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Role of Bacterial Phospholipases in Serum-Mediated Killing of *Escherichia coli*

DONALD L. KREUTZER,†* MARY VANDERMATEN, CLARENCE S. BULLER, DONALD C. ROBERTSON, AND ARTHUR A. HIRATA

Department of Microbiology, University of Kansas, Lawrence, Kansas 66044

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The importance of bacterial phospholipases during serum-mediated killing of *Escherichia coli* was examined by using wild-type DR$^+$ DS$^+$ and an isogenic phospholipase-deficient mutant DR$^-$ DS$^-$. No difference in serum sensitivity was observed when the parental DR$^+$ DS$^+$ and mutant DR$^-$ DS$^-$ strains were exposed to various concentrations of normal guinea pig serum. Examination of the free fatty acid (FFA) and lipid composition during serum-mediated killing of the two *E. coli* strains indicated that FFA release occurred only in the parental DR$^+$ DS$^+$ strain. No FFA release or lipid degradation was detected in the mutant DR$^-$ DS$^-$ strain during serum killing. The addition of heat-inactivated *E. coli* antiserum (rabbit) to normal guinea pig serum caused FFA release in both *E. coli* strains. This FFA release was found to be independent of serum-mediated killing and due to a highly active and heat-resistant rabbit serum phospholipase that hydrolyzed the bacterial lipids after serum killing. The data presented indicate that serum-mediated killing of *E. coli* is independent of FFA release and that activation of bacterial phospholipases and the resulting release of FFA are only a result rather than a cause of serum-mediated cell death.

The mechanism(s) by which serum exerts its bactericidal activity against gram-negative bacteria has long been of interest to scientists (12, 15). Numerous investigators have implicated antibody and complement in the bactericidal activity of serum via lethal alterations of either the inner (2, 9, 14) or outer membranes (2, 7) of the bacterial cell envelope. These envelope alterations have been attributed to modification or hydrolysis of membrane lipids, possibly by activation of bacterial or serum phospholipases (1, 2, 14). Work by Beckerdite-Quagliata et al. (1) has discounted serum phospholipases as a possible cause of death during serum-mediated killing of gram-negative bacteria.

This study was designed to determine the role of bacterial phospholipases in serum-mediated killing of gram-negative bacteria. For this purpose we examined the effect of serum on wild-type *Escherichia coli* K-12 DR$^+$ DS$^+$ and an isogenic phospholipase-deficient mutant DR$^-$ DS$^-$. The wild type contains normal outer membrane phospholipase A activity (DR$^+$) and normal inner membrane phospholipase A (DS$^+$) activity. The phospholipase-deficient mutant has been characterized by Nojima et al. as exhibiting less than 1% of the total phospholipase A activity of the wild type, but no difference in growth, phospholipid content and composition, or fatty acid composition (10).

The mutant and its wild-type parent thus provide an excellent model with which to probe the role of bacterial cell phospholipase in the serum-mediated killing of gram-negative bacteria.

MATERIALS AND METHODS

Bacteria. *E. coli* K-12 (λ) DR$^-$ DS$^-$ and its wild-type parent (DR$^+$ DS$^+$) were obtained from S. Nojima. The mutant is deficient in both detergent-resistant (DR) and detergent-sensitive (DS) phospholipase A (4, 10).

Serum and antibody. Guinea pig serum was purchased from Pel-Freez Biologicals, Inc., Rogers, Ark., and stored at −70°C until used. Heat-inactivated (56°C, 30 min) rabbit antiserum to *E. coli* K-235 was used in antibody studies.

Media and reagents. Hanks balanced salt solution (pH 7.4) was obtained from Grand Island Biological Co., Grand Island, N.Y. Tryptone broth, containing 1% tryptone (Difco Laboratories, Detroit, Mich.) and 0.1 M NaCl, was used as the growth medium. Plates for the viability assays contained tryptone broth and 1.5% agar (Difco). Diluting fluid for viability assays contained 0.1% (wt/vol) tryptone with 0.1 M NaCl, 1.0 mM $MgSO_4$, and 0.1 mM $CaCl_2$. Silica gel precoated plates were purchased from Matheson Scientific, Inc., Kansas City, Mo. [U-^{14}C]sodium acetate (56.2 mCi/mmol) and Liquifluor [2,5-diphenyloxazole-1,4-bis-(5-phenyloxazolyl)benzene toluene concentrate] were ob-

† Present address: Department of Pathology, University of Connecticut Health Center, Farmington, CT 06032.

tained from New England Nuclear Corp., Boston, Mass. Tris(hydroxymethyl)aminomethane was purchased from General Biochemicals Div. (Mogul Corp.), Chagrin Falls, Ohio.

Growth of bacteria, serum reactions, and viability assays. Bacteria were grown from a 1:100 dilution of an overnight culture in tryptone broth with aeration at 37°C until mid-log growth phase (2×10^8 cells per ml) was reached. The cells were collected by centrifugation and suspended in Hanks balanced salt solution. Reaction tubes contained bacteria (10^8 cells per ml) and various combinations of serum and/or antibody. The reaction tubes were incubated at 37°C with gentle agitation, and at various times samples were taken, diluted, and plated for viability counts. All data presented represent the mean of at least three independent experiments.

Labeling of phospholipids and assay for FFA. The acyl groups of *E. coli* phospholipids were labeled by growing the organisms in tryptone broth containing 0.2 μCi of [U-^{14}C]acetate per ml. The cells were incubated at 37°C for several generations, collected, and suspended as described above. At various times during the incubation, 1.0-ml samples were removed for estimation of free fatty acids (FFA) by using a modification of the procedure of Cronan and Wulff (3). These samples were transferred to screw-cap tubes containing 6.0 ml of chloroform-methanol (1:2, vol/vol) and 0.6 ml of carrier cells (10 g [dry weight] per ml). After incubation for at least 1 h at room temperature, the mixtures were centrifuged and the residues were again extracted with 6.0 ml of chloroform-methanol (2:1, vol/vol). The supernatants from the two extractions, containing the *E. coli* phospholipids and FFA, were combined and separated into two phases by adding 3.8 ml of water, and the chloroform phase was removed by aspiration. After concentration to dryness in a stream of N_2, the lipids were redissolved in 50 μl of chloroform and used for thin-layer chromatography. Thin-layer chromatography was performed by the methods of Skipski and Barclay (13). For separation of phospholipid classes, plates were developed in chloroform-methanol-water (65:25:4, vol/vol/vol). The FFA were separated from phospholipids by developing the plates in a solvent system containing isopropyl ether-acetic acid (96:4, vol/vol). Lipids were detected by exposing the developed plates to iodine vapors. The radioactivity of the individual spots was determined by quantitatively transferring the gel to vials for counting in a Tri-Carb liquid scintillation spectrometer. To assure complete recovery, all of the gels in each lane were routinely assayed by this procedure. The identities of the individual phospholipids were established by the methods of Peterson and Buller (11).

In the FFA and lipid distribution assays, the lipid extracts applied to the thin-layer chromatography plates contained between 4,000 and 10,000 dpm of ^{14}C radioactivity. The concentrations of FFA or individual lipids are expressed as the percentage of total radioactivity in the total fatty acid-lipid fraction. No attempts were made to assay for the presence of lysophospholipid products. All data presented represent the mean of at least three independent experiments.

Sonic oscillation. After growth, cells were suspended in 0.05 M tris(hydroxymethyl)aminomethane (pH 8) and subjected to sonic oscillation for 2 min (in 15-s intervals) at 4°C in a Bronwill Biosonik III (Bronwill Scientific Inc., Rochester, N.Y.).

Bacteriophage T4 ghost infection. Bacteriophage T4 was obtained from J. Emrich, and ghosts were prepared by the method of Duckworth (5). Bacteria were suspended in tryptone broth at 2×10^8 cells per ml and infected with T4 bacteriophage ghosts at an input ratio of 5 ghosts per bacteria.

RESULTS

Phospholipase activity. A variety of treatments, including heat, sonic oscillation, and bacteriophage ghost infection, were used to determine the relative phospholipase activity of both the wild-type and mutant *E. coli* strains. Heat and bacteriophage ghost infection (Table 1) activated phospholipases in the wild-type cell as determined by FFA release. Little FFA release was observed in the wild-type strain with sonic oscillation at 4°C, but when sonic extracts were incubated at 37°C for 60 min, extensive phospholipid degradation was observed. The lack of phospholipase activity at 4°C was most likely due to the decreased enzymatic activity at such a low temperature. Consistent phosphatidylethanolamine (PE) degradation was associated with the FFA increase in the wild-type cell. Since PE is, quantitatively, the major phospholipid in *E. coli*, its degradation would be expected. In the DR$^-$ DS$^-$ mutant, little or no FFA release

TABLE 1. *Effect of heat, sonic oscillation, and bacteriophage T4 ghost infection on free lipid composition of E. coli*[a]

Strain	% Total free lipid			
	FFA	CL[b]	PE[c]	PG[d]
Wild type				
Control	1	9	74	14
Heated	13	14	63	8
Ghost infected	14	14	63	8
Sonically oscillated, 0 min	3	4	67	22
Sonically oscillated, 60 min	29	6	39	17
DR$^-$ DS$^-$				
Control	1	9	75	12
Heated	2	17	73	6
Ghost infected	1	9	75	12
Sonically oscillated, 0 min	3	4	67	22
Sonically oscillated, 60 min	5	4	63	24

[a] Labeled cells were suspended in tryptone broth and heated at 55°C for 30 min before assaying. Sonically treated cells were extracted immediately after sonic oscillation (0 min) and after incubation at 37°C for 60 min. Cells infected with bacteriophage T4 ghosts were extracted at 15 min postinfection.

[b] CL, Cardiolipin.

[c] PE, Phosphatidylethanolamine.

[d] PG, Phosphatidylglycerol.

was observed under any of these experimental conditions, indicating that it contains very little phospholipase activity.

Bactericidal activity of normal guinea pig serum. Since it has been suggested that bacterial phospholipases may be important in serum-mediated killing (1), it seemed likely that a phospholipase-deficient mutant would show increased resistance to this activity when compared to its wild-type parent. However, bactericidal experiments with various concentrations of normal guinea pig serum against both strains of *E. coli* (Fig. 1) showed no difference in the killing curves at 10%, 50%, and 100% serum.

FFA release associated with bactericidal activity of normal guinea pig serum. To determine whether phospholipase activation occurred during serum-mediated killing, we examined the extent of FFA release in both wild-type and mutant *E. coli* during exposure to normal guinea pig serum. The wild-type bacteria exhibited FFA release accompanying cell death, reaching a maximum of 40% total lipid converted to FFA at 60 min (Fig. 2A). Heated guinea pig serum (56°C, 30 min) was nonbactericidal and did not induce FFA release from either cell type (data not shown)

Examination of phospholipid distribution in the wild-type cell (Table 2) after 30 min of incubation with normal guinea pig serum revealed that phosphatidylglycerol decreased as FFA increased, indicating that the former was the likely substrate for the phospholipase activity. When the phospholipase-deficient mutant was exposed to normal guinea pig serum, a similar killing pattern was observed (Fig. 2B), but

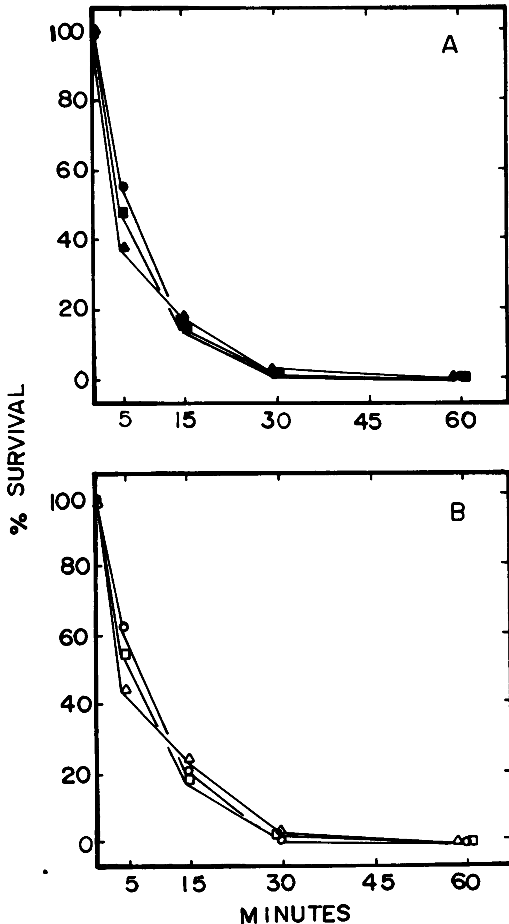

FIG. 1. *Bactericidal activity of normal guinea pig serum against wild-type (DR⁺ DS⁺; ▲, ●, ■) and mutant (DR⁻ DS⁻; △, ○, □) E. coli K-12 (λ). Bacterial inocula were prepared, and the viability was assayed after exposure to various concentrations of guinea pig serum. Symbols: ▲, △, 10% serum; ●, ○, 50% serum; and ■, □, 100% serum.*

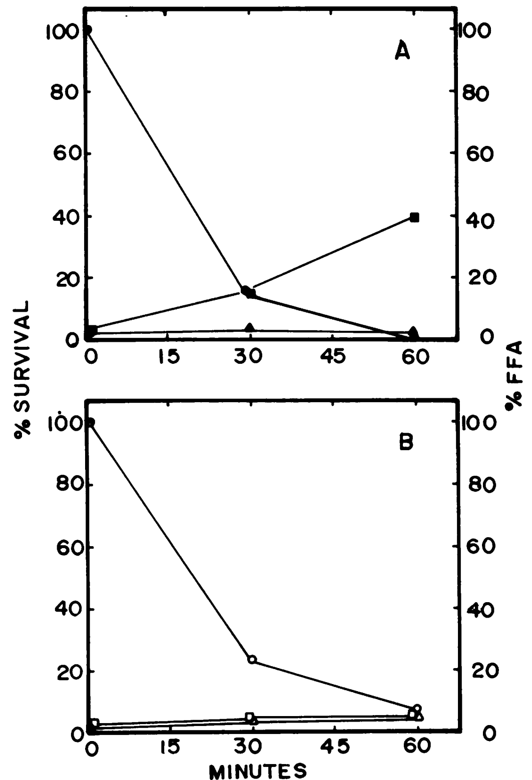

FIG. 2. *Bactericidal activity and FFA release in wild-type (DR⁺ DS⁺; ●, ▲, ■) and mutant (DR⁻ DS⁻; ○, △, □) E. coli K-12 (λ) during exposure to 10% normal guinea pig serum. Bacterial lipids were labeled by growth in [¹⁴C]acetate as described in the text. At the indicated times, samples were removed for assaying both viability and FFA release. Symbols: ●, ○ viability assay; ▲, △, control values for FFA release; and ■, □, experimental values for FFA release.*

TABLE 2. *Effect of serum and antibody on the free fatty acid content and composition of E. coli*[a]

Strain	% Total free lipid			
	FFA	CL[b]	PE[c]	PG[d]
Wild type				
Control	4	2	68	21
Serum	16	3	55	18
Serum plus antibody	27	5	39	17
DR⁻ DS⁻				
Control	4	3	65	20
Serum	5	3	65	19
Serum plus antibody	24	4	40	20

[a] Cells were incubated for 30 min with Hanks balanced salt solution (control), 10% guinea pig serum, or 10% guinea pig serum plus 1% rabbit antibody. The FFA and lipids were extracted and fractionated as described in the text.
[b] CL, Cardiolipin.
[c] PE, Phosphatidylethanolamine.
[d] PG, Phosphatidylglycerol.

no FFA release or change in phospholipid distribution was detected (Table 3). This suggests that the FFA release seen in the wild-type cell was due to activation of the bacterial phospholipases and not to serum phospholipases. If serum phospholipases were the cause of FFA release in the wild type, a similar release should have been seen in the mutant. Since the phospholipase-deficient mutant was killed with equal efficiency, but with no apparent FFA release, it is clear that FFA release occurs independently of serum-mediated killing (i.e., phospholipase activation is a result rather than a cause of serum-mediated killing in *E. coli.*).

FFA release during antibody-serum-mediated killing of *E. coli*. Since complement consumption is initiated by antigen-antibody complexes, the addition of a specific antibody enhances serum-complement-mediated killing. We examined bactericidal activity and FFA release when both *E. coli* strains were exposed to 1% heat-inactivated rabbit anti-*E. coli* K antiserum and guinea pig serum. The addition of a specific antibody enhanced bactericidal activity of the guinea pig serum in the wild-type and mutant *E. coli* strains (Fig. 3), with both strains showing a similar FFA release. Examination of the phospholipid distribution under these conditions revealed that both cell types exhibited a dramatic drop in PE associated with the FFA release (Table 2). That serum-mediated killing of the phospholipase-deficient *E. coli* could occur without FFA release (Fig. 2B) suggested that serum phospholipases in the rabbit antiserum were responsible for the FFA release.

FFA release with serum and autoclaved cells. To determine whether rabbit serum contained a heat-stable phospholipase that would account for the FFA release seen during antibody-serum-mediated killing, both *E. coli* strains were autoclaved to expose the bacterial phospholipids to exogenous phospholipases and to inactivate host phospholipases (1, 6). Figure 4 shows that FFA release occurred in both

TABLE 3. *Effect of serum and antibody on the free fatty acid content and composition of autoclaved E. coli*[a]

Strain	% Total free lipid			
	FFA	CL[b]	PE[c]	PG[d]
Wild type				
Autoclaved control	17	4	46	24
Serum	28	3	35	18
Serum plus antibody	44	7	15	11
DR⁻ DS⁻				
Autoclaved control	8	5	56	25
Serum	15	2	50	20
Serum plus antibody	41	5	17	12

[a] Portions of autoclaved cells were incubated with Hanks balanced salt solution (control), 10% guinea pig serum, or 10% guinea pig serum plus 1% rabbit antibody. Samples were taken at 30 or 60 min. The data shown are an average of several independent experiments.
[b] CL, Cardiolipin.
[c] PE, Phosphatidylethanolamine.
[d] PG, Phosphatidylglycerol.

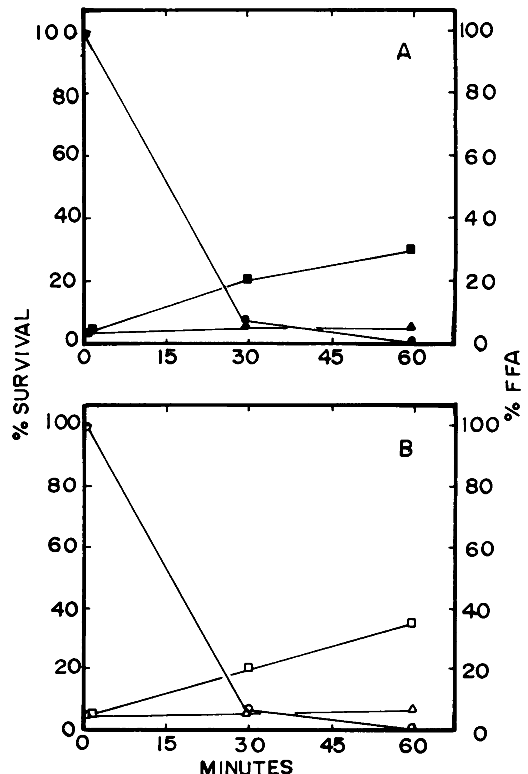

FIG. 3. *Bactericidal activity and FFA release in wild-type (DR⁺ DS⁺) and mutant (DR⁻ DS⁻) E. coli K-12 (λ) during exposure to 10% normal guinea pig serum and 1% rabbit antibody. See legend of Fig. 2.*

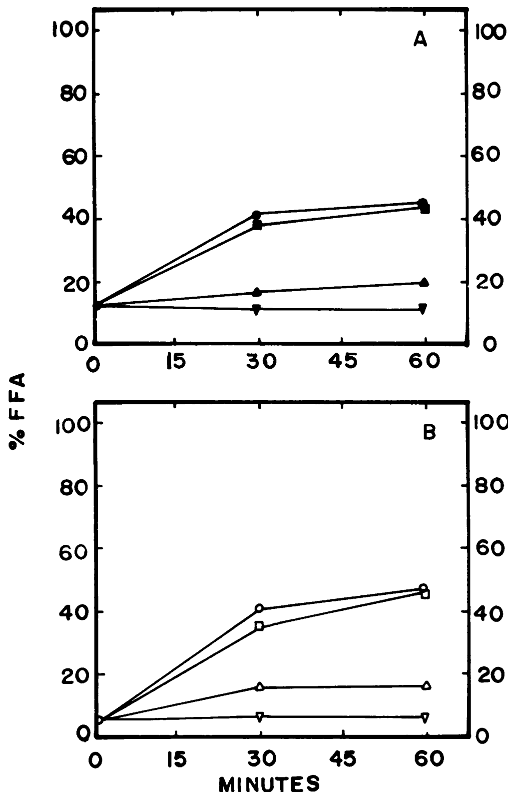

FIG. 4. *Effect of guinea pig and rabbit serum on FFA release in autoclaved wild-type (DR⁺ DS⁺; ▼, ▲, ■, ●) and mutant (DR⁻ DS⁻; ▽, △, □, ○) E. coli K-12 (λ). The cells were suspended in Hanks balanced salt solution and autoclaved for 15 min at 120°C. Cells were then added to incubation mixtures containing Hanks balanced salt solution only, 10% guinea pig serum, 1% rabbit serum, or 10% guinea pig serum plus 1% rabbit serum. Samples were removed for FFA estimation at the indicated time intervals. Symbols: ▼, ▽, control levels of FFA release; ▲, △, 10% guinea pig serum; ■, □, 1% rabbit serum; and ●, ○, 10% guinea pig serum plus 1% rabbit serum.*

strains when autoclaved cells were exposed to various combinations of serum and rabbit antibodies. Neither strain showed a significant level of FFA release in the presence of normal guinea pig serum. An appreciably higher FFA release was obtained when either strain was exposed to guinea pig serum plus heated rabbit antiserum or to heated rabbit antiserum alone. The distribution of phospholipids in the autoclaved bacteria was also examined before and after incubation with serum and serum-antibody. PE and phosphatidylglycerol, although degraded somewhat by guinea pig serum alone, were extensively degraded when rabbit antiserum was added to the guinea pig serum (Table 3). These data suggest that the FFA release seen when the mutant cell was exposed to serum and rabbit antibody was a result of a heat-stable phospholipase in the rabbit serum that hydrolyzed the phospholipids of the mutant during or after cell lysis. This is supported by Beckerdite-Quagliata et al. (1), who demonstrated by absorption of phospholipases from serum that serum phospholipases were not involved in serum-mediated killing of *E. coli*. Therefore, the FFA release observed in the mutant strain that was exposed to serum antibody was due to bacterial phospholipid hydrolysis by rabbit serum phospholipases. This release occurred independently of killing by the serum. The relatively low level of phospholipase activity of guinea pig serum accounts for the lack of FFA release after killing of the mutant by serum alone.

DISCUSSION

The bactericidal activity of serum against gram-negative bacteria has long been known, but relatively little is known about the molecular basis of this reaction. The alteration of membrane lipids has been suggested as one mechanism to explain the serum lytic activity, possibly by hydrolysis of the membrane lipids by serum or cellular phospholipases (1, 2, 8). The role of serum phospholipases in serum-mediated killing has been recently discounted (1). By using a wild-type and a phospholipase-deficient *E. coli* mutant, we demonstrated that cellular phospholipases are not required for serum-mediated killing of *E. coli*. It is also clear that the hydrolysis of fatty acids from membrane lipids is not required for this activity. This is supported by (i) the similar serum killing curves obtained for both the wild-type and phospholipase-deficient *E. coli* strains and by (ii) the lack of FFA release from the phospholipase-deficient mutant during serum killing. Since both the parental strain and the phospholipase-deficient mutant appear identical in other respects (10), it is unlikely that their serum-mediated killing mechanisms would differ.

In conclusion, the data clearly demonstrate that cellular phospholipase activity and FFA release are not required for serum-mediated killing of *E. coli*. It remains to be determined whether other modifications of membrane lipids occur during serum-mediated killing of gram-negative bacteria or whether any alteration of the cell membrane is required for this activity.

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