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Plasmid-Associated Resistance of *Salmonella typhimurium* to Complement Activated by the Classical Pathway

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The association of the virulence plasmid of *Salmonella typhimurium* with resistance to the bactericidal activity of human serum was studied in chromosomally isogenic pairs of strains differing in their virulence plasmid status. The presence of the plasmid correlated in three pairs of strains with resistance to serum. The absence of the plasmid correlated with increased sensitivity to serum, whereas the reintroduction of the plasmid to the cell resulted in the restoration of resistance to serum. Complement was activated by the classical and alternative pathways equally well by both strains of a pair, but the differential bactericidal action of serum was apparent only after the activation of complement by the classical pathway. No differences in the chemical compositions or in the molecular weight ranges of the lipopolysaccharides were apparent between paired strains. This work confirms the presence of a virulence plasmid-associated mechanism of resistance to serum and distinguishes it from lipopolysaccharide-mediated resistance.

The resistance of *Salmonella typhimurium* to the bactericidal effects of serum has long been attributed to a resistance to complement-mediated killing and to the composition of the lipopolysaccharides (LPS). Mutations from the smooth (S) to the rough (R) phenotype are related to increased sensitivity to serum, and there is a general correlation between the extent of the R defect and resistance to serum (8, 36). However, the degree to which the core moiety of the LPS is exposed and the number of O repeat units per core unit also influence resistance (13). The pathways involved in the activation of complement and the subsequent bactericidal activity of the complement also differ between R and S chemotypes. Smooth *Salmonella minnesota* strains, for example, activate complement by the alternate pathway (ACP), while the rough Re strains do not (1). In consequence, the S strains are slightly more susceptible than the Re strains are to killing by the ACP (4). The Re form, however, is much more susceptible than smooth salmonellae are to killing by the classical pathway (CCP) (4), even though both types of salmonellae activate the CCP to the same extent. The difference in susceptibility to the bactericidal effects of serum of S versus Re forms appears to reflect a difference in the extent or manner of binding of an early complement component, C1q (5), and the terminal membrane attack complex (21, 22) to the bacterial membrane.

While almost all studies of *S. typhimurium* resistance to serum have been conducted on known LPS mutants, other unmapped isolates are known in which sensitivity to serum is not associated with any detectable changes in LPS (35, 36). Determinants other than LPS have long been associated with resistance to serum in other organisms (18, 34, 45), and it is reasonable to expect that there may be genetic determinants other than the chromosomal LPS genes dictating resistance to serum in *S. typhimurium*. The identity of such determinants is complicated by genes that pleiotropically affect LPS structure (6) and by serum agents other than complement which affect the serum killing of *S. typhimurium* (2, 3) and of other members of the family *Enterobacteriaceae* (9).

The 95-kilobase (kb) plasmid of *S. typhimurium* has been associated with virulence (24), and it is reasonable to suppose that this may, in part, involve resistance to serum killing. In the present study, we report the association between the presence of an autonomous virulence plasmid and the resistance of the bacterium to the bactericidal effects of the CCP of human serum. This difference in susceptibility, furthermore, was independent of any detectable alterations in the composition or quantity of LPS.

(A preliminary report of a portion of these findings has been presented [J. L. VandenBosch and G. W. Jones, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, B91, p. 33].)

MATERIALS AND METHODS

Bacterial strains. All *S. typhimurium* strains used in this study are listed in Table 1. Virulent parental strains CR6600 and CR8500 and their respective avirulent mutant strains CR6260 and CR8100 have been described previously (24). After the completion of these studies, it was found that the plasmid exists in an integrated form in these mutant strains, which results in decreased plasmid gene expression (D. K. Rabert, Ph.D. thesis, University of Michigan, Ann Arbor, 1987). Strain CR0020 is a plasmid-free isolate (Rabert, Ph.D. thesis) obtained from the Centers for Disease Control, Atlanta, Ga. Spontaneous mutants resistant to 100 μg of nalidixic acid per ml (Nx^r) or to 1,000 μg of streptomycin per ml (Sm^r) were selected from each of these strains for use in the bactericidal assay (see below). Transconjugant strains CR7010 and CR7026 were constructed by mating mutant strains CR7007 and CR7024 with their respective parental strains (strains CR6610 and CR8510), which harbored Tn*10*-marked virulence plasmids (24) and the mobilizing plasmid F′*lac*. The Tn*10* insertion has no effect on adhesion to HeLa cells, virulence in mice, or resistance to serum (24; unpublished data). Transconjugants of strain CR7158 also were constructed by mating with the same plasmid donor strains. Tetracycline-resistant, non-lactose-fermenting transconjugates were selected on MacConkey agar plates containing nalidixic acid (100 μg/ml) and tetracycline (20 μg/ml). Plas-

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TABLE 1. Bacterial strains[a]

Strain	Virulence plasmid	Description (reference)
CR6600	pCF601	Virulent human isolate (24)
CR6260	—	Avirulent novobiocin-treated derivative of CR6600 (24)
CR7003	pCF601	Sm^r CR6600 (this study)
CR7007	—	Nx^r CR6260 (this study)
CR7010	pCF610	CR7007 × CR6610/F'*lac* transconjugant (24; this study)
CR8500	pCF801	Virulent FIRN biotype (24)
CR8100	—	Relatively avirulent ethidium bromide-treated derivative of CR8500 (24)
CR7020	pCF801	Sm^r CR8500 (this study)
CR7024	—	Nx^r CR8100 (this study)
CR7026	pCF810	CR7024 × CR8510/F'*lac* transconjugant (24; this study)
CR0020	ND	Obtained from Centers for Disease Control
CR7158	ND	Nx^r derivative of CR0020 (this study)
CR7162	pCF610	CR7158 × CR7005 transconjugant (this study)
SL1035	pSLT	*rfa-469* serum-sensitive *S. typhimurium* obtained from B. A. D. Stocker

[a] Strains CR6260 and CR8100 contain integrated plasmids (see text). ND, No plasmid sequences detectable by Southern hybridization.

mid content was confirmed on 0.7% agarose gels with lysates prepared by the method of Kado and Liu (25). Unless otherwise noted, bacteria were grown in L broth for 18 h at 37°C with gentle agitation.

Bacteriophages. Sensitivity to a set of S- and R-specific phages (50) was assessed by the drop-on-lawn technique (12).

Serum. Pooled normal human sera (PNHS) were obtained from groups of 10 normal adults by antecubital venipuncture. Blood was allowed to clot at room temperature for 15 min and then kept on ice for 1 h. After centrifugation, sera were pooled and filtered through a 0.22-μm-pore-size filter to remove residual cellular debris. The PNHS was aliquoted and stored at −70°C until used. Different batches of pooled sera varied in the level of bactericidal activity, but all batches demonstrated the same relative bactericidal activity toward the strains tested.

Serum treatment. Washed zymosan (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 25 mg/ml was incubated with PNHS for 30 min at 37°C and removed by centrifugation at 13,500 × *g* for 5 min. Cobra-venom-factor-treated PNHS was obtained by incubating 25 U of cobra venom factor (Cordis Laboratories, Inc., Miami, Fla.) per ml of PNHS at 37°C for 60 min (43). Controls for these treatments demonstrated no bactericidal effects. The CCP was inactivated by the addition of EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Sigma] to 8 mM and Mg^{2+} to 2 mM (23), and the ACP was inactivated by heating at 50°C for 15 to 20 min (15). Properdin-depleted PNHS was obtained by affinity chromatography (40) and was the kind gift of S. C. Ross. In each case of specific pathway inactivation, the inactivated pathway was reduced to less than detectable levels. The activity of the functional pathway is indicated in the pertinent Results section. All assays of the CCP were by $C'H_{50}$ (27), and assays of the ACP were by APH_{50} (23) hemolytic assays.

Bactericidal assays. Overnight cultures were diluted in Veronal buffer (Complement Fixation Test Diluent, BR16; Oxoid U.S.A., Inc., Columbia, Md.) plus 0.1% gelatin and inoculated into PNHS which had been prewarmed to 37°C and equilibrated in 10% CO_2. The final concentrations were 10^5 bacteria per ml and 90% PNHS. Test mixtures were incubated at 37°C in 10% CO_2 with gentle agitation, samples were removed at intervals, and dilutions were plated in triplicate. Percent survival was calculated from the mean viable count of survivors divided by the mean viable count of bacteria at time zero.

Complement activation. Overnight cultures diluted in Veronal buffer plus 0.1% gelatin were suspended in 90% PNHS at a final concentration of 10^8 bacteria per ml. Samples were taken after 1 and 2 h of incubation in 10% CO_2 at 37°C and centrifuged (13,500 × *g* for 5 min), and $C'H_{50}$ (27) and APH_{50} (23) hemolytic units in the serum supernatants were measured. The percent activation of complement attributable to the bacteria was determined from the reduction of hemolytic units in the presence of bacteria minus the reduction in the control, divided by the initial number of hemolytic units in the serum. This percentage was then normalized to the number of bacteria in the reaction mixture.

LPS analysis. Cells grown for 18 h in 20 ml of L broth were washed twice in 10 ml of phosphate-buffered saline (Oxoid) and suspended in 10 ml of phosphate-buffered saline. Samples for total bacterial counts and size determinations were fixed in 5% glutaraldehyde and analyzed on a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.) (20). Other samples for the serum bactericidal assay, for total counts (Petroff-Hauser counting chamber), and for viable counts were diluted in Veronal buffer plus 0.1% gelatin. LPS was prepared by a modification of the method of Goldman et al. (13) to allow for the removal of DNA with DNase I. Cells were pelleted, suspended in 200 μl of 1% sodium dodecyl sulfate in 0.06 M Tris hydrochloride (pH 6.8), and lysed by heating to 100°C for 10 min with vigorous vortexing every 2 min. Water was added slowly with vortexing, and then 0.5 ml of buffer (0.6 M Tris hydrochloride [pH 6.8], 0.1 M $MgSO_4$, 1 mM dithiothreitol) was added to bring the volume to 5 ml; samples were removed for protein analysis. The remainder was digested overnight at 37°C with 40 μg of RNase A (Sigma) per ml and 4 μg of DNase I (Sigma) per ml, followed by a 4-h digestion with 40 μg of proteinase K (Sigma) per ml. LPS was purified twice on a Sephadex G-25 column (Pharmacia, Inc., Piscataway, N.J.) equilibrated in 0.01 M ammonium acetate (pH 8.1). LPS collected in the void volume was lyophylized and redissolved in distilled water, and samples were analyzed for carbohydrates and on polyacrylamide gels for LPS chain length distribution.

Chemical analysis of LPS. The relative number of O repeat units was estimated by assaying abequose (38) against standards of D-digitoxose (Aldrich Chemical Co., Inc., Milwaukee, Wis.), which like abequose yields malondialdehyde upon oxidation, and by assaying rhamnose (7) with L-rhamnose (Pfanstiehl Laboratories, Waukegan, Ill.) as the standard. The relative number of core units was determined by a 2-keto-3-deoxyoctulosonic acid assay (38) with 2-keto-deoxyoctonate (Sigma) as the standard. Protein was assayed with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as the standard.

LPS polyacrylamide gel electrophoresis. LPS samples in buffer (2% sodium dodecyl sulfate, 2% 2-mercaptoethanol, 5% glycerol, 0.5 M Tris hydrochloride [pH 6.8], bromophenol blue) were heated at 100°C for 5 min and then resolved on 7 to 25% gradient polyacrylamide gels (14 by 30 cm) with a 5% stacking gel and a Laemmli buffer system (28) containing 0.1% sodium dodecyl sulfate. Electrophoresis was at 35 mA until the sample entered the separating gel and

then at 6 mA for about 20 h. The gels were treated with a silver stain (47) and photographed with Polaroid type 55 film, and the negatives were scanned with a Zeineh Soft Laser scanning densitometer model SL-TRFF in coordination with the Zeineh Videophoresis II program (Biomed Instruments, Inc., Fullerton, Calif.).

RESULTS

Association of the virulence plasmid with survival in serum. The inferior survival of the mutant strain CR7007 compared with that of the parental strain CR7003 in 90% PNHS is shown in Fig. 1. The mean ratio of strain CR7007 to strain CR7003 was 0.02 ± 0.006 (standard deviation) for all experiments, resulting in strain CR7003 surviving significantly better than the mutant strain CR7007 after 1 h in PNHS ($P < 0.0005$, $n = 7$; paired t test) (Table 2). Likewise, the parental strain CR7020 exhibited a 16% increase in viable numbers, compared with a 53% loss in viability of the mutant strain CR7024 after 1 h in PNHS ($P < 0.0005$, $n = 7$; paired t test) (Table 2). Strains with the opposite antibiotic resistance markers and the original strains behaved in a similar fashion, with the parental strains surviving better than the mutant strains.

The association of the virulence plasmid with resistance to the bactericidal effects of serum was confirmed by reintroducing the Tn*10*-tagged virulence plasmids into the Nx^r derivatives of mutant strains CR7007 and CR7026. These transconjugant strains (strains CR7010 and CR7026) had survival rates in PNHS equal to or greater than those of the parental strains (Table 2). Furthermore, the plasmid-free strain CR7158 became considerably more serum resistant upon receipt of the Tn*10*-marked virulence plasmid pCF610 to give strain CR7162 ($P < 0.01$, $n = 3$; paired t test) (Table 2).

Role of complement. The nature of the bactericidal agent was assessed by treating the serum in ways known to inactivate complement. Heating the serum at 60°C for 30 min, as well as treatment with zymosan and cobra venom factor, destroyed the bactericidal properties of PNHS and allowed the parental and mutant strains to survive equally well (Fig. 2 and Table 2), suggesting that complement is responsible for the bactericidal effects of PNHS. As might be expected, the performances of the plasmid-restored strains were similar to those of both the parental and mutant strains in these sera (Table 2).

Complement pathway determination. The involvement of the two pathways of complement activation was assessed by measuring the residual bactericidal activity of serum after the separate inactivation of each pathway.

The CCP was inactivated by the addition of 8 mM EGTA and 2 mM Mg^{2+} to eliminate the calcium necessary to maintain the quartenary structure of C1 while retaining the ACP activity (39). Serum treated in this manner retained 88 ± 7% of its ACP activity, while the CCP was completely eliminated. Although less than 1% of the inoculum of strain SL1035 survived after 1 h (data not shown), viable counts of neither the parental nor the mutant strain declined in this serum for 2 h (Fig. 3). Subsequent declines in viable counts were at equal rates. The killing curves, therefore, were not like those in untreated sera, demonstrating that the ACP was not responsible for the observed differences in survival of the strains in PNHS.

The ACP was completely eliminated by heating the serum at 50°C to degrade elements of the ACP more rapidly than those of the CCP (10, 15). In each experiment, different

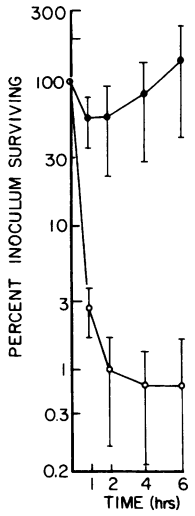

FIG. 1. Percentage of inoculum surviving in 90% PNHS. The Sm^r parental strain CR7003 (●) was incubated in 90% PNHS with the Nx^r mutant strain CR7007 (○). Bars represent the standard deviations of three experiments.

levels of CCP activity remained after heating. The parental strain was not killed in the presence of this residual CCP activity (Fig. 4). The mutant strain, in contrast, experienced a significant decline in viable numbers at CCP activity levels greater than 10% of that of untreated serum (Fig. 4), indicating that CCP activity was essential for the killing of the mutant strains.

The role of the CCP in the killing of the mutant strains was confirmed with PNHS that had been depleted of properdin, leaving 57% of the CCP activity intact (Table 3). The serum retained bactericidal activity sufficient to reduce the viable count of strain SL1035 by more than 99% in 1 h. After 2 h in the properdin-depleted serum, the serum-sensitive strain CR7007 showed significantly lower viable counts than did the parental strain CR7003 ($P < 0.01$, $n = 3$; paired t test) (Table 3).

Bacterial activation of complement. Differences in the ability of the parental and mutant strains of *S. typhimurium* to activate complement were assessed by measuring the reduction in the number of hemolytic units of the CCP ($C'H_{50}$) and ACP (APH_{50}) after 90% serum had been incubated with parental or mutant strains. The higher concentration of bacteria (10^8/ml) was required to elevate the amount of complement activated to a level more readily measured above background. Viable counts done on these test mixtures also showed better survival of the parental strains, consistent with the results of normal tests with 10^5 bacteria.

The percentages of total hemolytic units activated per 10^8 bacteria are shown in Table 4. There were no apparent differences in the ability of the parental or mutant strains of a pair to activate either pathway, suggesting that differential activation of complement is not responsible for differences in serum killing.

LPS determinations. Many studies have demonstrated that the increased sensitivity of salmonellae to serum is due to the rough phenotype. As certain plasmids are known to alter LPS composition, the possibility that virulence plasmid-mediated changes in LPS composition are responsible for sensitivity to serum was carefully considered.

Phage typing with bacteriophages known to bind to LPS (50) showed that no differences existed between strains of a

TABLE 2. Percent survival in sera after 1 h

Strain	Virulence plasmid[a]	% Survival in serum[b]:			
		Untreated	Heat inactivated	Zymosan treated	Cobra venom treated
CR7003	+	59 ± 18	102 ± 15	98 ± 23	121 ± 15
CR7007	−	3 ± 2	108 ± 33	107 ± 30	140 ± 35
CR7010	+	58 ± 17	108 ± 21	134 ± 21	NT
CR7020	+	116 ± 23	105 ± 40	118 ± 25	NT
CR7024	−	47 ± 25	89 ± 12	122 ± 41	NT
CR7026	+	108 ± 49	107 ± 7	149 ± 25	NT
CR7158	−	15 ± 12	NT	NT	NT
CR7162	+	91 ± 8	NT	NT	NT

[a] Autonomous plasmid determined by gel electrophoresis.
[b] Values are means ± standard deviations for three or more experiments. NT, Not tested.

pair, and hence, all strains were considered smooth (Table 5). It is worth noting that the plasmid-free strain CR7158 also was smooth by phage typing (data not shown).

The LPS was subjected to chemical analysis to determine the relative composition of the LPS molecules, as differences in the amount of LPS have been shown to alter bacterial sensitivity to serum (13, 46). These assays (Table 6) demonstrated no significant differences in the concentrations of either the unique core sugar (2-keto-3-deoxyoctulosonic acid) or the unique O repeat unit sugars rhamnose and abequose. All four strains had the same cell volume of 0.8 μm^3 and hence the same cell surface area, and it appeared, therefore, that the mean density of core and O repeat units per cell also was the same for both the parental and serum-sensitive mutant strains of each pair.

The possibility that the O repeat units might be differently distributed on the core units was studied by densitometer tracings of silver-stained gels of the same LPS preparations used in the chemical analyses. Evaluation by microdensitometry indicated that the bands remained in register and that the relative numbers of molecules of each molecular weight appear to be the same (Fig. 5). Indeed, quantitative analysis of the optical densities at various molecular weights, i.e., 0 to 15 O repeat units (zone 1), 16 to 50 O repeat units (zone 2), and >50 O repeat units (zone 3), was achieved by integration of the densitometric tracings. Such analysis demonstrated that the parental and mutant strains of each pair were indistinguishable (Table 7).

The 4-amino-4-deoxy-L-arabinose substituent on lipid A is associated with susceptibility to cationic proteins of neutrophils (42) and hence also may influence sensitivity to complement. Substitution has also been shown to increase resistance to the basic antibiotic polymyxin B 30- to 100-fold (48). All strains were inhibited by approximately 6 μg of polymyxin B per ml, however, indicating that there were no differences in 4-amino-4-deoxy-L-arabinose substitutions.

DISCUSSION

It has been shown that the 95-kb plasmid of *S. typhimurium* is associated with virulence, adhesive and invasive properties (24), and resistance to serum (Abstr. Annu. Meet. Am. Soc. Microbiol. 1985). In a worldwide survey of salmonella isolates, Helmuth et al. (17) also found a correlation between the presence of large plasmids and resistance to serum in *S. typhimurium* and showed that the same correlation exists in *S. enteriditis*, *S. dublin*, and *S. heidelberg*. In the present study, we extend our observations on the resistance of *S. typhimurium* to serum and show that the presence of the autonomous virulence plasmid correlates with increased resistance to the bactericidal activity of 90% PNHS.

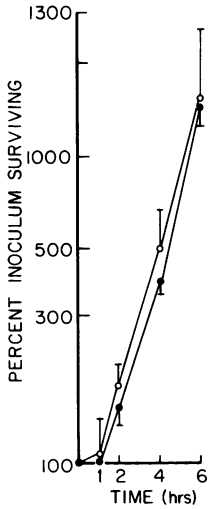

FIG. 2. Percentage of inoculum surviving in heat-inactivated PNHS. The Sm^r parental strain CR7003 (●) was incubated with the Nx^r mutant strain CR7007 (○) in 90% PNHS which had been heated at 60°C for 30 min. Bars represent the standard deviations of three experiments.

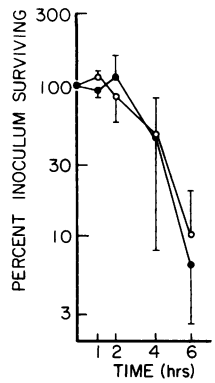

FIG. 3. Percentage of inoculum surviving in EGTA-Mg^{2+}-treated PNHS. The Sm^r parental strain CR7003 (●) and the Nx^r mutant strain CR7007 (○) were incubated together in 90% PNHS with 8 mM EGTA and 2 mM Mg^{2+}. The serum retained 88% of ACP activity but no detectable CCP activity. Bars represent the standard deviations of three experiments.

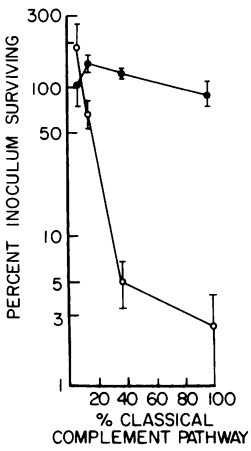

FIG. 4. Percentage of inoculum surviving in ACP-depleted PNHS after 60 min. The Sm^r parental strain CR7003 (●) and the Nx^r mutant strain CR7007 (○) were incubated together in 90% PNHS which had been completely depleted of ACP activity by heating at 50°C for 15 to 20 min. Values for survival at each residual CCP activity represent the mean and standard deviations of triplicate viable counts. Values for 100% CCP activity represent the means and standard deviations of the controls.

Subsequent to the completion of this work, mutant strains CR6260 and CR8100 were found to have integrated copies of the virulence plasmid (Rabert, Ph.D. thesis). It is unlikely that the observed decrease in virulence traits upon integration of the plasmid results from the interruption of chromosomal genes, as plasmid integration occurs in different chromosomal loci in different strain lines (Rabert, Ph.D. thesis) and the transconjugants containing the Tn*10*-marked virulence plasmid display full restoration of phenotypic traits (24) despite maintenance of the integrated copy. Furthermore, we have observed a restoration of resistance to serum by a 1-kb cloned fragment of the virulence plasmid (manuscript in preparation). This is considered strong evidence that the phenotypic lesion is due to the effect of integration upon the plasmid. Moreover, strain CR7158, in which no plasmid sequences can be detected by Southern hybridization (manuscript in preparation), became significantly more resistant upon receipt of the virulence plasmid pCF610.

Nx^r and Sm^r paired strains allowed the bactericidal assay to be conducted with mixed inocula and consequently with greater precision. The method greatly reduced variability between strains of a pair within a test and provided strict internal controls for normalization between experiments. The differences in susceptibility to serum could not be attributed to the antibiotic markers. Incubation in 10% CO_2 was absolutely essential for the maintenance of the appropriate pH and the proper functioning of the assay. In air, the pH of 90% serum increased to pH 9 by 2 h (data not shown), and at this pH, complement is less effective and bactericidal effects may be due to other factors (32).

TABLE 3. Survival of parental and mutant strains in treated sera after 2 h

Strain	Virulence plasmid[a]	% Survival in serum[b]:	
		Heat inactivated	Properdin depleted
CR7003	+	154 ± 19	137 ± 21[c]
CR7007	−	171 ± 38	89 ± 21[c]

[a] Autonomous plasmid determined by gel electrophoresis.
[b] Each value is the mean percentage of inoculum ± the standard deviation for three experiments.
[c] $P < 0.01$. P values of strain comparisons were calculated by paired t test ($n = 3$).

TABLE 4. Depletion of CCP and ACP by parental and mutant strains

Strain	Virulence plasmid[a]	% Reduction in[b]:	
		$C'H_{50}$[c]	APH_{50}[d]
CR6600	+	34 ± 14	18 ± 1
CR6260	−	29 ± 16	17 ± 2
CR8500	+	19 ± 7	14 ± 0
CR8100	−	20 ± 5	13 ± 4

[a] Autonomous plasmid determined by gel electrophoresis.
[b] Percent reduction = (reduction in complement units in the presence of bacteria − reduction in complement units in control)/initial complement units present; normalized to 10^8 bacteria per ml.
[c] Each value is the mean ± the standard deviation of three experiments.
[d] Each value is the mean ± the standard deviation of two experiments.

Killing of gram-negative bacteria in serum may be affected by either of the complement pathways or by other serum factors (44). This study demonstrates that the differential killing associated with loss of the plasmid results from the activation of complement by the CCP. The amount of killing seen in serum depleted of ACP at 50°C was directly related to the level of remaining CCP activity. Likewise, serum depleted of properdin retained its bactericidal specificity for the mutant strain. In contrast, serum in which a high level of ACP remained after EGTA-Mg^{2+} treatment had no apparent bactericidal activity over the first 2 h, during which time maximal killing of only the serum-sensitive strain by PNHS occurred. The subsequent equal reduction in viable counts of parental and mutant strains in the EGTA-Mg^{2+}-treated serum may be attributed to the toxicity of the EGTA (4), to alterations in the configuration of membrane structures, or to the removal of LPS, as occurs with EDTA at the same concentrations (29). Consistent with such membrane alterations, EGTA-Mg^{2+} caused equal decreases in the growth rates of both strains in heat-inactivated serum (data not shown).

Bactericidal activity mediated by the CCP also discriminates between the S and the Re phenotypes (4). Although both strains of each pair were smooth by phage sensitivity, it was possible that more subtle changes in the LPS could account for the sensitivity to serum. Since differences in the quantities of LPS per cell affect *Escherichia coli* resistance

TABLE 5. Sensitivity of parental and mutant strains to bacteriophages

Strain	Sensitivity[a] to phage:							
	Smooth specific			Smooth or rough specific	Rough specific			
	P22	KB41	9NA	FO	Br2	Br60	Ffm	6SR
SL1035[b]	−	−	−	−	+	+	+	+
LT2[c]	+	+	+	+	−	−	−	−
CR6600	−	+	±	+	−	−	−	−
CR6260	−	+	±	+	−	−	−	−
CR8500	−	+	+	+	−	−	−	−
CR8100	−	+	+	+	−	−	−	−

[a] −, Resistant; +, sensitive; ±, intermediate sensitivity.
[b] Reference rough strain (50).
[c] Reference smooth strain (50).

TABLE 6. LPS chemical analysis[a]

Strain	Virulence plasmid[b]	Protein (mg)	KDO[c] (nmol)	Rhamnose (nmol)	Abequose (nmol)
CR8500	+	2.9 ± 0.3	43.7 ± 16.8	583 ± 15	1,131 ± 199
CR8100	−	2.9 ± 0.5	47.6 ± 15.7	639 ± 48	1,285 ± 206
CR6600	+	3.4 ± 0.5	46.5 ± 9.4	585 ± 10	1,080 ± 146
CR6260	−	3.6 ± 0.3	52.6 ± 8.5	538 ± 57	1,148 ± 135

[a] All measurements were normalized to 10^{11} total bacteria. Each value is the mean ± the standard deviation for three assays.
[b] Autonomous plasmid determined by gel electrophoresis.
[c] KDO, 2-Keto-3-deoxyoctulosonic acid.

to serum (46), the comparison of paired serum-resistant and serum-sensitive salmonella strains required maximum recovery of LPS representative of the bacterial cell. The hot phenol-water extraction method, while providing highly purified LPS, preferentially isolates hydrophilic, longer-chain molecules (14, 26), whereas the petroleum ether-chloroform-phenol method preferentially isolates the shorter, more hydrophobic LPS species (14). The modified method (13) used to prepare LPS for this study seems, on the basis of polyacrylamide gel electrophoresis (13, 19), to recover a wider spectrum of LPS forms than do the more traditional methods. With the added purification step of DNase I treatment, the product is suitable for quantitative carbohydrate analysis. The molar concentrations of 2-keto-3-deoxyoctulosonic acid, rhamnose, and abequose were the same for both strains of a pair, indicating that resistance to serum was not due to differences in the actual number of core or O repeat units of LPS per cell.

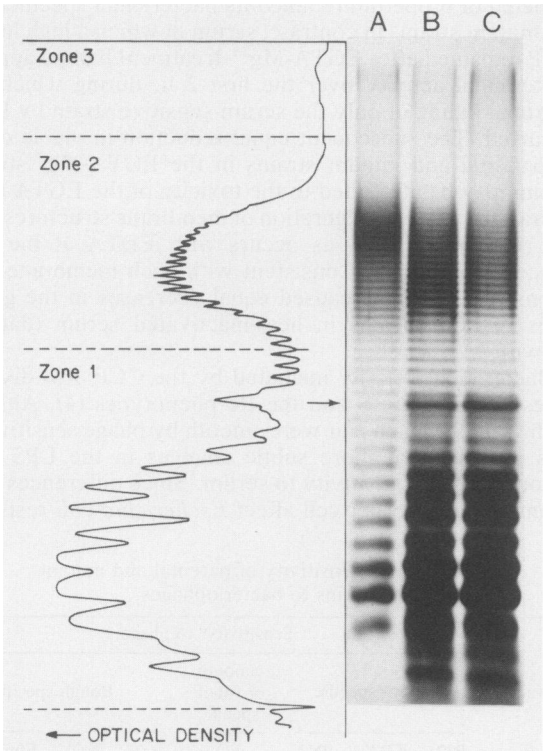

FIG. 5. Silver-stained 7.5 to 25% gradient polyacrylamide gel of LPS from *S. typhimurium* and densitometer tracing. Proteinase K-, DNase I-, and RNase A-digested cell lysates of the parental strain CR6600 (B) and the mutant strain CR6260 (C) were electrophoresed beside *S. typhimurium* LPS (A) prepared by the hot phenol-water method. The densitometer tracing of the strain CR6600 LPS illustrates the three groupings of LPS lengths: zone 1, 0 to 15 O repeat units; zone 2, 16 to approximately 50 O repeat units; and zone 3, >50 O repeat units. An unidentified protein (arrow) was apparent in equal quantities for each strain in most cell lysates.

Resistance to serum is affected not only by the absolute amount of LPS per cell but also by the differences in the lengths of the LPS molecules (13). Although the silver staining of LPS is less quantitative and provides less precision than do densitometric measurements with radiolabeled LPS (14, 38), it can be used for LPS from cells grown under many conditions, thereby avoiding LPS alterations due to medium composition (49). The method is well suited to comparisons of LPS extracted from chromosomally isogenic strains in which the LPS may differ only in chain length. Densitometer tracings of the silver-stained gels showed an LPS chain length distribution similar to those reported for phenol-water-extracted radiolabeled LPS (14, 37) and for chromatographically separated, commercially prepared LPS (38). The higher percentage of longer molecules reported in these other studies probably reflects the preferential isolation of the longer, hydrophilic molecules of the LPS by the extraction method used.

While no differences were seen in the LPS between members of tested pairs, it was noted that the more serum-resistant line, strain CR8500 and its derivatives, had proportionately more LPS molecules of higher molecular weight. Although these bacterial lines also differ in other respects, these differences in LPS chain length may result in the differences in the sensitivities to serum, a suggestion consistent with the observations of Goldman et al. (13) for *E. coli*. A resistance to serum that is dependent on the LPS then would be clearly distinct from those resistances associated with the autonomous virulence plasmid, even though both involve killing by the CCP.

The process by which the plasmid mediates resistance to serum is unclear. No major outer membrane protein has

TABLE 7. Percent distribution of LPS of different molecular lengths in parental and mutant strains[a]

Strains	Virulence plasmid[b]	% LPS[c]		
		Zone 1	Zone 2	Zone 3
CR6600	+	79 ± 6	19 ± 6	2 ± 1
CR6260	−	77 ± 9	20 ± 7	4 ± 3
CR8500	+	63 ± 4	31 ± 3	4 ± 1
CR8100	−	62 ± 7	31 ± 3	5 ± 1

[a] Values were calculated from densitometer scans (see Fig. 5 and text). Values are the means ± the standard deviations for three (strains CR8500 and CR8100) or seven (strains CR6600 and CR6260) analyses. *P* values for strains CR6600 and CR6260 compared with strains CR8500 and CR8100 by the Student *t* test: zone 1, $P < 0.001$; zone 2, $P < 0.001$; zone 3, $P < 0.25$.
[b] Autonomous plasmid determined by gel electrophoresis.
[c] Zone 1, 0 to 15 O repeat units; zone 2, 16 to 50 O repeat units; zone 3, >50 O repeat units.

been associated with a resistance-conferring cloned fragment of the virulence plasmid (unpublished data), and it is unlikely, therefore, to be by steric exclusion of antibody (41) or complement from the membrane. A specific alteration of a membrane target (e.g., *ompA* by *traT*), however, is possible. It is of note that *traT* probes have been shown to hybridize to the virulence plasmid (33). While activation of complement by LPS (30, 31) or other structures distal from the membrane could reduce the concentration of potentially reactive molecules near CCP-activating structures (11) at the membrane surface, there was no difference in either residual ACP or CCP activity following the incubation of PNHS with serum-resistant or serum-sensitive strains, implying that equal concentrations of antibody and complement should be able to reach the membrane in an active form. Inactivation of the CCP by excreted proteases is excluded by the observation that serum-sensitive mutant strains were not protected from the bactericidal effects of PNHS by the presence of serum-resistant parental strains. The plasmid may (i) act in concert with LPS to shed nascient membrane attack complexes (22, 23), (ii) alter the structure or quantity of membrane sites associated with CCP activation, (iii) modify the ability of complement to insert into the membrane, or (iv) affect the sequelae of complement insertion. Investigations to discriminate among these activities are currently being conducted. The gene products of a 1-kb fragment of the virulence plasmid that confers resistance to serum will be described in a subsequent publication.

In conclusion, the CCP seems to be the more relevant mechanism of serum killing of smooth *S. typhimurium*, and the resistance phenotype is closely associated with the virulence plasmid as well as with LPS. LPS and the plasmid-encoded products, however, appear to act independently in conferring resistance.

After the submission of this article, Hackett et al. (16) reported the cloning of *S. typhimurium* virulence plasmid genes which confer resistance to serum on rough *S. typhimurium* and *E. coli* strains. In contrast with their results, we saw no association of the virulence plasmid with LPS. Furthermore, their cloned resistance gene(s) appears distinct from the resistance gene cloned in this laboratory (manuscript in preparation).

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