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Further Characterization of Complement Resistance Conferred on *Escherichia coli* by the Plasmid Genes *traT* of R100 and *iss* of ColV,I-K94

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We have shown that the *traT* gene product was responsible for the complement resistance of the R100 plasmid. We compared this resistance with that specified by the *iss* gene of the ColV,I-K94 plasmid. The levels of resistance specified by the two genes were similar, and there was no additive effect on resistance when both genes were present together. Under conditions in which *traT* and *iss* conferred at least a 50- and 10-fold increase in survival, respectively, the consumption of C6, C7, C8, and C9 was the same for bacteria with and without the plasmid genes. This result indicated that it was the action of the terminal complex, not its formation, which was blocked by *traT* and *iss*.

Complement has the ability to opsonize and lyse a wide range of bacterial species, properties which are thought to constitute important host defense mechanisms (4). Support for this comes from the observations that individuals with genetic defects in their complement system are frequently predisposed to bacterial infections (3) and that bacteria causing generalized infections are frequently resistant to the action of complement (7).

Elucidation of the mechanisms which bacteria have evolved to avoid the bactericidal activities of complement is therefore important in understanding this area of bacterial pathogenicity (27, 28).

The presence of several outer membrane components has been correlated with serum resistance in *Escherichia coli*, including the prevalence of certain O and K antigens among serum-resistant strains (14, 18), changes in the protein profile (23), and changes in lipid (2) and lipopolysaccharide composition (24) of serum-resistant mutants.

Reports that various plasmids confer increased complement resistance on their hosts (11, 19–22) suggested to us that such plasmids provided an excellent opportunity to clone genes specifying complement resistance and to analyze the mechanisms of resistance in a genetically well-defined system.

Two plasmid genes specifying complement resistance have so far been identified (6, 16): the *traT* gene of the R6-5 plasmid which produces an outer membrane protein involved in surface exclusion (1) and the *iss* gene of the plasmid ColV,I-K94 (6). A cloned fragment containing the *iss* gene increased the virulance toward chicks of *E. coli* B188 approximately 100-fold (6).

Recombinant plasmids containing copies of *traT* and *iss* cloned into the plasmid vector pBR322 were used in this study to investigate the complement resistance they confer, and we report here that the R100 gene product conferring complement resistance was *traT* protein and that the resistances conferred by *traT* and the *iss* gene of ColV,I-K94 were similar. We have also shown that the consumption of components C6, C7, C8, and C9 was the same by bacteria in the presence and absence of the plasmid resistance genes. This result is discussed in terms of possible mechanisms of resistance. (Part of the work was presented at the International Conference on Bacterial Plasmids Jan. 5–9, 1981, Santo Domingo, Dominican Republic.)

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* K-12 strains J6-2 (*pro his trp lac*) and ED8739 (*met*) have been described previously (20, 26). Plasmid R100 in J6-2 was obtained from A. M. Reynard. Plasmid-containing derivatives of these strains, constructed by transformation (8), are shown in Table 1.

pBH6 contains *Eco*RI fragment A of pKH46-1::γδ (D. L. D. Davies, M. M. Binns, and K. G. Hardy, submitted for publication). pHA contains a 2-kilobase *Hin*dIII/*Ava*I fragment of R100 cloned into *Hin*dIII/*Ava*I-cleaved pBR322 (R. Ogata, C. Winters, and R. P. Levine, manuscript in preparation).

Sera, complement components, and buffers. Guinea pig serum was purchased from Pel-Freeze Biologicals, Inc., Rogers, Ark. Pooled normal human serum was a gift from J. Koplow. Normal rabbit serum was pre-

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TABLE 1. *E. coli* strains

Designation	Strain	Plasmid(s)
MB112	J6-2	
MB143	J6-2	R100
MB144	J6-2	pHA
MB115	J6-2	pBH6
MB145	J6-2	pBR322
MB146	J6-2	R100 and pHA
MB147	J6-2	R100 and pBH6
MB24	ED8739	
MB114	ED8739	pHA

pared by clotting freshly drawn blood for 1 h at room temperature and 3 h at 4°C. The clot was then removed by centrifugation. All sera were stored in 1-ml samples at −70°C until use.

Rabbit serum deficient in C6 was a gift from J. P. Atkinson. Human C8, C9, anti-human C7 antibody, and EAC1-7 were purchased from Cordis Laboratories, Miami, Fla.

Human R7 reagent (serum from which C7 has been removed) was prepared by passing normal human serum over a Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) column to which anti-human C7 antibody had been coupled by cyanogen bromide treatment as recommended by the manufacturers. R7 reagent prepared by this method did not lyse sensitized sheep erthrocytes.

VBS^{2+} contained 5 mm Veronal buffer, 0.15 M NaCl, 0.5 mM $MgCl_2$, and 0.15 mM $CaCl_2$ (pH 7.5).

Surface exclusion assay. The donor strain MB143 and the recipient strains MB24 and MB114 were grown in yeast tryptone medium (15) to optical density at 600 nm = 0.5. Equal volumes of the donor and recipient strain were mixed for each mating and incubated for 2 h, without shaking, at 37°C. Cells were collected by centrifugation, washed in VBS^{2+}, and serially diluted. We plated 0.1-ml samples onto selective media (Table 2) to estimate the numbers of donor, transconjugant, and recipient cells in each mating.

Identification of *traT* protein with anti-*traT* antibody. Cells from 10 ml of overnight culture (in yeast tryptone medium), were collected by centrifugation and resuspended in 0.05 M Tris-hydrochloride (pH 8.0) at a cell density of about 2×10^9/ml. We added 20 μl of 0.25 M EDTA and 1 ml of lysozyme (1 mg/ml). After incubation at room temperature for 20 min, the samples were sonicated, while kept in ice, for six 30-s periods with 1-min intervals. The envelope fraction was collected by centrifugation at 45,000 rpm for 1 h in a Sorvall AH-650 rotor on a Sorvall OTD-75 ultracentrifuge. The crude envelope fraction was then washed in 0.05 M Tris-hydrochloride (pH 7.5)–0.1 M NaCl–1 mM EDTA and suspended in 2% (wt/vol) Sarkosyl at a protein concentration of 5 mg/ml as determined by the method of Lowry et al. (12).

Anti-*traT* antibody (9), a gift from S. Levy, was diluted 2:1 with 0.05 M Tris-hydrochloride (pH 7.5)–0.1 M NaCl–1 mM EDTA, and 8 μl was added to the center well of an immunodiffusion plate (Hyland Diagnostic, Deerfield, Ill.). Samples of crude outer membrane preparations were added to the outer wells, and the plates were incubated at 4°C for 2 days, after which time precipitin bands were clearly visible.

Bactericidal assay. Bacteria grown in yeast tryptone medium at 37°C to an optical density at 600 nm = 0.2 to 0.4 were collected by centrifugation, washed once with VBS^{2+}, and suspended in the same buffer to an optical density at 600 nm = 0.5. Cells (30 μl) were incubated at 37°C for 1 h with serum and VBS^{2+} in a final volume of 1 ml. After incubation, samples were diluted in VBS^{2+} and plated onto L agar plates to determine the number of viable cells. Strains harboring plasmids pHA and pBH6 were also plated onto L agar plates containing ampicillin (120 μg/ml) to check for maintenance of the plasmids. The percentage of survival is expressed relative to that of bacteria incubated without serum.

Complement consumption assays. C6 assay. *E. coli* cells were prepared and incubated with 10% normal rabbit serum for 1 h at 37°C as in the bactericidal assay. The cells were then removed by centrifugation, and the residual C6 activity was assayed by incubating 0.2 ml of serial fivefold dilutions of the supernatants with 0.1 ml of sheep erythrocytes (1×10^8 cells per ml), and 0.2 ml of 5% C6-deficient rabbit serum at 37°C for 1 h. A total of 0.5 ml of VBS^{2+} was added, the tubes were centrifuged at 3,000 × *g* for 5 min., and the percent-

TABLE 2. Specification of surface exclusion by the 2-kilobase fragment of R100 conferring complement resistance cloned in pHA

Mating mixture[a]	Cell class	Selective medium[b]	No. of cells	
			Expt 1	Expt 2
MB24 × MB143				
	Donor	Pro, His, Trp, Tc	1×10^6	2.9×10^8
	Recipient and transconjugant	Met	1.1×10^7	8×10^7
	Transconjugant	Met, Tc	4.5×10^3	5.5×10^5
MB114 × MB143				
	Donor	Pro, His, Trp, Tc	3×10^6	2.3×10^8
	Recipient and transconjugant	Met, Amp	1.7×10^7	1.2×10^8
	Transconjugant	Met, Amp, Tc	1×10^2	2.5×10^4

[a] MB143 is J6-2 containing R100 and MB114 is MB24 containing pHA.

[b] Minimal agar plates supplemented with appropriate nutrients and antibiotics at the following concentrations: proline (Pro), histidine (His), tryptophan (Trp), and methionine (Met), 20 μg/ml; tetracycline (Tc), 25 μg/ml; and ampicillin (Amp), 120 μg/ml.

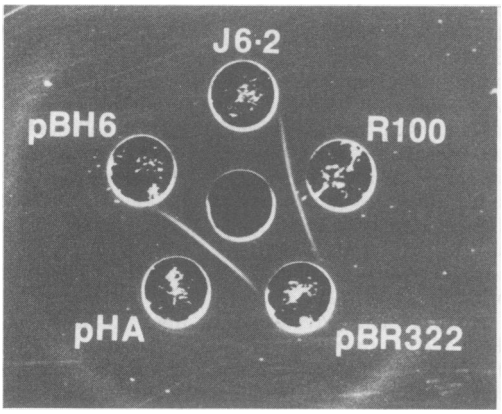

FIG. 1. Detection of *traT* protein with anti-*traT* antibody. Crude outer-membrane preparations isolated from cells of *E. coli* J6-2 carrying the plasmids indicated were obtained by the procedure given in Materials and Methods. We added 10-μl samples containing about 5 mg of protein per ml to the outer wells of the Ouchterlony plate. Anti-*traT* antibody was diluted 2:1 with 0.05 M Tris-hydrochloride (pH 7.5)–0.1 M NaCl–1 mM EDTA (10), and 8 μl was added to the center well. After incubation at 4°C for 48 h, precipitin lines were clearly visible.

age of lysis of the sheep erythrocytes was estimated by recording the absorbance at 414 nm.

C7, C8, and C9 assays. *E. coli* cells were prepared as for the bactericidal assay and 0.3 ml of cells incubated with 0.1 ml of normal rabbit serum and 0.6 ml of VBS^{2+} for 1 h at 37°C. The cells were removed by centrifugation, and fivefold serial dilutions of the supernatants made.

For the C7 assay, 0.1 ml of sheep erythrocytes (1×10^8 cells per ml) was incubated with 0.2 ml of 10% R7 reagent and 0.2 ml of the supernatant dilutions for 1 h at 37°C. A total of 0.5 ml of VBS^{2+} was added, the tubes were centrifuged at 30,000 × *g* for 5 min, and the absorbance at 414 nm was measured. Control tubes in which VBS^{2+} was substituted for the supernatant dilutions were included to measure background lysis of the sheep erythrocytes by the R7 reagent.

For the C8 assay, 0.1 ml EAC1-7 (10^8 cells per ml) were incubated with 0.2 ml of human C9 (50 U/ml) and 0.2 ml of the supernatant dilutions for 1 h at 37°C. Lysis was then measured as for the C7 assay. Control tubes were included to measure the lysis of EAC1-7 in the presence of C9.

C9 was assayed in the same way as C8, except that 0.2 ml of human C8 (50 U/ml) was substituted for C9 in the assay. Control tubes measured the lysis of EACl-7 in the presence of C8.

Lysis of sheep erythrocytes and EAC1-7 by dilutions of 10% normal rabbit serum were also measured as controls for the assays. All assays were done in duplicate.

RESULTS

Identification of *traT* as the R100 gene specifying complement resistance. Moll et al. (17) recently identified the *traT* gene product of the plasmid R6-5 as being responsible for the increase in serum resistance that this plasmid confers upon *E. coli* host bacteria.

The plasmids R100 and R6-5 are closely related (23), so it seemed probable that the complement resistance gene of R100 would also be *traT*.

By use of pHA, a recombinant plasmid containing 2 kilobases of R100 DNA, that specifies the production of only one R100 protein in maxicells (R. Ogata, personal communication), we have demonstrated that it is the *traT* gene of R100 which specifies complement resistance.

traT protein, along with *traS* protein, mediates the phenomenon of surface exclusion (1) by reducing the ability of a strain harboring a conjugative plasmid to act as a recipient in conjugation with closely related donor strains. The recipient abilities of MB24 and MB114 in a mating with MB143 were therefore compared. The recipient ability of MB114 (containing pHA) was reduced about 20-fold compared with MB24 (Table 2).

That pHA specifies production of *traT* protein was confirmed by using anti-*traT* antibody. Crude outer-membrane preparations from cells harboring R100 and pHA contained a protein antigenically similar to *traT* protein, whereas those from cells without plasmid or containing the vector pBR322 did not (Fig. 1).

Comparison of complement resistance specified by cloned *traT* and *iss*. We compared the levels of complement resistance conferred on MB112 by the plasmids pHA and pBH6 (Fig. 2). Although there are potential differences in the regulation of expression of the two genes cloned in pHA and pBH6, the levels of complement resistance that they conferred were remarkably similar.

It seems probable that the similar levels of resistance observed reflect a common mechanism of resistance for the two genes. This idea is supported by the results of experiments designed to determine whether complement resistance is increased in bacteria containing both *traT* and *iss* genes. The level of complement resistance was not increased by the presence of both genes, that of MB143 (containing R100) being about the same as that of MB147 (containing R100 and pBH6) (Table 3). The increase in the copy number of the *traT* gene from MB143 to MB144 to MB146 only slightly increased the level of complement resistance. (Figure 1 shows that MB144 produced more *traT* protein than MB143.) This result is consistent with the hypothesis that there are a limited number of sites at which *traT* can operate to confer complement resistance. It contrasts with the effect that *traT* has on surface exclusion where an increase in the gene copy number (and *traT* protein) is

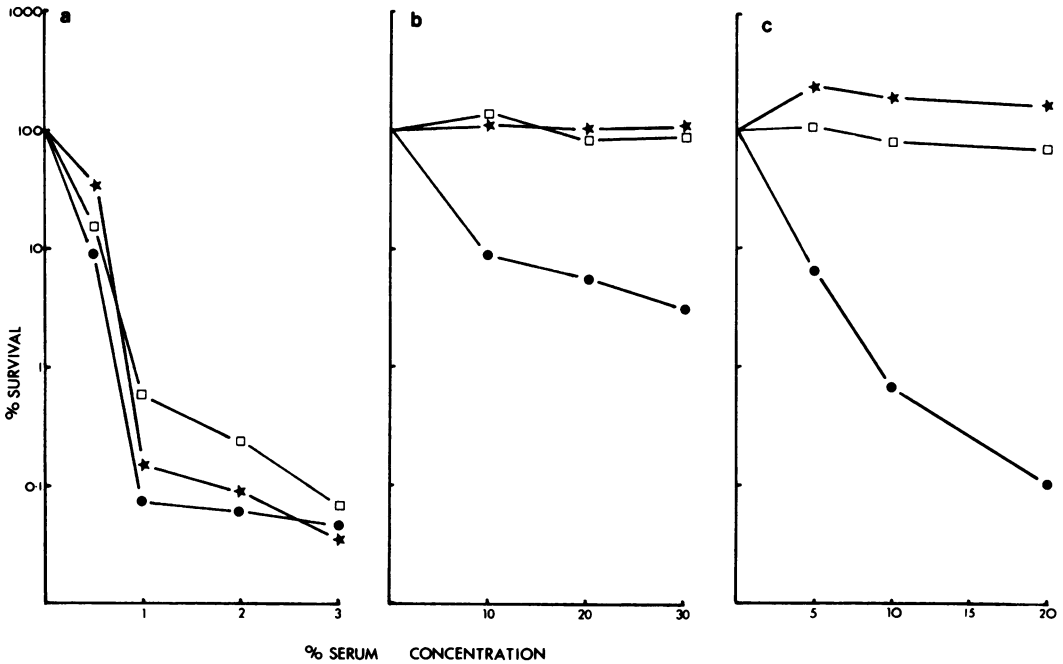

FIG. 2. Comparison of the complement resistance conferred on MB112 (●) by pHA (★) and pBH6 (□) to (a) normal human serum, (b) guinea pig serum, and (c) normal rabbit serum.

correlated with an increase in surface exclusion (1).

Complement consumption assays. The rationale behind the complement component consumption experiments was that the plasmid-specified block in the complement pathway should be reflected by higher concentrations of components after the block, in serum which had been incubated with plasmid-containing bacteria, relative to serum which had been incubated with plasmid-free bacteria.

Ogata and Levine (17) reported that the consumption of C4, C3, and C5 from normal human serum and guinea pig serum was the same for *E. coli* J6-2 with and without plasmid R100. The level of R100 complement resistance expressed under the assay conditions was relatively small, being 10-fold with normal human serum and 4-fold with guinea pig serum.

The level of resistance to normal human serum conferred by pHA is not as high as that reported for R100 (17), but we feel this result is due to the use of pooled normal human serum in this study, whereas serum from one individual was used in the previous study.

It was decided to assay the consumption of the terminal complement components by using normal rabbit serum. This had the advantage that, under the conditions used, pHA and pBH6 conferred at least a 50- and 10-fold increase in survival, respectively.

The consumption of C6, C7, C8, and C9 was the same for bacteria with and without plasmid complement resistance genes, and it is therefore likely that *iss* and *traT* operate at the same place in the complement system, namely by blocking the action of, but not the formation of, the terminal complex (Table 4).

DISCUSSION

Plasmids confer several phenotypes, e.g. alteration in nutrient uptake and utilization, antibiotic resistance, and adhesion antigens (for a general review, see reference 25), that enable their host organisms to survive under a range of adverse conditions. The complement resistance specified by the *traT* and *iss* genes is another property of plasmids which should be included in this category.

We present results which show that, as has been reported for the R6-5 plasmid (16), the gene product responsible for the complement resistance conferred on bacteria by R100 is the *traT* protein. This finding allows the structural studies on the *traT* proteins of R6-5, F, and R222 (9, 13, 16) to be correlated with the data on complement resistance reported by Ogata and Levine (17) and with that presented here. *traT* protein is an outer membrane protein of molecular weight 25,000 daltons, and it is one of the major ex-

posed proteins on the surface of cells harboring *traT* genes (13).

We report here that the consumption of terminal components of rabbit C6, C7, C8, and C9 is the same by bacteria with and without *traT* or *iss* genes. This result indicates that resistance operates at one of two places, either normally assembled terminal complexes are unable to cause membrane damage, or one or more of the five different terminal complex proteins are unable to bind to the bacterial membrane. They can, however form nonfunctional terminal complexes in solution. In the second case, it is questionable whether the consumption of components would be the same in the presence or absence of resistance genes.

The mechanism whereby complement proteins kill gram-negative bacteria, which possess two membranes, is obscure. The fact that an outer membrane protein, such as the *traT* protein, can protect cells from cytolysis, even though it is present in amounts that leave much of the cell surface exposed, suggests that there are specific sites at which functional terminal complexes are formed. The nature of these sites is uncertain, as it is well established that terminal complex assembly and function requires only a phospholipid bilayer (11).

The possibility that the preferred sites at which functional complexes are formed are the junctions between the inner and outer membranes (5) has been proposed (27). It may be that the *traT* and *iss* proteins occupy these sites and alter the physical properties of them in a way which prevents the formation of functional terminal complexes. Moll et al. (16) have reported that plasmids containing point mutations in the *traT* gene, which produce large amounts of *traT*-like protein, fail to protect bacteria from complement. This result suggests that any such interaction must be highly specific.

We have demonstrated that the *iss* and *traT* genes conferred similar levels of complement resistance and that they appeared to act by a similar mechanism. The *iss* gene has been mapped to a position outside the *tra* region of ColV,I-K94 (6). The *iss* gene did not specify the production of a protein which was antigenically the same as *traT* (Fig. 1). These observations, and the fact that the *iss* and *traT* genes do not hybridize in Southern blot experiments (R. Ogata and M. M. Binns, personal observations), provide good evidence that the two genes are distinct. It will, however, be interesting to compare the DNA sequences of the two genes to see if there are any common structural features of the two proteins which might explain why they both confer complement resistance.

TABLE 3. Assay to determine whether *traT* and *iss* have an additive effect on complement resistance when both are present in *E. coli* J6-2

Designation	Plasmid(s)	% Survival in 50% NRS[a]
MB112		0.2
MB144	pHA	98
MB115	pBH6	47
MB143	R100	93
MB146	R100 and pHA	110
MB147	R100 and pBH6	86

[a] Percentage of survival is expressed relative to that of bacteria incubated without serum in VBS^{2+}. Survival values well above 100% were observed in the assay.

TABLE 4. Complement component consumption by MB112, MB144, and MB115

Component	Control activity[a]	% Residual activity[b]		
		MB112	MB144	MB115
C6	150	75	70	77
C7	50	59	63	63
C8	600	67	63	61
C9	1,000	30	32	24

[a] Defined as the reciprocal of the dilution of 10% normal rabbit serum giving 63% lysis of sheep erythrocytes.

[b] Residual activity of serum incubated with bacteria relative to control serum incubated without bacteria.

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