyl groups (expressed as a percentage of the total) in lipid A of *Y. pestis* strains grown at 21°C

Fatty acid	Relative amt (% of total)				
	Y. pestis KIM6+	*Y. pestis* Δ*msbB*	*Y. pestis* Δ*msbB* (pLG*msbB*)	*Y. pestis* Δ*msbB* Δ*lpxP*	*Y. pestis* Δ*msbB* Δ*lpxP* (pLG*lpxP*)
C_{10}	4.8	9.0	9.0	15.7	9.6
C_{12}	17.3	0.0	16.6	0.0	0.0
C_{14}	0.7	1.5	0.6	0.5	0.7
2-OH-C_{14}	1.6	1.7	1.5	2.4	1.7
3-OH-C_{14}	51.0	53.5	47.1	79.2	55.3
C_{16}	1.5	5.4	1.3	2.1	2.0
$C_{16:1}$	23.1	28.9	23.9	0.0	30.7
Total	100	100	100	99.9	100

(*m*/*z* 1405), penta-acylated (*m*/*z* 1588), and C_{10}-containing hepta-acylated molecules (*m*/*z* 1977) (28). The hexa-acylated and hepta-acylated species were further modified by aminoarabinose addition (*m*/*z* 1955 and 2108, respectively [Fig. 2A]) (28). Fatty acid quantitation by GC corroborated the interpretation of the MALDI-TOF spectra (Table 3).

The MS spectra of the two mutant strains were different from that of the parental KIM6+ strain and from each other. Notably, lipid A from *Y. pestis* Δ*msbB* grown at 21°C contained no detectable C_{12} (0% compared to 17.3% for the parental strain; Fig. 2B, Table 3). Instead, a new predominant peak (*m*/*z* 1641) corresponding to a penta-acylated species that contained four 3-OH-C_{14} and one $C_{16:1}$ was observed. Minor peaks corresponding to lipid A forms containing C_{10} (*m*/*z* 1558, 1794) and aminoarabinose (*m*/*z* 1772, 1925) modification were not affected by the *msbB* mutation (Fig. 2B). When the *Y. pestis* Δ*msbB* mutant was complemented with a wild-type *msbB* gene controlled by its native promoter, the normal level of C_{12} addition to lipid A was restored and the wild-type hexa-acylated lipid A (*m*/*z* 1824) was again the predominant form (Fig. 2C, Table 3).

Lipid A produced by the *Y. pestis* Δ*msbB*Δ*lpxP* strain grown at 21°C lacked both C_{12} and $C_{16:1}$ and was primarily the tetra-acylated species containing four 3-OH-C_{14} (Fig. 2D). This is also the predominant form produced by the wild-type *Y. pestis* KIM6+ parent strain at 37°C (28). GC analysis confirmed that lipid A produced by the *Y. pestis* Δ*msbB* Δ*lpxP* strain contained <0.1% of either C_{12} or $C_{16:1}$ (Table 3). MS peaks consistent with C_{10} (*m*/*z* 1558), aminoarabinose (*m*/*z* 1536), or both modifications (*m*/*z* 1689) were also detected (Fig. 2D). As predicted, *Y. pestis* Δ*msbB* Δ*lpxP* complemented with a wild-type copy of *lpxP* synthesized primarily penta-acylated lipid A (*m*/*z* 1641) that contained 30.7% $C_{16:1}$, comparable to the wild-type level (Fig. 2E, Table 3). These results showed that the *Y. pestis* *msbB* and *lpxP* homologs add C_{12} and $C_{16:1}$, respectively, and account for the production of hexa-acylated lipid A from tetra-acylated lipid A when *Y. pestis* is shifted from 37 to 21°C (28).

In vitro and in vivo transcriptional regulation of *msbB* and *lpxP*. The previous observation that *Y. pestis* produces primarily tetra-acylated lipid A at 37°C and hexa-acylated lipid A at 21°C suggested that expression of late acyltransferase genes of *Y. pestis* is regulated by temperature (28). To test this hypothesis, transcription of *msbB* and *lpxP* relative to the housekeeping gene *proS* in bacteria grown at 37 and 21°C was analyzed by real-time quantitative RT-PCR. As a positive control, relative expression of *ymt*, a gene known to be regulated by temperature at the transcriptional level (11), was also assayed. Expression of the *msbB*, *lpxP*, and *ymt* genes was more than twofold higher in bacteria grown at 21°C (Fig. 3).

To determine if the *Y. pestis* late acyltransferases were expressed in the flea, transcript levels of *msbB*, *lpxP*, and *ymt* were determined from total RNA extracted from infected fleas. Transcription of all three genes was threefold higher in infected fleas than in cultures grown at 37°C ($P < 0.05$, Fig. 3).

Role of *Y. pestis msbB* and *lpxP* homologs in resistance to antimicrobial agents. Loss of C_{14} and $C_{16:1}$ modification of *E. coli* lipid A results in increased sensitivity to rifampin and vancomycin (37). We compared the susceptibility of the *Y. pestis* KIM6+, Δ*msbB*, and Δ*msbB* ΔlpxP strains to these antibiotics and to the CAMPs polymyxin B and cecropin A in bactericidal assays. Lack of C_{12} and/or $C_{16:1}$ modification of lipid A modestly increased the susceptibility of *Y. pestis* to cecropin A and deoxycholate (Fig. 4), but not to polymyxin B, vancomycin, or rifampin (data not shown).

In vivo phenotype of a *Y. pestis* Δ*msbB* Δ*lpxP* mutant. Fleas were infected with *Y. pestis* KIM6+ or with the Δ*msbB* Δ*lpxP* strain to determine if the ability to make hexa-acylated lipid A conferred protection in the flea gut environment. The *Y. pestis* Δ*msbB* Δ*lpxP* mutant was able to infect fleas as well as the *Y. pestis* KIM6+ parental strain (Fig. 5). The bacterial load in fleas infected with the two strains did not differ significantly during a 28-day period after the infectious blood meal, reaching a maximum of $\sim 10^5$ CFU/flea for the mutant and $\sim 2.2 \times 10^5$ CFU/flea for the parental strain. Furthermore, the percentage of fleas that developed proventricular blockage after infection with *Y. pestis* KIM6+ or *Y. pestis* Δ*msbB* Δ*lpxP* did not differ significantly (Fig. 5C).

DISCUSSION

The pathogenic yersiniae differ from *E. coli*, *Salmonella*, and other *Enterobacteriaceae* that have been examined in temperature-dependent lipid A acylation. At 37°C, *Y. pestis* synthesizes tetra-acylated lipid A and smaller amounts of penta-acylated forms modified with C_{10} or C_{12} acyl groups, and LPS from cells grown at 37°C has limited ability to induce tumor necrosis factor alpha (TNF-α) secretion from human and murine monocytes. At temperatures below 26°C, however, *Y. pestis* lipid A is hexa-acylated and more endotoxic, resembling the lipid A of other *Enterobacteriaceae* produced at temperatures above 30°C (23, 24, 28). The first aim of this study was to identify the genes in *Y. pestis* responsible for the temperature-dependent generation of hexa-acylated lipid A at 21°C, and to

FIG. 2. Negative-ion MALDI-TOF MS spectra of lipid A isolated from *Y. pestis* KIM6+ (A), *Y. pestis* Δ*msbB* (B), *Y. pestis* Δ*msbB* (pLG*msbB*) (C), *Y. pestis* Δ*msbB* Δ*lpxP* (D), and *Y. pestis* Δ*msbB* Δ*lpxP*(pLG*lpxP*) (E) grown at 21°C in LB. Peaks corresponding to lipid A species predicted to contain C_{10} (*), and aminoarabinose (†) are indicated (28). Acyl group positions are as proposed before (2, 23, 24).

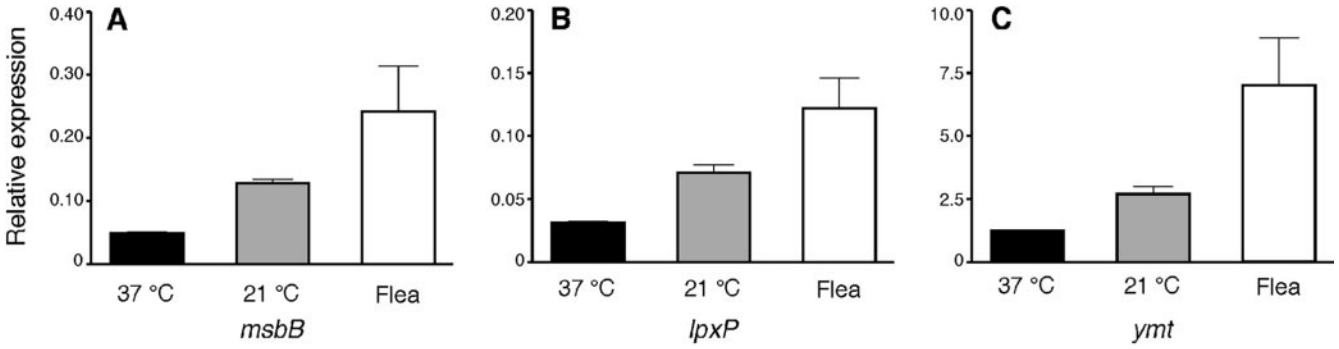

FIG. 3. Relative expression of *msbB* (A), *lpxP* (B), and *ymt* (C) genes in *Y. pestis*. Samples were obtained from *Y. pestis* KIM6+ grown in LB at 37°C (black bars), LB at 21°C (gray bars), and infected fleas maintained at 21°C (white bars). The mean and standard deviation of the relative transcript level compared to the transcript level of the *rpoS* housekeeping gene in three independent experiments are indicated.

determine if their expression is upregulated by a temperature shift from 37 to 21°C.

Homologs of the *lpxP* and *msbB* (*lpxM*) late acyltransferase genes of *E. coli* were identified in the *Y. pestis* chromosome, and loss-of-function deletion mutations were introduced into the two genes. Comparative MALDI-TOF and GC structural analyses showed clearly that the *Y. pestis msbB* and *lpxP* homologs encode acyltransferases responsible for the addition of C_{12} and $C_{16:1}$ acyl groups, respectively, to lipid A at 21°C (Fig. 2; Table 3). Notably, the lipid A spectrum of the *Y. pestis* Δ*msbB* Δ*lpxP* mutant grown at 21°C was identical to that of wild-type *Y. pestis* grown at 37°C (Fig. 2D) (28). Thus, the *msbB* and *lpxP* homologs are responsible for the temperature-dependent phenotypic variation from tetra- to hexa-acylated lipid A in *Y. pestis*.

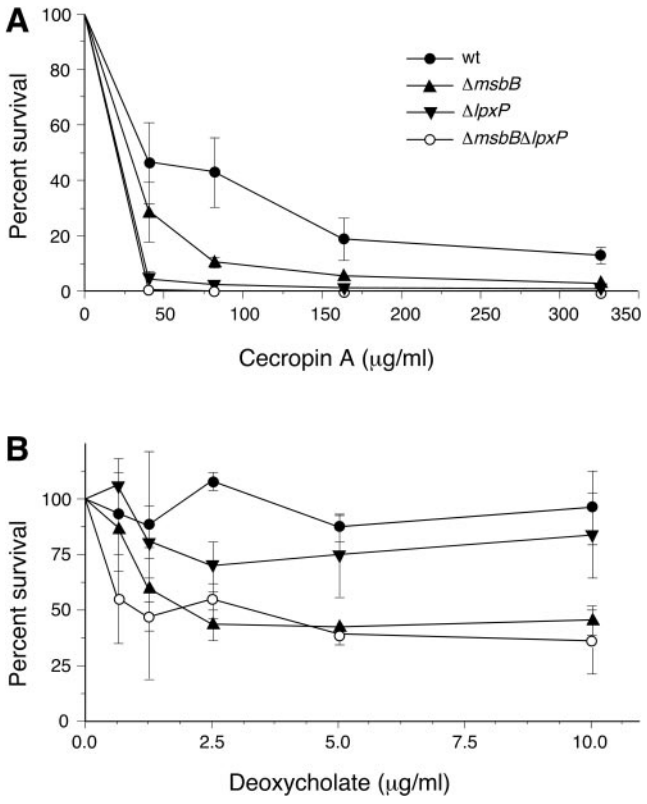

FIG. 4. Effect of lipid A acylation on *Y. pestis* viability after 2 h of exposure to increasing concentrations of (A) cecropin A or (B) deoxycholate.

RT-PCR analyses indicated that expression of these two genes was upregulated more than twofold in *Y. pestis* at 21°C both in vitro and in infected fleas (Fig. 3), suggesting that they are regulated by temperature. Transcript levels were low in all conditions, however. Thus, although decreased transcription is consistent with the lack of acylation at 37°C, temperature-sensitive catalytic activity or other posttranscriptional effects could also be responsible.

Y. pestis MsbB and LpxP likely transfer acyl groups to the 3′-3-OH-C_{14} and 2′-3-OH-C_{14} positions of the lipid A disaccharide, respectively, as in *E. coli* (2, 24). Some differences were apparent between the *Y. pestis* late acyltransferases and their *E. coli* counterparts, however. *Y. pestis* MsbB adds C_{12} only at growth temperatures below 26°C (23, 28), whereas *E. coli* MsbB adds a C_{14} group at all growth temperatures (8). Thus, although the sequence of *Y. pestis* MsbB is most similar to that of MsbB of *E. coli* and *S. enterica*, it more closely resembles that of *E. coli* HtrB in having C_{12} substrate specificity.

Bacterial acyltransferases can discriminate among acyl group substrates of different chain lengths via molecular measuring devices termed hydrocarbon rulers (1, 39), but the preferred chain length of homologous acyltransferases from different bacteria can differ as a result of even a single amino acid change (39). In addition, although *Y. pestis* LpxP adds a $C_{16:1}$ acyl group to lipid A, as does *E. coli* LpxP, this modification occurs only under cold shock conditions in *E. coli* (4). At growth temperatures above 12°C, a different *E. coli* late acyltransferase, HtrB (LpxL), adds a saturated C_{12} group (7, 8).

An *htrB* (*lpxL*) homolog could not be identified in *Y. pestis*. The lack of an *htrB* homolog and the temperature regulation of *msbB* may explain why *Y. pestis* produces primarily tetra-acylated lipid IV_A at 37°C; in *E. coli*, addition of C_{14} by MsbB follows C_{12} addition by HtrB, and in the absence of HtrB activity, MsbB catalysis is much less efficient (8). Consistent with this interpretation, complementation of the *Y. pestis* Δ*msbB* Δ*lpxP* strain with *msbB* did not result in C_{12}-modified, penta-acylated lipid A (R. Rebeil, unpublished data).

A second aim of this study was to test the hypothesis that the

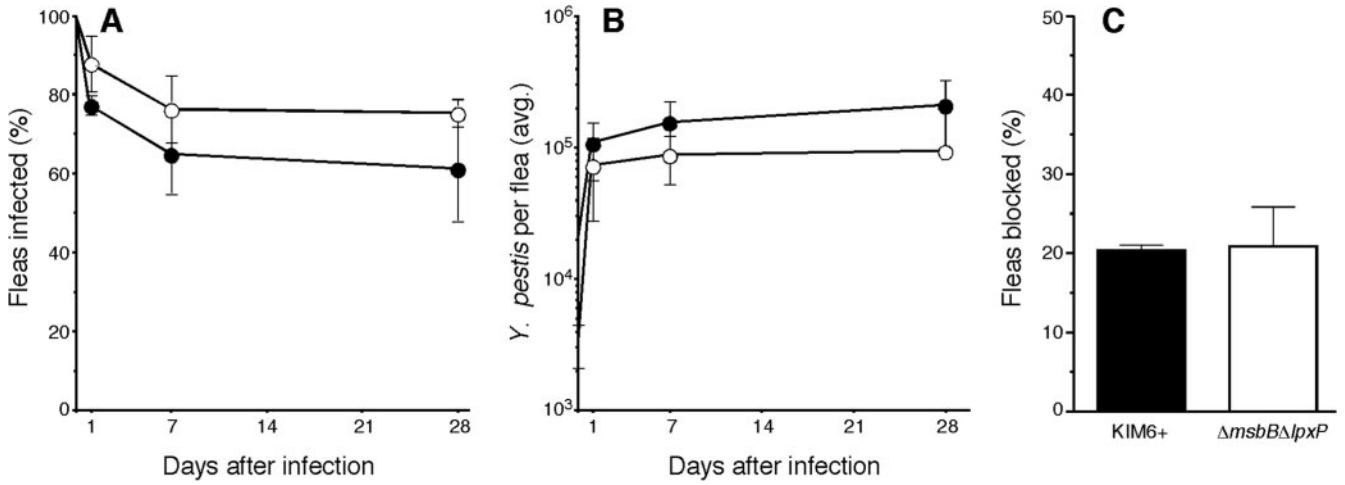

FIG. 5. Mutational loss of the *Y. pestis* acyltransferases MsbB and LpxP does not affect flea infection. The percentage of fleas infected (A), average *Y. pestis* CFU per infected flea (B), and percentage of fleas that developed proventricular blockage (C) were equivalent after infection with either *Y. pestis* KIM6+ (●) or *Y. pestis* Δ*msbB* Δ*lpxP* (○). The mean and standard deviation of two independent experiments are indicated.

hexa-acylated lipid A generated by *Y. pestis* at 21°C is required for outer membrane integrity and survival in the environment where *Y. pestis* would normally experience low temperature, the digestive tract of the flea vector. Of the agents used to assess increased outer membrane permeability, the detergent deoxycholate but not the hydrophobic antibiotics rifampin and vancomycin had increased bactericidal activity when fewer fatty acids were present in *Y. pestis* lipid A. In contrast, *E. coli msbB* and *lpxP* mutants are much more sensitive to rifampin and vancomycin (36, 37). Likewise the absence of $C_{16:1}$ and C_{12} modification of *Y. pestis* lipid A did not affect susceptibility to polymyxin B; slightly increased susceptibility to cecropin A was detected in the bactericidal assay (Fig. 4), but not in a MIC assay (data not shown). This was surprising, because wild-type *Y. pestis* is much more sensitive to both of these CAMPs in MIC assays at 37°C, when predominantly tetra-acylated lipid A lacking $C_{16:1}$ and C_{12} is produced, than at 21°C, when $C_{16:1}$- and C_{12}-containing hexa-acylated lipid A predominates (28).

Other temperature-dependent LPS modifications besides C_{12} and $C_{16:1}$ addition may account for the increased resistance of wild-type *Y. pestis* to CAMPs at 21°C. For example, aminoarabinose and C_{10} modification of *Y. pestis* LPS did not appear to be affected by *msbB* or *lpxP* mutation (Fig. 2). Positive surface charge associated with aminoarabinose addition to lipid A is critical for resistance to polymyxin B (13, 14), and aminoarabinose modification increases with lower growth temperature in *Y. pestis* (24). Alternatively, temperature-dependent differences in LPS core structure (24), or an as yet unknown temperature-mediated factor might be responsible for increased resistance at 21°C.

Ultimately, we wanted to test the hypothesis that the change from primarily tetra-acylated lipid A at 37°C to hexa-acylated lipid A at <26°C is important to the arthropod-borne life cycle of *Y. pestis*. To produce a transmissible infection in the flea vector, *Y. pestis* grows to large numbers in the flea midgut and eventually forms a biofilm that obstructs the proventriculus, a valve between the midgut and esophagus (20). Little is known about the flea gut environment. However, digestion and solubilization of the blood meal occurs in the midgut, which presumably contains proteases, lipases, and surfactants (34), and it seemed likely that full acylation of lipid A might enhance bacterial survival in that environment. Although it was not feasible to isolate sufficient quantities of LPS for structural analysis from infected fleas, the increased expression of the *Y. pestis* late acyltransferase genes indicates that hexa-acylated lipid A is produced in fleas (Fig. 3). Nevertheless, we observed no difference in the ability of *Y. pestis* KIM6+ and *Y. pestis* Δ*msbB* Δ*lpxP* to infect or block the proventriculus (Fig. 4), indicating that hexa-acylated lipid A is not necessary for survival in the *X. cheopis* digestive tract or to produce a transmissible infection. In contrast, the late acyltransferases are essential for normal in vitro and in vivo growth of *E. coli* and *Salmonella* spp. (21, 22, 30, 37).

The change from tetra-acylated lipid A at 37° to hexa-acylated lipid A at 21°C is characteristic of the enteropathogenic yersiniae as well as *Y. pestis* (18, 28). Thus, the hexa-acylated lipid A produced by *Y. pestis* at temperatures below 26°C may simply be an evolutionary inheritance from *Y. pseudotuberculosis* with a neutral effect in the flea. The fully acylated lipid A form made at ambient temperatures may be important for survival in the environment outside of a eukaryotic host, and downregulation of the late acyltransferases at 37°C to produce the less immunostimulatory tetra-acylated lipid A may be important for pathogenesis. This hypothesis remains to be tested.

The hexa-acylated lipid A by *Y. pestis* might also play a protective role immediately after flea-borne transmission. Upon entering a mammalian host, *Y. pestis* must survive the innate immune response and resist killing by dermal phagocytes until the expression of known antiphagocytic factors such as the F1 capsule and the Yop proteins is induced (5, 19, 33). In other gram-negative bacteria, increased acylation of lipid A confers protection against mammalian defensins (12, 15). Thus, the fully acylated lipid A produced by *Y. pestis* in the flea might help complete the transmission cycle by increasing resistance to phagocytes or defensins encountered in the skin upon transmission.

ACKNOWLEDGMENTS

This work was supported by the Division of Intramural Research, NIAID, NIH; the Ellison Medical Foundation (New Scholars Award in Global Infectious Diseases to B.J.H.); and by the WWAMI Regional Center of Excellence for Biodefense and Emerging Infectious Disease, PHS grant U54 AI057141 (R.K.E. and S.I.M.).

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