

Intraspecies and Temperature-Dependent Variations in Susceptibility of *Yersinia pestis* to the Bactericidal Action of Serum and to Polymyxin B

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Received 11 May 2005/Returned for modification 23 June 2005/Accepted 10 August 2005

Lipopolysaccharide (LPS) structure impacts the bactericidal action of cationic peptides, such as polymyxin B (PMB), and sensitivity to killing by normal human serum (NHS). Cultivation of different subspecies strains of *Yersinia pestis* isolated from unrelated geographic origins at various temperatures (mammals, 37°C; fleas, 25°C; or winter hibernation, 6°C) affects LPS composition and structure. We tested the susceptibilities of various strains of *Y. pestis* grown at these different temperatures to PMB and serum bactericidal killing. Both properties varied significantly in response to temperature changes. In *Y. pestis* subsp. *pestis* (the main subspecies causing human plague), high levels of resistance to PMB and NHS were detected at 25°C. However, at the same temperature, *Y. pestis* subsp. *caucasica* was highly sensitive to PMB. At both of the extreme temperatures, all strains were highly susceptible to PMB. At 25°C and 37°C, *Y. pestis* subsp. *caucasica* strain 1146 was highly susceptible to the bactericidal activity of 80% NHS. All *Y. pestis* strains studied were able to grow in heat-inactivated human serum or in 80% normal mouse serum. At 6°C, all strains were highly sensitive to NHS. Variations in the PMB resistance of different bacterial cultures related to both the content of cationic components (4-amino-4-deoxyarabinose in lipid A and glycine in the core) and a proper combination of terminal monosaccharides in the LPS. The NHS resistance correlated with an elevated content of *N*-acetylglucosamine in the LPS. Structural variation in the LPS of *Y. pestis* correlates with the organism's ability to resist innate immunity in both fleas and mammals.

Bubonic and pneumonic plague have in the past caused the loss of >200 million human lives, more than any other known infection. Although it is presently not a major public health problem, small outbreaks of plague continue to occur throughout the world and at least 2,000 cases of plague are reported annually. Plague has recently been recognized as a reemerging disease, and its causative agent, *Yersinia pestis*, is a category A biothreat agent that could potentially be used as a biological weapon. This gram-negative bacterium circulates in natural foci, which involve a rodent reservoir (gerbils, ground squirrels, marmots, voles, pikas, prairie dogs, guinea pigs, rats, etc.) and an insect vector (more than 80 flea species). Natural plague foci exist in Eurasia, Africa, and the Americas. The high lethality of plague in rodent reservoirs is necessary for its continued transmission in nature. Fleas ingesting infected blood during preagonal bacteremia must depart from the dead host and feed on a new rodent, which subsequently becomes an

infected source for further plague transmission (4–7, 12, 13, 16, 18, 19, 25, 28, 42, 43, 57).

In order to maintain its life cycle, *Y. pestis* must adapt to a broad range of environmental conditions with phenotypic changes commensurate with survival in these disparate environments. These conditions encompass a broad range of tolerated temperatures from 4°C to 41°C (12, 38, 42), allowing survival in winter-sleeping rodents during their hibernation and in the fleas associated with them (5°C) (30, 31, 40), survival and growth in fleas residing in rodent burrows or mammalian hair (21°C to 28°C), and survival and growth within the bodies and body fluids of infected mammals (37°C to 41°C) (7, 12, 42). In addition, *Y. pestis* must survive innate immune factors encountered in the flea vector intestinal tract, particularly the proventriculus and midgut, and innate and acquired immune effectors in the blood of susceptible rodents and accidentally infected hosts such as humans.

Temperature-dependent cellular variations must promote the virulence of *Y. pestis*, which results, in part, from its impressive ability to overcome the innate immune systems of both flea vectors and susceptible mammalian hosts, followed by massive microbial growth. However, *Y. pestis* strains isolated from enzootic foci are transmitted via different rodent and flea species, which has led to the assumption that the emergence of variant *Y. pestis* strains, classified variously as biovars (2, 7, 56),

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TABLE 1. Strains used in these studies

Strain	Biovar/subsp. ^a	Relevant characteristic(s)	Geographical origin of parent strain/reference
<i>Y. pestis</i> KM218	<i>Orientalis/pestis</i>	pFra ⁻ pCD ⁻ pPst ⁻ Δ <i>pgm</i> ; derived from the Russian vaccine strain EV line NIIEG	Madagascar
EV11M	<i>Antiqua/pestis</i>	pFra ⁻ pCD ⁻ pPst ⁻ Δ <i>pgm</i> ; derived from the Russian vaccine strain EV line NIIEG and carrying an undefined chromosomal mutation(s) resulting from multiple in vitro passages	Madagascar
KM260(11)	<i>Antiqua/pestis</i>	pFra ⁻ pCD ⁻ pPst ⁻ ; derived from the virulent strain 231	Aksai focus, Kirghizia
KIMD1	<i>Medievalis/pestis</i>	pFra ⁻ pCD ⁻ pPst ⁺ Δ <i>pgm</i>	Iran/Kurdistan
1146	<i>Antiqua/caucasica</i>	pFra ⁻ pCD ⁻ pPst ⁻ ; derived from the virulent strain 1146-Arm	Trans-Caucasian-highland focus, Caucasus
<i>E. coli</i> DH5α		<i>lacZΔM15 Δ(lacZYA-argF) recA1 endA1 hsdR17(r_k⁻ m_k⁺) phoA supE44 thi gyrA96 relA1</i>	51

^a For more detailed information on biovar-subspecies interrelations, see reference 7.

subspecies (7), ecotypes (7, 55), populations (1), or genotypes/genomovars (2, 33, 37, 45, 46, 56), is due to adaptation to specific factors within these different hosts. Variant strains of *Y. pestis* can be differentiated by fermentative activity, nutritional requirements, or ability to cause infectious bacteremia and death in diverse mammalian species, as well as via genome rearrangements. *Y. pestis* subsp. *pestis* strains, including those from biovars *antiqua*, *medievalis*, and *orientalis*, that are circulating all over the world are incapable of fermentation of rhamnose and as a rule are highly virulent for guinea pigs and humans (7, 55). Strains from each of these biovars are proposed to have been the cause of at least one pandemic (2). Within the most ancient natural plague foci in Asia, "rhamnose fermentation-positive" strains belonging to the so-called "pestoides group" or "microtus biovar," which consist of Russian subspecies other than *Y. pestis* subsp. *pestis* and 5 of 18 Chinese ecotypes, are of low virulence or are avirulent for guinea pigs and have caused only three reported cases of nonlethal human plague that were not accompanied by outbreaks of human-to-human transmission of infection (7, 55). While most studies have focused on the highly virulent *Y. pestis* subsp. *pestis* strains, particularly the clonally derived isolates distributed throughout North and South America (7), the availability of variant strains that efficiently cause plague in endemic rodent populations provides a means to gain a more complete understanding of the pathogenic potential of *Y. pestis* by contrasting the properties of the strains virulent for humans with those of enzootic strains with high virulence for some wild animals.

One factor known to vary greatly with environmental conditions, and likely to affect virulence, is the lipopolysaccharide (LPS) of *Y. pestis*. *Y. pestis* has a rough-type LPS (15, 27, 34, 35, 48, 49, 53, 54) lacking the O-polysaccharide chain due to inactivation of the O-antigen gene cluster by several frameshift mutations (49, 53). LPS seems to play a role in resistance of *Y. pestis* to serum-mediated lysis (44), which is necessary for survival and growth of the bacteria in mammalian blood and for transmission between insects and mammals (4–7, 12, 41, 42). LPS structure also determines bacterial resistance to cationic antimicrobial peptides (10), a key component of innate immunity in both mammals and insects (17). Recently, the structure of the *Y. pestis* LPS has been extensively analyzed.

The full LPS structure from representative strains of the various subspecies grown at 6°C, 21°C to 28°C, or 37°C have been published (8, 21, 27, 32, 34, 35, 50, 54). Variations in the LPS structure of *Y. pestis* were found to depend on the growth temperature, and prior reports suggest an important biological impact of the LPS structure related to virulence (32, 34, 35, 50). Rebeil et al. (50) showed that *Y. pestis* subsp. *pestis* was resistant to polymyxin B (PMB) when grown at 21°C but not when grown at 37°C and associated this phenotypic variation with substitution of the lipid A component of LPS with two molecules per glucosamine dimer of 4-amino-4-deoxyarabinose (Ara4N), a modification found to be dependent on the PhoP/PhoQ system. However, it has not been determined if structural variations in the LPSs of the various *Y. pestis* strains differing in epidemic potential and geographic origin contribute to serum sensitivity and resistance to antimicrobial peptides. In this report, we determined the serum sensitivities and susceptibilities to PBM of a variety of *Y. pestis* strains differing in subspecies or biovar classification and/or epidemic potential and correlated these results with the known structural variations in the LPS.

(This work was presented in part at the 104th General Meeting of the American Society for Microbiology, 23 to 27 May 2004, in New Orleans, La.)

MATERIALS AND METHODS

Bacterial strains. *Y. pestis* strain KIMD1 was kindly provided by M. Skurnik (University of Turku, Turku, Finland). Other *Y. pestis* strains used were obtained from the Russian Research Anti-Plague Institute "Microbe" (Saratov, Russia). Characteristics of the strains are given in Table 1. To guarantee the safety of the investigators, *Y. pestis* strains were attenuated by elimination of the virulence plasmid, pCD (42). None of the absent plasmids or missing parts of the genome of mutant strains contained genes for LPS biogenesis in any of the strains except for deep-rough mutant EV11M, which carries an unidentified chromosomal mutation(s). All parental strains containing pCD were virulent in both mice and guinea pigs, except for strain 1146-Arm, which, as are the majority of rhamnose fermentation-positive strains (7), is virulent for mice but avirulent for guinea pigs. Bacterial cultures were started from lyophilized stocks.

Growth of bacteria. For LPS isolation, *Y. pestis* strains were grown at 25°C or 37°C in New Brunswick Scientific fermentors with working volumes up to 10 liters. Liquid aerated medium was used and was composed of fish flour hydrolysate (20 to 30 g/liter), yeast autolysate (10 g/liter), glucose (3 to 9 g/liter), K₂HPO₄ (6 g/liter), KH₂PO₄ (3 g/liter), and MgSO₄ (0.5 g/liter) (pH 6.9 to 7.1).

A pH and partial O₂ pressure control was used, with the specified partial O₂ pressure value of >10%. *Y. pestis* biomasses were harvested by centrifugation after 48 h of incubation and then freeze-dried. When necessary, *Y. pestis* strains were grown on solid surfaces by using the same medium supplemented with 2% agar, pH 7.2. In some experiments, *Y. pestis* strains were grown at temperatures from 5°C to 40°C. *Escherichia coli* DH5 α was grown in the same medium at 37°C.

Isolation of LPS and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. LPS was extracted from dried cells with phenol-chloroform–light petroleum ether (20) and purified by repeated ultracentrifugation (105,000 \times g, 4 h) following enzymatic digestion of nucleic acids and proteins. The purity of the isolated LPS preparations was evident from the lack of protein and nucleic acid contaminants, as determined by sodium dodecyl sulfate-glycine polyacrylamide gel electrophoresis with silver staining of the gels (49) and gas-liquid chromatography of the derived alditol acetates (52), respectively. The LPS preparations from *Y. pestis* cultures of strain 1146 grown at 25°C and 37°C are designated LPS-25₁₁₄₆ and LPS-37₁₁₄₆, respectively.

Mass spectrometry. High-resolution electrospray ionization Fourier transform ion cyclotron resonance (ESI FTICR) mass spectrometry was performed in the negative-ion mode using an ApexII instrument (Bruker Daltonics, Billerica, MA) equipped with a 7-T actively shielded magnet and an Apollo electrospray ion source, as previously described (34). The content of each variable LPS constituent was estimated by relative intensities of mass peaks for molecules with and without the constituent in the mass spectra of the whole LPS. Capillary skimmer dissociation was induced by increasing the capillary exit voltage from –100 to –350 V.

Polymyxin resistance test. Determination of PMB MICs was performed essentially as described previously (27). The *Y. pestis* strains were grown on solid medium for 48 h at 5°C, 10°C, or 40°C or for 24 h at 15°C to 37°C. Dilutions of PMB (0.3 to 2,500 U/ml) were prepared in 96-well polypropylene microtiter plates in a volume of 50 μ l. Next, 200 μ l of the bacterial suspensions (10⁸ CFU/ml) were added and the microtiter plates were incubated at the different temperatures for the same periods as were used to prepare the inocula for the assay. At the end of the assays, the optical densities of the bacterial suspensions were measured at 595 nm by using a multichannel plate reader (LabSystem, Finland). The MIC was determined as the lowest concentration of PMB that did not result in measurable growth at the end of the experimental period. Each sample was run in triplicate, and experiments were repeated at least twice.

Binding of polymyxin B by LPS. LPS samples were suspended at different concentrations in 0.1 M sodium phosphate buffer, pH 7.4, and 100- μ l aliquots were mixed with 10 μ l of a solution containing 1,400 U PMB/ml and incubated at 37°C for 1 h. Following incubation, the mixtures were centrifuged at 14,000 \times g for 20 min, and the supernatants were tested for bactericidal activity in a MIC type assay against *Y. pestis* strain EV11M grown at 25°C, a strain containing only a disaccharide component for the LPS sugar substituents (34) and thus highly sensitive to PMB. EV11M cells were incubated with the twofold dilutions of supernatants at 25°C for 24 h, and the amount of unbound PMB left in the supernatants after initial incubation with the different *Y. pestis* LPS samples was calculated in comparison to a standard curve derived by adding known concentrations of PMB to *Y. pestis* strain EV11M.

Serum bactericidal assay. A pool of immune human serum (IHS) was obtained from eight healthy volunteers who were annually immunized with a Russian live-plague vaccine. A pool of normal human serum (NHS) was obtained from 10 nonimmunized healthy volunteers. Normal mouse sera were obtained from the Pushchino Branch of the Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Science. Sera were pooled, divided into aliquots, and stored at –70°C. Before use, sera were thawed on ice, and any remaining unused sera were discarded. The complement was inactivated by incubating sera at 56°C for 30 min.

Bactericidal properties of sera were studied essentially as described previously (9). Briefly, *Y. pestis* grown overnight on solid medium at 6°C, 25°C, or 37°C was suspended in phosphate-buffered saline. The optical density was determined at 600 nm, and bacteria were diluted to 10⁸ CFU/ml. CFU were determined by plating on solid medium. An aliquot of diluted bacteria (50 μ l) was put into a microcentrifuge tube, to which 200 μ l of serum that contained an intact or heat-inactivated complement system was added. The final serum concentration was 80%. When necessary, twofold dilutions from 80% to 1.25% of NHS in phosphate-buffered saline were used. The reaction mixtures were incubated for 1 h with shaking at 200 rpm in a 37°C incubator. The tubes were placed on ice for 5 min to stop the complement reaction. Serial 10-fold dilutions of the mixtures were prepared in phosphate-buffered saline, and CFU were determined by plating on solid medium. These were grown at 25°C, and colonies were counted on the second day. Bacterial counts are given as log₁₀ CFU/ml.

TABLE 2. Resistance of *Y. pestis* strains to PMB at various cultivation temperatures

Temp (°C)	MIC of PMB for <i>Y. pestis</i> strain (U/ml) ^a				
	KM218	KIMD1	KM260(11)	1146	EV11M
5	31.3 \pm 0	15.6 \pm 0	31.3 \pm 0	62.5 \pm 0	31.3 \pm 0
10	31.3 \pm 0	31.3 \pm 0	31.3 \pm 0	62.5 \pm 0	31.3 \pm 0
15	31.3 \pm 0	31.3 \pm 0	31.3 \pm 0	62.5 \pm 0	31.3 \pm 0
20	31.3 \pm 0	31.3 \pm 0	250 \pm 0	250 \pm 0	31.3 \pm 0
23	250 \pm 0	31.3 \pm 0	250 \pm 0	500 \pm 0	31.3 \pm 0
25	2,000 \pm 0	2,000 \pm 0	1,000 \pm 0	250 \pm 0	31.3 \pm 0
28	2,000 \pm 0	1,000 \pm 0	1,000 \pm 0	125 \pm 0	31.3 \pm 0
30	62.5 \pm 0	15.6 \pm 0	1,000 \pm 0	125 \pm 0	62.5 \pm 0
35	31.3 \pm 0	15.6 \pm 0	1,000 \pm 0	31.3 \pm 0	62.5 \pm 0
37	31.3 \pm 0	15.6 \pm 0	31.3 \pm 0	31.3 \pm 0	250 \pm 0
40	No data	1.9 \pm 0	15.6 \pm 0	No data	7.8 \pm 0

^a Standard deviations represent 95% confidence intervals; nonoverlapping 95% confidence interval standard deviations indicate a difference at a *P* value of <0.05. Boldface type indicates the highest levels of resistance to PMB.

RESULTS

Polymyxin resistance of bacteria and binding of polymyxin by LPS. The sensitivities of *Y. pestis* strains to PMB were tested using different doses of this cationic antimicrobial peptide (CAMP) to calculate the MIC as described previously (27). Table 2 shows that when *Y. pestis* subsp. *pestis* strains KM218, KIMD1, and KM260 (11), which produce a wild-type LPS, were grown at 37°C or 40°C, they were from 30 to 125 times more sensitive to PMB (MIC < 31 U/ml) than the same strains grown at 25°C or 28°C (MIC > 1,000 U/ml). *Y. pestis* subsp. *caucasica* strain 1146 was only 4 to 16 times more sensitive at 37°C than at 20°C to 30°C. In contrast, *Y. pestis* strain EV11M was highly susceptible to PBM at temperatures ranging from 5°C to 35°C (MIC, 31 to 63 U/ml) and only at 37°C showed a modest increase in the PBM MIC (250 U/ml). Overall, all strains, except for EV11M, showed their own optimal polymyxin resistance temperatures but they were all within the range of 20°C to 30°C (Table 2).

Adsorption of polymyxin B to LPS from *Y. pestis* strains grown at 25°C or 37°C. The LPS preparations from *Y. pestis* strains KM260 (11), KIMD1, and 1146 showed a greater adsorption of PMB than those from strains KM218 and EV11M (Fig. 1). The adsorption of PMB to the LPS preparations from wild-type strains increased when prepared from bacteria grown at 37°C compared to those grown at 25°C, consistent with the higher PMB susceptibility of the organisms grown at 37°C. Also consistent with the increased sensitivity to PMB when the LPS binds more of this CAMP, the increased sensitivity of strain EV11M grown at 25°C compared to that of cells grown at 37°C was reflected in the greater binding of PMB at the lower temperature. These findings mostly correlated with the contents of Ara4N and glycine in the LPS samples from all strains studied, except for *Y. pestis* strain 1146, which, while more resistant to PMB at 25°C than at 37°C, has comparable contents of Ara4N in LPS-25₁₁₄₆ and LPS-37₁₁₄₆ (34). However, LPS-25₁₁₄₆ has a markedly higher content of glycine than LPS-37₁₁₄₆ (34), which could account for the higher resistance of strain 1146 to PMB at the lower temperature.

Resistance to serum killing. *Y. pestis* subsp. *pestis* strains KM218, KM260 (11), and KIMD1 grown at either 25°C or

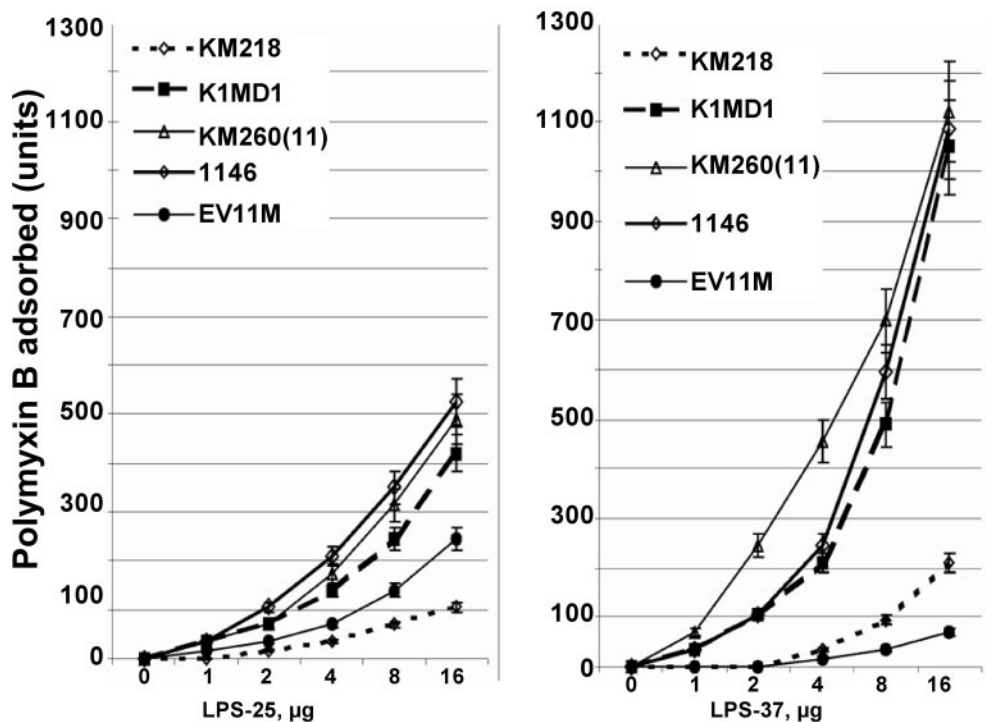


FIG. 1. PMB binding by *Y. pestis* LPS preparations isolated from organisms grown at 25°C or 37°C. The standard deviation at all points was less than 10% of the value.

37°C survived the action of complement present in 80% NHS or IHS (Fig. 2, lanes 2, 3, 5, 6, 8, and 9). CFU were not reduced significantly after treatment for 1 h with either NHS or IHS, having levels comparable to those incubated with heat-inactivated (HI) serum. However, when grown at 6°C, these strains were highly sensitive to the bactericidal action of NHS but not to that of HI-NHS (Fig. 2A, lanes 1, 4, and 7). Thus, the organisms undergo phenotypic changes resulting in susceptibility to the bactericidal activity of normal serum at a temperature at which host defenses of hibernating rodents appear to be suppressed, which may preclude normal immune responses from taking place (36, 47). Unlike wild-type *Y. pestis* subsp. *pestis* strains, mutant *Y. pestis* subsp. *pestis* strain EV11M and wild-type-LPS *Y. pestis* subsp. *caucasica* 1146 were killed by NHS but not by HI-NHS at cultivation temperatures of 25°C and 37°C (Fig. 2A, lanes 10 to 13). The strains were no more susceptible to killing by IHS at 25°C and 37°C than they were to killing by NHS at the same temperatures (Fig. 2B), indicating that the vaccine strain did not induce bactericidal antibodies in the human volunteers. These results confirm previous data that *Y. pestis* is serum resistant even in the presence of antibodies (42). Susceptibility of *Y. pestis* to killing in IHS at 6°C was not tested, as it would unlikely differ from the susceptibility to killing in NHS when organisms were grown at 6°C.

To determine if there was an association of LPS structure with susceptibility or resistance to the bactericidal activity of human serum, we compared the structural constituents among the most variable components of the LPS of *Y. pestis* subsp. *pestis* (serum resistant) and *Y. pestis* subsp. *caucasica* (serum sensitive). The variable components of LPS synthesized at growth temperatures of 6°C, 25°C, or 37°C for the *Y. pestis*

subsp. *pestis* strains (representative data from strain KM218) and the *Y. pestis* subsp. *caucasica* strain 1146 are presented in Table 3. *Y. pestis* strain EV11M lacks all of the components listed in Table 3 (34, 35). The strains with the greatest susceptibility to bactericidal killing have overall lower contents of GlcNAc in the LPS oligosaccharide side chain, as determined by ESI FTICR (34, 35). However, more-recent studies show that the low GlcNAc content in LPS is not the sole basis for serum sensitivity, as strains KM260 (11) and KM218 with mutations in genes *rfe* (YPO3866) or *waaL* (YPO0417) produce GlcNAc-free LPS but are still resistant to killing by serum (A. P. Anisimov, S. V. Dentovskaya, R. Z. Shaikhutdinova, B. Lindner, N. A. Kocharova, S. N. Senchenkova, and Y. A. Knirel, unpublished data). Thus, serum resistance appears to be a complex phenotype associated with changes beyond GlcNAc-dependent structural variation in the *Y. pestis* LPS.

Cells of all *Y. pestis* strains studied grown at any temperature, including 6°C, as well as *E. coli* DH5α were resistant to the bactericidal activity of 80% normal mouse serum or of 1.25% NHS (data not shown). These results confirm previous data that the concentration of bactericidal components in normal mouse serum is either much less than in NHS or structurally variant in ways that do not result in a bactericidal effect on *Y. pestis* (39).

DISCUSSION

The different environments that *Y. pestis* strains must traverse to maintain the enzootic cycle of transmission in the wild require the capacity to undergo phenotypic variations associated with growth and survival in these varied environments.

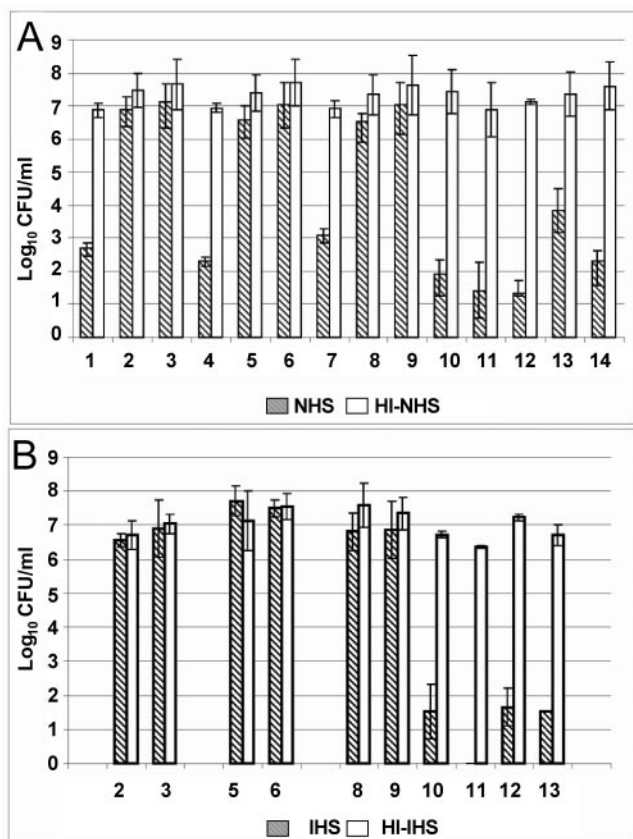


FIG. 2. Bactericidal activities of NHS (A) and IHS (B) versus those of HI-NHS and HI-IHS, respectively, toward *Y. pestis* strains grown at different temperatures. Lane 1, KM218 (6°C); lane 2, KM218 (25°C); lane 3, KM218 (37°C); lane 4, KIMD1 (6°C); lane 5, KIMD1 (25°C); lane 6, KIMD1 (37°C); lane 7, KM260 (11) (6°C); lane 8, KM260 (11) (25°C); lane 9, KM260 (11) (37°C); lane 10, 1146 (25°C); lane 11, 1146 (37°C); lane 12, EV11M (25°C); and lane 13, EV11M (37°C). Each bar depicts the mean of three independent determinations. Error bars represent 95% confidence intervals; a nonoverlapping 95% confidence interval error bar indicates a difference at a *P* value of <0.05.

Among environmental variables central to the *Y. pestis* life cycle are temperature, survival within flea vectors, and survival within mammalian blood. We hypothesized that temperature-dependent variations in the structure of the *Y. pestis* LPS, known to impact susceptibility of bacteria to CAMPs and serum, would correlate with bacterial susceptibility to these host factors. Here we evaluated the sensitivity to PMB of strains of *Y. pestis* with defined variabilities in their LPS structures that were obtained from divergent geographic locations. We used PMB as a representative of this class of molecule for evaluating the resistance levels of different strains of *Y. pestis* to CAMP. While the actual relatedness of PMB to insect CAMPs is not entirely clear, it has been shown that representatives of both pathogenic and symbiotic bacteria isolated from insects are resistant to PMB (11, 23, 29), suggesting PMB has a spectrum of activity similar to that of insect CAMPs. Secondly, though insect CAMPs are rather heterogeneous in their structures and mechanisms of action, some insect immune peptides, such as the cecropins, have a spectrum of antibacterial activity similar

to that of PMB (14). Thus, susceptibility to PMB likely mimics to some degree the antibacterial factors of insects.

Our data confirmed that the wild-type-LPS strains of *Y. pestis* subsp. *pestis*, representing the three biovars, are highly resistant to PMB when grown at 25°C, whereas at a growth temperature of 37°C their sensitivity to PMB increases more than 30-fold. The susceptibility of these strains to PMB correlates with the adsorption level of PMB by the LPS and is in inverse correlation with the content of the LPS cationic component, Ara4N, which is linked to the phosphate groups in lipid A. Ara4N content has been shown to relate to the PMB resistance of other enterobacteria (22), and more recently this phenomenon was also seen with a single strain of *Y. pestis* (50). Susceptibility to PMB was also inversely correlated with the amount of glycine present in the LPS core (34). Glycine is not a common component of enterobacterial LPS. Notably, the LPS of *Y. pestis* subsp. *caucasica* 1146 had comparable amounts of Ara4N when grown at 25°C and 37°C but was still approximately eightfold more resistant to PMB at 25°C than at 37°C. However, the LPS of *Y. pestis* subsp. *caucasica* grown at 25°C has a higher glycine content, which may contribute to the increased resistance to PMB of this strain.

Another temperature-dependent variation in the LPS of *Y. pestis* subsp. *pestis* is the content of Gal and that of DD-Hep, the former component being more highly expressed as the terminal sugar on the main oligosaccharide chain when *Y. pestis* subsp. *pestis* is grown at 6°C and the latter component at 37°C. The LPS of *Y. pestis* subsp. *pestis* grown at 25°C, the temperature at which these organisms are maximally resistant to PMB, possesses approximately equal amounts of LPS molecular variants with terminal Gal or with DD-Hep (34, 35). Also, DD-Hep is completely absent from the oligosaccharide of *Y. pestis* subsp. *caucasica* (34), a strain that is more susceptible to PMB at 25°C than is *Y. pestis* subsp. *pestis*. This suggests that another factor determining PMB resistance of *Y. pestis* is the ratio of these two monosaccharides at the nonreducing end of the main oligosaccharide side chain. This conclusion is in

TABLE 3. Cultivation temperature variability of the constituents in LPS of *Y. pestis* strains

Constituent	Proportion of LPS molecules positive for constituent in <i>Y. pestis</i> strain ^a					
	KM218			1146		
	37°C, type 1 LPS	25°C, type 3 LPS	6°C, type 3 LPS	6°C, type 4 LPS	37°C, type 2 LPS	25°C, type 3 LPS
Core						
GlcNAc	0.9	0.65	0.5	0.15	0.4	0.3
Gly	0.1	0.2	0.25	0	0.3	0.5
Lipid A						
Ara4N	0.4	0.95	1	0	0.95	0.95
12:0	0	0.3	0.45	0.5 ^b	0	0.3
16:1	0	0.15	0.25	1	0	0.1

^a Numbers indicate the proportions of the total number of LPS molecules (maximum of 1) that contain the particular constituent, as determined by ESI FTICR mass spectrometry of the whole LPS. For the structures of type 1 to type 4 LPS, see references 34 and 35; at 6°C, type 3 and type 4 LPS are produced in similar amounts (data of CSD ESI FTICR MS of the whole LPS).

^b In type 4 LPS, the amount of the 12:0 acyl group is dependent on the amount of the 3:OH 14:0 groups to which the C12:0 fatty acid is ester linked.

agreement with a recent finding that a *phoP* mutant of *Y. pestis* lacking Gal in the LPS was highly sensitive to PMB at 28°C (27).

Temperature-dependent and intraspecific variations in susceptibility to PMB and structure of the *Y. pestis* LPS could have significant biological implications. For instance, insects need adequate resistance to microbial parasites, a property essential both for their own survival and for the ability to transmit *Y. pestis* from infected to susceptible animals. In fleas, *Y. pestis* multiplies in the midgut and does not enter organs or hemolymph (4, 26, 41, 42), preventing *Y. pestis* from being eliminated by the insect's antibacterial killing mechanisms, such as melanotic encapsulation and phagocytosis. We speculate that among fleas that transmit plague between rodents, as has been shown for other insects, the gut epithelium and salivary glands can also produce CAMPs, whose synthesis and release is induced by microbial molecules like LPS (17). It has been shown that in *Xenopsylla gerbilli minax* flea intestine there is a non-lysozyme factor bactericidal for *Micrococcus lysodeikticus* and *Y. pestis* subsp. *pestis* grown at 37°C but harmless for *Y. pestis* subsp. *pestis* grown at 28°C (3). Though the flea antibacterial response has not been specifically characterized, it seems that in order for *Y. pestis* to survive in the flea midgut the microbe has to display resistance to the action of insect CAMPs, and here we report that strains of *Y. pestis* subsp. *pestis* are indeed highly resistant to PMB when grown at temperatures of 25°C. Our results are also in agreement with the data reported by Rebeil et al. (50) for *Y. pestis* subsp. *pestis* grown at 21°C. As for the somewhat lower resistance to PMB of strain *Y. pestis* subsp. *caucasica* 1146 grown at 25°C, it is possible that its flea vectors, *Callopsylla caspia* and *Nosopsyllus consimilis* (7), might produce lower levels of CAMPs. A different ability of strains of *Y. pestis* subsp. *pestis* and *Y. pestis* subsp. *caucasica* to survive within different flea species (4) may have evolved as a result of an adaptation of various *Y. pestis* populations to transmission between rodent hosts by different major flea vectors.

Another key component for maintaining the enzootic transmission cycle of plague is resistance of the organism to killing in serum. All *Y. pestis* subsp. *pestis* strains evaluated to date are able to grow in intact NHS, intact IHS, or 80% normal mouse serum, but *Y. pestis* subsp. *caucasica* 1146 was susceptible to the bactericidal activity of human sera. This may reflect the intrinsically low virulence of *Y. pestis* subsp. *caucasica* for humans. The resistance to bactericidal killing of this strain mediated by normal mouse serum suggests that within natural rodent hosts, *Y. pestis* subsp. *caucasica* is able to survive in rodent blood to maintain the transmission cycle. *Y. pestis* subsp. *caucasica* circulates in populations of common voles and is virulent for mice but displays a dramatically reduced virulence for guinea pigs and humans (7), consistent with the findings here regarding the susceptibility of this strain to human serum.

The main difference between the LPS structures in serum-resistant and serum-susceptible *Y. pestis* strains is the lack of oligosaccharide glycoforms substituted with DD-Hep and the much lower content of GlcNAc in the LPS of the NHS-susceptible strain *Y. pestis* subsp. *caucasica* 1146 (34). However, these two differences taken individually do not account for serum resistance of *Y. pestis* strains. For example, the lack of DD-Hep from the *Y. pestis* LPS is not likely solely responsible

for the susceptibility to NHS of strains other than *Y. pestis* subsp. *pestis*, since another *Y. pestis* subsp. *altaica* strain which is also deficient in DD-Hep (A. P. Anisimov, R. Z. Shaikhutdinova, B. Lindner, N. A. Kocharova, S. N. Senchenkova, and Y. A. Knirel, unpublished data) is resistant to killing by NHS (7). The GlcNAc content is another determinant or marker for serum sensitivity of *Y. pestis* strains, since the LPSs in all strains susceptible to killing by NHS, including *Y. pestis* subsp. *pestis* KM218 grown at 6°C (35) and *Y. pestis* subsp. *caucasica* 1146 (34), have low levels of GlcNAc. It has been proposed (34) that the terminal GlcNAc sugar in the main *Y. pestis* LPS oligosaccharide chain represents the acceptor sugar for the *Yersinia* O-antigen that is missing in *Y. pestis*, a finding supported by the lack of GlcNAc in a mutant strain deleted for the *waaL* O-antigen ligase (Anisimov, Dentovskaya, Shaikhutdinova, Lindner, Kocharova, Senchenkova, and Knirel, unpublished). In all serum-resistant cultures, including *Y. pestis* subsp. *pestis* KM218 (34) and *Y. pestis* subsp. *altaica* (7) grown at 25°C and 37°C, there is a high content of GlcNAc in LPS molecules (34). However, the presence of GlcNAc is not essential for serum resistance, as strains KM260 (11) and KM218, with mutations in *rfe* (YPO3866, a putative undecaprenyl-phosphate α -N-acetylglucosaminyltransferase) or *waaL* (YPO0417, an O-antigen ligase), produce GlcNAc-free LPS but are still resistant to killing by serum (Anisimov, Dentovskaya, Shaikhutdinova, Lindner, Kocharova, Senchenkova, and Knirel, unpublished). Thus, the GlcNAc content likely reflects a coassociated phenotype more directly related to serum resistance.

Y. pestis has only rarely been studied at temperatures below 20°C (24, 35, 38). However, 5°C is approximately the core body temperature of *Y. pestis* rodent hosts undergoing seasonal hibernation (marmots and ground squirrels), when their body temperature is maintained 1°C to 2°C above the ambient temperature (30, 31, 40). Taking into account the duration of the low-temperature period for the marmot and Eurasian ground squirrel, which are common hosts for maintaining natural plague foci (7), it appears that such low body temperatures are present for about half of the *Y. pestis* yearly life cycle. Thus, between 5 and 7 months a year, hibernating rodents and fleas do not maintain plague enzootics. Moreover, the death of the hibernating rodent in its burrow would cause termination of the transmissibility of *Y. pestis* from that individual host. Formerly, it was believed that rodents infected with *Y. pestis* just before entering a hibernation period in late autumn possessed an increased resistance to plague during the period of hibernation (18, 30). More recently, it was shown that host defense mechanisms appear to be downregulated during the hibernation season and preclude normal immune responses (36, 47); therefore, in this setting the plague pathogen has fewer host immune effectors to resist. Consistent with this hypothesis, *Y. pestis* grown at 5°C prior to infection has the lowest virulence for animals among all cultures tested that were grown within the temperature interval of 5°C to 41°C (38). It has also been noted that the main bacterial virulence factors responsible for *Y. pestis* pathogenicity, such as CafI, PsaA, Yops, Yscs, Pla, etc., as well as the insecticidal toxin complex TccC, were downregulated under cold growth conditions (24). Our data showing an increased susceptibility of *Y. pestis* grown at 6°C to the bactericidal action of innate immune factors are consistent with the development of a symbiotic interaction between the

microbe and mammalian host or flea vector for the period of winter hibernation, wherein bacteria are able to persist asymptotically while awaiting rodent awakening. After awakening, an acute infection can develop and further flea transmission of the disease can ensue.

Overall, our studies revealed a partial correlation between temperature-dependent variations in the LPS structures in representatives of various *Y. pestis* strains and their susceptibilities to killing by PMB and serum. The biological significance of all of the temperature-dependent LPS alterations described for *Y. pestis* subspecies is not completely clear, and one should take into account that growing bacteria under laboratory conditions may not accurately model the in vivo conditions. Therefore, for further studies of the biological properties of LPS forms, it is important to determine the exact structures of the LPS molecules that are synthesized primarily in fleas and mammals. This will enable selection of in vitro conditions that ensure production of LPS forms characteristic of each organism in the in vivo environments. Furthermore, knockout and complementation studies of the genes involved in LPS biosynthesis will clarify the contributions of different parts of the LPS molecules to *Y. pestis* pathogenesis in the context of resistance to mammal or flea immune effectors.

ACKNOWLEDGMENTS

This work was performed within the framework of the International Science and Technology Center (ISTC) partner project no. 1197, supported by the Cooperative Threat Reduction Program of the U.S. Department of Defense (ISTC partner). A.P.A., S.V.D., N.K.F., I.V.B., G.M.T., R.Z.S., and S.V.B. were also supported by contract no. 43.600.1.4.0031 from the Ministry for Industry, Science, and Technology of Russia, and B.L. was supported by the German Research Foundation (grant LI-448-1).

We thank I. A. Dunaitsev (Obolensk, Russia) for large scale biomass production and V. V. Amel'chenko (Obolensk, Russia) for LPS drying.

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Editor: J. B. Bliska