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Theodore J. Kottom, *North Dakota State University*

Lisa K. Nolan, *North Dakota State University*

Michael Robinson, *North Dakota State University*

John Brown, *University of Georgia*

Tom Gustad, *North Dakota State University*, et al.



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Author(s): Theodore J. Kottom, Lisa K. Nolan, Michael Robinson, John Brown, Tom Gustad, Shelley M. Horne and Catherine W. Giddings

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## Further Characterization of a Complement-sensitive Mutant of a Virulent Avian *Escherichia coli* Isolate

Theodore J. Kottom,<sup>A</sup> Lisa K. Nolan,<sup>AC</sup> Michael Robinson,<sup>A</sup> John Brown,<sup>B</sup> Tom Gustad,<sup>A</sup> Shelley M. Horne,<sup>A</sup> and Catherine W. Giddings<sup>A</sup>

<sup>A</sup>Department of Veterinary and Microbiological Sciences, North Dakota State University, Fargo, ND 58105

<sup>B</sup>Department of Medical Microbiology, College of Veterinary Medicine, University of Georgia, Athens, GA 30602

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**SUMMARY.** An attempt was made to characterize the mechanism of complement resistance operating in a virulent avian *Escherichia coli* isolate. Using flow cytometry to detect antibody to C3, we found that there was significantly more antibody bound to a complement-sensitive mutant of this wild type than to the parent organism, suggesting that more C3 subunits were bound to the wild type. Neither the wild type nor the mutant degraded C3. Further, the mutant was phagocytosed to a significantly greater degree than the wild type by cultured phagocytes in the presence of C5-deficient serum. These data suggest that the wild type is resistant to complement, at least in part, because of its ability to restrict C3 deposition on its surface. Therefore, the decrease in virulence seen in the mutant may be related to its increased sensitivity to complement-mediated bacteriolysis or its enhanced susceptibility to complement-opsonized phagocytosis or both.

**RESUMEN.** Caracterización adicional de una cepa virulenta, mutante y sensible al complemento, de *Escherichia coli* aviar.

Se hizo un intento para caracterizar el mecanismo de la resistencia al complemento de una cepa virulenta de *Escherichia coli*. Usando el citómetro de flujo para detectar el anticuerpo contra C3, se encontró que hubo significativamente más anticuerpo unido a una mutante sensible al complemento de esta cepa original, que al organismo de donde provino, sugiriendo que una mayor cantidad de subunidades C3 estaban unidas a la cepa original. Ni la cepa original ni la mutante degradaron el C3. Además, la mutante fue fagocitada en un grado significativamente mayor que la cepa original, mediante el cultivo de fagocitos en presencia de suero deficiente en C5. Estos datos sugieren que la cepa original es resistente al complemento, al menos en parte, debido a su capacidad de restringir la deposición de C3 en su superficie. Por lo tanto, la disminución en virulencia observada en la mutante puede estar relacionada a su aumento en la sensibilidad a la bacteriolisis mediada por el complemento, o a su aumento en susceptibilidad a la fagocitos opsonizada por el complemento, o a ambas.

**Key words:** avian *Escherichia coli*, avian colibacillosis, virulence, complement resistance, phagocytosis resistance

Abbreviations: BHI = brain-heart infusion; CFU = colony-forming units; DMEM = Dulbecco's minimal essential medium; FAB = fluorescent antibody buffer; LPS = lipopolysaccharide; OD = optical density; OMP = outer membrane protein; PBS = phosphate-buffered saline; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis; VBS = Veronal-buffered saline

Complement resistance is strongly associated with the virulence of *Escherichia coli* from extraintestinal locations (8,20,32,40). This trait is likely to be important to the virulence of avian *E. coli* because avian colibacillosis is usually ex-

traintestinal in nature (12). In spite of the likely importance of complement resistance to the pathogenesis of avian colibacillosis, the mechanisms of complement resistance employed by avian *E. coli* have not been closely scrutinized.

In previous work, a complement-sensitive mutant of a virulent, complement-resistant,

<sup>C</sup>Corresponding author.

wild-type avian *E. coli* isolate was generated in order to determine the role of complement resistance in the virulence of the wild-type *E. coli* (21,22). Except for the mutant's loss in virulence and its possession of a 16.2-kD outer-membrane protein (OMP) not present in the wild type, both organisms were similar in the parameters compared. These results suggested that complement resistance was an important factor in the virulence of the wild type. In the present study, this mutant was compared with the wild-type *E. coli* in an attempt to characterize the mechanism of complement resistance operative in the wild type but impaired in the mutant. Because bacterial interactions with C3 are central to complement's effects on bacterial infection and impinge on the interaction of bacteria with phagocytes (13,14,30,32), the mutant and wild type were examined for differences in C3 deposition on their surfaces, abilities to degrade C3, and uptake by cultured phagocytes. Also, the organisms were compared by their abilities to activate complement as measured by a bystander lysis assay (35).

## MATERIALS AND METHODS

**Organisms.** The *E. coli* used in this study included a virulent, complement-resistant wild-type isolate cultured from a chicken with systemic colibacillosis and a mutant of this wild type that was less complement resistant and virulent than the parent organism (21). These bacteria were maintained on nutrient agar (Difco Laboratories, Detroit, MI) or stored in brain-heart infusion (BHI; Difco) at  $-70^{\circ}\text{C}$  prior to testing. Also, *Porphyromonas gingivalis* W83 (Harvey A. Schenkein, Clinical Research Center for Periodontal Diseases, School of Dentistry, Virginia Commonwealth University, Richmond, VA), an anaerobe that secretes a C3-degrading protease, was used as a positive control in the C3-degradation assays (29).

**Bystander lysis.** The abilities of the mutant and wild-type *E. coli* to activate complement were compared using the assay described by Verduin and co-workers (35). In this assay, overnight cultures of the test bacteria were washed, resuspended in Veronal-buffered saline (VBS), and diluted to obtain various concentrations of bacteria. These concentrations were confirmed by viable counts (i.e., aliquots of each bacterial suspension were plated and incubated overnight at  $37^{\circ}\text{C}$ , the colony-forming units (CFU) were counted, and the counts were used to estimate starting concentrations in CFU per milliliter). Suspensions of the test bacteria were then mixed with 10% normal

human complement (Cappel of Organon Teknika, Durham, NC), and these mixtures were incubated at  $37^{\circ}\text{C}$  for 10 min to allow activation of the complement cascade. Then, 50  $\mu\text{l}$  VBS containing 10 mM ethylenediaminetetraacetic acid and  $2 \times 10^8$  chicken erythrocytes/ml was added to each mixture. After a 60-min period of incubation at  $37^{\circ}\text{C}$ , the intact erythrocytes and particulate debris were removed by centrifugation ( $2000 \times g$  for 5 min), and the mixtures were assessed for their hemoglobin content using spectrophotometry ( $A_{405}$ , as measured with the EL340 BioKinetics Reader, BioTek Instruments, Winooski, VT). These assays were repeated five times for each organism, and the results were averaged. The amount of free hemoglobin in these mixtures, as indicated by absorbance readings, was used as an indication of the extent of bystander lysis of the erythrocytes.

**C3 subunit deposition assay.** The deposition of C3 on bacterial surfaces was quantified with flow cytometry according to the principles of Donnelly and Baigent (6) and Raybourne and Bunning (27). Bacteria were grown in BHI for 18 hr at  $37^{\circ}\text{C}$  prior to analysis. Bacteria were washed three times in phosphate-buffered saline (PBS; 0.15 M sodium chloride and 0.15 M sodium phosphate, pH 7.4) and resuspended in VBS (10). Then, C5-deficient human complement (Sigma Chemical Co., St. Louis, MO) was added to each bacterial suspension and allowed to incubate at  $37^{\circ}\text{C}$  for 30 min to allow C3 deposition on the bacterial surfaces. Suspensions were then pelleted by centrifugation, washed two times with VBS, and diluted in VBS to obtain a concentration of approximately  $1 \times 10^6$  bacteria/ml. This number was confirmed with viable counts. A 1:100 dilution of goat anti-human C3 (Sigma) made with fluorescent antibody buffer (FAB; Dulbecco's phosphate-buffered saline, without calcium chloride and magnesium chloride plus 0.1% sodium azide, pH 7.3) was added to 100  $\mu\text{l}$  of the bacterial suspension. Binding of this primary antibody was allowed to occur for 1 hr on ice. This antibody, according to its manufacturer, recognizes C3 and is also likely to recognize C3 subunits such as C3b (Sigma Technical Service). The treated bacterial suspensions were pelleted, and the pellets were washed three times in FAB. Treated bacteria were incubated with fluorescein isothiocyanate-labeled rabbit anti-goat conjugate antibody (1:200) for 30 min at  $0^{\circ}\text{C}$  in the dark. After incubation, suspensions were pelleted, washed three times with FAB, and fixed with 300  $\mu\text{l}$  paraformaldehyde (1% v/v, final concentration). Samples were stored in the dark at  $4^{\circ}\text{C}$  until analyzed using flow cytometry. Analysis of cell suspensions was by FAC-Scan (Becton Dickinson, San Jose, CA) (24). For each sample, 10,000 bacteria were analyzed by fluorescence intensity with the intensity a measure of C3 deposition.

**Analysis of C3 degradation.** C3 degradation in the presence of the mutant and wild type was analyzed according to the method described by Schenkein *et al.* (29) except that *P. gingivalis* W83, the positive control organism, was grown under 100% CO<sub>2</sub> in BHI plus 0.4% (Na)<sub>2</sub>CO<sub>3</sub>. Briefly, bacteria were cultured at 37 C for 18 hr in BHI. Cultures were pelleted, washed three times in VBS, and resuspended in 20  $\mu$ l of C3 (1 mg/ml) (Sigma). These suspensions were incubated at 37 C for 30 min and then pelleted by centrifugation. Supernatants were removed to fresh tubes and placed on ice until assayed. Samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 10% separation gels to evaluate C3 degradation (15).

**Phagocytosis assays.** Phagocytic uptake of the mutant and wild type was measured using the method described by Lissner *et al.* (16). At 18 hr prior to performing this assay, cells of the J774.A1 murine monocyte–macrophage-like cell line (American Type Culture Collection, Rockville, MD) in Dulbecco's minimal essential medium (DMEM) plus 10% fetal bovine serum were seeded at a density of approximately  $5 \times 10^5$  cells/well of a 24-well tissue culture plate (Becton Dickinson, Lincoln Park, NJ) and incubated at 37 C. Immediately prior to use, cells were washed with fresh DMEM. Bacteria to be used in the assay were grown in BHI for 18 hr at 37 C, washed three times in PBS, and resuspended to original volumes in PBS. Bacterial suspensions were then diluted to obtain a concentration of approximately  $1 \times 10^7$  bacteria/ml. This concentration was confirmed with viable counts. Fifty microliters of each of the bacterial suspensions was mixed with 40  $\mu$ l of DMEM and 10  $\mu$ l of C5-deficient human complement and incubated for 30 min at 37 C to allow C3 opsonin deposition on the bacterial surfaces. Similar suspensions without complement were also included in the assays for comparative purposes. Immediately prior to the assays, each bacterial suspension was mixed with 900  $\mu$ l of DMEM, and these suspensions were added to the cultured phagocytes. Plates were spun at  $600 \times g$  for 5 min in a clinical centrifuge to synchronize exposure of the phagocytes to the bacteria. Plates were then incubated for 30 min at 37 C to allow uptake. The media overlays containing the test bacteria were then removed, and the phagocytes were washed three times with PBS to remove the extracellular, nonadherent bacteria. Fresh warm DMEM (37 C) containing 125  $\mu$ g/ml of gentamicin (Amresco, Solon, OH) was added to each well containing phagocytes, and these cultures were incubated for 90 min to kill extracellular bacteria not removed by washing (7). Phagocytes were then rinsed three times with PBS to remove the gentamicin, and the internalized bacteria were released from the phagocytes using 1% Triton-X-100 (Fisher Chemical, Fair

Lawn, NJ). Lysates were diluted, and viable counts of these lysates were performed. Each assay was repeated three times, and each test in an assay was performed in duplicate.

**Statistical analysis.** Percentage of fluorescence of bacteria obtained with flow cytometry was analyzed using a *Z*-test for proportions (17). Phagocyte uptake data were analyzed using a two-factor analysis of variance, and a follow-up Tukey multiple comparison test was used to locate the significance (17).

## RESULTS AND DISCUSSION

The reactions of each organism in the bystander lysis assay using 1% and 10% complement were compared to determine the appropriate complement concentration to be used in the remaining assays. The optical density (OD) readings for the mutant in 1% and 10% complement appeared to follow a similar pattern. However, the patterns of OD readings obtained with the wild type differed, suggesting that at a concentration of 1% complement becomes a limiting factor in the assay with the wild type. Therefore, in further assays of bystander lysis, 10% complement was used. At all concentrations of the test bacteria in 10% complement, the mutant was associated with a lower rate of bystander lysis than was the wild type (Fig. 1). When the interactions of the mutant and wild type with C3 were compared using flow cytometry, the proportion (89.43%) of mutant bacteria fluorescing was significantly greater than that of the wild type (45.45%;  $Z = 66$ ,  $P < 0.0001$ ). Because there is no precedent for direct C3 binding, this assay appears to measure the binding of C3 subunits, most probably C3b. Therefore, this flow cytometric assay indicated that the mutant had a significantly greater deposition of these C3 subunits on its surface than did the wild type (Table 1). This difference in deposition did not appear to be a result of C3 degradation because neither the wild type nor the mutant degraded C3 according to results of SDS-PAGE analysis (Fig. 2). Also, the difference in deposition occurring on the mutant and wild type was reflected by the interaction of the mutant and wild type with cultured phagocytes. Pretreatment of bacteria with C5-deficient serum (the source of C3) resulted in significantly greater uptake of both the wild type and mutant by cultured phagocytes ( $F = 24.1$ ,  $df = 1,68$ ,  $P < 0.001$ ) because for both organisms, the mean number of serum-

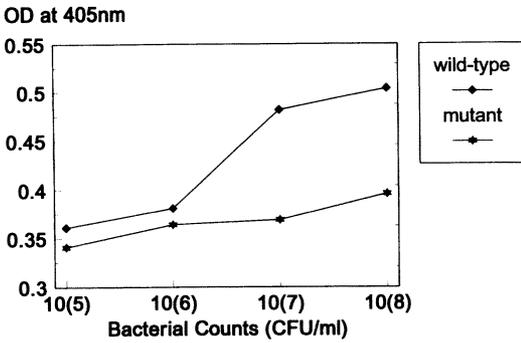


Fig. 1. Bystander lysis assay. 10% Guinea Pig Complement.

treated bacteria that were phagocytosed (in CFU) was significantly greater ( $P < 0.05$ ) than the mean number of untreated bacteria that were phagocytosed. Also, following pretreatment in C5-deficient serum, phagocytic uptake of the mutant bacteria was significantly greater than the uptake of the wild-type bacteria ( $F = 4.95$ ,  $df = 1,68$ ,  $P < 0.05$ ) because the mean number of wild-type bacteria phagocytosed was significantly lower than the mean number of the mutant bacteria phagocytosed ( $P < 0.05$ ; Table 2).

These results suggest that the wild type is resistant to complement, at least in part, because of the wild type's ability to limit C3 deposition on its surface. This difference in deposition may reflect a greater tendency for the mutant to bind complement components or a difference in the activation of complement by the two organisms. Data collected in this study cannot distinguish these two possibilities. These data also suggest that the decrease in virulence seen in the mutant may be related to its in-

Table 1. Flow cytometric analysis of C3 antiserum binding to wild-type and mutant *E. coli*.

Attribute	Wild type	Mutant
Fluorescence	4545	8943
No fluorescence	5455	1057
Total	10,000	10,000
Proportion fluorescing <sup>a</sup>	45.45%	89.43%

<sup>a</sup>The data were analyzed using a Z-test of proportions (17). The proportion (89.43%) of mutant bacteria fluorescing was significantly greater than the proportion (45.45%) of wild-type bacteria fluorescing ( $Z = 66$ ,  $P < 0.0001$ ).

creased sensitivity to complement-mediated bacteriolysis and/or its enhanced susceptibility to phagocytosis.

Because the genetic lesion responsible for the mutant's changed phenotype has not yet been described, alterations in DNA sequences known to encode complement resistance among *E. coli* may be responsible for this mutant's increased susceptibility to complement's effects and phagocytosis. Traits known for their association with the complement resistance of *E. coli* include a smooth lipopolysaccharide (LPS) (5,11), K1-capsular antigen (2,5,9,25) or other capsule types (28), and certain OMPs including TraT, Iss, and OmpA (3,4,18,19,23,26,31,33,34,36,37,38,39). Because both the mutant and wild type appear to have a smooth LPS layer, lack K1 antigen and capsule, but differ in OMP profiles (22), this OMP difference between the two organisms may deserve further attention in future studies of the wild type's mechanism of complement resistance.

Although the role of an OMP in the complement resistance of the wild type is purely speculative at this point, it is interesting to note that Agüero *et al.* (1) found that TraT in the outer membrane of *E. coli* resulted in decreased C3 deposition on the bacterial surface and decreased phagocytic uptake of serum-treated bacteria. Although the mechanism described by Agüero *et al.* (1) is similar to that described in this study, other workers have provided evidence that TraT acts at another, later, site in the complement cascade and not at the level of C3. Ogata and Levine (23) theorized that presence of the plasmid R100, which contains *traT* (26), results in an interruption of the complement cascade following C5 conversion. Pramoontjago and coworkers (26) postulated that TraT increased complement resistance by inhibiting formation of the C5b6 complex or by causing structural or functional alteration of this complex. Also, copy number of the *traT* gene has been linked to its phagocytosis-protective effect (1,31,36). That is, this protective effect might be an artificial manifestation of a cloned *traT* gene rather than a typical effect of *traT* in the wild-type situation. Conversely, the virulent isolate described in the present study exhibits a phagocytosis-protective effect in the wild-type condition. Iss, another OMP related to the complement resistance of *E. coli*, may block membrane attack complex function but

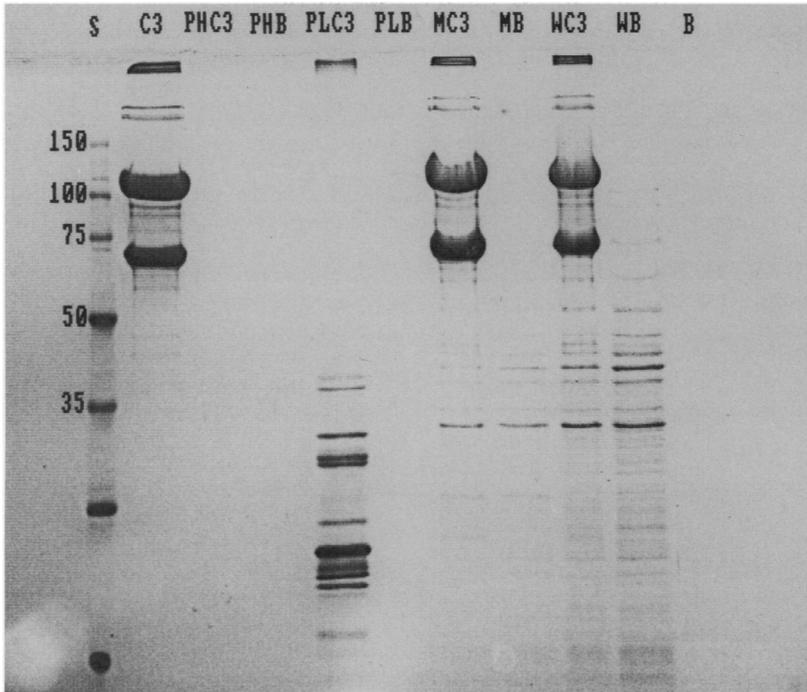


Fig. 2. C3 degradation by the mutant and wild-type *E. coli* as analyzed by SDS-PAGE. Lane S = molecular weight standard; Lane C3 = commercially obtained C3 that has not been exposed to the test bacteria; Lane PHC3 = C3 following exposure to approximately  $5 \times 10^8$  cells of *P. gingivalis* W83; Lane PHB = buffer control exposed to approximately  $5 \times 10^8$  cells of *P. gingivalis* W83; Lane PLC3 = C3 following exposure to approximately  $1 \times 10^8$  cells of *P. gingivalis* W83; Lane PLB = buffer control exposed to  $1 \times 10^8$  cells of *P. gingivalis* W83; MC3 = C3 following exposure to approximately  $1 \times 10^9$  cells of the mutant *E. coli*; Lane MB = buffer control following exposure to approximately  $1 \times 10^9$  cells of the mutant *E. coli*; Lane WC3 = C3 following exposure to approximately  $1 \times 10^9$  cells of the wild-type *E. coli*; Lane WB = buffer control exposed to approximately  $1 \times 10^9$  cells of the wild-type *E. coli*; Lane B = buffer only control.

not its formation (3,36). To the authors' knowledge, no reports describing the interaction of Iss with C3 and phagocytes are available, and OmpA's means of exerting its anticomplement effect have yet to be described.

In summary, this study has revealed that the wild type may employ a complement-resistance

mechanism similar to that ascribed to the OMP TraT by Agüero *et al.* (1), but further study of the wild type must be completed before it can be determined if an OMP, such as TraT, plays a role in this isolate's ability to resist the action of complement and phagocytes. Finally, it should be noted that the present study is distinguished from other studies of *E. coli* complement resistance because its subject is an avian *E. coli* isolate and not a mammalian one. At this time, it is not known if avian *E. coli* differ from mammalian isolates in their mechanisms of complement resistance and virulence, but it is known that complement resistance is an important virulence factor among avian *E. coli* that warrants further study.

Table 2. Analysis of phagocytic uptake.

Group	Mean CFU $\times 10,000^A$	
	Wild type	Mutant
Control (no C3)	27.78 <sup>a</sup>	27.11 <sup>a</sup>
Test (with C3)	38.44	57.89

<sup>A</sup>Within a row, values with the same lowercase superscript are *not* significantly different at the 5% level of probability. Within a column, values with the same lowercase superscript are *not* significantly different at the 5% level of probability.

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