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A Plasmid-Encoded Outer Membrane Protein, TraT, Enhances Resistance of *Escherichia coli* to Phagocytosis

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The presence of the outer membrane protein TraT, encoded by plasmid R6-5, reduces the sensitivity of *Escherichia coli* cells to phagocytosis by macrophages. This effect is independent of the bacterial capsule and is more evident in the presence of adsorbed normal human serum. The property of inhibiting phagocytosis is specifically abolished by anti-TraT protein antiserum and anti-TraT immunoglobulin G but not by Fab fragments. These results indicate that the TraT protein is a passive inhibitor of phagocytosis. Inhibition of phagocytosis is produced because the TraT protein antagonizes opsonization by complement, such that C3 deposition is reduced and altered in distribution.

Phagocytosis and the bacteriostatic and bactericidal properties of the serum constitute the first line of defense against invasive bacterial infections. The antibacterial activity of blood is effective in eliminating most normally encountered transient bacteremias and is the result of a constellation of specific (e.g., antibodies) and nonspecific (e.g., complement, iron-binding proteins) factors (7, 16).

The ability of bacteria to avoid host defenses and become invasive rests largely in cell surface components which are important in the preliminary steps of the infective process and crucial in determining its outcome (35). The role of surface components such as capsules, peptidoglycan, proteins, pili, and O-antigens in increasing the virulence of bacteria is well documented (34). Although most of these are encoded by genetic determinants located on the chromosome, recent reports testify to the importance of plasmid-encoded gene product modifications to the cell surface, which alter bacterial virulence (13–15, 31, 33, 34).

The *Escherichia coli* plasmid R6-5 carries a gene specifying resistance to the antibacterial activity of serum (31). This determinant, which has been identified as *traT*, is one of the genes of the *tra* operon, the genetic unit conferring conjugative ability upon the bacterial cell (1, 31). The *traT* gene directs the synthesis of a highly exposed outer membrane protein, which mediates resistance to serum. Since phagocytosis, the other main line of defense against invasive bacterial infections, involves complement, we explored the possibility that the TraT protein may confer upon bacteria the ability to resist phagocytosis. In this communication, we show that this is the case and that the protein interferes mainly with opsonization by the alternative pathway of complement by restricting and altering the pattern of C3 deposition.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids are listed in Table 1. Plasmid DNA, isolated by standard procedures, was introduced into bacterial strains by transformation or conjugation (45). Strains were stored at −20°C in 60% glycerol.

Molecular cloning procedures. Restriction endonuclease cleavage of plasmid DNA, ligation reactions, transformations, screening, and analysis by agarose gel electrophoresis were performed as described (45, 46).

Media. Bacterial strains were cultured in L broth with aeration or on L agar, at 37°C, with antibiotic selection when appropriate (8). Hanks balanced salt solution (HBSS; GIBCO Laboratories, Grand Island, N.Y.) plus calcium, magnesium, sodium bicarbonate, and phenol red was used in the phagocytosis assays (22). Phosphate-buffered saline (PBS) and PBS without calcium and magnesium (26) were used to wash bacterial cells and macrophages for phagocytosis assays and to dilute the bacterial suspensions.

Serum. Human blood was obtained from five healthy donors and was allowed to clot at room temperature for 1 h and for an additional 1 h at 4°C. Serum was separated by centrifugation, pooled, and stored in small portions at −70°C. Complement was inactivated by heating at 56°C for 30 min. This pool of normal human serum did not contain antibody against TraT protein as detected by immunoprecipitation. Antibodies were adsorbed basically as described by Horwitz and Silverstein (26). Briefly, 10^{11} exponential-phase bacteria were mixed with 1 ml of fresh normal serum in 1 ml of PBS and incubated on a rotary shaker at 4°C for 1 h. The process was repeated three times, and the serum was separated by centrifugation, filtered through 0.45-μm Millipore filters, and stored in small portions at −70°C. In the experiments performed with *E. coli* 59 *rif*, the serum was adsorbed with this strain and its isogenic derivative containing the plasmid pKT107. A similar process was followed in the experiments with *E. coli* FC004 and FC004(pKT107). *E. coli* strains under study, when opsonized with this adsorbed human serum preparation, did not show immunoglobulin G (IgG) on their surfaces as determined by immunofluorescence with fluorescein isothiocyanate-conjugated goat anti-human IgG immunoglobulins (Meloy Laboratories, Springfield, Va.). The anti-TraT protein antiserum was a gift from S. Levy and was prepared with partially purified TraT protein (17). This serum was adsorbed with bacterial *E. coli* outer membranes prepared from *E. coli* X984 and *E. coli* 59 *rif* cells to adsorb antibacterial antibodies. This adsorbed sera produced only one line of immune precipitation when reacted with outer membrane of $TraT^+$ cells. No precipitation line was apparent in the presence of *E. coli* outer

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TABLE 1. Bacteria and plasmids

Bacteria and plasmids	Characteristics	Source (reference)
E. coli 59	Wild-type *E. coli* isolated from feces; O:H2:K	(31)
E. coli 59 *rif*	rifampin-resistant derivative of *E. coli* 59	(31)
E. coli FC004	Capsuleless derivative of *E. coli*; O18ab:K1:H7	(2)
E. coli X984	*E. coli* K-12, employed to adsorb the antibacterial antibodies of the anti-*traT* antiserum	(17)
E. coli D1-7	*E. coli* K-12 containing the R222 plasmid; used to adsorb the anti-*traT* antibodies	(17)
pACYC184	High-copy-number cloning vector, copy number of 20	(10)
pSC101	Low-copy-number-cloning vector, copy number of 5	(11)
pKT107	Hybrid plasmid containing the *Eco*RI fragment E-7 of R6-5 cloned into the pACYC184 cloning vector	(31)
pKT118	Tn*3* insertional inactivation mutant of pKT107 unable to code for TraT protein	(31)
pFC021	Hybrid plasmid containing the *Eco*RI fragment E-7 of R6-5 cloned into the low-copy-number pSC101 cloning vector	This paper
R6-5, R222, R1-19	Genetically related, antibiotic-resistant conjugative plasmids of the FII incompatibility group	(41)
pB182	Natural conjugative low-copy-number plasmid encoding colicin B production	Agüero and Cabello (manuscript in preparation)

membranes lacking TraT protein. The anti-TraT antibodies were adsorbed with outer membranes of strain D1-7 and *E. coli* 59 *rif* (pKT107) cells (17).

Isolation of anti-TraT IgG and Fab fragments. The anti-TraT IgG was isolated from the heat-inactivated rabbit antiserum on a protein A-Sepharose column essentially as described by Miller and Stone (30). To avoid extremes of pH, it was eluted with a 0.1 M citrate gradient (pH 7.0 to 2.5). The eluate above pH 3.9 had 93% of the IgG. This eluate was further concentrated by ammonium sulfate precipitation and dialyzed against 0.1 M phosphate (pH 7.4). The Fab fragments were obtained by treating the IgG with mercuri-papain (Worthington Diagnostics, Freehold, N.J.) coupled to CNBr-Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), as will be described elsewhere (L. Aron and F. Cabello, manuscript in preparation). The digestion products were passed over a protein A-Sepharose column to remove Fc fragments and undigested IgG (18, 19). Their identity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoprecipitation with goat anti-rabbit Fab antiserum and goat anti-rabbit IgG antiserum.

Phagocytosis assays. Phagocytosis was assayed by using monolayers of mouse peritoneal macrophages prepared as previously described (2, 4, 21). Briefly, 5×10^6 *E. coli* cells were opsonized with different concentrations of the serum preparations at 37°C for 15 min. Then they were washed twice and suspended in HBSS before putting them in contact with monolayers containing macrophages. The reaction of the *E. coli* cells with anti-TraT Fab fragments was performed in the same way as the opsonization described above, with either 150 or 300 μg of Fab fragments per assay.

Fluorescence microscopy of C3-coated bacteria. Qualitative complement fixation by bacteria was assayed as described (26). Exponential-phase bacteria (5×10^6 cells) were incubated for 15 min at 37°C with 300 μl of 25% normal or adsorbed human serum in PBS, after which they were mixed with 1 ml of cold 10 mM EDTA in PBS and washed twice in PBS. The bacteria were then incubated at 37°C for 15 min in 0.1 ml of 1:10 diluted fluorescein isothiocyanate-conjugated goat anti-human C3 IgG (Meloy Laboratories) in PBS, washed twice, and resuspended in 100 μl of PBS. Slides were mounted and examined with a Leitz Dialux microscope.

Quantitation of C3 fixation. Quantitation of complement fixation by bacterial cells was done by the technique of Verbrough et al. (44). A suspension (0.2 ml) containing 5×10^8 bacteria per ml was incubated at 37°C for 15 min with 0.8 ml of different serum concentrations in Veronal saline buffer (ionic strength, 0.147; 0.15 mM Ca^{2+}, 1 mM Mg^{2+}, 0.1% [wt/vol] gelatin). The fixation of C3 was subsequently stopped by addition of 2.5 ml of 10 mM EDTA in PBS, and cells were washed three times with ice-cold PBS by centrifugation at $1,600 \times g$. The bacteria were incubated for 15 min at room temperature in 0.5 ml of 1:20 diluted fluorescein isothiocyanate-conjugated goat anti-human C3 IgG, washed three times with 2.5 ml of PBS, and suspended in 2.5 ml of PBS. Fluorescence was measured in an American Instrument Co. (Aminco) Bowman spectrofluorometer with an excitation wavelength cf 485 nm and emission wavelength of 525 nm. Results are given as percentages ranging from zero (control material incubated with buffer or inactivated serum, average actual value of 10) to 100% (maximal emission intensity in the series tested, average actual value of 75).

Immunoprecipitation. The immunoprecipitation assays were done as described by Ferraza and Levy (17). Crude envelopes of the different *E. coli* strains were used as antigens, and antiserum was the rabbit anti-TraT antibody provided by S. Levy.

Statistical analysis. The significance of the difference of the means was determined by Student's *t* test (12).

RESULTS

TraT protein interferes with phagocytosis. Envelope structures are known to contribute to the ability of bacterial cells to both evade and promote phagocytosis (26, 32, 37, 38).

The TraT protein encoded by the antibiotic resistance plasmid R6-5 is an outer membrane protein which confers upon *E. coli* resistance to the lethal activity of serum. This protective ability may result from inhibition of the bactericidal action mediated by both the classical and alternative complement pathways (31). Since the properties of the *traT* gene product are consistent with the hypothesis that they may also antagonize phagocytosis, it was of interest to investigate this possibility by using the cloned *traT* gene in various plasmid vectors.

TABLE 2. Phagocytosis of two wild-type *E. coli* strains and their isogenic derivatives diferring in the amount of TraT protein expressed in their outer membrane proteins[a]

Capsulated *E. coli* 59 *rif* derivatives	Phagocytic index[b]	Phagocytic index	Uncapsulated *E. coli* FC004 derivatives
59 *rif*	178.8	608.8	FC004
59 *rif*(pACYC184)	162.0	304.0[c]	
59 *rif*(pFC021)	152.6		FC004(pFC021)
59 *rif*(pR6-5)	118.8	$P < 0.01$	
	$P < 0.01$	229.3	FC004(pB182)
59 *rif*(pKT107)	44.9	230.4[c]	FC004(pKT107)
59 *rif*(pKT118)	153.0	564.0	FC004(pKT118)

[a] For all strains, 5×10^6 bacteria suspended in 0.5 ml of HBSS were opsonized with 25 μl (5%) of normal human serum for 15 min at 37°C. Then bacteria were washed twice and suspended in HBSS before putting them in contact with the monolayers containing the macrophages.

[b] The phagocytic index is the percentage of macrophages ingesting bacteria multiplied by the average number of *E. coli* cells ingested per macrophage.

[c] When percentages of macrophages ingesting bacteria are compared, *E. coli* FC004(pKT107) is significantly less phagocytized than FC004(pFC021) ($P < 0.025$).

Digestion of plasmid R6-5 with restriction endonuclease *Eco*RI generates 13 fragments, of which E-7 contains the *traT* gene (9). As previously reported (31), fragment E-7 was cloned into the high-copy-number vector pACYC184 generating recombinant plasmid pKT107, and in this laboratory this fragment was cloned into the low-copy-number vector pSC101, generating pFC021; pB182, a conjugative low-copy-number plasmid encoding colicin B production, was obtained from a clinical isolate (see Table 1). Plasmids R6-5, pKT107 pACYC184, pB182, and pKT118 were introduced into *E. coli* 59 *rif* or the FC004 capsuleless strain, or both, by conjugation or transformation. Their ability to promote the inhibition of phagocytosis was ascertained by visual assay for phagocytosis, with a monolayer of elicited mouse peritoneal macrophages. In the presence of normal serum, *E. coli* 59 *rif* and its derivative harboring the cloning vector pACYC184 were better engulfed, as indicated by the high proportion of macrophages associated with bacteria and the high phagocytic index (Table 2). In contrast, the isogenic strain harboring the plasmid pKT107 was poorly engulfed (Table 2), indicating that the presence of the *traT* gene on pKT107 does confer protection against phagocytosis. The difference in phagocytosis between these strains is mainly produced by differences in the percentage of macrophages ingesting bacteria, indicating that *E. coli* 59 *rif* is not easily opsonized.

Similar experiments performed with *E. coli* 59 *rif* containing the R6-5 plasmid indicate that this strain is slightly but not significantly less sensitive to phagocytosis than is its plasmidless counterpart. The decreased ability of plasmid R6-5 to confer the phenotypic ability to inhibit phagocytosis when compared with the plasmid pKT107 probably reflects the decreased production of TraT protein by *E. coli* cells harboring this low-copy-number plasmid, since in *E. coli* FC004 the plasmid pFC021, another low-copy-number recombinant plasmid carrying the *traT* gene, was also shown to confer decreased protection against opsonophagocytosis in the presence of normal serum (Table 2). The differing levels of inhibition of phagocytosis specified by pR6-5, pFC021, and pKT107 were shown by immunoprecipitation studies to correlate with the differing content of TraT protein in the outer membranes of cells harboring these plasmids, with pKT107-containing cells having a significantly higher amount of TraT protein in the outer membrane (Fig. 1). Furthermore, the high-copy-number plasmid pKT118, a transposition mutant of pKT107 that no longer expresses the TraT protein, fails to protect bacteria against phagocytosis (Table 2). It is also important to mention that only the capsulated *E. coli* strain which expresses elevated amounts of TraT protein is significantly less phagocytized than its isogenic capsulated derivatives. Concurrently, in the capsuleless strain, even low amounts of TraT protein are able to

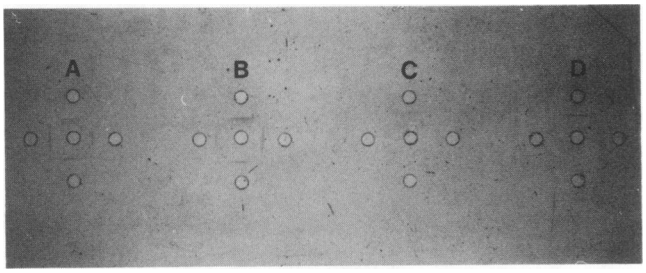

FIG. 1. Immunoprecipitation of outer membrane proteins of *E. coli* 59 *rif* bearing different TraT$^+$ plasmids. Envelopes were solubilized in 2% Sarkosyl as described (17). The precipitation was carried out in 1% agar gels containing 2% Sarkosyl. The center wells contained 17 μl of a 1:4 dilution of the adsorbed anti-TraT antiserum. Serial dilutions (17 μl each) of envelope extracts were placed in the test wells. Wells A, 59 *rif* pKT107 extract. Clockwise from top: undiluted, 1:2, 1:4, and 1:8 dilutions. Wells B, 59 *rif* pFC021 extract. Dilutions are as shown for wells A. Wells C, Clockwise from top: 59 *rif* pKT107 extract at 1:16 and 1:32 dilutions and 59 *rif* pFC021 extract at 1:16 and 1:32 dilutions. Wells D, 59 *rif* R6-5 extract. Dilutions are as shown for wells A. Undiluted samples were adjusted to 117 μg of protein per 17 μl. Comparison of the positions of the different precipitation lines indicates that *E. coli* cells harboring pFC021 and pKT107 express four and eight times more TraT protein, respectively, than cells harboring R6-5.

TABLE 3. Phagocytosis of the capsulated strain *E. coli* 59 *rif* (pKT107) in the presence of different opsonins

Opsonin	% Macrophages ingesting bacteria[a]	Phagocytic index
None	10.1	15.0
Normal human serum (5%)	21.4	44.9
Heat-inactivated human serum (5%)	13.9	13.3
Rabbit normal serum (5%)	14.5	30.5
Rabbit heat-inactivated serum (5%)	17.5	32.5
Rabbit anti-TraT antiserum (5%)	85.5	1,470.6
Rabbit anti-TraT IgG (0.3 mg)	84.0	705.0
Rabbit anti-TraT Fab (0.3 mg)[b] + normal human serum (5%)	22.0	51.7

[a] The values are the arithmetical mean of at least three experiments.

[b] Bacteria (5×10^6 cells) were first opsonized with 0.3 mg of the Fab fragment preparation, washed twice in HBSS, and then opsonized with 25 μl (5%) of normal human serum as described in the text.

TABLE 4. Plasmid-coded TraT protein protects *E. coli* from phagocytosis independently of the presence of capsule[a]

Opsonin	% Macrophages ingesting bacteria[b]			
	Capsulated *E. coli*[c]		Uncapsulated *E. coli*	
	59 *rif*	59 *rif*(pKT107)	FC004	FC004(pKT107)
Normal human serum	57.6 ± 2.2[c]	21.4 ± 9	87.2 ± 6[c]	53.5 ± 13.1
Heat-inactivated serum[d]	25.5 ± 10.7[c]	13.9 ± 6.6	21.4 ± 7.2[e]	23.5 ± 7.0[e]
Absorbed human serum[f]	29.6 ± 11.5[e]	18.0 ± 2.8[e]	71.8 ± 3.1[c]	36.5 ± 13.0

[a] Bacteria (5×10^6 cells) in 0.5 ml of HBSS were opsonized with 25 μl (5%) of normal and heat-inactivated human sera and 50 μl (10%) of adsorbed human serum as described in the text.

[b] A total of 100 to 200 macrophages were counted in each experiment. The results are the arithmetic mean of 10 independent experiments ± the standard deviation.

[c] Differences between pairs of values (phagocytosis of isogenic derivatives opsonized with the same serum preparation) are statistically significant at $P < 0.05$ (Student's *t*-text).

[d] The heat-inactivated serum lost the ability to generate complement opsonins.

[e] Differences between pairs of values are not statistically significant. $P > 0.05$.

[f] The adsorbed serum does not mediate opsonization by immunoglobulins or complement opsonins generated by activation of the classical pathway of complement.

protect against phagocytosis (Table 2). These data indicate that protection against opsonophagocytosis is dependent on the presence of TraT protein in the bacterial outer membrane and correlates with the amount of TraT protein present.

Reaction with antibodies against TraT protein block the inhibition of phagocytosis. Reaction of *E. coli* 59 *rif* cells carrying pKT107 with specific antibodies directed against TraT protein influenced the phagocytosis of these cells as expected, i.e., pKT107-containing cells were readily phagocytized in the presence of anti-TraT protein antiserum, as evidenced by the high percentage of macrophages ingesting bacteria (Table 3). Similar results were obtained when the bacterial cells were opsonized with purified anti-TraT rabbit IgG. When the anti-TraT protein antiserum was adsorbed with the $TraT^+$ strain D1-7, no phagocytosis was observed, indicating that phagocytosis in the presence of antiserum was mainly due to the opsonizing effect of the TraT protein-specific antibody or to blocking by anti-TraT antibodies of the phagocytosis-inhibiting effect of the TraT protein. The lack of phagocytosis when using the absorbed rabbit antiserum could be due to its low concentration of complement.

Effect of Fab fragments on opsonization by normal human serum. To answer the question of whether the TraT protein acts as a passive or active inhibitor of phagocytosis, we determined the effect of the presence of Fab fragments on the phagocytosis of $TraT^+$ *E. coli* cells in the presence of normal human serum (29). If the TraT protein has an active inhibitor effect, we should expect an increase in the phagocytosis of the $TraT^+$ *E. coli* cells reacted with Fab fragments because they will block its inhibitory effect upon the macrophages (29).

We observed that the reaction of *E. coli* cells expressing TraT protein, with 0.15 and 0.3 mg of anti-TraT Fab fragments, does not increase their phagocytosis in the presence of human serum, indicating that the TraT protein is a passive inhibitor of phagocytosis (Table 3). The opsonization of the capsulated $TraT^+$ cells (Table 4) with different human serum preparations is mediated by antibodies against other bacterial surface structures other than the TraT protein, as evidenced by the decrease in phagocytosis of the $TraT^+$ strain in the presence of normal or inactivated human sera. No significant difference was observed between phagocytosis of the capsulated *E. coli* 59 *rif* and the isogenic *E. coli* 59 *rif*(pKT107) when the opsonin was absorbed human serum, indicating that the capsule interferes with the activation of complement. Alternatively, experiments performed with the capsuleless strain *E. coli* FC004 indicate that this strain, which is highly phagocytized in the presence of normal human serum, seems to be opsonized mainly by the activation of complement in the absence of antibodies; this explains the important difference in phagocytosis between FC004 and FC004(pKT107) in the presence of adsorbed serum (Table 4). These findings support the conclusion that the TraT protein does not actively inhibit phagocytosis, because its inhibitory effect can be overcome by antibodies against either the TraT protein or other surface antigens (29). These results also agree with previous results that indicate that the TraT protein confers upon *E. coli* serum resistance by a mechanism that does not involve degradation or inactivation of the components of the complement system (47).

Ability of the TraT protein to interfere with phagocytosis is independent of the bacterial capsule. Since the bacterial capsule is a surface structure capable of increasing bacterial resistance to serum (2, 27, 43, 47), it was of interest to determine the possible influence of the presence of a bacterial capsule on the inhibition of phagocytosis mediated by the TraT protein. Plasmid pKT107 was therefore introduced by transformation into the capsuleless strain *E. coli* FC004. Comparison of capsulated and capsuleless *E. coli* strains

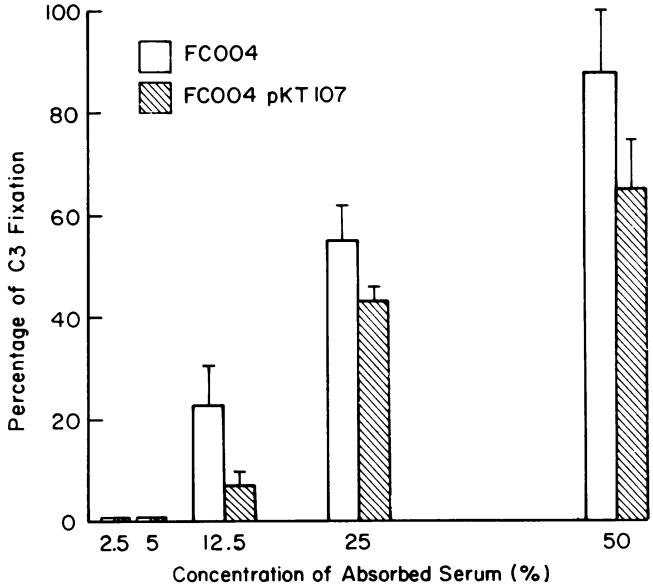

FIG. 2. The effect of serum concentration on the amount of C3 bound to *E. coli* FC004 and FC004(pKT107). A total of 10^8 bacteria was incubated at 37°C for 15 min with 0.8 ml of different concentrations of absorbed human serum, and the C3 deposited was measured by spectrofluorometry as described in the text. The results are the arithmetic mean of three experiments ± the standard deviation. These differences were not statistically significant ($P > 0.05$).

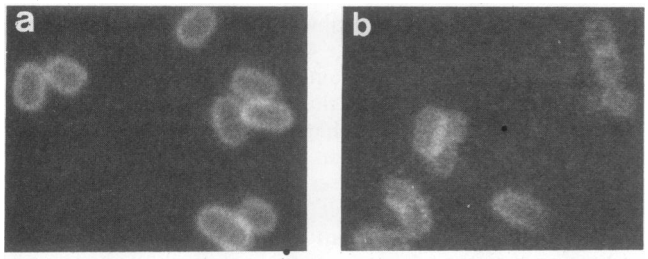

FIG. 3. Complement fixation of the capsuleless strain FC004 (a) and FC004(pKT107) (b). Complement deposit was evidenced by fluorescein isothiocyanate-conjugated goat anti-human C3 IgG, and the bacteria were examined by fluorescence microscopy. Note the light and irregular pattern of fluorescence of the strain expressing TraT protein. ×500.

containing pKT107 in the visual assay for phagocytosis indicated that both were capable of antagonizing phagocytosis (Tables 2 and 4). The decrease in the phagocytosis of the FC004(pKT107) strain when the opsonin was inactivated or adsorbed serum was used indicates that antibody and complement play a role in the opsonization of this strain by normal human serum (Table 4). These results also suggest that the protective effect of the TraT protein may be at least partially independent of O-antigen structure since the capsulated strain, *E. coli* 59 *rif*, and the capsuleless strain, *E. coli* FC004, used in these experiments have different O-antigen composition. Because we were unable to isolate a capsuleless mutant of *E. coli* 59 *rif*, these experiments were performed with *E. coli* FC004; use of the capsulated isogenic variant of strain FC004 was not possible due to difficulties in introducing DNA into this wild-type strain by conjugation or transformation.

TraT protein interferes with opsonization by the alternative complement pathway. The data reported above suggest that the TraT protein is a passive inhibitor of phagocytosis that interferes mainly with opsonization by the alternative complement pathway, since *E. coli* FC004 cells harboring pKT107 were significantly less phagocytized ($P < 0.005$) in the presence of adsorbed serum, in which case only the activation of the alternative complement pathway can effect deposition of the C3 opsonin (Table 4). Qualitative complement fixation assays suggested that *E. coli* cells expressing TraT protein deposited less complement in the presence of adsorbed serum. To confirm this finding these experiments were repeated wtih the more sensitive fluorometric immunofluorescence assay described in the text.

Since preliminary results suggested that the capsule present in *E. coli* 59 *rif* interfered with the detection of small differences in deposited complement, the capsuleless *E. coli* strain FC004 containing pKT107 was used for these studies, in which cells exposed to concentrations of normal and absorbed human serum ranging from 2.5 to 50% were assayed for complement deposition. This strain has a smooth O18ab antigen and is relatively resistant to serum (2). These characteristics rule out the possibility of a nonimmune activation of the classical pathway of complement (3, 39). Cells in which the *traT* gene product was present sustained less deposition of complement, as compared with control cells, over the entire concentration range of adsorbed serum used in this experiment (Fig. 2). The significant differences in phagocytosis between the $TraT^+$ and $TraT^-$ *E. coli* cells in the presence of adsorbed serum could not be totally explained by the nonsignificant differences in complement deposition in the presence of adsorbed serum. Because previous reports indicated that surface proteins can restrict the distribution of complement deposition on bacterial surfaces, we decided to investigate the pattern of complement deposition on $TraT^+$ *E. coli* FC004(pKT107) cells (28). It can be seen in Fig. 3 that cells in which the TraT protein is expressed exhibited an irregular deposition of complement, indicating that complement distribution is altered by the presence of the *traT* gene product. The irregular deposition of complement is more striking at high concentrations of serum, suggesting that the phenomenon is not due to differences in the amount of complement deposited. Opsonophagocytosis experiments also demonstrated that increased concentrations of serum do not augment the phagocytosis of $TraT^+$ cells, underlining the need for a homogenous complement deposition to have efficient phagocytosis (data not shown).

DISCUSSION

The bacterial cell surface has become the focus of efforts to understand the pathogenesis and epidemiology of bacterial disease. An understanding of the factors which contribute to bacterial virulence is highly germane to the development of new preventative and therapeutic measures. As demonstrated in this report, the use of molecular cloning techniques and the subsequent testing of bacteria harboring these recombinant molecules in vitro and in vivo models of infection constitute a powerful method for identifying various pathogenic factors and discerning their relevance. The data presented here demonstrate that an R plasmid gene is capable of modifying the cell surface to favor bacterial invasiveness; this modification may act per se or in cooperation with chromosomally specified structures to increase bacterial virulence.

The results presented above and previous reports demonstrating the resistance of R plasmid-containing cells to the bactericidal activity of serum clearly indicate that the presence of TraT protein in the *E. coli* outer membrane strongly influences the interaction of bacterial cells with the host defense systems (5, 31). When the *traT* gene is present on a low-copy-number plasmid and the levels of *traT* expression are low, it confers serum resistance and effects some protection against phagocytosis in capsuleless *E. coli* strains. However, when the *traT* gene is expressed from a high-copy-number vector, its ability to antagonize phagocytosis is increased, as is the presence of TraT protein in the outer membrane and the expression of the *traT* gene-specified conjugative function resulting in surface exclusion (1). The experiments performed with the capsuleless strain *E. coli* FC004 and its derivatives indicate that the presence of the TraT protein results in preferential interference with opsonization mediated by the alternative complement pathway, since its ability to antagonize opsonophagocytosis is amplified in the presence of adsorbed serum and abolished in the presence of heat-inactivated serum. Expression of the *traT* gene product results in the restriction of C3 deposition and also affects opsonin distribution such that complement deposition is diffuse and irregular. The latter observation supports the idea that homogeneous deposition of complement on the bacterial surface is required for the "zipper effect" which facilitates phagocytosis (20).

Antibodies directed against outer membrane proteins in *Haemophilus influenzae* type b are known to exert a protective effect for the challenged host during infection by these microorganisms (23, 24). Similarly, when cells which express the TraT protein are reacted with specific antibodies

directed against the *traT* gene product, their ability to antagonize phagocytosis is eliminated. However, when these cells are reacted with adsorbed anti-TraT protein antibody, the protective effect conferred by *traT* gene expression continues to be evident. The inability of the anti-TraT protein Fab fragments to block the inhibition of phagocytosis and the fact that antibodies present in the normal human serum directed to other bacterial structures can opsonize TraT$^+$ *E. coli* cells indicate that TraT protein is a passive inhibitor of phagocytosis. In this respect the TraT protein exerts a shielding effect similar to the effect of the capsules of some bacteria and fungae (2, 29).

The antiopsonic effect exerted by the TraT protein is independent of the presence of a bacterial capsule. This suggests that the ability of some capsules to interfere with phagocytosis may be further augmented by outer membrane components such as the TraT protein. This potential for enhanced protection is consistent with the hypothesis that the bacterial capsule is a heterogeneous cell surface covering, leaving exposed patches of outer membrane which can interact with opsonins and macrophages.

Bacterial resistance to the bactericidal activity of serum and to phagocytosis, which has been classically ascribed to the presence of polysaccharide capsules and O antigens, may be mediated by other cell surface structures (40, 42). This is evident not only from the recent elucidation of TraT protein function in *E. coli* but also from a recent report concerning the function of M protein, a surface structure common to group A streptococci (28). The presence of M-protein restricts complement deposition, alters the pattern of complement distribution, and increases the pathogenicity of streptococci (6, 28, 36). Similarly, clinical and laboratory data on gonococcal disease indicate that outer membrane proteins of gonococci are also responsible for the ability to produce disseminated infection and to resist the antibacterial activity of serum (25). Thus, it has become increasingly evident that outer membrane proteins are surface structures of considerable and general importance in bacterial pathogenicity. Experiments to be reported elsewhere indicate that, as compared with control cells, plasmid-containing *E. coli* cells capable of *traT* gene expression have an increased ability to cause disease in mice in tests measuring either bacteremia or lethality. These experiments and other results show that *traT* gene expression in natural isolates containing low-copy-number plasmids, such as ColV, also causes *E. coli* cells to have increased pathogenic characteristics (C. Parada, M. E. Fernandez, M. Binns, and F. C. Cabello, submitted for publication). These results suggest that antibiotic resistance plasmids may carry genes which have the potential to influence the natural history of a bacterial infection independently of the success or failure of antibiotic therapy. If antibiotic-resistant bacteria are indeed potentially more pathogenic than drug-sensitive strains, imprudent use of antibiotics will constitute a selective process for bacterial virulence. The results communicated here indicate that the antibiotic resistance plasmid R6-5 does specify a gene product which has the potential to increase *E. coli* pathogenicity by at least two mechanisms: serum and phagocytosis resistance.

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