BACTERIAL PATHOGENESIS

The role of fimbriae and flagella in the adherence of avian strains of *Escherichia coli* O78:K80 to tissue culture cells and tracheal and gut explants

R. M. LA RAGIONE, W. A. COOLEY and M. J. WOODWARD

Bacteriology Department, Veterinary Laboratories Agency (Weybridge), New Haw, Addlestone, Surrey KT15 3NB

To investigate the role of fimbriae and flagella in the pathogenesis of avian colibacillosis, isogenic insertionally inactivated mutant strains of *Escherichia coli* O78:K80 strain EC34195 defective in the elaboration of type-1 and curli fimbriae and flagella were constructed by allelic exchange. Single and multiple non-fimbriate and non-flagellate mutant strains were compared to the wild-type *in vitro* in adherence assays with a HEp-2 cell line, a mucus-secreting cell line HT2916E, a non-mucus-secreting cell line HT2919A, tracheal explant and proximal gut explant. Mutant strains defective in the elaboration of type-1 fimbriae were significantly less adherent – in the order of 90% reduction – than the wild-type strain in all assays. Mutant strains defective in the elaboration of flagella were generally as adherent as the wild-type strain except when assayed with the mucus-secreting cell line HT2916E, for which a significant reduction of adherence – of the order of 90% – compared with the wild-type strain was observed. Mutant strains defective for the elaboration of curli fimbriae adhered as well as the wild-type strain in all assays, except when assayed in tests with gut explant tissue for which a significant reduction of adherence – of the order of 80% – compared with the wild-type strain was observed. Adherence to explants was to epithelial, not serous, surfaces and was 10-fold greater to tracheal than to gut explants. Together, these data support the hypothesis that type-1 fimbriae are significant factors in adherence, aided by flagella for penetration of mucus and curli fimbriae for adherence to the gut.

Introduction

Escherichia coli is an important cause of disease in mammals, birds and reptiles [1]. In domestic poultry, avian colibacillosis is frequently associated with *E. coli* strains of serotypes O78:K80, O1:K1 and O2:K1 [2]. Infections commonly occur *via* the respiratory tract, often after a primary bacterial or viral infection or as a result of poor husbandry practices. The disease generally affects broilers between 3 and 10 weeks of age and is associated with high morbidity and mortality. Carcass condemnation at slaughter is common [3]. The most severe manifestation of avian colibacillosis is septicaemia which is characterised by pericarditis, perihepatitis, air sacculitis and salpingitis [4–7].

Adherence is a necessary prerequisite for bacterial colonisation and pathogenesis. To withstand the mechanical cleansing and bulk flow associated with intestinal mucosal surfaces, bacteria bind specifically to epithelial and mucosal surfaces [8], often by proteinaceous surface appendages called fimbriae. Avian isolates of *E. coli* commonly express type-1, P and curli fimbriae and commonly possess flagella which confer motility [9]. Type-1 fimbriae confer mannose-sensitive haemagglutination, whereas P fimbriae confer mannose-resistant haemagglutination. Both type-1 and P fimbriae have been cited as important factors for adherence to epithelial cell surfaces *in vitro* and *in vivo* [3, 10–14] and type-1 fimbriae are also thought to be important in the initial stages of infections of poultry by *E. coli* strains of serogroups O1 and O2 [8, 11, 15–17]. The role of curli fimbriae in poultry infection by *E. coli* remains unstudied, although it has been demonstrated that *in vitro* they mediate binding to fibronectin, laminin, plasminogen, plasminogen activator proteins and type-1 collagen [18–22]. The role of flagella in the pathogenesis of

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Corresponding author: Professor M. J. Woodward (e-mail: mwoodward.cvl.wood@gtnet.gov.uk).

avian colibacillosis is unclear, because isolates are often non-flagellate.

Avian strains of *E. coli* adhere to tracheal sections and epithelial cell lines [7, 10, 11, 15, 23, 24]. Other tissue-culture and organ-explant approaches have been used to study the initial events of colonisation of different pathotypes of *E. coli in vitro* [25–29]. Recent studies of avian colibacillosis have focused on the role of individual kinds of surface antigens [3, 7, 11]. By contrast, the aim of the present study was to investigate the combined role of fimbriae and flagella in pathogenesis. As a first step, a reverse genetics approach was used to produce a library of non-fimbriate and non-flagellate single and multiple isogenic mutant strains of a clinical isolate of an avian strain of *E. coli* O78:K70. In this paper the construction of the library and its use in various tissue-culture and organ-explant adherence assays are described.

Materials and methods

Bacterial isolates and media

Wild-type strains of *E. coli* O78:K80 were obtained from the *E. coli* Reference Laboratory, VLA, Weybridge (Table 1). Strain EC34195 was used in all mutagenesis studies. Bacterial cultures were maintained on Dorset's egg slopes at 20°C and stored at –80°C in heart infusion broth (HIB) supplemented with glycerol 30% v/v. Working cultures were maintained at 4°C on sheep blood (5%) agar, supplemented with antibiotics as appropriate. Luria Bertani (LB) Agar (Oxoid),

supplemented with Xgal (Promega) 25 mg/ml and ampicillin (Penbrithin, Beecham) 100 µg/ml, was used in all cloning procedures. Minimal medium was M9 salts medium supplemented with glucose 0.2% w/v and 10 mM magnesium sulphate. Bacteria for tissue-culture adhesion and invasion assays were cultured statically in HIB at 37°C for 48 h. They were prepared for adhesion by centrifugation at 4000 rpm for 10 min at ambient temperature and resuspended in phosphate-buffered saline (PBS) to an optical density (540 nm) of 1.2 absorbance units. Bacteria for tissue-explant adhesion assays were cultured as for tissue-culture assays and were resuspended in PBS to an optical density (550 nm) of 0.6 absorbance units. In some experiments, bacteria were cultured aerobically in Colonisation Factor Antigen (CFA) Broth (Oxoid) at 25°C for 72 h.

Phenotypic analysis of *E. coli* isolates

Procedures for mannose-sensitive and -resistant haem-agglutination, motility, agglutination reactions, antibiotic resistance, fibronectin and Congo red binding, curli fimbriae ELISA and transmission and scanning electron microscopy were as described previously [32, 33, 36–38].

Genetic methods

Genetic methods, including plasmid and chromosomal DNA extraction and purification, restriction endonuclease digestion, agarose gel electrophoresis, ligation, preparation of electro-competent bacterial cells,

Table 1. Plasmids and bacteria used

Plasmids and strains	Comments	Source
pBLUESCRIPT	Cloning vector	Commercially available (Stratagene)
pCRSRIPT	Direct PCR cloning vector	Commercially available (Stratagene)
pBSL30	Source of str ^r	[30]
pBSL141	Source of gen ^r	[30]
	Source of cam ^r	Commercially available (Stratagene)
	Source of kan ^r	Commercially available (Pharmacia)
pJP5603	kan ^r suicide vector	[31]
pERFORM K	pJP5603 with modified MCS	[32, 33]
pERFORM C	pJP5603 with modified MCS and kan ^r replaced by cam ^r	[32, 33]
pERFORM G	pERFORM C with cam ^r replaced by gen ^r	This study
<i>E. coli</i> JM109 λ <i>pir</i>	Cloning strain. Genotype: e14 [–] (mcrA [–]) recA 1 end A1 gyrA96thi 1 hsd R17 (r _k -m _k ⁺) supE44 relA1Δ (lac-proAB) [F ⁺ traD36 proAB lac ^q z4M15] λ <i>pir</i>	[31]
<i>E. coli</i> S-17 <i>pir</i>	Mobilising strain. Genotype: pro res- mod ⁺ RP42 [–] Tc::Mu [–] Km::Tn7 λ <i>pir</i>	[34]
EC43297	Human urinary tract isolate	This study
EC54597	Wild-type avian <i>E. coli</i> O78:K80 strain	VLA Weybridge
EC5197	Wild-type avian <i>E. coli</i> O78:K80 strain	VLA Weybridge
EC24197	Wild-type avian <i>E. coli</i> O78:K80 strain	VLA Weybridge
EC24297	Wild-type avian <i>E. coli</i> O78:K80 strain	VLA Weybridge
EC24397	Wild-type avian <i>E. coli</i> O78:K80 strain	VLA Weybridge
EC39597	Wild-type avian <i>E. coli</i> O78:K80 strain	VLA Weybridge
EC43197	Wild-type avian <i>E. coli</i> O78:K80 strain	VLA Weybridge
EC47897	Wild-type avian <i>E. coli</i> O78:K80 strain	VLA Weybridge
EC30896	Wild-type avian <i>E. coli</i> O78:K80 strain	VLA Weybridge
EC26296	Wild-type avian <i>E. coli</i> O78:K80 strain	VLA Weybridge
EC34195	Wild-type avian <i>E. coli</i> O78:K80 strain	[35]
EC2597	Wild-type avian <i>E. coli</i> O78:K80 strain	VLA Weybridge
EC5497	Wild-type avian <i>E. coli</i> O78:K80 strain	VLA Weybridge

electroporation, lysis of bacterial colonies for colony dot-blot hybridisation, Southern hybridisation and autoradiography were as described previously [39]. Probes for radiolabelled probing were either purified PCR products or entire plasmids.

PCR

Primers were designed to amplify *fimC*, *fliC*, *papA* and *csgA* (Table 2). PCR reactions were set up in 50- μ l volumes containing water, Thermo DNA polymerase reaction buffer (Promega), 200 μ M dNTPs (Pharmacia), 1.5 mM MgCl₂, thermostable *Taq* polymerase (Promega) 2.5 units, 10 pmols of each primer and total genomic DNA 1 ng from *E. coli* O78:K80 strain EC34195. Reactions were overlaid with mineral oil (Promega) 50 μ l and cycling was carried out in a thermo-cycler (Biometra) with the following programme: initial denaturation at 95°C for 5 min followed by 30 cycles of denaturing for 2 min at 95°C, annealing at 56°C for 1.5 min, extension at 72°C for 2 min, and thereafter by a final extension at 72°C for 10 min. PCR products were stored at 4°C until required.

Nucleotide sequencing and analysis

The BigDye terminator cycle sequencing kit (Perkin Elmer) was used in all reactions. Individual reaction mixtures 20 μ l contained plasmid DNA 400 ng or PCR amplified DNA 200 ng, primer 3.2 pmol and Ready Reaction Mix (Perkin Elmer) 8 μ l in Eppendorf tubes. Reactions were overlaid with mineral oil, extensions were performed in a thermo-cycler (Perkin-Elmer) and prepared for analysis on a 377 automated DNA sequencer following the manufacturer's instructions (Applied Biosystems) Trace data were analysed and assembled with the use of DNA Star software (DNASar).

Cloning and insertional inactivation of fimbrial and flagellar genes

Mutagenesis was carried out by allelic exchange [32, 33, 36] and the preparative stages for each mutational event are listed in Table 3. In brief, DNA fragments encoding the *fimC*, *csgA* and *fliC* genes, each flanked by immediately adjacent sequences, were amplified by PCR with total genomic DNA extracted

from *E. coli* strain EC34195 of O78:K80 as a template. Amplicons were cloned into pCR-Script plasmid vector (Stratagene) with *E. coli* K12 strain DH5 α (Gibco BRL) as host. Plasmids were mapped by restriction endonuclease digestion and DNA nucleotide sequences were confirmed as described above. Antibiotic resistance cassettes were cloned into appropriate unique restriction sites in the centre of each gene. Insertionally inactivated genes were subcloned into appropriate pPERFORM plasmid vectors [33] and electroporated into the permissive auxotrophic host *E. coli* K12 strain S-17 λ *pir* with selection for both the antibiotic resistance marker encoded by the resistance cassette and the antibiotic resistance marker conferred by the suicide vector antibiotic gene.

Conjugation

The pPERFORM vectors harbouring antibiotic resistance-marked, insertionally inactivated genes were transferred by conjugation from the permissive host *E. coli* K12 strain S-17 λ *pir* into the non-permissive *E. coli* O78:K80 recipient strain EC34195 by filter mating as described before [33] with selection made for the antibiotic resistance marker conferred by the cassette used to inactivate the targeted gene.

Selection of mutant strains

Individual colonies from conjugation selective plates were streaked on to fresh glucose minimal medium supplemented with appropriate antibiotic (i.e., the antibiotic to which resistance was encoded by the insert of the inactivated gene) and cultured overnight at 37°C. Individual colonies were tested for loss of the antibiotic resistance conferred by the suicide vector. Putative double cross-over mutant strains were streaked to single colonies three times before confirmatory genotypic and phenotypic testing. Mutant strains are listed in Table 4.

Pathogenicity of strain EC34195 in 1-day-old chicks

Eighteen 1-day-old SPF white Leghorn chicks were separated randomly into two groups of nine birds each and housed in separate isolators. All birds in one group

Table 2. The sequences and sources of primers used in mutagenesis studies

Primer identity	Sequence 5'–3'	Position (bp)	Accession number
<i>fimC</i> 1	TTT <u>CAGCTG</u> AATGCGGATGCGACCTTCAAG	3501–3521	Z37500
<i>fimC</i> 2	AAAC <u>CAGCTG</u> CATCACGCGTTGCCATATAAC	5248–5227	Z37500
<i>csgA</i> 1	TTT <u>AGGCCT</u> TATTGATCGCACACCTGACAG	2498–2518	X90754
<i>csgA</i> 2	AAA <u>AGGCCT</u> CCAAGGGTTGTGTTATCCATA	4665–4645	X90754
<i>fliC</i> 1	TTT <u>CAGCTG</u> CATGGCACAAAGTCATTAATACC	101–122	Z36877
<i>fliC</i> 2	AAA <u>CAGCTG</u> GATAAGCGCAGCGCATA	1861–1846	Z36877
<i>papA</i> 1	AACTATTCCACAGGGGCA	1834–1851	X61239
<i>papA</i> 2	CAGGTTGAAATTCGCAAC	2291–2308	X61239

Underlined sequences denote engineered restriction enzyme sites (*fimC*, *PvuII*; *csgA*, *StuI*; *fliC*, *PvuII*).

Table 3. Cloning and mutagenesis strategy

Appendage	Target gene	Associated protein	Primers used	Size of amplicon (bp)	Cloned amplicon construct	Marker gene cassette and location of insertional inactivation*	Cloned amplicon and antibiotic resistance marker	Suicide vector construct
Type-1 fimbriae	<i>fimC</i>	Fimbrial chaperone	FimC1 and FimC2	1747	pRLR1	kan ^r – <i>MunI</i> site at 4504 bp	pRLR1.1	pRLR1.2
Curli fimbriae	<i>csgA</i>	Subunit gene	CsgA1 and CsgA2	2167	pRLR2	cam ^r – <i>MunI</i> site at 3636 bp	pRLR2.1	pRLR2.2
Flagella	<i>flhC</i>	Subunit gene	FlhC1 and FlhC2	2078	pRLR6	str ^r – <i>EcoRI</i> site at 1121 bp	pRLR6.1	pRLR6.2

*kan, kanamycin; cam, chloramphenicol; str, streptomycin.

[†]Primers were designed (FlhC 1 and FlhC 2) on K12 sequence (Z36877) to amplify a PCR product of 1760 bp for *flhC* and flanking DNA from strain EC34195. However, a product of 2078 bp was amplified. Confirmatory nucleotide sequencing was carried out and the sequence deposited (Accession number AF079163).

were dosed by intra-gastric intubation with EC34195 bacteria (0.1 ml containing 1×10^9 cfu) and all birds in the other group received a similar dose subcutaneously. Birds were given standard rations and water *ad libitum*. Three birds were randomly selected from each group and killed by cervical dislocation at 24, 48 and 168 h for detailed examination at necropsy. Histological examination of tissues was done after staining with haematoxylin and eosin and Giemsa [40].

Tissue-culture adherence and invasion assays

The mucus-secreting cell line HT2916E [41] was sown 2×10^5 cells in 24-well plates in Dulbecco's modified Eagle's medium (Sigma) supplemented with fetal calf serum 10%, non-essential amino acids ($\times 100$) 1%, 2 mM L-glutamine and gentamicin (Sigma), 50 μ g/ml. Monolayers were incubated for 5 days and were fed on alternate days. By day 5, a confluent mucus layer was detectable on the surface of HT2916E cells. Before use, monolayers were washed twice with Hanks's balanced salts solution (HBSS) to remove cell debris and residual gentamicin. The bacterial inoculum was added (1×10^8 cfu/ml) in incomplete Dulbecco's medium (without antibiotics or fetal calf serum) and added in 1-ml volumes to each well. Monolayers were then incubated at 37°C supplemented with CO₂ 5% for 2 h. The inoculum was removed and the monolayer was washed six times with 1 ml of HBSS to remove non-adherent bacteria. The monolayer was then disrupted for 10 min with a solution of Triton 100 (Sigma) 1% and a 12-mm magnetic stirrer. After disruption for 10 min, 10-fold serial dilutions were plated on to LB agar and incubated overnight at 37°C to determine the count (cfu/ml). A non-mucus-secreting cell line, HT2919A, was used as a control [41, 42]. HEP-2 cells were prepared as above except that they were grown for 48 h in Eagle's Minimal Essential Medium (EMEM; Sigma). All assays were repeated at least twice on at least two separate occasions.

For invasion assays, bacteria were allowed to adhere to HEP-2 or HT2916E cells for 2 h, at which time monolayers were washed three times with HBSS before adding EMEM containing gentamicin 100 μ g/ml. Plates were incubated at 37°C in CO₂ 5% for 2 h and washed three times with HBSS. Monolayers were then disrupted with Triton 100 solution 1% and the number of cfu was determined [37].

Tracheal and proximal gut explant adhesion assay

One-day-old SPF white Leghorn chicks were killed by cervical dislocation. Lengths (*c.* 2 cm) of the trachea and proximal gut (duodenal loop) were removed aseptically to sterile Krebs'-Ringer's solution. Fatty tissue and mesentery were removed from the gut sections and tissues were cut to expose the villiated surfaces (*c.* 1 cm²) for use in adherence assays as described above. Sections were gently washed twice in

Table 4. Library of fimbrial and flagellar mutant strains of *E. coli* O78:K80 strain EC34195

Strain no.	Genotype	Antibiotic resistance ($\mu\text{g/ml}$)	Phenotype
RML1	<i>fimC::kan^r</i>	Kanamycin (25)	MSHA ⁻
RML2	<i>csgA::cam^r</i>	Chloramphenicol (10)	Lacy ⁻ , curli ELISA ⁻ , Congo red ⁻ and fibronectin ⁻
RML3	<i>fimC::kan^r</i>	Kanamycin (25)	MSHA ⁻ and Lacy ⁻ , curli ELISA ⁻ , Congo red ⁻ and fibronectin ⁻
	<i>csgA::cam^r</i>	Chloramphenicol (10)	
RML6	<i>fliC::str^r</i>	Streptomycin (25)	Non-motile
RML7	<i>fimC::kan^r</i>	Kanamycin (25)	MSHA ⁻ and non-motile
	<i>fliC::str^r</i>	Streptomycin (25)	
RML8	<i>csgA::cam^r</i>	Chloramphenicol (10)	Lacy ⁻ , curli ELISA ⁻ , Congo red ⁻ , fibronectin ⁻ and non-motile
	<i>fliC::str^r</i>	Streptomycin (25)	
RML9	<i>csgA::cam^r</i>	Kanamycin (25)	Lacy ⁻ , curli ELISA ⁻ , Congo red ⁻ fibronectin ⁻ MSHA ⁻ and non-motile
	<i>fimC::kan^r</i>	Chloramphenicol (10)	
	<i>fliC::str^r</i>	Streptomycin (25)	

pre-warmed Krebs'-Ringer's solution and immersed individually in fresh sterile Krebs'-Ringer's solution in a 50-ml Falcon tube (BDH) to which 1 ml of the bacterial inoculum (1×10^8 cfu/ml) was added. Incubation was at 37°C with orbital rotation at 225 rpm for up to 180 min. At timed intervals, sections were rinsed three times in fresh sterile pre-warmed Krebs'-Ringer's solution to remove all non-adherent bacteria and then homogenised. Serial dilutions of homogenates were plated in triplicate on to LB agar plates which were incubated at 37°C overnight and bacterial counts were determined. All assays were repeated at least twice on at least two separate occasions.

Statistical analyses

Bacterial counts were converted to log₁₀ values for statistical analysis. Repeated measures analyses of variance were done with the Statistica or Genstat 5 software packages. The means, SEMs and significances (p) of strain differences were calculated.

Results

Characterisation of avian isolates of *E. coli* O78:K80

To select one isolate of *E. coli* O78:K80 for study, specific criteria were established, namely that the

isolate should express relevant surface appendages, be sensitive to antibiotics and be pathogenic. Thirteen isolates of *E. coli* O78:K80 were tested for haemagglutination (type-1 and P fimbriae), motility (flagella), colony morphology, Congo red and fibronectin binding (curli fimbriae) and observed by transmission electron microscopy for the presence of these and other surface appendages. None of the isolates elaborated mannose-resistant haemagglutination, but considerable variability in the elaboration of flagella and type-1 and curli fimbriae was noted (Table 5). Each isolate was tested against a panel of 16 antibiotics (VLA, Weybridge Enteric Bacteria Testing Section) and three antibiotic-sensitive isolates – EC30896, EC5197 and EC34195 – which possessed type-1 fimbriae, curli fimbriae and flagella were selected for further study. Their *fliC*, *fimC* and *csgA* genes were amplified successfully by PCR and these amplicons were cloned, mapped by restriction endonuclease digestion and confirmed by DNA sequence analysis. PCR with *pap* primers failed to generate an amplicon for any of the isolates tested, whereas amplicons were derived successfully by PCR from a control strain (EC43297) of *E. coli* isolated from a patient with urinary tract infection.

Strain EC34195 was tested in the day-old SPF chick pathogenicity model. Birds were inoculated subcutaneously or by the oral route and selected at random from both dosage groups for analysis at necropsy 24,

Table 5. Phenotypic characters of *E. coli* O78:K80 isolates

Strain no.	Elaboration of curli fimbriae	Colony morphology	Congo red binding	Fibronectin binding	Elaboration of type-1 fimbriae	Haemagglutination of chicken or guinea-pig erythrocytes	Elaboration of flagella	Motility	Flagellar antigen
EC54597	—	S	—	±	+	+	+	+	51
EC5197	+	L	+	+	+	+	+	+	9
EC24197	+	L	+	+	+	+	—	—	—
EC24297	+	L	+	+	+	+	—	—	—
EC24397	+	L	+	+	+	+	—	—	—
EC39597	+	S/L	+	+	+	+	+	+	51
EC43197	+	L	+	+	—	—	+	—	51
EC47897	+	S/L	+	+	+	+	+	+	9
EC30896	+	S/L	+	+	+	+	+	+	9
EC26296	+	S/L	+	+	—	—	—	—	—
EC34195	+	L	+	+	+	+	+	+	9
EC2597	—	S	—	±	—	—	+	+	11
EC5497	—	S	—	±	—	—	+	+	11

L, Lacy; S, smooth colonies; ±, binding to fibronectin at low level.

48 and 168 h after infection. Histological examination of tissues from diseased birds showed generalised congestion of the heart, with suppurative pericarditis and epicarditis. Examination of liver tissues revealed congestion with mild multifocal cellular infiltration and focal hepatocyte vacuolation.

Construction of isogenic mutant strains of *E. coli* O78:K80 EC34195

Single and multiple mutant strains were constructed in strain EC34195 as described above. Southern hybridisation analysis of each mutant strain and of the wild-type progenitor strain EC34195 were compared. Hybridisation with the native target gene PCR product showed an increase in size of the target gene commensurate with the insertion of an antibiotic resistance cassette (Fig. 1). The antibiotic resistance cassette used as probe hybridised with the target mutant gene only, whereas detectable hybridisation was not observed when the pPERFORM suicide plasmid DNA alone was used as a probe.

Strain RML1 (a type-1 non-fimbriate mutant, *Fim*⁻) failed to agglutinate guinea-pig erythrocytes. Strain RML2 (a curli non-fimbriate mutant, *Crl*⁻) did not generate convoluted colonies, failed to bind either Congo red or fibronectin and was negative in an indirect curli ELISA. Strain RML6 (a non-flagellate mutant, *Fla*⁻) was non-motile and failed to agglutinate in H9 antiserum. Multiple-mutant strains were tested similarly and behaved as anticipated. All mutant strains were shown to have the same growth rates *in vitro* as the wild-type strain when cultured in LB broth (data not shown).

Adherence of non-fimbriate and non-flagellate mutant strains to tissue-culture cells

Single and multiple isogenic non-fimbriate and non-flagellate mutant strains were compared with the isogenic wild-type strain in HEP-2, HT2916E and HT2919A cell-adhesion assays (Table 6). In tests with HEP-2 cells, mutant strains defective in the elaboration of type-1 fimbriae (strain RML1, *Fim*⁻; strain RML3, *Fim*⁻ *Crl*⁻; strain RML7, *Fim*⁻ *Fla*⁻; strain RML9,

Fig. 1. Southern hybridisation of *E. coli* O78:K80 strain EC34195 and isogenic derivatives to demonstrate insertional inactivation of target fimbrial and flagellar genes. (a) Lanes 1, 3, 5, wild-type strain and lanes 2, 4, 6, strain RML1 (*fimC*::kan^r); DNA digested to completion with *Hind*III (1, 2), *Eco*RV (3, 4) or *Pvu*II (5, 6) and hybridised against *fimC*. (b) Lanes 1, 3, 5, 7, wild-type strain and lanes 2, 4, 6, 8, strain RML2 (*csgA*::cam^r); DNA digested to completion with *Sca*I (1, 2), *Pst*I (3, 4), *Cla*I (5, 6) or *Sma*I (7, 8) and hybridised against *csgA*. (c) Lanes 1, 3, 5, 7, wild-type strain and lanes 2, 4, 6, 8 strain RML6 (*fliC*::str^r); DNA digested to completion with *Pst*I (1, 2), *Hind*III (3, 4), *Hpa*I (5, 6) and *Bgl*I (7, 8) and hybridised against *fliC*.

Table 6. Adherence of wild-type, single and multiple mutant strains of *E. coli* to different cell lines

Cluster*	Strain	HEp-2 [†]			HT2916E [†]			HT2919A [†]		
		Mean [†] log ₁₀	(SEM)	Significance (p) [‡]	Mean [†] log ₁₀	(SEM)	Significance (p) [‡]	Mean [†] log ₁₀	(SEM)	Significance (p) [‡]
I	Wild-type	5.88	(0.09)	—	5.93	(0.11)	—	5.99	(0.13)	—
	RML1	5.26	(0.09)	<0.001	4.68	(0.13)	<0.001	5.00	(0.13)	0.013
	RML2	5.91	(0.10)	0.823	5.74	(0.13)	0.273	6.00	(0.13)	0.976
	RML6	5.40	(0.56)	0.527	4.73	(0.13)	<0.001	5.33	(0.13)	0.038
II	Wild-type	6.01	(0.56)	—	5.93	(0.11)	—	6.18	(0.06)	—
	RML3	5.36	(0.11)	0.006	4.83	(0.18)	<0.001	5.00	(0.06)	<0.001
III	Wild-type	6.03	(0.17)	—	5.93	(0.11)	—	6.13	(0.03)	—
	RML7	5.03	(0.20)	0.019	4.73	(0.18)	<0.001	4.94	(0.03)	<0.001
	RML8	5.16	(0.20)	0.030	4.84	(0.18)	<0.001	5.94	(0.03)	0.011
	RML9	5.21	(0.20)	0.035	4.75	(0.18)	<0.001	4.94	(0.03)	<0.001

*For ease of manipulation, and to provide multiple values for wild-type strains for statistical analysis, assays were performed in three clusters (I–III).

[†]Mean bacterial counts obtained after culture of inoculum in HIB at 37°C for 48 h.

[‡]Significance values calculated with reference to wild-type strains (wild-type *versus* mutant).

Fim[−] CrI[−] Fla[−]) showed a reduction in adherence of the order of 90% ($p < 0.001$, $p = 0.006$, $p = 0.019$, $p = 0.035$, respectively) when compared with the wild-type strain. The curli non-fimbriate mutant strain RML2 (CrI[−]) showed no reduction in adherence and the non-flagellate mutant strain RML6 (Fla[−]) showed a reduced adherence of *c.* 50% compared with the wild-type strain, although this difference was not statistically significant ($p = 0.527$). It was of interest that the curli non-fimbriate, non-flagellate mutant strain RML8 (CrI[−], Fla[−]) was significantly less adherent (*c.* 90%) than the wild-type strain ($p = 0.030$).

At least 80% of the HT2916E cells secreted mucus and the adherence of strain RML6 (Fla[−]) to this cell line was reduced by >90% compared with the wild-type strain ($p < 0.001$). Other mutant strains, lacking either type-1 fimbriae or flagella, also showed reduced adherence of the order of 90% when compared with the wild-type strain; again, these findings were statistically significant. Adherence of strain RML2 (CrI[−]) was not reduced significantly ($p = 0.273$).

With the non-mucus-secreting cell line HT2919A, all mutant strains lacking type-1 fimbriae showed reduced adherence (*c.* 90%) compared with the wild-type strains and this difference was statistically significant. Strains RML6 (Fla[−]) and RML8 (Fla[−] CrI[−]) both showed *c.* 40% reduction in adherence compared with the wild-type strain ($p = 0.038$, $p = 0.011$, respec-

tively) but the curli non-fimbriate mutant, strain RML2 (CrI[−]) adhered as well as the wild-type strain.

Invasion of HEp-2 and HT2916E cells by non-fimbriate and non-flagellate mutant strains

Invasion assays were set up for mutant strains RML1 (Fim[−]), RML2 (CrI[−]) and RML6 (Fla[−]) only in HEp-2 and HT2916E cells (Table 7). With HEp-2 cells, invasion of strain RML1 (Fim[−]) was reduced to *c.* 0.01–0.05% of adherent bacteria compared with 0.1% for the wild-type strain, a significant finding ($p = 0.027$). The reduction in invasion by strains RML2 (CrI[−]) and RML6 (Fla[−]) was not statistically significant ($p = 0.285$, $p = 0.230$, respectively). About 0.005–0.01% of adherent wild-type bacteria invaded HT2916E cells, and this level of invasion was less than that for similar experiments with HEp-2 cells. Strains RML1 (Fim[−]), RML2 (CrI[−]) and RML6 (Fla[−]) were less invasive than the wild-type strain ($p < 0.001$, $p = 0.010$, $p < 0.001$, respectively), although the extent of invasion in these assays was directly proportional to the extent of adhesion.

Development of chick tracheal and gut explant adhesion models

Bacterial growth was not detectable when tracheal and proximal gut samples (before incubation with test bacteria) were homogenised and plated on LB agar

Table 7. Invasion* of HEp-2 and HT2916E cells by wild-type and single mutant strains of *E. coli*

Strain	HEp-2			HT2916E		
	Mean log ₁₀	(SEM)	Significance (p) [†]	Mean log ₁₀	(SEM)	Significance (p) [†]
Wild-type	3.27	(0.06)	—	2.91	(0.03)	—
RML1	3.03	(0.06)	0.027	2.25	(0.03)	<0.001
RML2	3.17	(0.06)	0.285	2.67	(0.03)	0.010
Wild-type	2.69	(0.16)	—	2.91	(0.03)	—
RML6	2.29	(0.16)	0.230	2.03	(0.03)	<0.001

*Mean bacterial counts obtained after culture of inoculum in HIB at 37°C for 48 h.

[†]Significance values calculated with reference to wild-type strains (wild-type *versus* mutant).

with aerobic incubation overnight at 37°C. Tissues showed signs of disaggregation after incubation for 4 h, and, for convenience, sampling was done 3 h after inoculation. After exposure to bacteria, tissues were fixed and examined by scanning electron microscopy (Fig. 2a and b). Counts of bacteria from 10 randomly selected fields per tissue surface showed preferential adherence to the ciliated epithelial rather than the serous surface (ratio 80:1). Furthermore, bacteria adhered singly and not as aggregates or clumps. Significant differences in adherence were not seen between different areas of the gut or trachea when *E. coli* strain EC34195 was incubated with similarly sized sections of tissues of proximal, mid and distal trachea, proximal, mild and distal gut, caecum or rectum.

Adhesion of non-fimbriate and non-flagellate mutant strains to explant tissue

The adherence to tracheal explant tissue (Table 8) of mutant strains defective in the elaboration of type-1 fimbriae (strain RML1, Fim⁻; strain RML3, Fim⁻ CrI⁻; strain RML7, Fim⁻ Fla⁻; strain RML9, Fim⁻ CrI⁻ Fla⁻) was reduced by *c.* 60–90% compared with that of the wild-type strain; these differences were significant ($p < 0.001$, $p = 0.016$, $p = 0.036$, $p = 0.042$, respectively). There was no significant reduction in adherence for either the curli non-fimbriate strain RML2 (CrI⁻) or the non-flagellate strain RML6 (Fla⁻), although the adherence of strain RML8 (Fla⁻ CrI⁻) was reduced by *c.* 75% compared with that of the wild-type strain, a finding which was significant ($p = 0.036$).

The adherence to gut explant tissue (Table 8) of strains RML1 (Fim⁻) and RML2 (CrI⁻) was reduced by *c.* 90% and 80%, respectively, compared with that of the wild-type strain ($p = 0.002$, $p = 0.005$, respectively). It was surprising that the adherence of strains RML3, defective in the elaboration of both type-1 and curli fimbriae, was reduced by *c.* 70% compared with that of the wild-type strain, a reduction that was not statistically significant ($p = 0.496$). In this model, the role of flagella was found to be equivocal with strains RML6 (Fla⁻), RML8 (CrI⁻, Fla⁻) and RML9 (Fim⁻, CrI⁻, Fla⁻) showing only marginally less adherence than the wild-type strain ($p = 0.086$, $p = 0.068$, $p = 0.123$, respectively), although adherence of strain RML7 (Fim⁻ Fla⁻) was reduced by 90% compared with that of the wild-type strain ($p = 0.016$).

After incubation with bacteria for 3 h both gut and tracheal explant tissues were shown by electron microscopy to have lost much of the mucus. That may have influenced the findings.

Influence of culture of inoculum on curli fimbriae-mediated adherence to tissue culture

Curli fimbriae were not detectable by electron microscopy on wild-type bacteria grown in HIB at 37°C for

48 h, although evidence was presented that curli non-fimbriate mutant strains grown under these conditions were less adherent to gut explants than the wild-type strain. When inocula were prepared by growth in CFA broth at 25°C for 72 h without shaking, conditions known to support expression of curli but not type-1 fimbriae [30, 38], adherence of strain RML2 (CrI⁻) to both mucus-secreting and mucus-non-secreting cell lines was reduced by >80% compared with that of the wild-type strain ($p = 0.003$ and $p = 0.026$, respectively). Similar reductions were observed for all other mutant strains prepared under these conditions (Table 9) and of particular note was the reduction in adherence to the mucus cells of >90% for mutant strains defective in the elaboration of flagella ($p < 0.001$).

Discussion

Non-fimbriate and non-flagellate mutant strains were constructed in *E. coli* O78:K80 strain EC34195 by allelic exchange and evidence was gained that these strains possessed an antibiotic resistance cassette in the targeted genes and possessed the desired phenotypic changes. Allelic exchange is an established technique and it was considered unlikely that other, less overt phenotypic changes might have resulted from the mutagenesis strategy used. Considerable phenotypic diversity was shown by the small panel of avian, colibacillosis-causing isolates of O78:K80 examined and an additional question arose as to whether strain EC34195 was typical of avian colibacillosis strains. P fimbriae are elaborated by many avian isolates of *E. coli* [17, 43], but the isolates tested in this study did not elaborate mannose-resistant haemagglutination and did not possess *pap* genes as determined by PCR. Electron microscopy confirmed the absence of P fimbriae or any other, hitherto undescribed surface elaborated structures and so it was reasonable to assume that differences observed in adherence and invasion assays were likely to result from the phenotypic outcome of each mutation.

All adhesion assays indicated that type-1 fimbriae were of particular significance in mediating adhesion of *E. coli* O78:K80 to epithelial cells. This finding was consistent with studies with *E. coli* of many other serotypes including other avian colibacillosis serotypes such as O1:K1 and O2:K1 [10, 15]. Again, evidence was presented that *E. coli* O78:K80 strain EC34195 adhered to tracheal explants more effectively than to gut explants. This may reflect the density of receptors – largely type-1 fimbrial adhesin, mannose-bearing glycoproteins [44, 45] – or innate tropism for tracheal tissues which may also relate to the specific adhesin-receptor interactions.

Flagella have been cited as mediating penetration of mucus before epithelial adherence mediated by type-1,

Fig. 2. (a) Scanning electron micrograph of tracheal tissue explant showing ciliated luminal surface after exposure to bacteria of *E. coli* O78:K80 strain EC34195 for 3 h. Magnification shown by white bar, marker. (b) Scanning electron micrograph of tracheal tissue explant showing fibroblastic surface after exposure to bacteria of *E. coli* O78:K80 strain EC34195 for 3 h. Magnification shown by white bar, marker.

Table 8. Adherence of wild-type, single and multiple mutant strains of *E. coli* to tracheal and gut explant tissue

Strain	Tracheal tissue			Gut tissue		
	Mean* log ₁₀	(SEM)	Significance (p) [†]	Mean* log ₁₀	(SEM)	Significance (p) [†]
Wild-type	5.96	(0.10)	—	4.93	(0.08)	—
RML1	5.10	(0.10)	<0.001	3.89	(0.08)	0.002
Wild-type	6.08	(0.07)	—	4.93	(0.08)	—
RML2	6.15	(0.07)	0.526	4.11	(0.08)	0.005
Wild-type	5.93	(0.01)	—	4.23	(0.33)	—
RML3	5.44	(0.01)	0.016	3.85	(0.33)	0.469
Wild-type	5.54	(0.13)	—	4.93	(0.08)	—
RML6	5.50	(0.13)	0.845	4.66	(0.08)	0.086
Wild-type	5.10	(0.27)	—	5.11	(0.19)	—
RML7	4.36	(0.27)	0.302	4.09	(0.19)	0.160
Wild-type	5.08	(0.03)	—	4.83	(0.06)	—
RML8	4.41	(0.03)	0.036	4.03	(0.06)	0.068
Wild-type	5.33	(0.04)	—	4.91	(0.09)	—
RML9	4.47	(0.04)	0.042	4.24	(0.09)	0.123

*Mean counts of adherent bacteria after culture of inoculum in HIB at 37°C for 48 h.

[†]Significance values calculated with reference to wild-type strains (wild-type *versus* mutant).

Table 9. Adherence of wild-type and single mutant strains of *E. coli* to different cell lines

Strain	HEp-2*			HT2916E*			HT2919A*		
	Mean log ₁₀	(SEM)	Significance (p) [†]	Mean log ₁₀	(SEM)	Significance (p) [†]	Mean log ₁₀	(SEM)	Significance (p) [†]
Wild-type	6.71	(0.046)	—	6.64	(0.061)	—	6.47	(0.117)	—
RML1	5.97	(0.046)	0.001	5.96	(0.061)	0.004	5.71	(0.117)	0.020
RML2	6.41	(0.046)	0.020	5.87	(0.061)	0.003	5.78	(0.117)	0.026
RML6	6.52	(0.046)	0.213	5.45	(0.061)	<0.001	5.71	(0.117)	0.019

*Mean bacterial counts after culture of inoculum in CFA broth at 25°C for 72 h.

[†]Significance values: see footnotes to Table 8.

and other, fimbrial and non-fimbrial surface antigens [46]. The data generated in this work support that concept in that non-flagellate mutant strains adhered less well to the mucus secreting cell line HT2916E than did the wild-type strain. It was of interest that non-flagellate mutant strains adhered less well to the non-secreting cell line HT2919A, indicating that factors other than flagella and mucus was important in adherence to the HT2916-type cell lines. Whether the use of tissue explants gave a clearer understanding of the role of flagella is also open to debate, especially as non-flagellate mutant strains were not significantly less adherent than the wild-type strain. Gut and tracheal explant tissues from day-old chicks may lack fimbrial adhesin receptors because of immaturity [47], and that may have influenced results. Electron microscopic analysis of tissues after incubation with bacteria indicated that mucus was absent and it is probable that mucus was lost by the shear forces associated with the agitation used during incubation. In addition, that agitation may have provided sufficient contact between bacteria and tissue such that any chemotactic role associated with adherence was not apparent.

Curli non-fimbriate mutant strains grown in HIB at 37°C for 48 h showed only a marginal and statistically equivocal reduction in adherence to explants, but no reduction of adherence to tissue culture cells *in vitro*.

When the bacterial inoculum was prepared in CFA broth at 25°C for 72 h, conditions which induce the production of curli, but not type-1, fimbriae [38, 48], differences in adherence between mutant and wild-type strains were accentuated and were statistically significant. It was of interest that curli fimbriae were not seen by electron microscopy on HIB-grown bacteria, although differences in adherence between curliate and non-curliate derivatives were observed in gut explant assays. Thus, it is possible that either sufficient curli fimbriae were elaborated in HIB to enable observation of differences or contact with explants stimulated their elaboration. Alternatively, other adhesins may have been elaborated under these conditions. Curli fimbriae mediate aggregation of curliate bacterial cells [49, 50], but in this study cell clumping at the epithelial cell surface was not observed by scanning electron microscopy. It was probable that differences in bacterial counts reflected actual adherence values not apparent reductions due to clumping, especially as Triton, which disaggregates bacterial clumps, was a component of the diluent used for the enumeration of adherent bacteria. Bacterial cultural and tissue-culture assay conditions influenced bacterium–host cell interactions, findings in common with those of Duguid and Gillies [51], who showed that anaerobiosis induced fimbriation. Again, Ernst *et al.* [52] showed that anaerobiosis enhanced invasion. A comprehensive

comparable study of adherence to a number of cells and tissues has been presented and has indicated that a role in adherence *in vitro* is associated with each of the structures investigated: i.e., type-1 fimbriae, curli fimbriae and flagella.

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