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Association of *iss* and *iucA*, but Not *tsh*, with Plasmid-Mediated Virulence of Avian Pathogenic *Escherichia coli*

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Avian pathogenic *Escherichia coli* (APEC) is an economically important respiratory pathogen of chickens worldwide. Factors previously associated with the virulence of APEC include adhesins, iron-scavenging mechanisms, the production of colicin V (ColV), serum resistance, and temperature-sensitive hemagglutination, but virulence has generally been assessed by parenteral inoculation, which does not replicate the normal respiratory route of infection. A large plasmid, pVM01, is essential for virulence in APEC strain E3 in chickens after aerosol exposure. Here we establish the size of pVM01 to be approximately 160 kb and show that the putative virulence genes *iss* (increased serum survival) and *tsh* (temperature-sensitive hemagglutinin) and the aerobactin operon are on the plasmid. These genes were not clustered on pVM01 but, rather, were each located in quite distinct regions. Examination of APEC strains with defined levels of respiratory pathogenicity after aerosol exposure showed that both the aerobactin operon and *iss* were associated with high levels of virulence in APEC but that the possession of either gene was sufficient for intermediate levels of virulence. In constrast, the presence of *tsh* was not necessary for high levels of virulence. Thus, both the aerobactin operon and *iss* are associated with virulence in APEC after exposure by the natural route of infection. The similarities between APEC and extraintestinal *E. coli* infection in other species suggests that they may be useful models for definition of the role of these virulence genes and of other novel virulence genes that may be located on their virulence plasmids.

Avian pathogenic *Escherichia coli* (APEC) has been identified as an emerging problem in the poultry meat industry, contributing to the complex respiratory disease syndrome that primarily affects broiler chickens between the ages of 4 and 6 weeks (4). Avian colibacillosis is responsible for a significant proportion of the mortality in poultry flocks, contributing to extensive economic losses (12).

Several factors have been associated with the virulence of *E. coli* for avian hosts, but no specific virulence factor has been discovered that contributes entirely to the pathogenicity of APEC. In addition, while a number of factors have been associated with virulence in epidemiological studies, confirmation of their role has generally been limited to models of infection that do not reproduce the natural route of exposure, inhalation of aerosols of *E. coli*. These factors include iron-scavenging mechanisms such as aerobactin production, serum resistance, temperature-sensitive hemagglutination, adhesins, and the production of colicin V (ColV). It is thought that a combination of these characteristics increases the pathogenicity of *E. coli* (25, 34). Previous studies have found that many APEC isolates carry large plasmids (5), some of which encode potential virulence factors (15). It is notable that many of the factors that have been associated with virulence of APEC are also associated with virulence in extraintestinal *E. coli* in other species, suggesting that the basis of virulence in extraintestinal *E. coli* may be somewhat similar in a range of different species and that clarification of the role of virulence factors in APEC may also contribute to understanding the pathogenesis of colibacillosis in other species.

The contribution of siderophores to the pathogenicity of APEC strains has been examined previously. The hydroxamate siderophore aerobactin has previously been associated with the pathogenicity of APEC strains (4, 18, 19, 25, 34). Iron scavenging has been recognized as a potentially important virulence factor in *E. coli* for a number of years, although siderophores have not been directly implicated in virulence.

Adherence to epithelial cells is likely to be a fundamental requirement for colonization of the respiratory tract by *E. coli*. The *tsh* gene, encoding a temperature-sensitive hemagglutinin, was isolated and characterized by Provence and Curtiss (28) and may act as an adhesin, particularly in the initial stages of colonization of the respiratory tract. The *tsh* gene region of APEC strain χ7122 was sequenced by Dozois et al. (6) and contains the 4.133-kb *tsh* gene. It is not known if temperature-sensitive hemagglutination contributes to pathogenesis, although it has been suggested that it may play a part in determining the virulence of a strain (6, 23, 25). The contribution of ColV plasmids to virulence has been debated extensively (29, 31), and previous studies have shown that ColV-type plasmids carry the *tsh* gene (6) and frequently also carry genes for the aerobactin iron uptake system (35).

The *iss* gene, encoding increased serum survival in human *E. coli* isolates, was amplified from an APEC strain isolated from a chicken with systemic colibacillosis and sequenced (14). It was more prevalent in APEC isolates than in *E. coli* isolates

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TABLE 1. Morbidity rates and virulence genes in experimentally infected birds for each of the field isolates.

Strain	Morbidity rate (%)[a]	Virulence genes
E3	75	*iucA*, *tsh*, *iss*
E30	59	*iucA*, *iss*
E133	25	*iss*
E956	88	*iucA*, *iss*
E1043	9	ND[b]
E1292	31	*iucA*[c]
E3/2.4	12	ND

[a] Percentage of inoculated birds that developed air sac lesions.
[b] ND, none detected.
[c] By Southern blot hybridization only.

from healthy birds (27), and it has been suggested that the *iss* gene and its outer membrane protein product, Iss, are potential targets for the detection and control of APEC (14). An association between pathogenicity and serum resistance has been previously demonstrated (18, 34), although a recent study found that the *iss* gene did not play a major role in resistance to serum in strain χ7122 (24). Previous work in our laboratory investigated the virulence of five strains that were isolated from chickens with different disease status (10). In this study, strains E3, E30, and E133 were isolated from lesions in diseased broilers while strains E956 and E1043 were isolated from cloacal swabs from healthy broiler breeders. When the virulence of these strains was assessed by a novel aerosol exposure method, strains E3, E30, and E956 were found to be highly virulent and strains E133 and E1043 were found to be less virulent (10).

Strain E3 is an O nontypeable:H28 field isolate, isolated in pure culture from the pericardium of a 40-day-old broiler chicken with colibacillosis (10). It is a virulent strain of avian *E. coli* that contains six plasmids and produces a colicin(s) and a hydroxamate siderophore. Plasmid curing by subculturing at high temperatures led to the derivation of a series of strains with different plasmid combinations. Using aerosol exposure (10) to test the pathogenicity of plasmid-cured E3 strains, it was found that the loss of the largest plasmid, pVM01, was correlated with the loss of virulence and an inability to colonize the respiratory tract (11). The introduction of a Tn*phoA*-tagged pVM01 plasmid into the plasmid-cured, avirulent strain E3/2.4 restored the virulent phenotype (11).

The aim of this study was to determine the coding potential of pVM01 by calculating the size of the plasmid and to identify putative virulence genes carried on the plasmid, including the aerobactin operon and the *tsh* and *iss* genes that have been reported in the literature. We also examined other APEC strains of known pathogenicity for the presence of these genes and assessed the association of possession of multiple putative virulence genes with pathogenicity of the isolate.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* strains used in this study were field isolates E3, E30, E133, E956, and E1043 (10) and E1292 (9) and derivatives of field isolate E3 with different combinations of plasmids (11). The pathogenicity of five of the field isolates and the plasmid-cured derivatives of E3 had been determined previously using an aerosol challenge method (10). The virulence genes and morbidity rates of the field isolates are shown in Table 1 and the plasmid profiles, virulence genes and phenotypic characteristics of the E3 derivatives are listed in Table 2. *E. coli* strain DH5α was provided by N. Ficorilli (School of Veterinary Science, The University of Melbourne). All strains were grown in Luria-Bertani (LB) broth or on LB agar at 37°C overnight with the appropriate antibiotic selection (ampicillin, 50 μg ml^{-1}; kanamycin, 100 μg ml^{-1}) unless otherwise mentioned. The identity of strains was confirmed by antimicrobial sensitivity testing and plasmid profiling.

Preparation of plasmid DNA. Plasmid DNA from avian *E. coli* was prepared using the Qiagen (Clifton Hill, Victoria, Australia) plasmid midi kit (Qiagen-tip 100) as recommended by the manufacturer, except that *E. coli* was grown in 50 ml of brain heart infusion broth (Oxoid, Heidelberg West, Victoria, Australia). The modifications recommended by the manufacturer to obtain higher yields of low-copy-number plasmids were also used. Plasmids were separated by pulsed field gel electrophoresis (PFGE).

PFGE. PFGE was carried out in a CHEF-DR III system (Bio-Rad, Regents Park, New South Wales, Australia). Plasmids were separated in 1.0% (wt/vol) DNA grade agarose (Progen, Darra, Queensland, Australia) containing 0.5× TBE buffer (1× TBE is 90 mM Tris, 90 mM boric acid, and 2 mM EDTA). A lambda ladder (Bio-Rad) and HindIII-digested lambda DNA were used as molecular size standards. Electrophoresis was carried out for 20 h at 6 V/cm with a switch time of 1 to 20 s and an included angle of 120° or for 11 h with a switch time of 1 to 6 s to visualize the smaller fragments. Gels were stained for 30 min in 1× TBE containing 0.5 mg of ethidium bromide ml^{-1} and then destained in 1× TBE for 30 min. DNA was visualized on a TFX 35M UV transilluminator (Gibco BRL, Melbourne, Victoria, Australia) and photographed using a Kodak Digital Science DC120 zoom digital camera (Eastman Kodak, Rochester, N.Y.).

Polyacrylamide gel electrophoresis. The Bio-Rad Mini Protean 3 gel system was used as specified by the manufacturer to visualize small DNA fragments (<4 kb) not resolved by PFGE. DNA was separated in either 5 or 7.5% (wt/vol) polyacrylamide. The following stock solutions of electrophoresis reagents were used: upper gel buffer (0.5 M Tris-HCl [pH 6.8]), lower gel buffer (1.5 M Tris-HCl [pH 8.8]), electrode running buffer (0.19 M glycine, 0.025 M Tris), and 30% acrylamide-bisacrylamide solution 37.5:1 (Bio-Rad). PstI- and HindIII-digested lambda DNAs were used as molecular size standards. Electrophoresis was carried out for approximately 1 h at a constant 200 V. DNA was visualized by silver staining the polyacrylamide gel (13).

Restriction endonuclease digestion of plasmid pVM01. Restriction endonuclease digestion of pVM01 DNA with the restriction enzymes NotI, SpeI, and XbaI was carried out as specified by the manufacturer (New England Biolabs, Beverly, Mass.). The digested DNA was separated by PFGE, and the sizes of the DNA fragments were calculated using Kodak Digital Science 1D image analysis software. Smaller fragments were visualized by polyacrylamide gel electrophoresis, and the size of the DNA fragments was calculated using the Gel Frag Sizer program (8).

Preparation of genomic DNA for PCR and Southern blotting. Genomic DNA from avian *E. coli* isolates was prepared by phenol-chloroform extraction (30).

PCR. Tn*phoA*, 16S rRNA, and the putative virulence genes were amplified by PCR using the primers listed in Table 3. The PCR amplification was performed using 1.25 U of *Taq* DNA polymerase (Roche, Castle Hill, New South Wales, Australia) in a 25-μl reaction mix containing 25 pmol each of the forward and reverse primers, 5 nmol of each deoxynucleoside triphosphate, and 1 μl DNA template in 1× buffer. The PCR conditions were as follows: 94°C for 3 min for 1 cycle; 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min for 26 cycles; and a final extension at 72 °C for 10 min using a Hybaid (Franklin, Mass.) Omnigene PCR thermocycler. PCR products were visualized on 2% (wt/vol) agarose gels containing 0.5× TPE (1× TPE is 30 mM $NaH_2PO_4 \cdot 2H_2O$, 36 mM Tris, and 1 mM EDTA) buffer and 0.1 μg of ethidium bromide ml^{-1}, run at 70 V for 2 h. The 1-kb PLUS DNA ladder (Gibco BRL) was used as a molecular size marker.

TABLE 2. Plasmid profiles, phenotypic characteristics, and virulence genes of the E3 derivatives

Strain	Plasmid(s)	Characteristics[a]	Virulence genes
E3	pVM01, pVM02, pVM03, pVM04	Col^+ Hyd^+	*iucA*, *tsh*, *iss*
E3/2.4	pVM02, pVM03	Col^+	ND[b]
E3/2.4/1	pVM01::Tn*phoA*, pVM02, pVM03	Col^+ Hyd^+	*iucA*, *tsh*, *iss*
DH5α/5	pVM01::Tn*phoA*	Hyd^+	*iucA*, *tsh*, *iss*
DH5α	None isolated	ND	ND

[a] Col^+, colicin production; Hyd^+, hydroxamate siderophore production.
[b] ND, none detected.

TABLE 3. Primers used for PCR amplification of genes

Primer[a]	Gene specificity (expected size [bp])	Sequence (5′→3′)	Accession no. (reference)[b]
Sid	*iucA* (1,482 bp)	ATGAGAATCATTATTGACATAATT CTCACGGGTGAAAATATTTT	X76100 (22)
Tsh	*tsh* (642 bp)	GGTGGTGCACTGGAGTGG AGTCCAGCGTGATAGTGG	AF218073 (6)
Iss	*iss* (762 bp)	GTGGCGAAAACTAGTAAAACAGC CGCCTCGGGGTGGATAA	AF042279 (14)
16S	16S rRNA (253 bp)	GCTGACGAGTGGCGGACGGG TAGGAGTCTGGACCGTGTCT	AJ404543 (17)
Tn*pho*A	*phoA* (658 bp)	GGGTTGGTACACTGTCATTAC TACCGGGCAATACACTCACTA	U25548 (21)

[a] Custom-made primers (Geneworks and Life Technologies).
[b] GenBank accession number of sequence, from which primers were designed, followed by reference in parentheses.

The design of the Sid primers was based on the 4.475-kb sequence of the aerobactin operon carrying the *iucABC* genes on the enterobacterial plasmid pColV-K30 (22). They were designed to amplify the first 1,482 bases of the operon, which carry part of the *iucA* gene. The Tsh primers were previously designed by Dozois et al. (6) to amplify a 642-bp segment of the 4.133-kb *tsh* gene. The Iss primers were designed by Horne et al. (14) to amplify a 762-bp segment that includes the 310-bp *iss* gene. The 16S rRNA primers were designed as part of this study to be used as a positive control for the presence of *E. coli* DNA in PCR amplifications. The Tn*phoA* primers were designed to amplify a 658-bp segment of the *phoA* component of the 7.733-kb synthetic transposon Tn*phoA*, which had been introduced into plasmid pVM01.

Cloning PCR products. PCR products were purified from low-melting-temperature agarose gels by the QIAEX II method (Qiagen) and ligated to the pGEM-T vector (Promega, Annandale, New South Wales, Australia) as specified by the manufacturer. The products of the ligation reaction were used to transform JM109 cells by electroporation (Gene Pulser; Bio-Rad). Transformants were selected on LB agar containing ampicillin (50 μg ml^{-1}), isopropyl-β-D-thiogalactopyranoside (IPTG; 40 μg ml^{-1}) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 μg ml^{-1}). The identity of the cloned PCR products was confirmed by DNA sequencing.

DNA sequencing. Plasmid DNA template was prepared using the cetyltrimethylammonium bromide precipitation method (3) and purified using a QIAquick PCR purification kit (Qiagen). Sequencing reaction mixtures were prepared with the ABI PRISM Big Dye Terminator cycle-sequencing ready reaction kit (Perkin-Elmer, Rowville, Victoria, Australia). Sequencing gels were run at the Australian Genomic Research Facility at the Walter and Eliza Hall Institute (Parkville, Victoria, Australia). The sequences were compared with those in the GenBank database by using the ClustalX program (32).

Construction of the Sid, Iss, Tsh, and Tn*phoA* probes. The PCR products Sid, Tsh, Iss, and Tn*phoA* amplified from pVM01::Tn*phoA* DNA were purified from a low-melting-point agarose gel using a QIAEX II gel extraction kit, and the DNA was used to produce a hybridization probe.

Southern blot hybridization. Genomic DNA from *E. coli* strains was digested with HindIII, and the fragments were separated by agarose gel electrophoresis. Following agarose gel electrophoresis of either genomic DNA fragments or plasmid fragments DNA was transferred from the gel to a nylon membrane (Hybond-N^{+}; Amersham Pharmacia Biotech, Castle Hill, New South Wales, Australia) by capillary transfer (30). DNA probes were labeled with [α-^{32}P]dATP using a random-primed DNA-labeling kit (Roche). Prehybridization and hybridization were carried out in Church buffer (0.5 M Na_2HPO_4 [pH 7.4], 7% sodium dodecyl sulfate, 1 mM EDTA, 1% bovine serum albumin BSA) (2) overnight at 55 °C. Membranes were washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 55°C three times for 30 min each and autoradiographed with Kodak BioMax MS film at −70°C.

Pathogenicity testing. The virulence of *E. coli* strain 1292 was assessed using the aerosol exposure method previously used to determine the virulence of the other six strains used in this study (10). Briefly, 1-day-old specific-pathogen-free White Leghorn Hybrid chicks were given an intranasal inoculation of infectious bronchitis virus vaccine (Webster's, Vic S) at 10× the immunizing dose and were then exposed to an aerosol of *E. coli* for 20 min. Further aerosols of *E. coli* were administered to the birds on days 4 and 7 of the experiment, and surviving birds were killed on day 11 with an intravenous injection of barbiturate. The birds were examined for airsacculitis, pericarditis, perihepatitis and peritonitis.

Statistical analysis. Multiple regression was used to assess associations between possession of any two or all three of the putative virulence genes and the virulence of the seven strains of *E. coli* in the aerosol exposure model of avian colibacillosis. The VassarStats website at http://faculty.vassar.edu/lowry/multU.html was used to determine the regression coefficients and the significance of the regression equation.

RESULTS

Determination of the size of pVM01. To determine the coding potential of virulence plasmid pVM01, the size of the plasmid was estimated by restriction endonuclease digestion. Linear fragments of the plasmid that could be accurately compared to linear DNA molecular size markers were obtained by digestion of pVM01::Tn*phoA* DNA extracted from DH5α/5 with the restriction enzymes NotI, SpeI, and XbaI.

Restriction endonuclease digestion of pVM01::Tn*phoA* with NotI, SpeI or XbaI resulted in the mean fragment sizes seen in Table 4, which were calculated from between two and six measurements. The fragments obtained by restriction endonuclease digestion of pVM01::Tn*phoA* with the enzymes NotI, SpeI, and XbaI were consistent with the linear size of the plasmid being approximately 160 kb.

Identification of three putative virulence genes on pVM01. To identify whether the putative virulence genes reported in the literature were carried on the virulence plasmid pVM01,

TABLE 4. Fragment sizes from single restriction enzyme digestion of pVM01

Fragment	Size (kb) (mean ± SD)		
	NotI	SpeI	XbaI
A	92.9 ± 2.24[a,c,d]	95.5 ± 1.85[b,d]	104.5 ± 3.65[a,d]
B	64.3 ± 1.81[b]	41.1 ± 2.75[a]	55.9 ± 2.88[b,c]
C	4.67 ± 0.14	18.0 ± 1.19[c]	2.09 ± 0.07
D		6.65 ± 0.13	

[a] Sid probe hybridized to this fragment.
[b] Tsh probe hybridized to this fragment.
[c] Iss probe hybridized to this fragment.
[d] Tn*phoA* probe hybridized to this fragment.

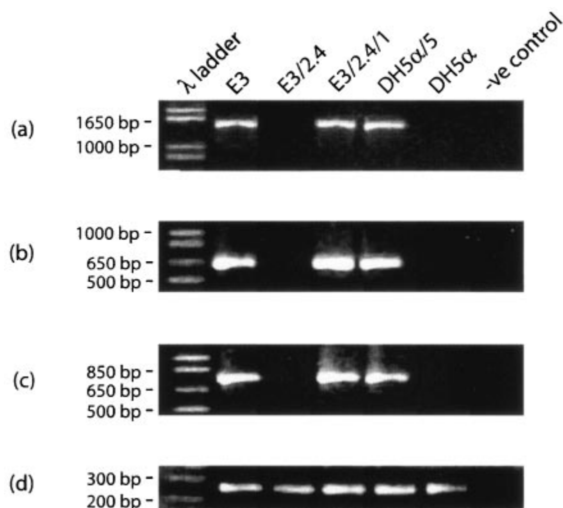

FIG. 1. PCR amplification of the putative virulence genes from strain E3 and its derivatives. The genes *iucA* (a), *tsh* (b), and *iss* (c) were amplified from all E3 derivatives carrying plasmid pVM01; primers for the 16S rRNA gene (d) were included as a positive control, and the gene was amplified from all five strains. Products were 1,482 bp (a), 642 bp (b), 762 bp (c), and 253 bp (d).

PCR amplification of the *iss*, *tsh*, and *iucA* genes was performed. The three putative virulence genes were amplified from strains E3 and E3/2.4/1 and not from strain E3/2.4 (Fig. 1). Thus *iucA*, *iss*, and *tsh* were amplified from the E3 derivatives carrying pVM01 and none of these putative virulence genes were amplified from the E3 derivatives that did not carry pVM01. This established that all three genes were carried on the virulence plasmid pVM01. Amplification of the putative virulence genes from strain DH5α/5 and not DH5α further confirmed their presence on pVM01. Conclusive evidence that *iucA*, *iss*, and *tsh* were carried on pVM01 was provided by amplification of the genes from pVM01::Tn*phoA* DNA purified from DH5α/5. The nucleotide sequences obtained for each of the PCR products confirmed that the *iucA*, *iss*, and *tsh* genes had been amplified.

Physical mapping of plasmid pVM01. Restriction endonuclease digestion with combinations of NotI, SpeI, and XbaI and Southern blot hybridization using the Sid, Tsh, Iss, and Tn*phoA* probes enabled a physical map of the virulence plasmid pVM01 to be constructed (Fig. 2). The plasmid is approximately 160 kb in size and has at least three restriction endonuclease cleavage sites for both NotI and XbaI and four cleavage sites for SpeI.

To establish the proximity of the putative virulence genes to each other and to determine the location of the Tn*phoA* insert, digested DNA fragments were transferred from a pulsed-field gel to a nylon membrane, which was subsequently used for Southern hybridization analysis. The Southern blot of restriction endonuclease-digested pVM01::Tn*phoA* DNA was probed with the PCR products Sid, Iss, Tsh, and Tn*phoA*. The sizes of the fragments to which the probes hybridized are indicated in Table 4; however, it is important in solving the map to note that the 4.6-kb NotI fragment is part of the Tn*phoA* insert located to the right of the Tn*phoA* probe which hybridized to a SpeI-NotI fragment that was approximately 24.9 kb in size. The Sid probe hybridized to the 41.1-kb SpeI fragment, while Iss was found on the 16.7-kb NotI-SpeI fragment and Tsh was found on the 27.2-kb XbaI-SpeI fragment. These data, with the known cleavage sites, were used to solve the map of pVM01 illustrated in Fig. 2.

Association of virulence genes with pathogenic strains. The significance of *iucA*, *iss*, and *tsh* in contributing to virulence was further assessed by examining field isolates of known pathogenicity for the presence of these genes by PCR. A further strain, E1292, was assessed for virulence by aerosol exposure of chickens. Morbidity rates were calculated from the proportion of birds that developed lesions after experimental aerosol exposure to the *E. coli* strains (10). Strains E3, E30, and E956 were the most virulent strains, causing morbidity in 75, 59, and 88% of exposed birds, respectively, while the less virulent strains, E133, E1043, and E1292, caused morbidity in 25, 9, and 31% of experimentally exposed birds, respectively. Strain E3/2.4, a plasmid-cured derivative of E3, caused morbidity in 12% of exposed birds (11). PCR products were analyzed on an agarose gel and can be seen in Fig. 3. The *iucA* gene was amplified only from the three most virulent strains, E3, E30, and E956 (Fig. 3a). The *tsh* gene was detected only in strain E3 (Fig. 3b), while the *iss* gene was amplified from E3, E30, E956, and the less virulent strain E133 (Fig. 3c). The presence of each the virulence genes in the strains was further tested by Southern blot hybridization of genomic DNA to each of the probes used to map the positions of the genes on the pVM01 virulence plasmid. The findings from Southern blot hybridization agreed with those obtained by PCR with one exception: the *iucA* gene was detected in strain E1292 only by Southern blotting (Table 1). Multiple regression was used to assess the association be-

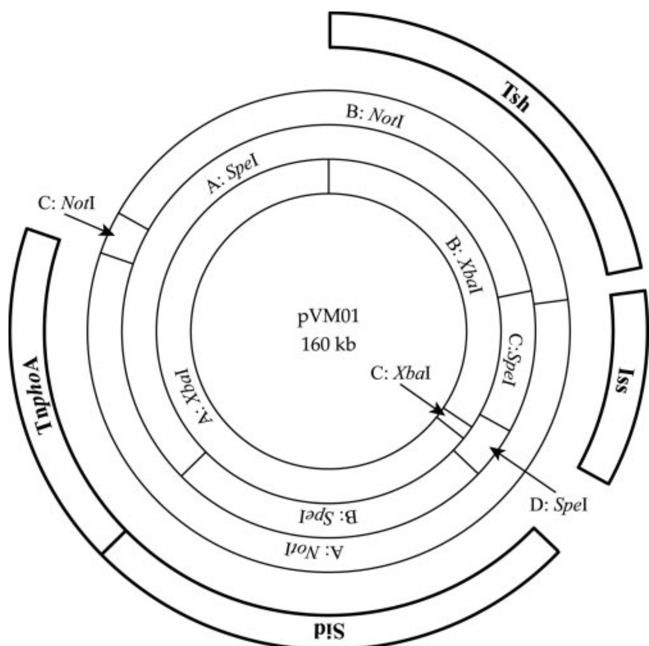

FIG. 2. Physical map of plasmid pVM01::Tn*phoA* illustrating the NotI, SpeI, and XbaI restriction endonuclease cleavage sites and the locations of the Tn*phoA* insert and the putative virulence genes, *iucA*, *iss*, and *tsh.* Designation of fragments (A,B,C, or D) corresponds to the designations in Table 4.

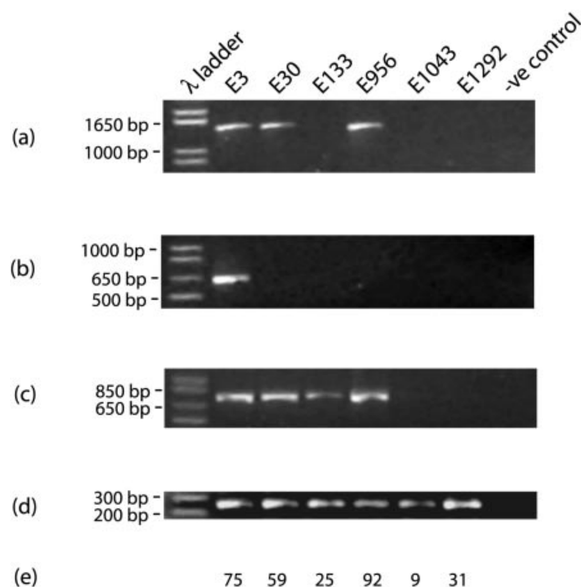

FIG. 3. (a to c) PCR amplification of the putative virulence genes *iucA* (a), *tsh* (b), and *iss* (c) in the field isolates. (d) Primers to the 16S rRNA gene were included as a positive control, and the gene was amplified from all six strains. (e) Morbidity rate after experimental infection with each strain. The *iucA* (a) and *iss* (c) genes were amplified from all field isolates with a morbidity rate higher than 50%. The *iss* gene was also amplified in strain E133, whilst the *tsh* gene was amplified only from strain E3. Products are 1,482 bp (a), 642 bp (b), 762 bp (c), and 253 bp (d). Southern blot hybridization confirmed the PCR results, with the exception that *iucA* was detected in strain E1292.

tween carriage of different combinations of the three putative virulence genes and the pathogenicity of the seven strains. Only a model including the detection of *iucA* and the detection of *iss* yielded a significant regression (adjusted multiple r^2 = 0.8184, P = 0.0147). Thus, these data suggested a strong association between the level of virulence and the carriage of the *iss* and/or *iucA* gene, but not of the *tsh* gene.

DISCUSSION

A number of genes carried by *E. coli* strains isolated from diseased birds have been suggested to be putative virulence genes. Not only was strain E3 isolated from a diseased bird, but also its pathogenicity was confirmed in an aerosol exposure model of disease (10). In addition, the virulence of strain E3 is dependent on carriage of plasmid pVM01 (11). In this study we have characterized the physical structure of pVM01 and examined whether it possessed one or more of the putative virulence genes, *iss*, *tsh*, and the aerobactin operon gene *iucA*.

The restriction endonuclease digestion data obtained were consistent with the size of the virulence plasmid pVM01 being approximately 160 kb. This corresponds to a previous study, which determined that APEC isolates frequently carry large plasmids in the range of 90 to 220 kb that carry virulence-related genes, including some of the genes involved in aerobactin biosynthesis (5).

The three putative virulence genes examined were amplified from strain E3 and from derivatives that carried pVM01. In contrast, none of these genes were amplified from the E3 derivatives that lacked pVM01. This indicates that the *iss*, *tsh*, and *iucA* genes are carried on the virulence plasmid pVM01. While it was known that pVM01 encoded a hydroxamate siderophore, it was not known if the hydroxamate siderophore was aerobactin. Using the Sid primers that were designed as part of this study, a 1.482-kb segment of the aerobactin operon was amplified from all strains carrying pVM01. It is assumed that the presence of *iucA* is representative of the entire aerobactin operon being encoded on pVM01. This assumption could be confirmed by PCR amplification of the other three biosynthesis genes, *iucBCD*, and the transport gene *iutA*, which make up the 8.3-kb aerobactin operon.

Work undertaken by Ginns et al. (11) found that carriage of plasmid pVM01 is necessary for colonization of the respiratory tract. We detected the presence of *tsh*, which has previously been suggested to be involved in the preliminary binding of *E. coli* to the respiratory tract (28), on pVM01. A 642-bp segment of the *tsh* gene was amplified from all strains carrying pVM01 by using the primers designed by Dozois et al. (6), leading to the assumption that the entire 4.133-kb gene and its flanking regions are located on pVM01. In addition, a 762-bp region encompassing the 310-bp *iss* gene was amplified from the E3 derivatives carrying pVM01. The Iss primers were designed to DNA flanking the *iss* gene and could potentially amplify a segment of DNA that does not contain the *iss* gene. The presence of the *iss* gene within the amplified region from pVM01 was confirmed by sequencing the PCR product.

The finding that both *iucA* and *iss* were present on plasmid pVM01 is consistent with the published observations of Ike et al. (15), who identified a large plasmid that encoded both serum resistance and aerobactin-mediated iron uptake, as determined by biological assays, from an APEC strain. Recently, the role of *iss* in serum resistance has been questioned by the results of a study showing that its presence in strains rendered serum sensitive by deletions either in the K1 capsule gene or in lipopolysaccharide biosynthesis genes did not protect against the bactericidal effects of chicken serum in vitro (24). However, since these strains were less sensitive than a strain lacking both the *iss* and *traT* genes, it was suggested that Iss and TraT may play a limited role in protection from complement. Furthermore, these studies do not preclude a role for Iss in strains that do not carry deletions in the capsule or lipopolysaccharide biosynthesis genes, and they do not rule out a role for Iss in vivo.

A physical map of pVM01::Tn*phoA* could be constructed from the data obtained in this study, illustrating the restriction sites for the endonucleases NotI, SpeI, and XbaI and the locations of the Tn*phoA* insert and the putative virulence genes *iss*, *tsh*, and *iucA*. Southern hybridization enabled the fragments with which each probe hybridized to be accurately placed within the pVM01 map.

A series of APEC strains, from both diseased and healthy birds, of defined virulence, based on morbidity in chickens after experimental exposure to an aerosol of the strain, were examined to establish if there was any correlation between the three pVM01-associated putative virulence determinants and the more virulent APEC isolates. It was anticipated that this study would confirm the association of each gene with the virulence of APEC. The *iucA* gene located on plasmid pVM01 was found in the three highly virulent strains and in one of the intermediately virulent strains. Thus, in this series of APEC

isolates, the presence of the *iucA* gene and, presumably the aerobactin operon, correlated with high levels of virulence (10). These data are consistent with previous findings (19) showing that the presence of aerobactin genes was highly correlated with virulence, as determined by a lethality test in 1-day-old chickens and add support to the hypothesis that aerobactin may be a virulence factor in APEC strains.

The *iss* gene was found in the three highly virulent strains and also in one of the intermediately virulent strains. This suggests that it may also play an important role in virulence. However, it should be noted that the presence of only *iss* or *iucA* was associated with intermediate levels of virulence. This is consistent with the findings by Pfaff-McDonough et al. (27) that the presence of *iss* alone might not be sufficient to identify a virulent APEC strain, since they found that some isolates from healthy birds contained *iss*. Indeed, the study suggests that both *iss* and *iucA* are required for the highest levels of virulence. While previous studies have examined the association of these genes with virulence, they were based on demonstrating an epidemiological association between strains and disease rather than direct experimental determination of the level of virulence after respiratory exposure.

The *tsh* gene was amplified only from strain E3 and not from any of the other field isolates. This indicates that temperature-sensitive hemagglutination is not essential for high levels of virulence, since strains E30 and E956 were highly virulent but neither of them carried the *tsh* gene. While this conflicts with the suggestion by Dozois et al. (6) that Tsh is an essential virulence factor, it is possible that alternative adhesins can substitute for Tsh.

The identification of potential virulence genes in complex diseases such as avian colibacillosis can be attempted by using different, complementary approaches. The majority of investigations conducted previously have been epidemiological, where the prevalence of different virulence-associated genes in relatively large numbers of isolates of *E. coli* from birds with disease has been compared with that in normal fecal isolates. While this approach can lead to the identification of putative virulence genes and may indicate their prevalence among *E. coli* isolates from diseased birds, the role of cofactors, such as other infectious agents and environmental conditions, in the occurrence and severity of each outbreak of disease prevents any conclusion being drawn about the relative contribution of these different genes to the level of virulence of avian *E. coli*.

Another approach is to select a single strain and generate isogenic mutants that have specific virulence-associated genes deleted or disrupted and then to experimentally infect birds with these mutants. While this approach will provide specific information about the role of each gene in a specific strain, the time and effort required for such mutational approaches is likely to limit the ability to assess many strains, and thus it will provide little information about how important these genes are across a range of strains. This approach has been used to determine that the *tsh* gene plays a role in the χ7122 strain (7), but, as shown in our study, this role is not necessarily replicated in other strains, and hence the *tsh* gene does not appear to be a unique marker of virulence in APEC.

The approach used in this study is to reproduce disease under controlled, reproducible experimental conditions, allowing the relative virulence of a number of epidemiologically distinct strains to be compared, and then to assess each of these strains for the presence of different virulence-associated genes, permitting an estimate to be made of the relative contribution of each gene to virulence across a population, rather than to that of a single strain. Since a wider range of strains can be assessed, the likely existence of alternative virulence genes in some members of the populations can be detected. It is likely that a full picture of the significance of different virulence genes in the broader population of APEC will require synthesis of information from all three investigative approaches.

While previous studies have found the genes *tsh*, *iss*, and the aerobactin operon in isolates from diseased birds and have suggested that they contribute to virulence, no study has previously shown an association between these genes. Our study has shown not only that these genes are associated with each other but also that they can be carried on the same virulence plasmid. This provides a solid background for examining the virulence of E3 derivatives lacking some or all of these putative virulence genes. In addition, this study supports the use of *iss*- and *iucA*-derived probes to detect APEC in clinical samples.

In this study, the *iucA*, *tsh*, and *iss* genes have been identified on plasmid pVM01 and might be anticipated to encompass approximately 8.9 kb of its sequence. However, at 160 kb, there is the potential for this plasmid to also carry many other virulence genes. Further research is needed to better define the role of *iss*, *tsh*, and the aerobactin operon in the virulence plasmid pVM01 and in other APEC strains. Therefore, it would be useful to further dissect the virulence plasmid pVM01 in order to identify other genes that may contribute to virulence and that, along with *iss* and the aerobactin operon, may also be exploited in the development of diagnostic tools or employed as vaccine targets.

The fact that similar factors have been associated with virulence in extraintestinal *E. coli* strains from a number of species, including the hemoglobin protease (Hbp) autotransporter protein found in extraintestinal *E. coli* infection of humans, which is identical to Tsh (26), adhesins like type 1 fimbriae and P fimbriae, encoded by *papG*, which is commonly associated with virulence in both avian and urinary *E. coli* isolates (16, 33), and aerobactin, which has long been associated with extraintestinal *E. coli* infections in humans and other species (1, 20, 36), suggests that elucidation of the role of specific genes on the virulence plasmids of APEC strains may have broad significance for our understanding of the pathogenesis of colibacillosis in a range of animal species.

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