INFECTION AND IMMUNITY, Feb. 2008, p. 612–622 Vol. 76, No. 2
0019-9567/08/$08.00+0 doi:10.1128/IAI.01125-07

Resistance of *Yersinia pestis* to Complement-Dependent Killing Is Mediated by the Ail Outer Membrane Protein▽

Sara Schesser Bartra,[1,5] Katie L. Styer,[2] Deanna M. O'Bryant,[3] Matthew L. Nilles,[3] B. Joseph Hinnebusch,[4] Alejandro Aballay,[2] and Gregory V. Plano[1]*

Department of Microbiology and Immunology, University of Miami Miller School of Medicine, Miami, Florida 33101[1]; Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, North Carolina 27710[2]; Department of Microbiology and Immunology, University of North Dakota School of Medicine and Health Sciences, Grand Forks, North Dakota 58202[3]; Laboratory of Zoonotic Pathogens, Rocky Mountain Laboratories, NIH, NIAID, Hamilton, Montana 59840[4]; and Department of Molecular Biology, Umeå University, 901 87 Umeå, Sweden[5]

Received 13 August 2007/Returned for modification 11 September 2007/Accepted 30 October 2007

***Yersinia pestis*, the causative agent of plague, must survive in blood in order to cause disease and to be transmitted from host to host by fleas. Members of the Ail/Lom family of outer membrane proteins provide protection from complement-dependent killing for a number of pathogenic bacteria. The *Y. pestis* KIM genome is predicted to encode four Ail/Lom family proteins. *Y. pestis* mutants specifically deficient in expression of each of these proteins were constructed using lambda Red-mediated recombination. The Ail outer membrane protein was essential for *Y. pestis* to resist complement-mediated killing at 26 and 37°C. Ail was expressed at high levels at both 26 and 37°C, but not at 6°C. Expression of Ail in *Escherichia coli* provided protection from the bactericidal activity of complement. High-level expression of the three other *Y. pestis* Ail/Lom family proteins (the y1682, y2034, and y2446 proteins) provided no protection against complement-mediated bacterial killing. A *Y. pestis ail* deletion mutant was rapidly killed by sera obtained from all mammals tested except mouse serum. The role of Ail in infection of mice, *Caenorhabditis elegans*, and fleas was investigated.**

Yersinia pestis is the etiologic agent of plague, an often fatal disease of mammals (30). The pathogenicity of *Y. pestis* results largely from its ability to thwart the defenses of its host and to overwhelm it with massive growth (7). Transmission of plague normally occurs via the bite of an infected flea (16). Regurgitated bacteria initially journey to the draining lymph nodes, which enlarge to form the characteristic buboes. Bacteria subsequently spread to the blood and colonize the spleen, liver, and other organs. Eventually, a terminal, high-density septicemia occurs, leading to shock, disseminated intravascular coagulation, peripheral gangrene, and death.

The ability to survive in blood is critical for *Y. pestis* to cause disease, to infect its flea vector, and to be transmitted from host to host. To survive in diverse mammalian tissues and blood, *Y. pestis* actively blocks bacterial phagocytosis and resists complement-mediated bacteriolysis (30). A plasmid pCD1-encoded type III secretion system plays a key role in preventing bacterial phagocytosis (41); however, the mechanism by which *Y. pestis* evades complement-mediated lysis is not fully understood.

The enteropathogenic yersiniae (*Y. enterocolitica* and *Y. pseudotuberculosis*) are fully resistant to complement when they are grown at 37°C but not when they are grown at 26°C (5, 30). In contrast, *Y. pestis* is constitutively resistant to complement at both 26 and 37°C, but it is sensitive when it is grown at 6°C (1). Resistance to complement in *Y. enterocolitica* has been studied in detail and has been shown to involve YadA, Ail, and lipopolysaccharide (LPS) O antigen, with YadA playing a dominant role (4). Both YadA and Ail are outer membrane proteins that function as adhesins/invasins, as well as complement resistance proteins. Interestingly, *Y. pestis* does not express YadA and expresses only rough LPS (30). Previous studies have suggested that *Y. pestis* LPS may mediate serum resistance (34); however, there is no evidence of a direct role for LPS in serum resistance.

Members of the Ail/Lom family, which include Ail of *Y. enterocolitica* and *Y. pseudotuberculosis*, Rck and PagC of *Salmonella enterica*, and OmpX of *Escherichia coli*, are outer membrane proteins that share significant amino acid sequence similarity and identity and are predicted to have similar membrane topologies (15, 24, 26, 36, 42, 44). These proteins are all predicted to possess eight transmembrane amphipathic β-strands and four extracellular loops, with the regions of greatest amino acid sequence similarity and identity concentrated in the membrane-spanning segments. Amino acid residues located in extracellular loops 2 and 3 have been shown to play a critical role in Ail-mediated attachment, invasion, and serum resistance of *Y. enterocolitica* (25). Although members of this family of proteins exhibit diverse functions, several of them, including Ail of *Y. enterocolitica* and *Y. pseudotuberculosis*, as well as Rck of *S. enterica*, function, at least in part, to protect bacteria from complement-mediated lysis. The Rck protein has been shown to protect *S. enterica* from complement by inhibiting C9 polymerization and subsequent assembly of a functional membrane attack complex (14). Other Ail/Lom family members are required for survival in macrophages (PagC) or have no confirmed function (OmpX and Lom).

In this study, we investigated the role of Ail/Lom family members in the pathogenesis of *Y. pestis*. The genomes of *Y. pestis* KIM and CO92 both contain four genes predicted to

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Miami Miller School of Medicine, Miami, FL 33101. Phone: (305) 243-6310. Fax: (305) 243-4623. E-mail: gplano@med.miami.edu.

▽ Published ahead of print on 19 November 2007.

TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristics[b]	Reference
Y. pestis strains[a]		
KIM5 (Pla$^+$ parent)	pCD1, pPCP1, pMT1	40
KIM5 (Δy1324) (Δ*ail*)	pCD1, pPCP1, pMT1 Δ*ail*	This study
KIM5 (Δy1682)	pCD1, pPCP1, pMT1 Δy1682	This study
KIM5 (Δy2034)	pCD1, pPCP1, pMT1 Δy2034	This study
KIM5 (Δy2446)	pCD1, pPCP1, pMT1 Δy2446	This study
KIM8-E (Δ*yopE-sycE*::*dhfr*) (Pla$^-$ parent)[c]	pCD1 (*sycE yopE*::Km), pPCP1$^-$, pMT1	2
KIM8-E (Δy1324) (Δ*ail*)	pCD1 (*sycE yopE*::Km), pPCP1$^-$, pMT1 Δ*ail*	This study
KIM8-E (Δy1682)	pCD1 (*sycE yopE*::Km), pPCP1$^-$, pMT1 Δy1682	This study
KIM8-E (Δy2034)	pCD1 (*sycE yopE*::Km), pPCP1$^-$, pMT1 Δy2034	This study
KIM8-E (Δy2446)	pCD1 (*sycE yopE*::Km), pPCP1$^-$, pMT1 Δy2446	This study
KIM8-E (Δ4) (Δy1324 Δy1682 Δy2034 Δy2446)	pCD1 (*sycE yopE*::Km), pPCP1$^-$, pMT1 Δy1324 Δy1682 Δy2034 Δy2446	This study
KIM6+	pCD1$^-$, pPCP1, pMT1	37
KIM6+ Δ*ail*	pCD1$^-$, pPCP1, pMT1 Δ*ail*	This study
E. coli DH5α	F$^-$ *recA endA gyrA thi hsdR supE relA* Δ(*lacZYA-argF*) *deoR* Φ80*lac*(Δ*lacZ*)*M15*	6

[a] All *Y. pestis* strains except KIM6+ and KIM6+ Δ*ail* are avirulent due to deletion of the *pgm* locus (40). *Y. pestis* KIM6+ and derivatives of this strain have been cured of plasmid pCD1.

[b] The native plasmids of *Y. pestis* include pCD1 (31), pPCP1 (35) encoding the outer membrane plasminogen activator (Pla) protease that has been shown to degrade exported Yops, and pMT1 encoding the capsular protein (21).

[c] *Y. pestis* KIM8-E was previously designated KIM8-Δ4 (2).

encode Ail/Lom family proteins (11, 29). We demonstrated that one of these proteins, designated Ail, is required for resistance to complement-mediated killing at both 26 and 37°C. The Ail protein was expressed at high levels at both 26 and 37°C; however, expression of Ail was minimal at 6°C. Serum-sensitive *E. coli* DH5α transformed with an Ail expression vector became serum resistant, confirming that the Ail protein can provide serum resistance independent of other *Y. pestis* virulence factors. A *Y. pestis ail* deletion mutant was rapidly killed by exposure to all animal sera tested except mouse serum. In accordance with these findings, a *Y. pestis* KIM *ail* deletion mutant was fully virulent in an intravenous mouse model of infection. Interestingly, the *ail* mutant was attenuated in a *Caenorhabditis elegans* infection model, but it exhibited no defect in colonization or blockage of the flea digestive tract.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in this study are described in Table 1. All *Y. pestis* KIM strains except *Y. pestis* KIM6+ and derivatives of this strain are Pgm$^-$ and avirulent when peripheral routes of infection are used (40). *Y. pestis* KIM6+ lacks the pCD1 virulence plasmid that is required for virulence in mammals (37). These strains and their derivatives were routinely grown in heart infusion broth (HIB) or on tryptose blood agar (TBA) base plates (Difco, Detroit, MI). For serum survival experiments *Y. pestis* and *E. coli* strains were grown at 6, 26, or 37°C in HIB in the presence of 2.5 mM calcium chloride. *E. coli* DH5α (6) was used for routine cloning experiments.

Deletion of *Y. pestis* KIM5, KIM6+, or KIM8-E (2) chromosomal DNA sequences encoding amino acid residues 20 to 122 of the y1324 (*ail*) protein, amino acid residues 17 to 121 of the y1682 protein, amino acid residues 37 to 144 of the y2034 protein, and amino acid residues 48 to 154 of the y2446 protein and insertion of a kanamycin resistance (Km^r) or chloramphenicol resistance (Cm^r) cassette flanked by FLP recognition sequences were accomplished using lambda Red-mediated recombination essentially as described by Datsenko and Wanner (10). PCR products used to construct gene replacements were generated using template plasmids pKD3 (Cm^r) and pKD4 (Km^r). Oligonucleotide primers used for amplification of the PCR products are listed in Table 2. PCR products were gel purified, ethanol precipitated, and resuspended in 10 μl of distilled H_2O. *Y. pestis* strains carrying plasmid pKD46, encoding the Red recombinase, were induced with 0.2% L-arabinose for 2 h prior to harvest. Electrocompetent cells were electroporated with the purified PCR products and plated on TBA plates containing chloramphenicol (20 μg/ml) or kanamycin (50 μg/ml). Plasmid pCP20, which encodes the FLP recombinase, was electroporated into the single-deletion strains to facilitate removal of the FLP recognition target-flanked cassettes. In addition to the single-deletion mutants, a strain with all four identified genes deleted was constructed using lambda Red-mediated recombination to sequentially delete the y1682, y2034, and y2446 genes from KIM8-E (Δy1324). Plasmids pKD46 and pCP20 were cured from the *Y. pestis* strains by overnight growth at 39°C. All gene replacements were confirmed by PCR analysis using CTL primers listed in Table 2.

Construction of Ail/Lom family expression plasmids. Plasmids pAil and pTRC-Ail were constructed by inserting a ca. 0.6-kb PCR fragment generated with oligonucleotide primers y1324-KpnI and y1324-XbaI (Table 2) into KpnI- and XbaI-digested pBluescript KS(−) and pTRC99a, respectively. PCR fragments containing the entire y1682, y2034, and y2446 genes, including at least 50 bp upstream of the start codon, were amplified with oligonucleotide primer pairs y1682-KpnI/y1682-XbaI, y2034-KpnI/y2034-XbaI, and y2446-KpnI/y2446-XbaI (Table 2). The resultant ca. 0.6-kb PCR fragments were digested with KpnI and XbaI and inserted into KpnI- and XbaI-digested pTRC99a, generating plasmids pTRC-y1682, pTRC-y2034, and pTRC-y2446.

Serum survival assay. Normal human serum (NHS) was pooled from at least five healthy volunteers. Animal sera were purchased from Valley Biomedical Products and Services, Inc. (Winchester, VA) or Sigma (St. Louis, MO). Heat-inactivated serum was used as a control in all experiments. Harvested and washed *Y. pestis* or *E. coli* was diluted in phosphate-buffered saline (PBS) (pH 7.4) to obtain an optical density at 620 nm (OD_{620}) of 0.2 (~1×10^8 bacteria/ml). Fifty microliters of the diluted bacteria (~5×10^6 bacteria) was added to 200 μl of NHS, normal goat serum (NGS), normal sheep serum (NSS), normal rabbit serum (NRS), normal mouse serum (NMS), normal guinea pig serum (GPS), heat-inactivated serum (HIS), or PBS (final concentration of serum, 80%). The samples were incubated at 37°C with shaking for 1 h. The number of surviving bacteria (CFU) in each sample was determined by dilution and plating on TBA plates at 30°C for 1 or 2 days. The percent survival was calculated as follows: average number of bacteria that survived exposure to NHS at 1 h/number of bacteria that survived exposure to HIS at 1 h × 100.

Outer membrane preparations. Outer membranes preparations were obtained essentially as described by Biedzka-Sarek et al. (4). Bacteria were grown overnight in HIB at 26 or 37°C. Bacterial cultures (5 ml) were harvested by centrifugation at 8,000 × *g* for 10 min at 4°C. Pellets were resuspended in 2 ml of 10 mM Tris-HCl–5 mM $MgCl_2$ (pH 8.0) (buffer A), and the bacteria were lysed by two passages through an ice-cold French pressure cell. Unlysed cells and large debris were removed by centrifugation as described above. Bacterial membranes were collected by ultracentrifugation at 100,000 × *g* for 1 h at 4°C. Membrane pellets were resuspended in buffer A containing 2% Triton X-100. The Triton X-100-resistant outer membrane protein fraction was pelleted by ultracentrifugation and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence
y1324-P1	TCTCTTCTTTAATTGCATGTTTATCAATTGCGTCTGTTATGTGTAGGCTGGAGCTGCTTC
y1324-P2	TTTACTTCTACTCTCTGATTGACCAAATATTGAGGAAAACATATGAATATCCTCCTTAGT
y1682-P1	ATTGCATGTCTTTCAGCGGTAGCAGCGTGTGTGTTAGCCTGTGTAGGCTGGAGCTGCTTC
y1682-P2	GGTGAAGCCGTAGTCACTGGTGCTGTGTTTATCACCAACCATATGAATATCCTCCTTAGT
y2034-P1	CAGTGATGCTAGCAACACGGTATCTTTCGGTTACGCTCATGTGTAGGCTGGAGCTGCTTC
y2034-P2	AGCTGTATCAATAGCAACGTGTTTTACCGGATTGAACTGCATATGAATATCCTCCTTAGT
y2446-P1	CTCAGGGAGATGTAAGACTCGGTGATGGAAATCGAAAAGTGTGTAGGCTGGAGCTGCTTC
y2446-P2	AGTTTTGTATACTCGTATGAAGCATCAATAGCAATATTGCATATGAATATCCTCCTTAGT
y1324-KpnI	TTTGGTACCTACTGTATTAGGTATTGTTATAAC
y1324-XbaI	TTTTCTAGAAGGACGTTAGAACGGTAACCC
y1682-KpnI	TTTGGTACCTTGTGTCACTCAATAATTTGCGTC
y1682-XbaI	TTTTCTAGATCAGGCTATCACTTAGAAAGTGTA
y2034-KpnI	TTTGGTACCCCTTATCAAAAATTAACTTTTAAT
y2034-XbaI	TTTTCTAGAATCAACAGAATATTAGAAGCGGTA
y2446-KpnI	TTTGGTACCTCTAAGATAATAATTTAAGGATAA
y2446-XbaI	TTTTCTAGAATAGAAAGTATTTTATTAAAAACG
y1324-CTL1	TACTGTATTAGGTATTGTTATA
y1324-CTL2	AGGAGGACGTTAGAACCGGTA
y1682-CTL1	TTTGGTACCTTGTGTCACTCAATAATTTGCGTC
y1682-CTL2	TTTTCTAGATCAGGCTATCACTTAGAAAGTGTA
y2034-CTL1	GAAAATTGGAAAAGATAATAAAGG
y2034-CTL2	ACAGTGTTGCTTACCATACGCGGC
y2446-CTL1	CCATAAGTTGAGTGAAATGTTCGG
y2446-CTL2	AATTGCATCCCAACACCATATGCC

SDS-PAGE analysis of *Y. pestis* and *E. coli* proteins. Volumes of cellular fractions corresponding to equal numbers of bacteria or equal amounts of protein were mixed 1:1 (vol/vol) with 2× electrophoresis sample buffer and analyzed by SDS-PAGE essentially as previously described (33). SDS-PAGE-separated proteins were stained with Coomassie brilliant blue R-250 or silver stained.

Mouse infection assays. Female 6- to 8-week-old Swiss-Webster mice were used for infections. *Y. pestis* KIM5 and KIM5 Δ*ail* were grown overnight in HIB at 26°C. Fresh cultures were inoculated to obtain an OD_{620} of 0.2 and grown with shaking to an OD_{620} of 1.0. Bacterial cultures were harvested by centrifugation and resuspended in PBS. Mice were inoculated with *Y. pestis* via the retroorbital sinus using blunt feeding needles. Plate counting was performed to verify the number of CFU in the infectious doses.

***C. elegans* infection assays.** Groups of 40 *C. elegans* N2 (wild-type) young adults were exposed to *Y. pestis* strains grown on modified NG agar plates at 25°C. Worm mortality was scored over time. *C. elegans* survival was plotted using the PRISM computer program. A survival curve was considered significantly different from the control when the *P* value was <0.05.

Flea infection assays. *Xenopsylla cheopis* fleas were infected with *Y. pestis* KIM6+ or KIM6+ Δ*ail* using an artificial feeding system essentially as described previously (17). One group of 50 male and 50 female fleas that took an infected blood meal were saved and examined for blockage immediately after they were allowed to feed on an uninfected mouse on days 6, 9, 13, 16, 20, 23, and 27 after infection. Additional samples of 20 female fleas were taken at the beginning of the experiment (time zero), 9 days after infection, and at the end of the experiment (day 28) to determine the infection rate and the average bacterial load per positive flea by CFU counting (17).

RESULTS

Generation of *Y. pestis* strains deficient in expression of Ail/Lom family proteins. Previous analyses of available *Y. pestis* genome sequences identified four genes (the *Y. pestis* KIM y1324, y1682, y2034, and y2446 genes) predicted to encode Ail/Lom family proteins (11, 29). All four of the predicted gene products share significant amino acid sequence similarity and identity with each other and with Ail of *Y. enterocolitica* (Fig. 1). The *Y. pestis* y1682 gene encodes a protein that is 71% identical to the *E. coli* OmpX protein. In contrast, the y1324, y2034, and y2446 gene products share only 41, 41, and 31% amino acid sequence identity with OmpX, respectively. The y1682 gene and the *E. coli ompX* gene are also both located adjacent to the *glnH*, *dps*, and *rhtA* genes in the *Y. pestis* KIM and *E. coli* K-12 chromosomes, respectively. Thus, the y1682 gene appears to encode a *Y. pestis* OmpX homolog.

To investigate the role of the y1324, y1682, y2034, and y2446 gene products in the serum resistance and virulence of *Y. pestis* KIM, we used lambda Red-mediated recombination (10) to create *Y. pestis* strains KIM5 (Pla^+) and KIM8-E (Pla^- Δ*yopE-sycE*::*dhfr*) specifically deficient in expression of the four Ail/Lom family proteins (Table 1). The DNA fragments encoding amino acid residues 20 to 122 (y1324), 17 to 121 (y1682), 37 to 144 (y2034), and 48 to 154 (y2446) were removed, and a Km^r cassette flanked by FLP recognition sequences was inserted. Plasmid pCP20, which encodes the FLP recombinase (10), was electroporated into the single-deletion strains to facilitate removal of the FLP recognition target-flanked Km^r cassette. In addition to the individual deletion mutants, a strain with all four identified genes deleted was constructed using lambda Red-mediated recombination to sequentially delete the y1682, y2034, and y2446 genes from KIM8-E (Δy1324).

***Y. pestis* Ail (y1324) is required for resistance to complement-mediated killing.** The abilities of the parent *Y. pestis* KIM8-E strain and the isogenic mutants lacking one or all four of the genes encoding Ail/Lom family proteins to survive in NHS or HIS were determined. Bacteria were grown at 26 or 37°C and diluted in PBS to obtain an OD_{620} of 0.2 (approximately 1×10^8 bacteria/ml), and 50 μl of bacteria (approximately 5×10^6 bacteria) was added to 200 μl of NHS, HIS, or PBS. The samples were incubated at 37°C for 60 min, and the numbers of surviving bacteria (CFU) were determined by dilution and plating on TBA base plates at 30°C.

The parent *Y. pestis* strain and the isogenic strains with deletions in the y1682, y2034, or y2446 gene were completely resistant to the killing action of NHS at both 26 and 37°C (Fig. 2A). In contrast, the *Y. pestis* strain with a deletion in the y1324 gene and the strain with all four genes deleted (Δ4) were susceptible to complement-mediated killing at both temperatures, suggesting that the y1324 gene product was required for

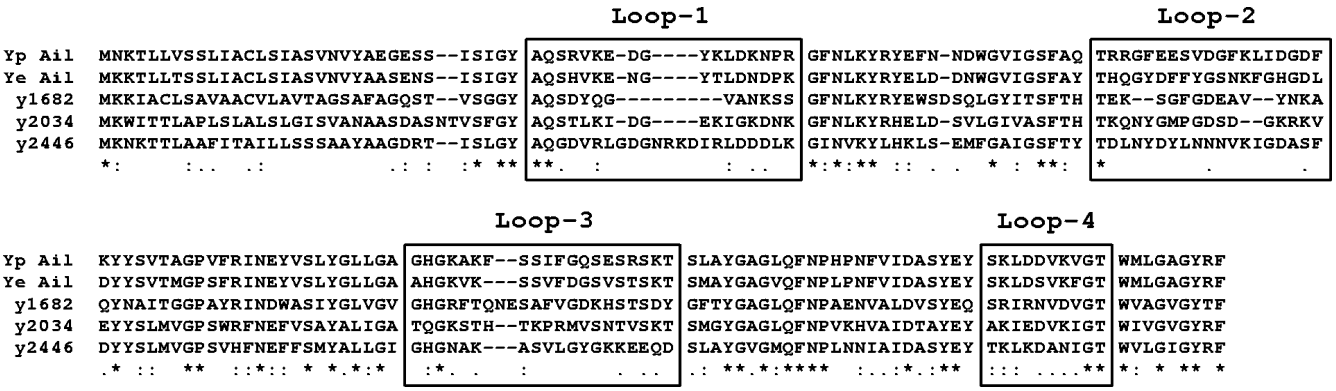

FIG. 1. Amino acid sequence alignment of *Y. pestis* KIM Ail with other *Yersinia* Ail/Lom family proteins. The amino acid sequences of *Y. pestis* KIM Ail (Yp Ail) and *Y. enterocolitica* Ail (Ye Ail) and the predicted amino acid sequences of the *Y. pestis* KIM Ail/Lom family y1682, y2034, and y2446 proteins are aligned. Amino acid residues in the four predicted surface-exposed loops are enclosed in boxes (loops 1 to 4). Identical residues are indicated by asterisks, strongly similar residues are indicated by colons, and weakly similar residues are indicated by periods. The alignment was generated using the CLUSTALW multiple-sequence alignment program (39).

survival in NHS. The survival of each of the strains was unaffected by incubation with HIS or PBS. To confirm that the y1324 gene product was required for serum resistance, the y1324 gene was PCR amplified and inserted into pBluescript KS(−) (Stratagene) downstream of the vector P_{lac} promoter, and the resulting plasmid was moved into the y1324 deletion strain and into the multiple-deletion strain (Δ4). Providing the y1324 gene in *trans* completely restored the ability to survive complement-mediated killing at both 26 and 37°C in these strains, confirming that the y1324 gene is required for serum resistance of *Y. pestis*. Use of NHS treated with 10 mM EGTA and 5 mM $MgCl_2$ (which inactivates the Ca^{2+}-dependent classical and lectin pathways) instead of NHS provided essentially identical results (data not shown), suggesting that the alternative pathway was sufficient to mediate killing of the serum-sensitive *Y. pestis* mutants.

***Y. pestis* Ail is highly expressed at 26 and 37°C but not at 6°C.** The outer membrane protein profile of each of the strains was examined to determine the expression of the Ail/Lom family proteins essentially as described by Biedzka-Sarek et al. (4). Isolated total membrane fractions from 5-ml overnight cultures of each strain grown at either 26 or 37°C were treated with 2% Triton X-100, and the Triton X-100-insoluble fractions were analyzed by SDS-PAGE and silver staining (Fig. 2B). A highly expressed putative outer membrane protein with an apparent molecular mass of ca. 20 kDa was found in the parent strain and in the strains having deletions in the y1682, y2034, and y2446 genes but not in the y1324 gene deletion strain or in the strain with all four genes deleted (Δ4). Complementation with the y1324 gene expression plasmid (pAil) restored expression of the ca. 20-kDa protein, confirming that this protein was the y1324 gene product. No consistent protein band corresponding to the y1682, y2034, or y2446 gene product was observed, suggesting that these proteins were not highly expressed under the growth conditions used. The y1324 gene product was required for serum resistance of *Y. pestis* at both 26 and 37°C and exhibited 77 and 98% amino acid sequence identity to the *Y. enterocolitica* and *Y. pseudotuberculosis* Ail proteins, respectively. Furthermore, the chromosomal locations of the *Y. pestis* y1324 gene and the *Y. pseudotuberculosis* *ail* gene were strictly conserved; therefore, the y1324 protein was designated the *Y. pestis* Ail protein.

The high-level expression of *Y. pestis* Ail at both 26 and 37°C provides an explanation for the ability of *Y. pestis* to resist the killing action of complement at both temperatures. Interestingly, Anisimov et al. (1) demonstrated that *Y. pestis* strains that are serum resistant when they are grown at 25 or 37°C become serum sensitive when they are grown at 6°C. We confirmed that *Y. pestis* KIM8-E and KIM8-E Δ*ail* were both serum sensitive when they were grown at 6°C (Fig. 3A). Analysis of whole-cell proteins from *Y. pestis* strains grown at 6, 26, and 37°C (Fig. 3B) confirmed that Ail was expressed well at both 26 and 37°C; however, Ail was not as highly expressed in strains grown at 6°C. Thus, serum resistance of *Y. pestis* explicitly correlated with high-level expression of Ail.

***E. coli* DH5α expressing Ail is serum resistant.** To confirm that expression of Ail alone is sufficient to mediate serum resistance, we moved the Ail expression plasmid (pAil) into a serum-sensitive strain of *E. coli*. *E. coli* DH5α strains carrying no plasmid, pBluescript KS(−) (vector control), or pAil were grown overnight at 37°C, and the serum resistance of the strains was determined as described above for the *Y. pestis* strains (Fig. 4A). DH5α alone and DH5α carrying pBluescript KS(−) were both rapidly killed by NHS but not by HIS. In contrast, DH5α carrying the pAil plasmid was resistant to the bactericidal action of NHS. Analysis of whole-cell proteins by SDS-PAGE and Coomassie blue R-250 staining confirmed that DH5α carrying pAil expressed the ~20-kDa Ail protein (Fig. 4B). These results confirm that the *Y. pestis* Ail protein can confer resistance to complement-dependent killing independent of other *Y. pestis*-specific virulence factors.

High-level expression of the Ail/Lom family y1682, y2034, and y2446 proteins does not protect *Y. pestis* from complement-mediated killing. *Y. pestis* strains defective in expression of the Ail protein were sensitive to complement-mediated killing at both 26 and 37°C, whereas strains having a deletion in one of the genes encoding other Ail/Lom family proteins were serum resistant. These results suggest that the y1682, y2034, and y2446 genes were not expressed under the conditions used or that the expressed proteins were unable to mediate resistance to complement-di-

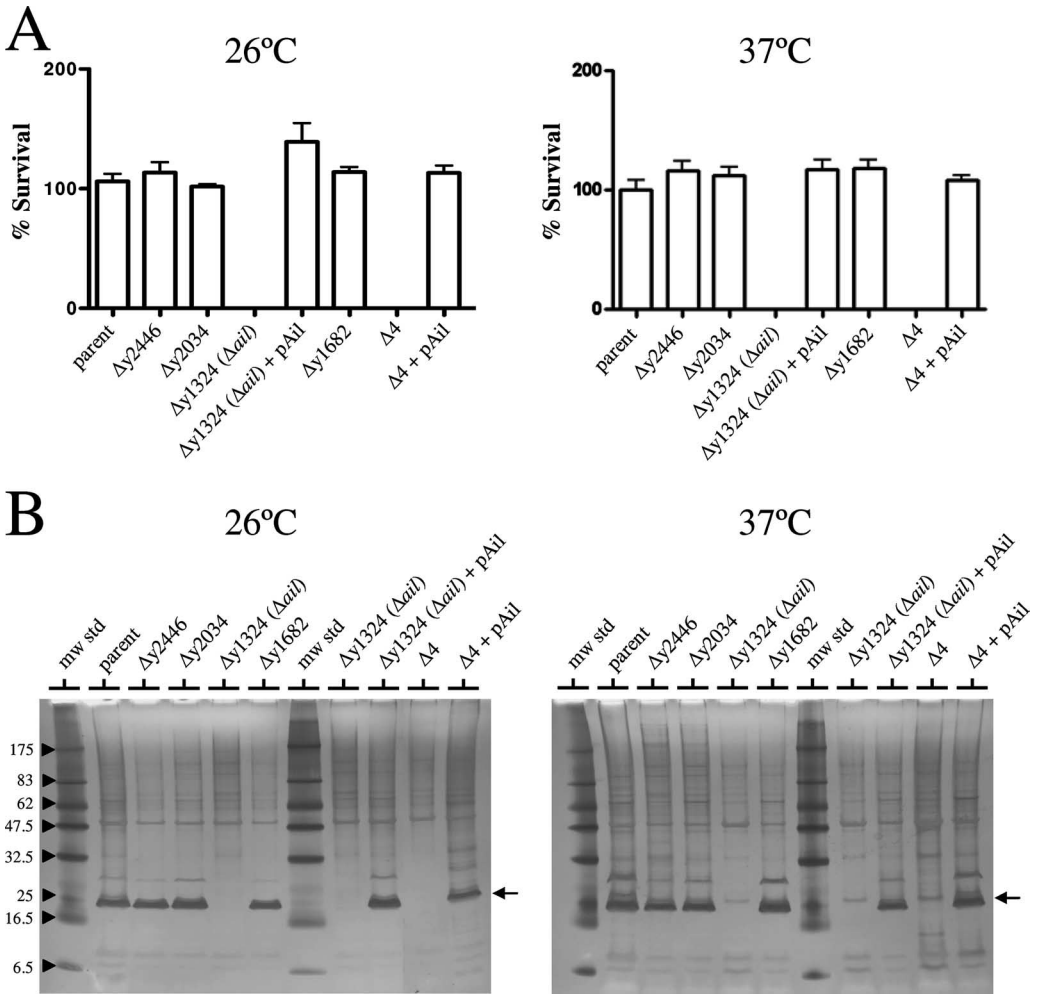

FIG. 2. *Y. pestis* Ail is highly expressed and required for survival in NHS. (A) Survival of wild-type and mutant strains of *Y. pestis* in 80% NHS. Bacteria grown overnight at 26 or 37°C (~5×10^6 bacteria) were incubated with 80% NHS or HIS for 1 h at 37°C. Aliquots were diluted and plated on TBA plates at 30°C. The percent survival was calculated as follows: average number of bacteria that survived exposure to NHS at 1 h/number of bacteria that survived exposure to HIS at 1 h × 100. (B) Silver-stained SDS-PAGE gel of outer membrane proteins isolated from the parent and mutant strains of *Y. pestis*. The location of the Ail (y1324) protein is indicated by an arrow. The positions of molecular mass standards (in kilodaltons) are indicated on the left. The strains used were *Y. pestis* strains KIM8-E (parent), KIM8-E Δy2446, KIM8-E Δy2034, KIM8-E Δy1324 (Δ*ail*), KIM8-E Δy1324 (Δ*ail*)/pAil, KIM8-E Δy1682, KIM8-E Δ4, and KIM8-E Δ4/pAil. mw std, molecular mass standards.

rected bacteriolysis. Analysis of the proteins expressed by these strains (Fig. 2 and 3) indicated that the y1682, y2034, and y2446 proteins were not expressed at a level that allowed detection under the experimental conditions utilized.

To determine if the y1682, y2034, and y2446 gene products were capable of mediating resistance to complement-mediated killing, expression vectors for each *Y. pestis* Ail/Lom family protein were constructed. The *ail*, y1682, y2034, and y2446 genes were inserted downstream of the strong *trc* promoter in plasmid pTRC99a (Amersham Biosciences). The resulting constructs, designated pTRC-Ail, pTRC-y1682, pTRC-y2034, and pTRC-y2446, were moved into the serum-sensitive multiple-Ail/Lom-deletion mutant (*Y. pestis* KIM8-E Δ4), and the resulting strains were grown at 26°C in the presence or absence of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce expression of the Ail/Lom family proteins. SDS-PAGE and Coomassie blue R-250 staining demonstrated that each of the Ail/Lom family proteins was highly expressed following induction with IPTG (Fig. 5A). Selective solubilization studies (data not shown) demonstrated that the majority of each Ail/Lom family protein was present in the *Y. pestis* outer membrane.

The ability of each of the *Y. pestis* strains expressing the different Ail/Lom family proteins (IPTG-induced cultures) to survive in NHS was determined (Fig. 5B). The parent strain and the Δ4 strain expressing the Ail protein were resistant to complement-mediated killing. In contrast, the Δ4 strain and the Δ4 strain carrying plasmid pTRC-y1682, pTRC-y2034, or pTRC-y2446 were sensitive to complement-mediated killing. These results suggest that the *Y. pestis* Ail protein is the sole Ail/Lom family protein responsible for the serum resistance of *Y. pestis*. However, these results do not rule out alternative roles for the other Ail/Lom family proteins in the pathogenesis of *Y. pestis*.

***Y. pestis* Ail mediates resistance to the bactericidal activity of a wide variety of animal sera.** Sera obtained from different

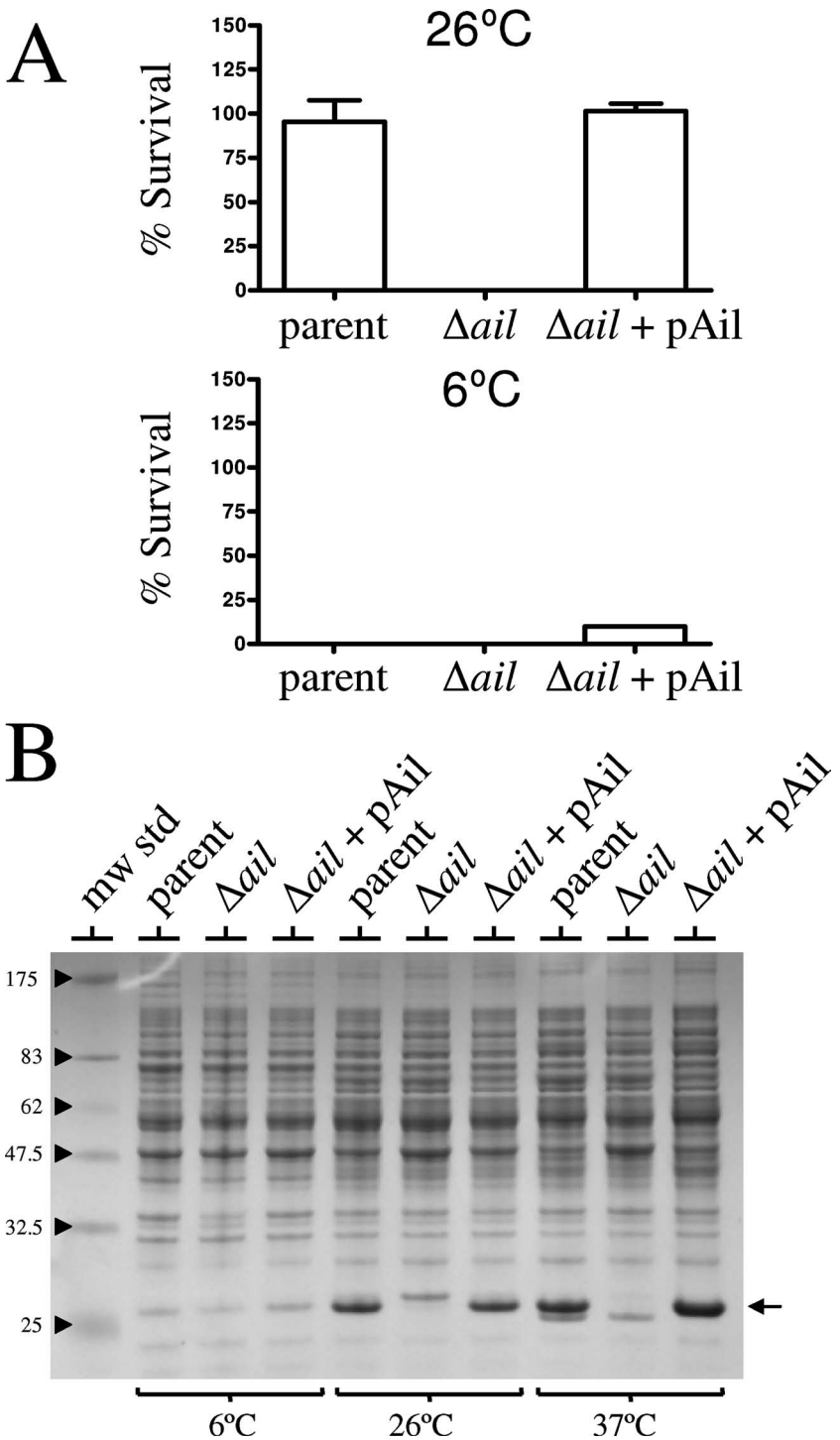

FIG. 3. Ail is not expressed by *Y. pestis* strains grown at 6°C. (A) Survival of wild-type and mutant strains of *Y. pestis* in 80% NHS. Bacteria grown overnight at 6 or 26°C (~5×10^6 bacteria) were incubated with 80% NHS or HIS for 1 h at 37°C. Aliquots were diluted and plated on TBA plates at 30°C. The percent survival was calculated as follows: average number of bacteria that survived exposure to NHS at 1 h/number of bacteria that survived exposure to HIS at 1 h × 100. (B) Coomassie blue R-250-stained SDS-PAGE gel of whole bacterial cell proteins isolated from wild-type and mutant strains of *Y. pestis*. The location of the Ail protein is indicated by an arrow. The positions of molecular mass standards (in kilodaltons) are indicated on the left. The strains used were *Y. pestis* strains KIM8-E (parent), KIM8-E Δy1324 (Δ*ail*), and KIM8-E Δy1324 (Δ*ail*)/pAil. mw std, molecular mass standards.

species of mammals possess different levels of bactericidal activity for gram-negative bacteria. For example, previous studies have shown that NMS is not bactericidal for *E. coli* or *Y. enterocolitica* (13, 23, 43). Furthermore, NMS had no effect on a *Y. enterocolitica ail* or *yadA* deletion mutant (43). The abilities of *Y. pestis* parent and *Y. pestis ail* deletion mutant strains to survive in sera obtained from various mammals were determined essentially as described above for NHS. *Y. pestis*

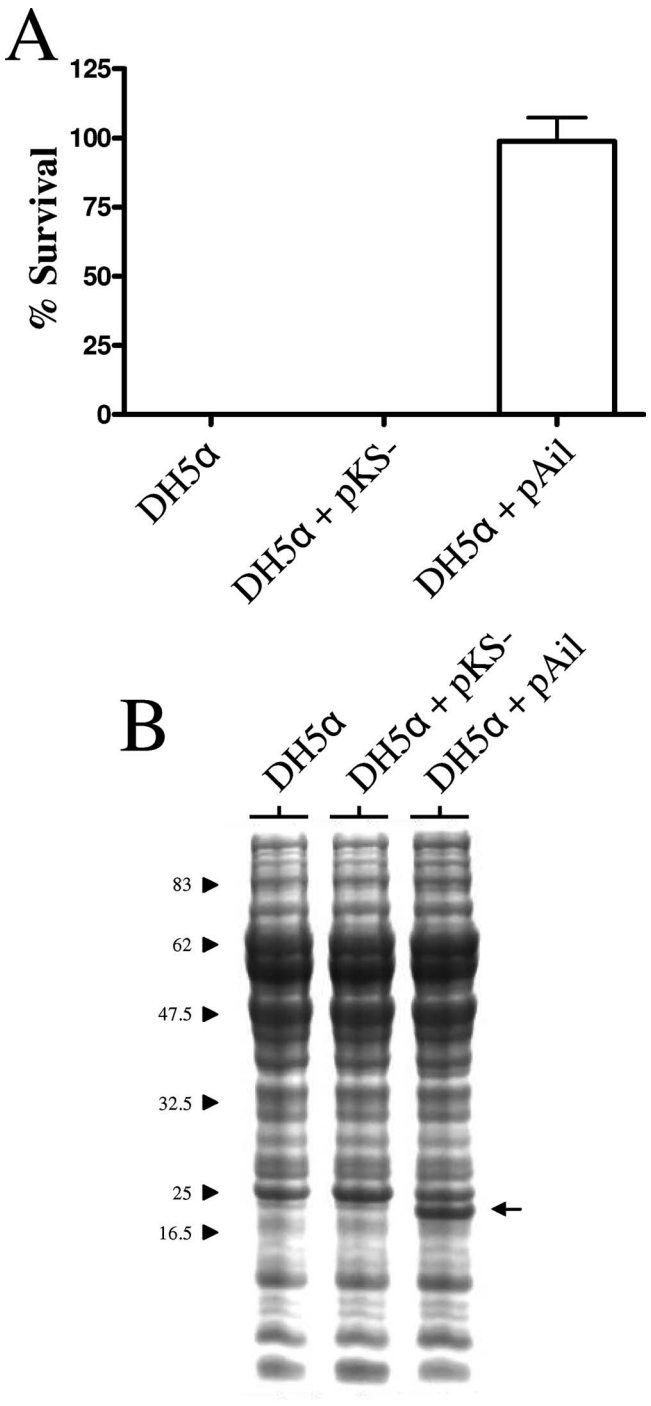

FIG. 4. Expression of Ail in *E. coli* provides protection from complement-dependent killing. (A) Survival of *E. coli* with or without plasmid pAil in 80% NHS. Bacteria grown overnight at 37°C (~5×10^6 bacteria) were incubated with 80% NHS or HIS for 1 h at 37°C. Aliquots were diluted and plated on LB plates at 37°C. The percent survival was calculated as follows: average number of bacteria that survived exposure to NHS at 1 h/number of bacteria that survived exposure to HIS at 1 h × 100. (B) Coomassie blue R-250-stained SDS-PAGE gel of whole bacterial cell proteins isolated from *E. coli* with or without plasmid pAil. The location of the Ail protein is indicated by an arrow. The positions of molecular mass standards (in kilodaltons) are indicated on the left. The strains used were *E. coli* strains DH5α, DH5α/pBluescript KS(−) (pKS$^-$) (vector control), and DH5α/pAil.

KIM8-E and KIM8-E Δ*ail* that had been grown overnight at 26°C were prepared as described above and incubated with sera obtained from humans, goats (NGS), sheep (NSS), rabbits (NRS), guinea pigs (GPS), and mice (final concentration of serum, 80%) for 1 h at 37°C (Fig. 6). The viability of the parent strain *Y. pestis* KIM8-E was unaffected by any of the sera tested. The NHS, NGS, NSS, NRS, and GPS efficiently killed the *ail* deletion mutant. In contrast, the NMS had no affect on the parent or the *ail* deletion mutant. These results confirm that the *ail* deletion mutant is serum sensitive; however, as reported previously, NMS is not bactericidal, and exposure of the *ail* deletion mutant to NMS did not alter the viability of this strain in vitro.

***Y. pestis* Ail is not required for virulence in an intravenous mouse model of plague.** To begin to evaluate the role that *Y. pestis* Ail plays during an infection, preliminary virulence studies were conducted using a murine model of plague. Although NMS is not bactericidal, Ail could have alternate functions or additional roles that are important for *Y. pestis* virulence in mice. Intravenous injection of approximately 100 CFU (six mice) or 1,000 CFU (six mice) of the *Y. pestis* KIM5 *ail* deletion mutant (Pgm$^-$) resulted in 100% fatality by day 6 postinfection. As expected, almost all mice inoculated with the *Y. pestis* KIM5 parent strain (Pgm$^-$) also succumbed to the infection (five of the six mice inoculated with 100 CFU and all six mice inoculated with 1,000 CFU). These data indicate that that the Ail protein is not required for virulence in mice infected via an intravenous route; however, these results do not rule out a role for Ail in mice infected via alternate routes. In addition, Ail is likely required to cause infection in other higher mammals, such as humans, that possess a complement system capable of rapidly killing Ail-deficient strains of *Y. pestis*.

***Y. pestis* Ail is required for virulence in a *C. elegans* infection model.** Previous studies have demonstrated that *Y. pestis* can kill the nematode *C. elegans* by both biofilm-dependent and biofilm-independent mechanisms (9, 38). *Y. pestis* strains that carry the *hmsHFRS* genes produce a biofilm that is required for flea blockage and subsequent transmission of plague (17). The production of biofilm also blocks feeding of *C. elegans*. In contrast, *Y. pestis* strains that lack the *hmsHFRS* genes (for example, Pgm$^-$ strains) are capable of killing *C. elegans* by a biofilm-independent process that involves direct ingestion of the pathogen and accumulation of bacteria in the intestine (38).

To determine if any of the *Y. pestis* Ail/Lom family proteins play a role in the biofilm-independent infection of *C. elegans*, we exposed groups of 40 *C. elegans* N2 young adults to the parent strain (KIM8-E) and to each of the Ail/Lom family deletion mutants (Fig. 7). As shown previously (38), ingestion of the Pgm$^-$ parent strain produced a lethal infection at 25°C that resulted in the death of all 40 worms by day 8 of infection. Similar results were obtained when the worms were fed *Y. pestis* strains specifically deficient in expression of the y1682, y2034, or y2446 gene. In contrast, worms exposed to the Δ*ail* strain or to the strain with all four *ail*-like genes deleted (Δ4) showed significantly increased survival ($P < 0.0001$). Providing the *ail* gene in *trans* (pAil) to the Δ*ail* or Δ4 strain restored the ability of these strains to efficiently kill *C. elegans*. These results indicate that the *Y. pestis* Ail protein plays an important role in the biofilm-independent killing of *C. elegans*. Exposure of *C.*

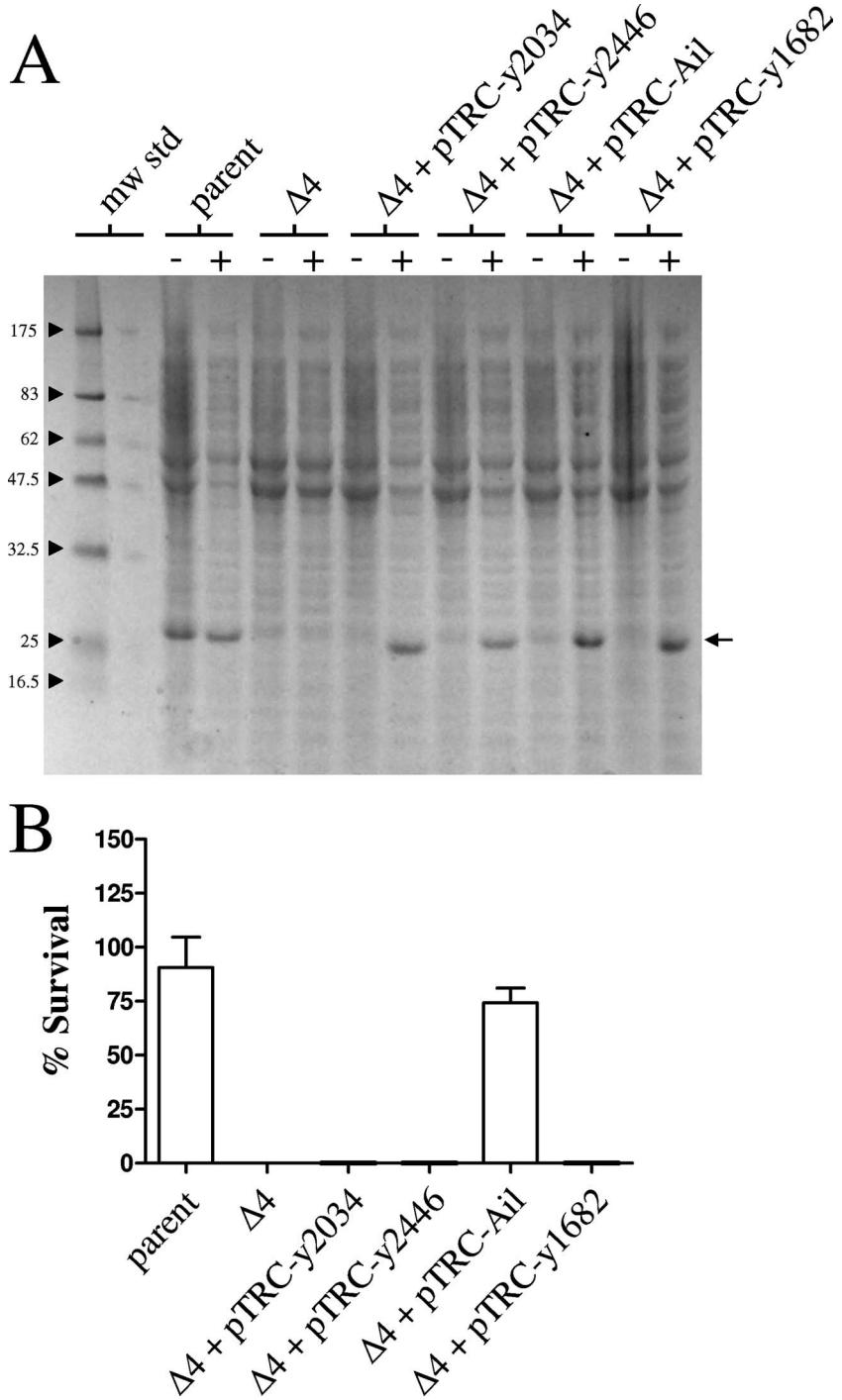

FIG. 5. Expression of Ail, but not expression of other *Y. pestis* Ail/Lom family proteins, provides protection against complement-dependent killing. (A) Coomassie blue R-250-stained SDS-PAGE gel of whole bacterial cell proteins isolated from *Y. pestis* strains induced (lanes +) or not induced (lanes −) with 1 mM IPTG. The location of the various Ail/Lom family proteins is indicated by an arrow. The positions of molecular mass standards (in kilodaltons) are indicated on the left. The strains used were *Y. pestis* strains KIM8-E (parent), KIM8-E Δ4, and KIM8-E Δ4 containing pTRC-y2034, pTRC-y2446, pTRC-Ail, or pTRC-y1682. mw std, molecular mass standards. (B) Survival of the parent strain, the Δ4 strain, and the Δ4 strain carrying plasmid pTRC-y2034, pTRC-y2446, pTRC-Ail, or pTRC-y1682 in 80% NHS. Bacterial cultures were grown at 26°C and induced or not induced with 1 mM IPTG 3 h prior to harvest. Bacteria (~5×10^6 cells) were incubated with 80% NHS or HIS for 1 h at 37°C. Aliquots were diluted and plated on TBA plates at 30°C. The percent survival was calculated as follows: average number of bacteria that survived exposure to NHS at 1 h/number of bacteria that survived exposure to HIS at 1 h × 100.

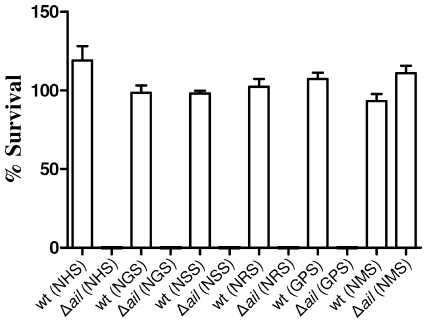

FIG. 6. Ail is required for survival in sera obtained from many diverse mammals but not for survival in mouse serum: survival of the parent (wt) and Δ*ail* strains of *Y. pestis* in 80% animal sera. Bacteria grown overnight at 26°C (~5×10^6 bacteria) were incubated with 80% animal serum or HIS for 1 h at 37°C. Aliquots were diluted and plated on TBA plates at 30°C. The percent survival was calculated as follows: average number of bacteria that survived exposure to NHS at 1 h/number of bacteria that survived exposure to HIS at 1 h × 100.

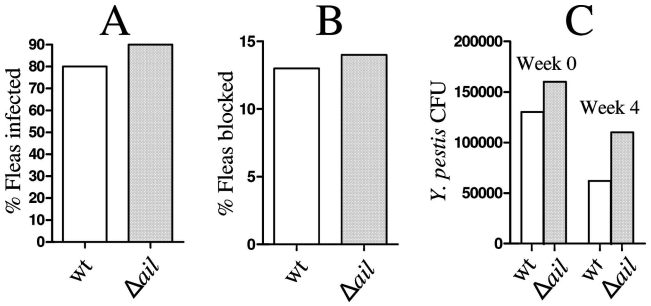

FIG. 8. Ail is not required for infection or blockage of fleas. (A) Colonization of fleas after an infected-blood meal. (B) Flea blockage during a 4-week period following a single infected-blood meal. (C) *Y. pestis* CFU associated with infected fleas on day 0 (week 0) and day 28 (week 4). wt, parent strain KIM6+; Δ*ail*, strain KIM6+ Δ*ail*.

elegans to biofilm-competent (Pgm^+) *Y. pestis* KIM6+ or KIM6+ Δ*ail* resulted in rapid killing of the nematodes, indicating that Ail plays no significant role in the biofilm-dependent killing of *C. elegans* (data not shown).

***Y. pestis* Ail is not required to infect or block fleas.** Previously, the *C. elegans* infection model has been used to identify and/or characterize gene products that are important for *Y. pestis* to infect and/or block fleas (8, 9). *C. elegans* infection experiments are performed at 25°C; therefore, *Y. pestis* gene products expressed in the *C. elegans* infection model are likely to also be expressed in the flea (21°C). Therefore, we investigated if the *Y. pestis* Ail protein is required to infect or block fleas.

X. cheopis rat fleas were infected with mouse blood containing *Y. pestis* KIM6+ or an isogenic *ail* deletion mutant (KIM6+ Δ*ail*) (Fig. 8). Infected fleas were monitored for 4 weeks following the initial infection. During this time, 13% of the fleas infected with KIM6+ and 14% of the fleas infected with KIM6+ Δ*ail* became blocked. Similarly, no significant difference in the infection rate or bacterial load per flea was observed between the two strains, as determined by a *t* test. These data indicate that Ail does not play a significant role in the infection or blockage of fleas.

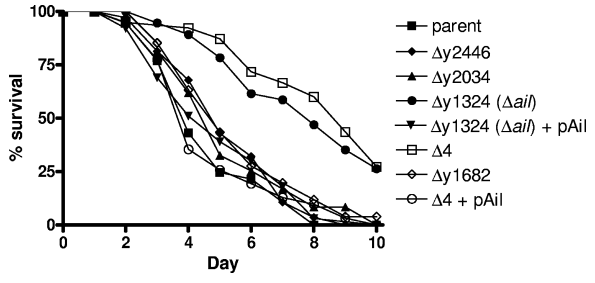

FIG. 7. Ail is required for efficient biofilm-independent killing of *C. elegans*. Groups of 40 *C. elegans* N2 young adults were exposed to *Y. pestis* KIM8-E (parent), KIM8-E Δy2446, KIM8-E Δy2034, KIM8-E Δy1324 (Δ*ail*), Δy1324 (Δ*ail*)/pAil, KIM8-E Δ4, KIM8-E Δ4/pAil, and Δy1682. Animal survival was plotted using the PRISM computer program.

DISCUSSION

The success of *Y. pestis* as a vector-borne pathogen is dependent upon its ability to produce high-level septicemia in its host. The ability to reliably generate bacterial loads in the blood that can approach 10^9 bacteria/ml ensures that a feeding flea ingests a sufficient number of bacteria to establish an infection (12, 16, 22). To survive in the blood of its mammalian host and in the blood meal of its flea vector, *Y. pestis* must be resistant to complement-dependent killing at both 26 and 37°C. We determined that the *Y. pestis* Ail protein is required for resistance to complement-dependent killing at both temperatures. Thus, Ail is an important virulence factor that would be expected to be essential for infection of mammalian hosts that possess a fully functional bactericidal complement system. Indeed, Ail may represent one of the key factors that has enabled *Y. pestis* to infect a wide range of mammals, including humans.

Anisimov et al. (1) demonstrated that *Y. pestis* strains that are resistant to complement at 26 and 37°C become sensitive to complement when they are grown at 6°C. It has been hypothesized that this response represents part of a coordinated down regulation of virulence attributes that enables *Y. pestis* to coexist with its host during periods of winter hibernation. Our studies indicate that strong down regulation of expression of Ail at low temperatures is likely responsible for the dramatic increase in the susceptibility of the bacteria to serum when they are grown at 6°C; however, other factors, including temperature-dependent changes in LPS structure, may also be involved in regulating serum resistance in response to low temperature (1).

Previous studies have indicated that LPS may play a critical role in the resistance of *Y. pestis* to complement-mediated killing (1, 34); however, the fact that *Y. pestis* strains deficient in expression of Ail are extremely sensitive to complement supports the hypothesis that *Y. pestis* LPS provides little or no intrinsic protection against complement. Alternatively, alterations in the structure of LPS may have dramatic effects on the expression, surface exposure, or activity of Ail. Indeed, in *Y. enterocolitica* alterations in LPS structure have been demonstrated to alter both Ail expression and Ail function (3, 32). Thus, the structure of *Y. pestis* LPS is likely optimized to promote Ail function and may be modified, at least in part, to

regulate Ail function in response to changes in environmental conditions.

Initial infection experiments indicated that Ail is not required to produce a lethal infection in mice when an intravenous route of infection is used. This was not surprising, considering that mouse serum is not bactericidal for *Yersinia* spp. (13) and thus had no effect on the *ail* deletion mutant. In contrast, sera from guinea pigs, rabbits, sheep, goats, and humans were all able to rapidly kill the *ail* deletion mutant. These results strongly indicate that the *ail* deletion mutant should be unable to survive in these animals and indicate that Ail is an important virulence factor; however, confirmation of the role of Ail during an infection will likely require an alternative animal model. Guinea pigs have previously been used to evaluate the virulence of *Y. pestis* (18) and may represent an alternative host for evaluation of the role of Ail during an infection.

Surprisingly, the *ail* deletion mutant exhibited a significant defect in virulence in the biofilm-independent *C. elegans* infection model (38). Interestingly, the *C. elegans* genome appears to encode only a limited number of putative complement components (28), such as factor H, a regulator of the alternative pathway (20). However, the unique combinations of mammalian complement domains required for a functional pathway are not found in nematodes, suggesting that Ail may provide protection against other antibacterium products or that Ail has alternative functions that are important in *C. elegans* virulence. Interestingly, the *Y. enterocolitica* Ail protein functions as an adhesin/invasin, in addition to its role in serum resistance (27). However, no role in attachment to, or invasion of, eukaryotic cells was associated with the *Y. pseudotuberculosis* Ail protein (44). Future studies will be aimed at determining the specific role of Ail in *C. elegans*.

Y. enterocolitica Ail was originally identified as an outer membrane protein that promoted bacterial invasion of eukaryotic cells (26). Subsequent studies revealed that Ail also plays a role in serum resistance (5). The mechanism by which *Y. enterocolitica* Ail protects the bacterium from complement-dependent lysis is not understood. In fact, studies with *Y. enterocolitica* have suggested that Ail alone is not sufficient to fully protect *Y. enterocolitica* from complement-dependent killing in vitro (4). Instead, evidence suggests that the *Y. enterocolitica* YadA protein is the dominant serum resistance factor expressed by this bacterium. In contrast to serum resistance in *Y. enterocolitica*, in *Y. pestis* Ail appears to be the sole complement resistance factor required to fully protect the bacterium from complement in vitro (44). This suggests that the *Y. pestis* Ail protein functions differently, is more efficient, or is expressed at a higher level than the *Y. enterocolitica* Ail protein.

The *Y. enterocolitica* Ail and *Y. pestis* Ail proteins share significant amino acid sequence identity throughout their lengths (75% overall identity); however, the level of amino acid sequence identity in the four surface-exposed loops is much less (53%) than the level in the regions located outside these loops (87%). Results of site-directed mutagenesis studies of *ail* of *Y. enterocolitica* suggest that the residues located in loop 2 are particularly important for Ail function; however, only 5 of the 19 residues found in loop 2 are identical to residues found in loop 2 of *Y. pestis* Ail (Fig. 1). Therefore, even though preliminary experiments suggest that the *Y. pestis* Ail protein is expressed at a higher level than the Y. *enterocolitica* Ail protein (data not shown), it is also possible that amino acid sequence differences in the surface-exposed loops account, in part, for the ability of the *Y. pestis* Ail protein to fully protect the bacterium from complement-dependent killing. It is also possible that the *Y. pestis* Ail protein, which closely resembles Ail of *Y. pseudotuberculosis*, represents a protein optimized and dedicated to serum resistance. Indeed, previous studies have suggested that the *Y. pseudotuberculosis* Ail protein has no role in bacterial attachment or invasion; however, recent studies of Kolodziejek et al. (19) indicated that the *Y. pestis* Ail protein functions as both an adhesin and an invasin. These studies suggested that *Y. pestis* Ail, like *Y. enterocolitica* Ail, is a multifunctional protein with roles in serum resistance, attachment, and invasion.

ACKNOWLEDGMENTS

We thank Hans Wolf-Watz for helpful discussions and Lisa Plano for advice and technical assistance.

This work was funded by grants R01 AI039575 and R21 AI074820-02 from the National Institutes of Health to G.V.P.

REFERENCES

1. **Anisimov, A. P., S. V. Dentovskaya, G. M. Titareva, I. V. Bakhteeva, R. Z. Shaikhutdinova, S. V. Balakhonov, B. Lindner, N. A. Kocharova, S. N. Senchenkova, O. Holst, G. B. Pier, and Y. A. Knirel.** 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.
2. **Bartra, S. S., M. W. Jackson, J. A. Ross, and G. V. Plano.** 2006. Calcium-regulated type III secretion of Yop proteins by an *Escherichia coli hha* mutant carrying a *Yersinia pestis* pCD1 virulence plasmid. Infect. Immun. **74:**1381–1386.
3. **Bengoechea, J. A., H. Najdenski, and M. Skurnik.** 2004. Lipopolysaccharide O antigen status of *Yersinia enterocolitica* O:8 is essential for virulence and absence of O antigen affects the expression of other *Yersinia* virulence factors. Mol. Microbiol. **52:**451–469.
4. **Biedzka-Sarek, M., R. Venho, and M. Skurnik.** 2005. Role of YadA, Ail, and lipopolysaccharide in serum resistance of *Yersinia enterocolitica* serotype O:3. Infect. Immun. **73:**2232–2244.
5. **Bliska, J. B., and S. Falkow.** 1992. Bacterial resistance to complement killing mediated by the Ail protein of *Yersinia enterocolitica*. Proc. Natl. Acad. Sci. USA **89:**3561–3565.
6. **Cambau, E., F. Bordon, E. Collatz, and L. Gutmann.** 1993. Novel *gyrA* point mutation in a strain of *Escherichia coli* resistant to fluoroquinolones but not to nalidixic acid. Antimicrob. Agents Chemother. **37:**1247–1252.
7. **Cornelis, G. R.** 2000. Molecular and cell biology aspects of plague. Proc. Natl. Acad. Sci. USA **97:**8778–8783.
8. **Darby, C., S. L. Ananth, L. Tan, and B. J. Hinnebusch.** 2005. Identification of *gmhA*, a *Yersinia pestis* gene required for flea blockage, by using a *Caenorhabditis elegans* biofilm system. Infect. Immun. **73:**7236–7242.
9. **Darby, C., J. W. Hsu, N. Ghori, and S. Falkow.** 2002. *Caenorhabditis elegans*: plague bacteria biofilm blocks food intake. Nature **417:**243–244.
10. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97:**6640–6645.
11. **Deng, W., V. Burland, G. Plunkett III, A. Boutin, G. F. Mayhew, P. Liss, N. T. Perna, D. J. Rose, B. Mau, S. Zhou, D. C. Schwartz, J. D. Fetherston, L. E. Lindler, R. R. Brubaker, G. V. Plano, S. C. Straley, K. A. McDonough, M. L. Nilles, J. S. Matson, F. R. Blattner, and R. D. Perry.** 2002. Genome sequence of *Yersinia pestis* KIM. J. Bacteriol. **184:**4601–4611.
12. **Engelthaler, D. M., B. J. Hinnebusch, C. M. Rittner, and K. L. Gage.** 2000. Quantitative competitive PCR as a technique for exploring flea-*Yersina pestis* dynamics. Am. J. Trop. Med. Hyg. **62:**552–560.
13. **Hanski, C., M. Naumann, A. Grutzkau, G. Pluschke, B. Friedrich, H. Hahn, and E. O. Riecken.** 1991. Humoral and cellular defense against intestinal murine infection with *Yersinia enterocolitica*. Infect. Immun. **59:**1106–1111.
14. **Heffernan, E. J., S. Reed, J. Hackett, J. Fierer, C. Roudier, and D. Guiney.** 1992. Mechanism of resistance to complement-mediated killing of bacteria encoded by the *Salmonella typhimurium* virulence plasmid gene *rck*. J. Clin. Investig. **90:**953–964.
15. **Heffernan, E. J., L. Wu, J. Louie, S. Okamoto, J. Fierer, and D. G. Guiney.** 1994. Specificity of the complement resistance and cell association phenotypes encoded by the outer membrane protein genes *rck* from *Salmonella typhimurium* and *ail* from *Yersinia enterocolitica*. Infect. Immun. **62:**5183–5186.

16. **Hinnebusch, B. J.** 2005. The evolution of flea-borne transmission in *Yersinia pestis*. Curr. Issues Mol. Biol. **7:**197–212.
17. **Hinnebusch, B. J., R. D. Perry, and T. G. Schwan.** 1996. Role of the *Yersinia pestis* hemin storage (*hms*) locus in the transmission of plague by fleas. Science **273:**367–370.
18. **Jones, S. M., K. F. Griffin, I. Hodgson, and E. D. Williamson.** 2003. Protective efficacy of a fully recombinant plague vaccine in the guinea pig. Vaccine **21:**3912–3918.
19. **Kolodziejek, A. M., D. J. Sinclair, K. S. Seo, D. R. Schnider, C. F. Deobald, H. N. Rohde, A. K. Viall, S. S. Minnich, C. J. Hovde, S. A. Minnich, and G. A. Bohach.** 2007. Phenotypic characterization of OmpX, an Ail homologue of *Yersinia pestis* KIM. Microbiology **153:**2941–2951.
20. **Krushkal, J., C. Kemper, and I. Gigli.** 1998. Ancient origin of human complement factor H. J. Mol. Evol. **47:**625–630.
21. **Lindler, L. E., G. V. Plano, V. Burland, G. F. Mayhew, and F. R. Blattner.** 1998. Complete DNA sequence and detailed analysis of the *Yersinia pestis* KIM5 plasmid encoding murine toxin and capsular antigen. Infect. Immun. **66:**5731–5742.
22. **Lorange, E. A., B. L. Race, F. Sebbane, and B. Joseph Hinnebusch.** 2005. Poor vector competence of fleas and the evolution of hypervirulence in *Yersinia pestis*. J. Infect. Dis. **191:**1907–1912.
23. **Marcus, S., D. W. Esplin, and D. M. Donaldson.** 1954. Lack of bactericidal effect of mouse serum on a number of common microorganisms. Science **119:**877.
24. **Mecsas, J., R. Welch, J. W. Erickson, and C. A. Gross.** 1995. Identification and characterization of an outer membrane protein, OmpX, in *Escherichia coli* that is homologous to a family of outer membrane proteins including Ail of *Yersinia enterocolitica*. J. Bacteriol. **177:**799–804.
25. **Miller, V. L., K. B. Beer, G. Heusipp, B. M. Young, and M. R. Wachtel.** 2001. Identification of regions of Ail required for the invasion and serum resistance phenotypes. Mol. Microbiol. **41:**1053–1062.
26. **Miller, V. L., J. B. Bliska, and S. Falkow.** 1990. Nucleotide sequence of the *Yersinia enterocolitica ail* gene and characterization of the Ail protein product. J. Bacteriol. **172:**1062–1069.
27. **Miller, V. L., and S. Falkow.** 1988. Evidence for two genetic loci in *Yersinia enterocolitica* that can promote invasion of epithelial cells. Infect. Immun. **56:**1242–1248.
28. **Nonaka, M., and F. Yoshizaki.** 2004. Primitive complement system of invertebrates. Immunol. Rev. **198:**203–215.
29. **Parkhill, J., B. W. Wren, N. R. Thomson, R. W. Titball, M. T. Holden, M. B. Prentice, M. Sebaihia, K. D. James, C. Churcher, K. L. Mungall, S. Baker, D. Basham, S. D. Bentley, K. Brooks, A. M. Cerdeno-Tarraga, T. Chillingworth, A. Cronin, R. M. Davies, P. Davis, G. Dougan, T. Feltwell, N. Hamlin, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Leather, S. Moule, P. C. Oyston, M. Quail, K. Rutherford, M. Simmonds, J. Skelton, K. Stevens, S. Whitehead, and B. G. Barrell.** 2001. Genome sequence of *Yersinia pestis*, the causative agent of plague. Nature **413:**523–527.
30. **Perry, R. D., and J. D. Fetherston.** 1997. *Yersinia pestis*—etiologic agent of plague. Clin. Microbiol. Rev. **10:**35–66.
31. **Perry, R. D., S. C. Straley, J. D. Fetherston, D. J. Rose, J. Gregor, and F. R. Blattner.** 1998. DNA sequencing and analysis of the low-Ca^{2+}-response plasmid pCD1 of *Yersinia pestis* KIM5. Infect. Immun. **66:**4611–4623.
32. **Pierson, D. E.** 1994. Mutations affecting lipopolysaccharide enhance *ail*-mediated entry of *Yersinia enterocolitica* into mammalian cells. J. Bacteriol. **176:**4043–4051.
33. **Plano, G. V., and S. C. Straley.** 1995. Mutations in *yscC*, *yscD*, and *yscG* prevent high-level expression and secretion of V antigen and Yops in *Yersinia pestis*. J. Bacteriol. **177:**3843–3854.
34. **Porat, R., W. R. McCabe, and R. R. Brubaker.** 1995. Lipopolysaccharide-associated resistance to killing of yersiniae by complement. J. Endotoxin Res. **2:**91–97.
35. **Protsenko, O. A., P. I. Anisimov, O. T. Mozharov, N. P. Konnov, and A. Popov Iu.** 1983. Detection and characterization of the plasmids of the plague microbe which determine the synthesis of pesticin I, fraction I antigen and "mouse" toxin exotoxin. Genetika **19:**1081–1090. (In Russian.)
36. **Pulkkinen, W. S., and S. I. Miller.** 1991. A *Salmonella typhimurium* virulence protein is similar to a *Yersinia enterocolitica* invasion protein and a bacteriophage lambda outer membrane protein. J. Bacteriol. **173:**86–93.
37. **Sikkema, D. J., and R. R. Brubaker.** 1987. Resistance to pesticin, storage of iron, and invasion of HeLa cells by yersiniae. Infect. Immun. **55:**572–578.
38. **Styer, K. L., G. W. Hopkins, S. S. Bartra, G. V. Plano, R. Frothingham, and A. Aballay.** 2005. *Yersinia pestis* kills *Caenorhabditis elegans* by a biofilm-independent process that involves novel virulence factors. EMBO Rep. **6:**992–997.
39. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
40. **Une, T., and R. R. Brubaker.** 1984. In vivo comparison of avirulent Vwa^- and Pgm^- or Pst^r phenotypes of yersiniae. Infect. Immun. **43:**895–900.
41. **Viboud, G. I., and J. B. Bliska.** 2004. *Yersinia* outer proteins: role in modulation of host cell signaling responses and pathogenesis. Annu. Rev. Microbiol. **59:**69–89.
42. **Vogt, J., and G. E. Schulz.** 1999. The structure of the outer membrane protein OmpX from *Escherichia coli* reveals possible mechanisms of virulence. Structure **7:**1301–1309.
43. **Wachtel, M. R., and V. L. Miller.** 1995. In vitro and in vivo characterization of an *ail* mutant of *Yersinia enterocolitica*. Infect. Immun. **63:**2541–2548.
44. **Yang, Y., J. J. Merriam, J. P. Mueller, and R. R. Isberg.** 1996. The *psa* locus is responsible for thermoinducible binding of *Yersinia pseudotuberculosis* to cultured cells. Infect. Immun. **64:**2483–2489.

Editor: A. J. Bäumler