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Lipopolysaccharide Phase Variation Determines the Complement-Mediated Serum Susceptibility of *Coxiella burnetii*

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Phase variation of *Coxiella burnetii* is due to variation of the lipopolysaccharide (LPS), a phenomenon analogous to smooth-to-rough LPS variation of gram-negative enteric bacteria. Virulent enterobacteria usually have a smooth LPS and resist serum killing, whereas avirulent rough LPS mutants are sensitive to complement-mediated serum killing. Like gram-negative enterobacteria, smooth LPS phase variants of *C. burnetii* are virulent, whereas the rough LPS variants are avirulent. We therefore studied the effects of human serum on the LPS variants of the Nine Mile strain of *C. burnetii*. Analogous to gram-negative enterobacteria, the smooth and intermediate LPS *C. burnetii* phase variants were resistant to complement-mediated serum killing, whereas the rough LPS variants were killed by serum complement. Although the smooth and intermediate LPS variants were serum resistant, they differed in their interactions with the complement system. The smooth LPS variant activated complement poorly and did not bind C3b; however, the intermediate LPS variant activated complement and bound C3b. The rough LPS variant activated complement via the alternative pathway, whereas the intermediate LPS variant activated the classical pathway. These results provide an explanation for the avirulent nature of the rough LPS variant of *C. burnetii* and suggest that differences in *C. burnetii* LPS structure influence the interactions of the LPS phase variants with the complement system.

Gram-negative enteric bacteria which cause bacteremic infections are usually resistant to complement-mediated serum killing (21). These serum-resistant bacteria are often smooth strains that have a lipopolysaccharide (LPS) with complete polysaccharide side chains (27, 29). Conversely, rough strains that lack complete polysaccharide chains are often serum sensitive and are unable to cause systemic infections. Thus, smooth-to-rough LPS variation is one factor that determines the susceptibility of gram-negative bacteria to the bactericidal effects of serum complement.

Coxiella burnetii, the obligate intracellular bacterium that causes Q fever, also undergoes an LPS phase variation analogous to the smooth-to-rough LPS variation seen in enterobacteria (22, 24). Isolates of *C. burnetii* from animals, insects, or infected hosts are wild-type phase I strains. These organisms are able to persist in infected hosts, sometimes for many years. Avirulent phase II variants are derived by serial passage in yolk sacs or in tissue culture (reviewed in reference 2). Earlier studies have shown that a major structural difference between the phase variants lies in their LPS—phase I organisms have a smooth LPS, while phase II variants have a rough LPS (7, 22). A mutant with an LPS chemotype intermediate between the smooth and rough variants has also been identified (7). Recent studies from this laboratory examined the virulence of *C. burnetii* Nine Mile (9mi) in an animal model system (16). The phase I variant (9mi/I) and the intermediate phase variant (9mi/Cr) caused infections in guinea pigs, but only 9mi/I persisted in their spleens for 30 days after infection. Thus, 9mi/Cr was intermediate in LPS chemotype and virulence. The phase II variant (9mi/II) was avirulent. Whereas differences in virulence and LPS structure are known, the susceptibilities of virulent and avirulent organisms to complement-mediated serum killing have not been critically examined. We hypothesized that, like gram-negative enteric bacteria, virulent *C. burnetii* with smooth LPS are serum resistant, whereas avirulent organisms with rough LPS are serum sensitive. We tested this hypothesis with *C. burnetii* 9mi and found that phase II organisms were indeed sensitive to normal human serum; phase I and 9mi/Cr organisms were serum resistant. Serum-resistant 9mi/I and 9mi/Cr organisms differed in their interactions with the complement system and resisted serum killing by blocking the complement pathway at different steps.

MATERIALS AND METHODS

Bacterial strains. *C. burnetii* 9mi/I, 9mi/II, and 9mi/Cr were used. Purification procedures (30), passage history, and propagation (7) have been previously described.

Reagents and media. Serum samples from two healthy adult male volunteers were pooled, stored immediately at −70°C, and thawed just before use. These sera, when examined by a microagglutination assay (4), were devoid of anti-*C. burnetii* antibodies. Human serum depleted of C9 (C9D), mouse anti-human C3c monoclonal antibody (anti-C3c MAb), mouse anti-human C3d MAb (anti-C3d MAb), and the purified human complement component C9 were purchased from Cytotech Inc., San Diego, Calif. Sheep and rabbit erythrocytes were obtained from the animal care facility at the Rocky Mountain Laboratories, stored in Alsever solution, and processed as described before (5, 20). Rabbit anti-sheep erythrocyte immunoglobulin M antibody was donated by M. D. P. Boyle, University of Florida, Gainesville, Fla. Rabbit antisera to *C. burnetii* have been described before (7). Fluorescein-conjugated goat anti-rabbit immunoglobulin antiserum was purchased from Cooper Biomedical, Inc., West Chester, Pa. The buffers used included Hanks balanced salt solution (HBSS), HBSS with 0.15 mM $CaCl_2$ and 1.0 mM $MgCl_2$ (HBSS++), Veronal-buffered saline with 0.1% gelatin (GVBS), GVBS with 0.15 mM $CaCl_2$

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and 1.0 mM $MgCl_2$ (GVBS++), GVBS with 2.0 mM $MgCl_2$ and 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (GVBS-Mg-EGTA), Eagle minimal essential medium with 10% fetal bovine serum and 10 μg of gentamicin per ml (MEM-10), and phosphate-buffered saline, pH 7.2.

Radioiodination. $Na^{125}I$ was purchased from New England Nuclear Corp., Boston, Mass. Anti-C3c and -C3d MAbs were radioiodinated by the chloramine T procedure (10).

Effect of serum on bacterial viability. *C. burnetii* 9mi/I, 9mi/Cr, and 9mi/II phase variants were suspended in HBSS++ to a final concentration of 10^{10}/ml. Reaction mixtures were prepared that contained 4×10^9 bacteria, 10 or 50% pooled normal human serum (PNHS) or human serum heated at 56°C for 30 min (HIS), and HBSS++ in a final volume of 1 ml in 1.5-ml Microfuge tubes and incubated for 60 min at 37°C with constant rotation. At zero time and at 60 min, bacteria were pelleted by centrifugation in a Microfuge 11 (Beckman Instruments, Inc., Fullerton, Calif.) at 12,000 rpm for 5 min at room temperature, and the serum was removed. Bacterial pellets were suspended in MEM-10, and 100 μl of dilutions of the suspension was inoculated in duplicate on Vero cell monolayers grown on 12-mm-diameter glass cover slips placed in 24-well tissue culture plates. An equal volume of MEM-10 was added, and the plates were incubated for 30 min at 37°C. The medium was then removed, and 1 ml of fresh MEM-10 was added to each well. The plates were incubated at 37°C for 96 h, the media were removed, and the cells were fixed with absolute methanol. The wells were then washed three times with phosphate-buffered saline, treated with 200 μl of a 1:100 dilution of rabbit anti-*C. burnetii* antiserum, and incubated for 30 min at 37°C. The wells were washed again with phosphate-buffered saline, treated with 200 μl of a 1:100 dilution of fluorescein-conjugated goat antisera against anti-rabbit immunoglobulins for 30 min at 37°C, and washed three times with phosphate-buffered saline and once with distilled water. The cover slips were examined by fluorescence microscopy in a blind fashion for inclusion-forming units (IFU) at ×40 magnification. Inclusions in 15 fields were counted for 9mi/II and 9mi/Cr, and the total number of inclusions on the cover slips were counted for 9mi/I, which does not grow abundantly in tissue culture. At zero time, there were approximately 100 IFU per field for 9mi/II and 9mi/Cr and approximately 100 IFU per cover slip for 9mi/I organisms. The viability of the 9mi/II phase variant in C9D was determined similarly. Depletion of C9 in C9D was confirmed in sensitized sheep erythrocyte (EA) hemolysis assays with purified C9 according to manufacturer (Cytotech) instructions.

Whole-complement consumption assays. Bacteria were incubated with 10% PNHS as described for the IFU assays. At 15, 30, and 60 min, 200 μl was removed, and the bacteria were pelleted by centrifugation. The hemolytic activity remaining in the supernatants was measured in 50% hemolytic complement units by using EA cells in a total reaction volume of 1.5 ml by the technique of Rapp and Borsos (20). Whole-complement hemolytic activity was determined and considered to be 100% for PNHS that had been incubated similarly without bacteria. Complement consumption by bacteria was determined by subtracting complement hemolytic activity remaining in supernatants incubated with bacteria from the control PNHS values and calculating the percentage of activity lost.

Binding of complement component C3 to bacteria. To determine binding of C3 to *C. burnetii* phase variants, 9.9×10^8 bacteria were incubated in HBSS++ containing 10% PNHS in a final reaction volume of 200 μl for 60 min at 37°C with constant rotation. At various times, bacteria were pelleted by centrifugation in a Microfuge at 12,000 rpm for 5 min at room temperature and washed three times in HBSS–1% gelatin. The pellets were suspended in HBSS containing ca. 100,000 cpm of ^{125}I-labeled anti-C3c MAb or ^{125}I-labeled anti-C3d MAb and incubated for 60 min at 37°C with constant rotation. Reaction mixtures were centrifuged at 12,000 rpm for 5 min at room temperature, and the bacterial pellets were washed three times in HBSS–1% gelatin. Radioactivity in the final pellets was determined by counting in a Beckman Gamma 4000 counter. Controls included bacteria incubated in 10% HIS and bacteria incubated in buffer without serum before incubation with ^{125}I-labeled anti-C3 antibodies.

Pathway of complement activation. To determine the pathway of complement activated by the bacteria, PNHS was treated with 2.0 mM $MgCl_2$ and 10 mM EGTA for 10 min at 37°C (PNHS-Mg-EGTA) to block the classical pathway of complement activation (CPC) (14). Bacteria were incubated in PNHS, PNHS-Mg-EGTA, or HIS, and the degree of C3 binding was determined by using ^{125}I-labeled anti-C3c MAb as described above. For bacteria incubated in PNHS and HIS, GVBS++ was used as a buffer, and for bacteria incubated in PNHS-Mg-EGTA, GVBS-Mg-EGTA was used as a buffer. To confirm that the CPC was blocked in PNHS-Mg-EGTA, whole-complement activity was determined by the EA assay (20). To determine that the alternative pathway of complement activation (APC) was intact in PNHS-Mg-EGTA, hemolysis of rabbit erythrocytes was measured by the technique of Nelson and Ruddy (18).

RESULTS

Effect of serum on bacterial viability. The viability of 9mi phase variants in 10 and 50% PNHS was determined in IFU assays. In preliminary experiments, 10% PNHS did not affect the viability of any of the phase variants at bacterial inocula required to give optimal IFU counts. Therefore, viability of the organisms was examined in detail in experiments using a 50% serum concentration. Incubating smooth LPS 9mi/I and the intermediate LPS phase variant 9mi/Cr for 60 min in PNHS did not significantly affect viability, whereas incubating rough LPS 9mi/II organisms in PNHS reduced their viability by 80% (Table 1). The viability of 9mi/II organisms incubated in C9D for 60 min was reduced by only 22.6%, evidence that all of the terminal complement components were required for efficient serum killing. Incubation in HIS did not decrease the viability of any of the 9mi phase variants. Thus, the smooth and intermediate LPS *C. burnetii* phase variants were serum resistant, whereas the rough LPS

TABLE 1. Effect of serum on viability of *C. burnetii* phase variants[a]

Strain/phase	Mean ± SD % decrease in viability caused by:		
	PNHS	HIS	C9D
9mi/I	14.63 ± 12.69	1.5 ± 2.7	ND[b]
9mi/Cr	7.9 ± 11.2	1.13 ± 1.13	ND
9mi/II	80.25 ± 12.75	5.2 ± 8.9	22.6 ± 9.9

[a] Bacteria were incubated in 50% sera at 37°C for 60 min. At zero time and at 60 min, they were pelleted by centrifugation, washed, suspended in MEM-10, and inoculated onto Vero cells. Viability was determined in an IFU assay described in the text. The results are based on duplicate experiments repeated at least twice.

[b] ND, Not done.

variant was susceptible to complement-mediated serum killing.

Consumption of complement. To measure consumption of whole complement by *C. burnetii* phase variants, equal numbers of organisms were incubated in 10% serum for 15, 30, and 60 min, residual whole-complement hemolytic activity was determined, and complement consumption was expressed as the percentage of hemolytic activity lost from the whole-complement hemolytic activity of PNHS incubated without bacteria. The serum-sensitive 9mi/II and serum-resistant 9mi/Cr variants consumed complement more efficiently than did the serum-resistant 9mi/I variant (Fig. 1). Therefore, although these 9mi/Cr and 9mi/I variants were both serum resistant, they differed in their abilities to activate complement.

Bacterial binding of C3. Using ^{125}I-labeled anti-C3c MAb to detect the abilities of these organisms to bind C3, we found that *C. burnetii* 9mi/I did not bind C3b (Fig. 2). However, the serum-sensitive 9mi/II and serum-resistant 9mi/Cr phase variants bound C3b. These findings provided further evidence that the two serum-resistant strains blocked different reactions in the complement cascade. Since 9mi/I may have bound C3b which was subsequently degraded to the C3d fragment, which is incapable of forming C5 convertase, we tried to identify bound C3d by using similar assays with ^{125}I-labeled anti-C3d. We did not detect C3d on the surface of 9mi/I (Table 2), evidence that this organism did not bind the C3b molecule.

Pathway of complement activation. We examined the two phase variants, 9mi/Cr and 9mi/II, which activated complement and bound C3b to determine whether they activated complement via the APC or the CPC. The bacteria were incubated in PNHS or PNHS-Mg-EGTA for 60 min, and the deposition of C3b on the bacterial surface was measured as described above. There were no differences in bound C3b when 9mi/II was incubated in PNHS or PNHS-Mg-EGTA, showing that this strain activated complement via the APC (Table 3). In contrast, considerably less C3b was detected on the 9mi/Cr phase variant after incubation in PNHS-Mg-EGTA, evidence that this strain activated complement mainly via the CPC.

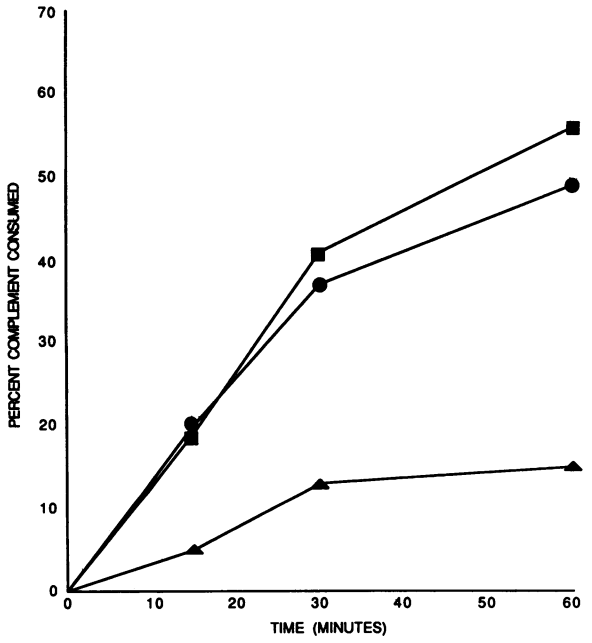

FIG. 1. Consumption of whole-complement hemolytic activity by *C. burnetii* phase variants. Bacteria were incubated at 37°C with 10% sera for 15, 30, and 60 min and pelleted by centrifugation, and consumption of whole complement was measured by assaying complement hemolytic activity in the supernatants. Sera incubated similarly without bacteria were used as controls. The results represent the mean of two experiments done in duplicate. Symbols: ▲, *C. burnetii* 9mi/I; ●, 9mi/Cr; ■, 9mi/II.

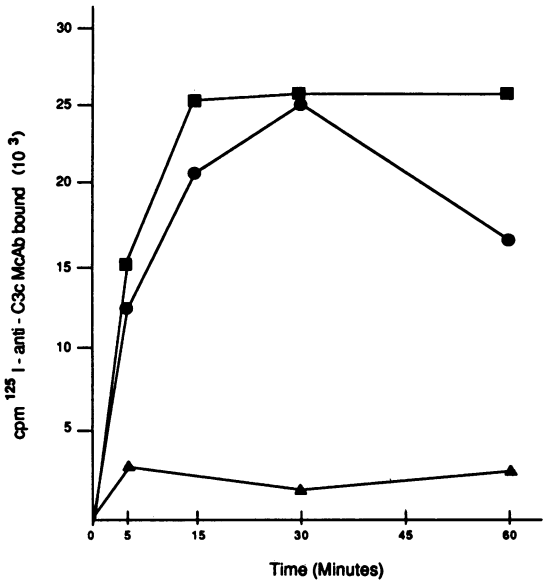

FIG. 2. Binding of C3b to *C. burnetii*. Bacteria were incubated at 37°C for various times, and bound C3b was determined as described in text. Binding of ^{125}I-labeled anti-C3c MAb to all bacteria suspended in buffer only or incubated with HIS for 60 min was ca. 2×10^3 cpm. Symbols: ▲, *C. burnetii* 9mi/I; ●, 9mi/Cr; ■, 9mi/II. The differences between counts per minute bound to 9mi/I and to the other two variants were statistically significant by Student's *t* test at all time points ($P < 0.001$). The differences in counts per minute bound to 9mi/II and 9mi/Cr were not statistically significant at any time point.

DISCUSSION

C. burnetii phase variants mimic enterobacterial LPS variants in their virulence capabilities; i.e., the phase I variants with smooth LPS are virulent, whereas the phase II variants with rough LPS are avirulent. Like the smooth-to-rough LPS variation of enterobacteria, phase variation of *C. burnetii* correlates with the extent of oligosaccharide side chain substitution of the LPS (22). In this study, we found that phase II organisms were serum sensitive, whereas phase

TABLE 2. Binding of C3d to *C. burnetii*[a]

Strain/phase	Mean ± SD cpm of ^{125}I-labeled anti-C3d MAb bound at:		
	5 min (PNHS)	60 min (PNHS)	60 min (HIS)
9mi/I	1,978 ± 514	3,613 ± 1,765	1,641 ± 489
9mi/II	6,135 ± 3,238	31,080 ± 2,283	2,753 ± 398

[a] Bacteria (9.9×10^8) were incubated with 10% PNHS for 60 min at 37°C, centrifuged, washed, and then incubated with ca. 100,000 cpm of ^{125}I-labeled anti-C3d MAb. Bacteria were centrifuged and washed again, and pellets were counted in a gamma counter. The results are based on duplicate experiments repeated twice.

TABLE 3. Pathway of complement activation by *C. burnetii*[a]

Conditions	Mean ± SD cpm of ^{125}I-labeled anti-C3c MAb bound	% Inhibition
9mi/II + PNHS	20,346 ± 4,449	—[b]
9mi/II + PNHS-Mg-EGTA[c]	18,803 ± 1,914	8
9mi/Cr + PNHS	21,791 ± 3,611	—
9mi/Cr + PNHS-Mg-EGTA	6,782 ± 3,818	69

[a] Bacteria (9.9×10^8) were incubated in 10% PNHS or 10% PNHS–Mg–EGTA for 60 min. Bound C3c was identified as described in text. The results are based on three experiments done in triplicate.

[b] —, None.

[c] Blocking of the CPC was confirmed by EA hemolytic assays, and the integrity of the APC was confirmed by rabbit erythrocyte hemolytic assays.

I and intermediate 9mi/Cr organisms were serum resistant. To determine the role of complement in mediating serum killing of 9mi/II organisms, we tested the ability of C9D to decrease the viability of the bacteria in IFU assays. Under similar experimental conditions, PNHS reduced the viability of 9mi/II by 80%, whereas C9D decreased viability by only 22.6%, evidence that the decrease in viability seen with PNHS required the participation of all of the terminal complement components and therefore occurred mainly through complement-mediated killing. These results suggest that complement killing may explain the inability of 9mi/II to infect animals and the inability to isolate these strains from naturally or experimentally infected hosts.

The bactericidal activity of the complement system proceeds from initial activation through the APC or CPC, resulting in the formation of C3 convertase, which catalyzes the deposition of C3b on the bacterial surface. In the next series of reactions, bound C3b participates in formation of C5 convertase, followed by final activation of the terminal components C5b through C9, resulting in the formation of a membrane attack complex (MAC). The MAC is then stably inserted through the bacterial cell wall, causing osmotic instability, membrane damage, and finally, bacterial death (11, 25). We investigated the abilities of *C. burnetii* phase variants to activate complement and bind C3b. Serum-resistant 9mi/I consumed significantly less complement than did the other phase variants. In addition, we could not detect C3b on 9mi/I organisms after incubation in serum. Our inability to detect C3b by using ^{125}I-labeled anti-C3c antibody could have been due to the lack of deposition of this molecule on the bacterial surface or degradation of deposited C3b to the C3d fragment, which is incapable of forming C5 convertase. However, we could not detect C3d on this phase variant in assays using ^{125}I-labeled anti-C3d MAb. Thus, phase I organisms appear to resist complement-mediated killing because they activate complement poorly and because C3b is not deposited on their surfaces. However, the other serum-resistant phase variant, 9mi/Cr, activated complement and bound C3b on its surface. Minor changes in the LPS of other bacteria have been shown to change their abilities to activate complement (6). Schramek et al. have shown that 9mi/I LPS has unusual sugars, including virenose and dihydroxystreptose (23). They and others have also shown recently that there are compositional differences between the 9mi/I and 9mi/Cr LPS (1; H. Mayer, J. Radziejewska-Lebrecht, and S. Schramek, *in* A. Wu, ed., *Molecular Immunology of Complex Carbohydrates*, in press). Therefore, the differences in the abilities of *C. burnetii* 9mi/I and 9mi/Cr to activate complement may be due to differences in the chemical compositions of their LPS.

The precise mechanism(s) by which 9mi/Cr evades complement-mediated killing is unclear. Using a wild-type serum-resistant smooth LPS *Salmonella minnesota* strain and a rough LPS serum-sensitive mutant, Joiner et al. have shown that both strains allow the deposition of early and late complement components on their surfaces (12). However, the C5b-C9 MAC is not stably inserted into the cell wall of the smooth strain and is subsequently shed from the bacterial surface (13). The smooth LPS of the *S. minnesota* strain thus prevents stable insertion of the MAC through its cell wall. A similar mechanism has been shown to occur in other serum-resistant gram-negative bacteria (14, 26). In addition, serum-resistant *Neisseria gonorrhoeae* appears to evade complement-mediated killing because the molecular configuration of the MAC formed on its surface differs from that formed on serum-sensitive strains (15). Since 9mi/Cr allows deposition of C3b on its surface and activates complement, it may use similar mechanisms to resist serum killing; i.e., the MAC may be formed on its surface but shed, or the MAC may be stably deposited on the surface but in a form that cannot lyse the bacteria. Another possibility is a defect in the series of reactions leading from the deposition of C3b to formation of the MAC.

We found that 9mi/II activated complement through the APC, whereas the intermediate 9mi/Cr activated complement mainly through the CPC. Early studies suggested that the LPS of gram-negative enteric bacteria activated both pathways in the absence of anti-LPS antibody, the lipid A subunit activating the CPC and smooth LPS activating the APC (17). The pathway of complement activation by enterobacterial LPS may also be determined by the presence of certain oligosaccharides. Specifically, Vukajlovich et al. recently showed that the monosaccharide L-glycero-D-mannoheptose linked to the terminal 2-keto-3-deoxyoctulosonic acid of LPS confers the ability to activate the APC (28). There is also evidence that some polysaccharide-containing preparations of LPS and some smooth enterobacteria can activate the CPC without the participation of anti-LPS antibody (3, 19). Similarly, the pathway of complement activated by these phase variants could be determined by differences in the oligosaccharide structure of the 9mi/II and 9mi/Cr described (Mayer et al., in press). Also, the phase II organisms have surface-exposed proteins that are sterically hidden by the smooth LPS in phase I variants (7), and it is possible that activation of complement is initiated by these molecules rather than the LPS. Minor changes in LPS structure between 9mi/I and 9mi/Cr appear to enable them to resist the lytic effects of complement by different mechanisms. Since complement does not kill these phase variants, other host defenses must be important in controlling infections caused by these organisms. Cell-mediated immunity is probably the major host defense against these organisms (8, 9). However, after infection, 9mi/Cr organisms are cleared, whereas 9mi/I organisms are able to persist (16). The presence of a complete smooth LPS on 9mi/I may enable these organisms to partially evade cell-mediated immunity and allow them to persist. The absence of a smooth LPS renders the 9mi/II phase variants susceptible to complement-mediated serum killing, thus suggesting a simple explanation for their inability to cause infections.

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