Role of Avian Pathogenic *Escherichia coli* Virulence Factors in Bacterial Interaction with Chicken Heterophils and Macrophages

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Avian pathogenic *Escherichia coli* (APEC) cause extraintestinal disease in avian species via respiratory tract infection. Virulence factors associated with APEC include type 1 and P fimbriae, curli, aerobactin, lipopolysaccharide (LPS), K1 capsular antigen, temperature-sensitive hemagglutinin (Tsh), and an uncharacterized pathogen-specific chromosomal region (the 0-min region). The role of these virulence factors in bacterial interaction with phagocytes was investigated by using mutants of three APEC strains, each belonging to one of the most predominant serogroups O1, O2, and O78. Bacterial cell interaction with avian phagocytes was tested with primary cultures of chicken heterophils and macrophages. The presence of type 1 fimbriae and, in contrast, the absence of P fimbriae, K1 capsule, O78 antigen, and the 0-min region promoted bacterial association with chicken heterophils and macrophages. The presence of type 1 and P fimbriae, O78 antigen, and the 0-min region seemed to protect bacteria against the bactericidal effect of phagocytes, especially heterophils. The tested virulence factors seemed to have a limited role in intracellular survival for up to 48 h in macrophages. Generally, opsonized and nonopsonized bacteria were eliminated to the same extent, but in some cases, unopsonized bacteria were eliminated to a greater extent than opsonized bacteria. These results confirm the important role of type 1 fimbriae in promotion of initial phagocytosis, but nevertheless indicate a role for type 1 fimbriae in the protection of bacteria from subsequent killing, at least in heterophils. The results also indicate a role for K1 capsule, O78 antigen, P fimbriae, and the 0-min region in initial avoidance of phagocytosis, but demonstrate an additional role for O78 antigen, P fimbriae, and the 0-min region in subsequent protection against the bactericidal effects of phagocytes after bacterial association has occurred.

Avian pathogenic *Escherichia coli* (APEC) strains cause extraintestinal disease in chickens, turkeys, and other avian species. The most common form of colibacillosis is characterized as an initial respiratory infection (airsacculitis), which is frequently followed by a generalized infection (perihepatitis, pericarditis, and septicemia). *E. coli* cells in poultry often enter the host by the respiratory tract. Sites of entry into the bloodstream are the gas-exchange region of the lung (2, 11, 46, 51) and the air sacs (46), which are relatively vulnerable to colonization and invasion by bacteria due to a lack of resident macrophages (56). Biological and environmental stresses such as viral or mycoplasmal infections, overcrowding, and poor ventilation predispose birds to *E. coli* infections (23).

APEC strains belong to limited clones and serogroups, the most common and widespread serogroups being O1, O2, and O78 (7, 12, 17). Several potential virulence factors have been associated with APEC, including type 1 (F1A) and P (F11) fimbriae, curli, the aerobactin iron-sequestering system, K1 capsular antigen, temperature-sensitive hemagglutinin (Tsh), and resistance to the bactericidal effect of serum (13). Genetic approaches have identified additional regions of the chromosome likely to be associated with the virulence of APEC strains (8).

APEC strains adhere to chicken epithelial cells of the pharynx and trachea by means of type 1 fimbriae, comprising a major structural subunit, FimA, and a minor subunit, FimH, that mediates the attachment to D-mannose residues (44). Type 1 fimbriae are mostly expressed by bacteria colonizing the trachea, lungs, and air sacs, but not those colonizing deeper tissues or blood (15, 48). P fimbriae, produced by some APEC strains, are expressed by bacteria that colonize the air sacs, lungs, and internal organs, but not expressed by those that colonize the trachea (48). Receptor specificity of P fimbriae is conferred by the adhesin PapG, which recognizes different isoreceptors of the globoseries of glycolipids (37). Curli, possessing a major subunit, CsgA (42), promote binding to the major histocompatibility complex class I (MHC-I), extracellular matrix and serum proteins (27, 43), and avian intestinal cells (31, 32) and erythrocytes (9), suggesting that they may contribute to APEC infection. Province and Curtiss (49) have described a Tsh in an APEC strain; Tsh belongs to the serine protease autotransporter family of virulence-associated proteins present in numerous pathotypes of *E. coli* and in *Shigella*...
APEC, particularly of serogroups O1 and O2. Pourbakhsh et al. suggest a possible role for Tsh in the early stages of respiratory inflammation as the first line of cellular defense, followed by macrophages (20, 21, 22). The tsh gene is associated with APEC (16, 38) and is located on CoIV-related virulence plasmids. The results of experimental infection studies of chickens infected with a wild-type strain or an isogenic tsh knockout mutant suggest a possible role for Tsh in the early stages of respiratory infection (16). The K1 antigen is frequently associated with APEC, particularly of serogroups O1 and O2. Pourbakhsh et al. (47) showed that APEC K1− strains were more resistant to the bactericidal effect of serum than APEC strains expressing other K antigens.

Avian air sacs have no resident cellular defense mechanisms and must rely on an inflammatory influx of heterophils as the first line of cellular defense, followed by macrophages (20, 21, 60, 61). In vivo experiments showed that APEC cells were present in macrophages, but occasionally were also free in the air sac lumen and interstitium of infected chickens. In the airways, bacteria were free within the lumen and mixed with heterophils, erythrocytes, and fibrin (46).

Pourbakhsh et al. (47) showed that E. coli strains of high pathogenicity are more capable of invading the host than those with low pathogenicity. In the case of highly pathogenic strains, bacterial cells were often associated in vivo with macrophages or were present within macrophages in the air sacs and lungs of infected birds, in contrast to less-pathogenic strains, for which bacteria were rarely observed associated with macrophages. In addition, the pathogenic strains resisted killing by chicken macrophages in vitro to a greater extent than the less-pathogenic strains.

Although the potential roles of various APEC virulence factors in pathogenesis of avian colibacillosis have been established, little is known about their interaction with innate immune resistance.

This report examines the role of various virulence factors, including curli, type 1 and P fimbriae, Tsh, O antigen polysaccharide, K antigen, and plasmid pAPEC-1 in resistance to avian innate immune defenses (heterophils and macrophages). This was achieved by analyzing the interaction of avian phagocytes with wild-type and mutant APEC strains. Three wild-type APEC strains, each belonging to one of the most predominant serogroups (O1, O2, and O78), and their respective isogenic mutants lacking different virulence factors were examined for their interaction with avian heterophils and macrophages.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The strains used in this study are listed in Table 1. In order to obtain derivatives of APEC strain TK122 that express different O antigens instead of the native O78 antigen, strain TK179, an O78− histidine-requiring hisG::Tn10 auxotroph of strain TK122 (Table 1), was used as the recipient for bacteriophage P1 cm clb100-mediated transduction. Because hisG is closely linked to the rfb O antigen-encoding DNA region, some transduced derivatives would acquire both prototrophy and an O antigen-encoding rfb gene cluster. Briefly, P1 phage lysates of strains TK122 (O1), TK206 (O26), and TK206 (O111) were used to transduce strain TK179 as previously described (8). Transductants were selected for prototrophy by growth on minimal medium containing glucose, and loss of tetracycline resistance mediated by Tn10 was verified. O antigen-positive clones were confirmed by slide agglutination with O antigen-specific antisera. The O1-, O2-, and O111-expressing derivatives of strain TK179 were named TK179, TK176, and TK176, respectively.

**Strains were grown under conditions (media and temperature) that allowed optimal expression of the appropriate virulence factors, as described in the literature:**

(i) Strain TK3 and its papG mutant were subjected to three passages of 18 h on tryptic soy agar (TSA) at 37°C for optimal production of P fimbriae and minimal production of type 1 fimbriae (18).

(ii) Strain MT78 and its mutants were subjected to three overnight consecutive passages in tryptic soy broth (TSB) at 37°C to allow a high level of expression of type 1 fimbriae. (iii) Strains TK172, TK273, TK274, and TK176 were grown on colonization factor antigen agar at 26°C for 48 h for optimal expression of Tsh and curli (50, 55).

(iv) Strains TK172, TK176, TK176, and TK176 were grown in TSB at 37°C for 24 h for optimal expression of O antigens. Strain TK176 was grown under the same conditions as for the O1 serotype mutants. Ampicillin (100 μg ml−1), kanamycin (25 μg ml−1), chloramphenicol (25 μg ml−1), nalidixic acid (12.5 μg ml−1), and tetracycline (10 μg ml−1) were used as required at the indicated concentrations unless stated otherwise. Serotyping was done by standard slide and tube agglutination techniques (45).
Opsonization of strains. Bacteria (10^6 CFU/ml) were opsonized by incubation in 50% serum from nonimmunized specific-pathogen-free chickens for 15 min at 37°C and were washed twice with phosphate-buffered saline (PBS) at pH 7.4.

Resistance to phagocytosis. Resistance of strains to phagocytosis was examined in primary cultures of heterophils, monocyte-derived macrophages, and peritoneal macrophages.

(i) Isolation of heterophils and monocytes. Peripheral blood was collected from the wing vein of 4- to 6-week-old specific-pathogen-free chickens (commercial Ross×Ross broiler chickens) with heparin-coated syringes and pooled. Avian heterophils and monocytes were isolated with discontinuous gradients with Ficoll (Pharmacia) diluted to an osmolality of 340 mosmol of H2O per kg by adding 9 parts (vol/vol) of 1.5 M NaCl. Further 90, 80, and 70% dilutions of the Percoll solution were made with 0.15 M NaCl. Blood diluted in an equal volume of Hanks balanced salt solution (HBSS) was applied to the top of gradients. The test tubes were centrifuged for 15 min at 450 × g at room temperature.

Heterophils were removed from a band at the interface between the 80 and 70% layers. The cells were washed twice in HBSS. Precipitates were taken with heterophil manipulations as recommended by Andreasen and Latimer.

Monocytes were removed from the cloudy region extending from the 80 and 90% layers. The cells were washed twice in HBSS and resuspended in RPMI 1640 medium (Gibco BRL), supplemented with 2 mM L-glutamine, 10% denatured fetal bovine serum, and antibiotics (100 U of penicillin per ml, 100 μg of streptomycin per ml). A volume of 200 μl of cell suspension containing approximately 10^4 cells was added to wells of 96-well tissue culture plates containing macrophages, cells were applied to a Percoll gradient as described for isolation of macrophages. Harvested cells were washed twice with HBSS and incubated at 40°C in a 5% CO2-humidified air atmosphere for 2 h to allow the monocytes to adhere to the plates. The plates were then washed with HBSS to remove all nonadherent cells. Complete medium with antibiotics was then added. To obtain monocyte-derived macrophages, the medium was replaced daily with fresh culture medium for 4 days. The viability of noninfected cells was determined by trypan blue exclusion. Because of problems with multinucleation and an inability to survive for long periods, the monocyte-derived macrophages were not used in all experiments.

(ii) Isolation of peritoneal macrophages. A suspension of Sephadex G-50 (3%) in 0.85% saline was injected into the peritoneal cavity of 4- to 6-week-old chickens (1 ml/100 g of body weight) to elicit macrophages. Two days later, the birds were euthanized, and the peritoneal cavities were aseptically washed with 40 ml of heparinized HBSS to obtain cells in peritoneal exudate. To obtain pure macrophages, cells were applied to a Percoll gradient as described for isolation of monocytes. Harvested cells were washed twice with HBSS and suspended in complete RPMI medium with antibiotics. Cells were added to each well of 96-well tissue culture plates containing macrophages and incubated at 40°C in a 5% CO2-humidified air atmosphere for 2 h to allow macrophages to adhere to the plates. Nonadherent cells were removed by three washes with HBSS.

(iii) Bacterial infection of cells. Prior to infection with bacteria, cells were washed and resuspended in medium without antibiotics. Bacterial suspensions in HBSS, opsonized or not, were then added to cell cultures at a multiplicity of infection of 10 bacteria per cell for both macrophages and heterophils. Plates containing macrophages were incubated for 1 h at 40°C in a 5% CO2-humidified air atmosphere. Plates containing heterophils were centrifuged for 15 min at 200 × g to improve bacterial adherence and then incubated for 45 min at 40°C in a 5% CO2-humidified air atmosphere.

(iv) Association of bacteria with cells. Macrophage suspensions were added to the wells of Lab-Tek slides, and heterophils were added to the wells of 24-well plates containing Thermax (Nalge Nunc International) coverslips. Following bacterial infection and incubation as described above, cells were washed twice with HBSS. Lab-Tek slides and coverslips were air dried, and cells were fixed and stained with the Diff-Quick kit (Dade Behring) according to the manufacturer’s instructions. One hundred individual cells per sample were randomly selected for counting of associated bacteria by light microscopy.

(v) Viability of bacteria associated with cells. Viability of associated bacteria was determined by Live/Dead BacLight bacterial viability kit (Molecular Probes, Eugene, Ore., U.S.A.). Heterophils were washed twice with HBSS to eliminate nonadherent bacteria. The fluorescent dye mixture was added to yield final concentrations of 10 μM SYTO 9 and 60 μM propidium iodide, and plates were incubated for 15 min at room temperature in the dark and then examined under fluorescence with a Leica Diaplan microscope (Leica Microsystems, Canada, Inc.). One hundred individual heterophils that had nuclei staining orange-red were examined per field. Live and dead macrophages could be distinguished by propidium iodide and SYTO 9, respectively. Cells stained green from staining with SYTO 9, whereas dead bacteria stained orange-red due to the propidium iodide. The number of associated bacteria that stained green or orange-red were counted, and the percentage of live (green) bacteria was determined.

Peritoneal macrophages seeded in eight-well Lab-Tek slides were infected with bacteria and incubated under the same conditions as described before and then stained with the Live/Dead kit as described for heterophils, with the exception that they were treated with 0.1% Triton X-100 for 30 s before staining to permeabilize the cell membrane.

(vi) Intracellular survival. Internalized bacteria surviving within macrophages were enumerated by the standard gentamicin protection assay. Peritoneal macrophages were infected with opsonized or nonopsonized bacteria at a ratio of 1:10 and incubated at 40°C in a 5% CO2-humidified air atmosphere for 1 h. The cells were washed three times with HBSS and incubated for another 2 h in fresh complete RPMI containing 200 μg of gentamicin per ml. At this time (t0) and at different incubation times (3, 6, 12, 24, and 48 h), cells were washed three times with HBSS and lysed with 0.1% Triton X-100 in PBS for 5 min at room temperature. Bacteria were serially diluted and enumerated by colony count on TSA plates. Experiments were repeated three to five times, and in each experiment, tests were repeated two to four times.

Statistical analyses. Data are presented as the mean number of at least three experiments ± the standard deviation. Student’s t test was performed to compare pairs of group means.

RESULTS

Ability of wild-type strains and their isogenic mutants to associate with heterophils and macrophages. In general, under the conditions in this study (i.e., low multiplicity of infection [10 bacteria per cell]), bacteria associated with phagocyte cells more greatly with time, as measured by Diff-Quick, at least up to 1 h after inoculation (data at 30 min not shown), and following opsonization with normal chicken serum. There was generally less bacterial association with heterophils than with macrophages (Fig. 1).

(i) Type 1 fimbriae promote association with phagocytes. Wild-type strain MT78, grown under conditions that allow a high level of expression of type 1 fimbriae, demonstrated a greater association with phagocytes than mutant strains PA68 (jmlH) (Fig. 1A and 2) and DM34 (jml) (Fig. 1A). Compared to wild-type strain MT78, mutants DM34 and PA68 were significantly less associated with heterophils, whereas for association with macrophages, the difference was significant for DM34, but not for PA68 (Fig. 1A).

(ii) K1 capsule, O78 antigen as well as the 0-min region, and PapG are unfavorable for association of strains MT78, χ7122, and TK3, respectively, with phagocytes. The mutant BEN2694 (K1−) showed a slightly greater association with macrophages than wild-type strain MT78, following contact for 60 min, although the difference was not significant (Fig. 1A and 2). Wild-type strain χ7122 associated poorly with phagocytes, even when opsonized, when grown under the conditions used for this study. Following opsonization, mutant χ7145 (O78−) (Fig. 1B and 2), O antigen replacement derivatives of χ7122, χ7193 (O1) and χ7167 (O111), and the 0-min replacement mutant χ7146 (Fig. 1B) all demonstrated a greater association with heterophils and macrophages than the wild-type strain, χ7122. In contrast, strain χ7168 (O26) was highly associated with macrophages, but not with heterophils (Fig. 1B). Nonopsonized bacteria of all mutant strains were poorly associated with phagocytes, with the exception of mutant χ7146 (0 min).

The wild-type strain TK3, grown under conditions that allow high expression of F fimbiae, associated poorly with phagocytes, even when opsonized. The mutant TK37G (papG) demonstrated a significantly greater association with macrophages than the wild-type parent strain TK3; however, the difference
between the strains was not significant for heterophils (Fig. 1D).

(iii) The other virulence factors tested did not seem to affect bacterial association with phagocytes. The mutants \(\chi T7273\) (\(tsh\)), \(\chi T7274\) (pAPEC-1 negative), and \(\chi T7186\) (\(csgA\)) associated with heterophils and macrophages to a similar extent as the wild-type parent strain, \(\chi T7122\), grown under the same culture conditions (Fig. 1C).

Viability of phagocyte-associated bacteria of wild-type strains and their respective mutants. The viability of bacteria associated with phagocytes was examined with the Live/Dead test. Bacterial association observed in these experiments (data not shown) was similar to that observed in the Diff-Quick test, although greater association was observed with the latter.

For each of the wild-type pathogenic strains MT78, \(\chi T7122\), and TK3, \(>63\%\) of heterophil- and macrophage-associated bacteria remained alive after 1 h of contact, whether bacteria were opsonized (Table 2) or not (data not shown). On the other hand, for the nonpathogenic strain 862, \(<34\%\) of heterophil-associated bacteria and \(50\%\) of macrophage-associated bacteria remained alive after 1 h of contact (data not shown).

(i) Fim and K1, O78 antigen as well as the 0-min region, and PapG protect bacteria against bactericidal effect of heterophils and macrophages at 1 h of contact for strains MT78, \(\chi T7122\), and TK3, respectively. For the mutant strains DM34 (\(fim\)) (Table 2) and BEN2694 (K1\(^{−}\)) (Table 2 and Fig. 2B), the percentage of phagocyte-associated bacteria remaining alive after 1 h of contact was less than that for the wild-type parent strain MT78 (Table 2 and Fig. 2B). In contrast, in the absence of \(fim\)H, mutant strain PA68 was as resistant to phagocytes as its wild-type parent strain, MT78 (Table 2 and Fig. 2B). The absence of O78 antigen (Fig. 2B) or its substitution by

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**FIG. 1.** Association of opsonized (■) and nonopsonized (□) APEC with heterophils and macrophages. (A) APEC strain MT78 and its mutants PA68 (\(fimH\)), DM34 (\(fim\)), and BEN2694 (K1\(^{−}\)). (B) APEC strain \(\chi T7122\) and its mutants \(\chi T7193\) (O1), \(\chi T7167\) (O111), \(\chi T7168\) (O26), \(\chi T7145\) (O78\(^{−}\)), and \(\chi T7146\) (0 min). (C) APEC strain \(\chi T7122\) and its mutants, \(\chi T7273\) (\(tsh\)), \(\chi T7274\) (pAPEC-1 negative), and \(\chi T7186\) (\(csgA\)). (D) APEC strain TK3 and its mutant, TK37G (PapG). Each group of strains was grown under conditions described in Materials and Methods. The results represent the means ± standard deviation of at least three experiments. Asterisks show significant difference versus the wild-type strain (*, \(P < 0.05\); **, \(P < 0.005\)).
another O antigen resulted in a higher sensitivity of strains \((H9273\,7145, H9273\,7193, H9273\,7167,\) and \((H9273\,7168)\) to the bactericidal effects of heterophils and macrophages, than for the wild-type parent strain \((H9273\,7122)\) (Table 2). Also, in the absence of the 0-min region corresponding to strain \((H9273\,7122)\), strain \((H9273\,7146)\) was more sensitive to the bactericidal effects of phagocytes. For macrophages, the difference from the wild-type strain was significant for the \(O111\)-antigen substituted, \(O78\)-negative, and 0-min mutants when bacteria were opsonized (Table 2) and for the \(O111\) and \(O26\) antigen-substituted mutants when bacteria were not opsonized (data not shown). For heterophils, the viability of the wild-type strain was significantly higher than the viability of all \(O\) antigen polysaccharide mutants when bacteria were opsonized (Table 2).

A null mutation in the \(papG\) gene (strain \(TK37G\)) resulted in a decrease in the resistance of strain \(TK3\) to the bactericidal effect of heterophils and macrophages. This was more apparent in the case of heterophils, since the difference was significant for the former, but not the latter (Table 2).

(ii) Other virulence factors tested did not greatly affect the

FIG. 2. Representative photos demonstrating the association and viability of APEC strains and their respective mutants. Shown are APEC strain \(MT78\) and its mutants, \(PA68\) (\(jimH\)) and \(BEN2794\) (\(K1\)), as well as APEC strain \(\chi7122\) and its mutant, \(\chi7145\) (\(O78\)). (A) Diff-Quick stain of bacteria associated with heterophils and macrophages. (B) Fluorescence stain of bacteria associated with heterophils and macrophages using SYTO 9 and propidium iodide (Live/Dead test). Green indicates live bacteria, and orange-red indicates dead bacteria.

498 MELLATA ET AL. INFECT. IMMUN.
bacterial sensitivity to phagocytes. A mutation in the tsh gene (strain χ7273) or the absence of the pAPEC-1 plasmid (strain χ7274) resulted in a decrease in the resistance of strains to the bactericidal effects of heterophils and macrophages, although the differences versus the wild-type strain were not significant.

Tested virulence factors do not appear to affect bacterial persistence in macrophages, although nonopsonic phagocytosis is more efficient than opsonic phagocytosis in some cases. We examined intracellular survival of APEC wild-type strains and mutant derivatives in macrophages. Our results show that despite differences in association of bacteria of each strain with macrophages as observed above, the number of live intracellular bacteria at $T_0$ were approximately the same for all strains tested.

There was little apparent difference between wild-type strains and their respective mutant strains with respect to their persistence in macrophages, although nonopsonized mutants DM34 (FimH-), and BEN2694 (K1-) were completely eliminated between 24 and 48 h, whereas wild-type strain MT78 persisted more than 48 h in macrophages, whether opsonized or not (Fig. 3B). When opsonized, the O26 antigen-substituted mutant (χ7168) persisted less well than the wild-type strain χ7122 or its other mutants at 24 and 48 h (data not shown).

Interestingly, bacteria of some of the strains persisted less well following nonopsonic phagocytosis than following opsonic phagocytosis. This was observed for mutant strains χ7145 (O78-) and χ7146 (0 min-) of χ7122, PA68 (FimH-) and BEN2694 (K1-) of MT78, and APEC strain TK3 and its mutant, TK37G (PapG-) (Fig. 3). The differences were significant between 0 and 24 h for χ7145 and χ7146 and between 24 and 48 h for PA68, TK3, and TK37G. The other strains tested were generally eliminated to the same extent, whether opsonized or not.

**DISCUSSION**

Many virulence factors have been associated with APEC strains, although their role in pathogenesis is not well known. Extrapolation from mammalian results would not be appropriate because of particularities of the avian immune system. In fact, the inflammatory response in avian species more closely resembles the reptilian response than that of mammals (39). This is the first study in which the role of different virulence factors, including O serotype, F1 and F pilus, curli, Tsh, and pAPEC-1, in bacterial interaction with heterophils and macrophage phagocytes was investigated by using mutants of three APEC strains belonging to the most predominant serogroups, O1, O2, and O78.

In this study, the three APEC strains examined and their isogenic mutants, grown under conditions favoring expression of the appropriate virulence factors, behaved differently with respect to specific stages of phagocytosis, such as opsonization, attachment, internalization, and bacterial killing.

The ability to express fimbriae allows bacteria to attach to and colonize epithelial surfaces. In vitro and in vivo studies have shown that MT78 adheres to tracheal and pharyngeal cells, whereas its mutants DM34 (fim) and PA68 (fimH), which no longer produce type 1 fimbriae or adhesin FimH, respectively, are unable to adhere to these cells (4, 35). Similarly, our results showed that MT78 was associated highly with phagocytic cells. Even when the bacteria are not opsonized, type 1 fimbriae are known to promote adhesion to phagocytic cells by lectin-carbohydrate interactions (41). Experiments carried out in the absence of complement opsonization allowed us to distinguish between phagocytosis mediated by fimbriae and that mediated by complement. This is an important observation, because opsonization may obscure fimbria-related events that are important in conditions where normal opsonic activity is impaired (6, 54). In the absence of type 1 fimbriae, bacteria of mutants PA68 (fimH) and DM34 (fim) associated poorly with macrophages and opsonization had little effect on them, possibly due to the presence of K1 capsule. In fact, according to Horwitz and Silverstein (28), nonimmune serum is a poor opsonin for strains carrying the K antigen, because the capsule may prevent association simply due to its hydrophobicity and negative charge. Indeed, the K1-mutant strain was highly associated with phagocytic cells. It is probable that the importance of K1 capsule would have been more apparent in the absence of F1 fimbriae. The use of a double K1- and fim mutant would help to resolve this question. It would also be interesting to examine the role of the different variants of type 1 present on other APEC serotypes, such as O78, in phagocytic adherence, because such variation in the FimA protein may affect the binding affinity of the type 1 adhesin (33). Based on serological, nucleotide sequence, and amino acid composition differences in the FimA subunit, a number of closely related variants of type 1 fimbriae have been identified in _E. coli_...
strains. Marc and Dho-Moulin (36) identified four variable regions in the fimA sequence of MT78 (O2:K1), including a fragment of gene fimA, which is specific for most O2 strains.

Some microorganisms escape the host immune system by remaining inaccessible to it. Bacteria may avoid phagocytic cells by the repulsion effect of P fimbriae via electrostatic properties of their PapG adhesin, as has been demonstrated for human E. coli strains (59) or due to a lack of membrane glycolipid receptors for P fimbriae on host cells (57, 58). Some APEC strains also express P fimbrial adhesin, although its implication in the pathogenic process has not yet been elucidated. In this study, the papG mutant strain TK37G was more greatly associated with phagocytes, especially with macrophages, than its wild-type parent. Our results agree with those of Tewari et al. (59), who used a papG mutant from a human serotype O6:H5:H1102 strain.

This low bacterial association with phagocytes could result from a combination of several virulence factors that prevent association. Among these factors, the K1 capsule, as demonstrated with strain MT78, reduces association of bacteria with phagocytes. Hence, the role of P fimbriae in O1:K1 strain TK3 would probably be more apparent in the absence of K1 capsule.

APEC strain χ7122, grown under two different conditions to promote the expression of different surface components, associated poorly with phagocytic cells, even when opsonized. Similarly, Rosenberd-Arska et al. (52) had noted that a human O78:K80 strain was poorly associated with phagocytes when treated with nonimmune serum and required specific antibodies for effective opsonization. In the present study, we showed that deletion of the rfb locus encoding O78 antigen or replacement of the O78 antigen in strain χ7122 by antigens of other O serotypes (O1, O26, and O111) allowed bacteria to associate more extensively with phagocytic cells, particularly when bac-

FIG. 3. Persistence of opsonized (O) and nonopsonized (NO) APEC in macrophages (gentamicin test). The values are shown as log CFU per milliliter, and the results are the means of at least three experiments. (A) APEC strain χ7122 and its mutants, χ7145 (O78−) and χ7146 (0 min). (B) APEC strain MT78 and its mutants, PA68 (fimH) and BEN2694 (K1−). (C) APEC strain TK3 and its mutant, TK37G (PapG−). Asterisks indicate significant difference (P < 0.05) between opsonized and nonopsonized bacteria.
teria were opsonized. Similarly, Burns and Hull (10) showed that absence of the O75 antigen from a uropathogenic O75:K5 E. coli strain resulted in greater ingestion by polymorphonuclear leukocytes (PMNs) than the wild-type strain. Hence, accessibility of opsonin to the bacterial surface and effectiveness of complement fixation appear to depend on the presence and composition of lipopolysaccharide (LPS) sugar chains. For instance, the O antigen polysaccharides tested (O1, O26, O111, and O78) differ in their sugar compositions (24, 25, 29, 34).

Tsh, pAPEC-1, and curli mutant derivatives of APEC strain χ7122 (Table 1) were also only weakly associated with phagocytes, denoting the low importance of Tsh and curli in bacterial association with phagocytic cells for χ7122. However, it has been shown that curli-expressing strains bind much more human (43) and murine (30) MHC-I and β2m than curli-deficient mutant strains. This difference in results may be explained by the presence on APEC strain χ7122 of certain surface structures, such as K80 capsule (8), which may prevent interaction of curli with MHC-I.

In in vivo studies, apparently viable bacteria of highly pathogenic isolates were often observed associated with or located within macrophages in the air sacs and lungs of inoculated birds (46). In the present study, the role of various virulence factors in the viability of bacteria associated with heterophils and macrophages was assessed in vitro by fluorescent dye exclusion at 1 h postinfection. This test is of double interest, because it determines bacterial association with phagocytes and viability of both extracellular and intracellular bacteria associated with the phagocytes. Bacterial association data, as determined with this test, confirmed those observed with the Diff-Quick test, although greater bacterial association was observed with the latter, probably because cells were fixed in the latter test, but not in the former.

The different infection patterns suggest that the various virulence attributes of the three isolates probably mediated different bacterial mechanisms of resistance to the innate immune system. Our results demonstrate that several of the tested virulence factors tested—namely type 1 fimbiae and K1 capsule in strain MT78, O antigen and the 0-min region in strain χ7122, and PapG in strain TK3—seemed to protect bacteria against killing by phagocytic cells, especially heterophils, following 1 h of interaction (Table 2). The role of type 1 fimbiae in pathogenicity is controversial. Our results agree with those of in vivo tests that revealed that mutant DM34 (fim) was less able to survive in the lungs of chickens than the parent strain, MT78 (35). Hence, it appears that the type 1 fimbiae, when expressed, would promote bacterial phagocytosis, but would nevertheless protect the phagocyte-associated bacteria from subsequent killing, although the mechanism for this is not known. The FimH adhesin does not appear to be involved in this protection, because the fimH mutant strain was as resistant to killing as the wild-type strain. For O78 antigen, K1 capsule, and the 0-min region of the chromosome, our phagocyte viability results agree with the in vivo chicken inoculation results, in which the appropriate mutant strains were less invasive than their respective wild-type strains (Mellata et al., submitted for publication). These virulence factors and PapG adhesin may downregulate heterophil and macrophage antimicrobial functions. In fact, Tewari et al. (59) demonstrated that a papG mutant E. coli strain elicited a considerably higher oxidative burst response from neutrophils than that evoked by its wild-type parent. Examination of host response, such as the oxidative, nitric oxide, and cytokine responses could help to elucidate the mechanisms used by APEC bacteria to resist chicken phagocytes.

Several bacterial species have developed survival mechanisms that prevent killing of bacteria after they have been phagocytosed by macrophages (53). In the present study, intracellular persistence of bacteria was assessed in macrophages rather than in heterophils, because invasive bacteria generally target host cells that have a longer life span.

The virulence factors tested in this study play a limited role in persistence of bacteria in macrophages, because the mutants behaved similarly to their parent wild-type strains. However, since K1 and O78 antigen mutant strains were sensitive to the first steps of the innate immune response (i.e., the bactericidal effects of serum and heterophils) (Mellata et al., submitted; this study), they would probably have been eliminated before they reached the macrophages.

Depending in their ability to persist in macrophages in the presence or absence of opsonization, two groups of bacterial strains were distinguished: those for which persistence was affected by opsonization and those for which it was not. These results suggest that, similarly to mammalian phagocyte cells, chicken macrophages possess the appropriate machinery to mediate different ways of phagocytosis. Our unexpected finding was the persistence of some strains to a greater extent when opsonized than when nonopsonized. Although nonopsonic phagocytosis is considered as an important arm of host defense, it is generally less efficient than opsonic phagocytosis (40). Our results suggest that nonopsonic phagocytosis, characterized by a primitive holdover ingestion mechanism, may, in certain circumstances, be more efficient than opsonic phagocytosis in chickens. The finding that nonopsonic phagocytosis was more efficient in the absence of O78 antigen or 0-min region for χ7122 and FimH and K1 capsule for MT78 or for APEC strain TK3 (Fig. 3) indicates that the nature of the surface molecular structure of the bacteria may orient the way that bacteria will be phagocytosed. Further investigation of the biochemical mechanisms of the response of chicken macrophages under both conditions is essential for a better understanding of the pathogenesis of the infection process and may also provide a basis for improved methods for treatment and prevention of APEC infection.

Taken together, our results show that different virulence factors associated with APEC strains determine the behavior of these strains in the presence of phagocytes. Type 1 fimbiae in MT78 promote bacterial association with phagocytes, whereas K1 capsule in MT78, O78 antigen and the 0-min region in χ7122, and P fimbiae in TK3 allow bacteria to avoid phagocytosis, indicating different mechanisms used by APEC strains in their interaction with phagocytic cells. APEC strains are able to resist the bactericidal effects of phagocytes to a much greater extent than a nonpathogenic strain. We have demonstrated a role for some virulence factors (Fim and K1 capsule in MT78, O78 serotype and 0-min region in χ7122, and P fimbiae in TK3) in resistance to this bactericidal effect of phagocytes, and this is more apparent in the case of heterophils. These results are particularly interesting, since heterophils form the first line of avian cellular defense against
invading pathogens in the lungs and air sacs, where resident macrophages are lacking (20, 21, 60, 61). Heterophil activity would restrict bacterial multiplication to a level permitting more efficient elimination of bacteria by the subsequent host defenses. E. coli strains possessing Fim, K1 capsule, O78 serotype, the 0-min region, or P fimbrae would more successfully combat this heterophil activity and subsequently invade the host. The virulence factors tested in this study seem to play a limited role in persistence of APEC strains within macrophages. We are currently looking for other genes potentially involved in this step of pathogenesis.

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