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The role of fimbriae and flagella in the colonization, invasion and persistence of *Escherichia coli* O78:K80 in the day-old-chick model

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SUMMARY

To understand the role of flagella and fimbriae of *Escherichia coli* O78:K80 in avian colibacillosis, day-old chicks were dosed orally with defined afimbriate and or aflagellate mutants and colonization, invasion and persistence compared with that of the wild-type. In an invasion model, chicks were dosed with 1×10^5 c.f.u. of a single strain and mutants defective for type 1 fimbriae, curli fimbriae or flagella colonized livers by 24 h although the numbers of bacteria present were significantly less than the wild-type. Mutants colonized between 50 and 75% of spleens whereas the wild-type colonized 100% of spleens. Additionally, the numbers of mutant bacteria in colonized spleens were significantly less than the wild-type. Surprisingly, mutants defective for the elaboration of more than one appendage were no more attenuated than single mutants. In a persistence model, chicks were dosed with 1×10^2 c.f.u. of a single strain and mutants defective for type 1 or curli or flagella or any combination thereof persisted as assessed by cloacal swabbing for 5 weeks of the experiment less well than the wild-type. In an additional persistence model, chicks were dosed with 5×10^2 c.f.u. of each of wild-type and one mutant together. All mutants were significantly less persistent than the wild-type ($P < 0{\cdot}001$) and one mutant which lacked type 1, curli and flagella, was eliminated within 2 weeks. Analysis of the trends of elimination indicated that flagella contributed to persistence more than curli, which contributed more than type 1 fimbriae. Here was evidence for a major role in colonization, invasion and persistence played by type 1, curli and flagella.

INTRODUCTION

Avian colibacillosis is an economically important infectious disease of domestic poultry. The aetiological agent responsible for colibacillosis is *Escherichia coli*, with the most commonly implicated serotypes being O1:K1, O2:K1 and O78:K80. The most severe clinical manifestation of *E. coli* infections in poultry is colisepticaemia, which often begins as an upper respiratory infection following a primary mycoplasmal or viral infection, leading to infiltration of the blood and internal organs and development of pericarditis, perihepatitis, airsacculitis and salpingitis [1, 2].

Several putative virulence factors have been associated with avian pathogenic *E. coli*, such as the presence of an iron chelating system, expression of type 1 fimbriae, bacterial motility, encapsulation, lipopolysaccharide (LPS) and resistance to killing by complement whilst the ability to bind Congo red is regarded as a marker of virulence [3–11].

The most studied of these virulence determinants of avian *E. coli* are type 1 and P fimbriae [12–16]. Of these, type 1 fimbriae which confer mannose sensitive haemagglutination are elaborated readily *in vitro* and

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in vivo and have been shown to be expressed in the respiratory tract of chickens and rats implicating these appendages as important in attachment to host tissues at these sites [14–17]. Additionally, type 1 fimbriae activate both neutrophils and mast cells and modulate phagocytosis in mice [18–21]. Some avian *E. coli* isolates elaborate P fimbriae which mediate mannose resistant haemagglutination and are regarded essential for uropathogenic *E. coli* virulence in urinary tract infections [22] although their role in avian colibacillosis is equivocal. Both P and type 1 fimbrial types enhance epithelial cell cytokine responses with the secretion of chemokines stimulated and neutrophil recruitment *in vivo* [23–25].

Whilst the role of type 1 fimbriae of various pathogenic *E. coli* serotypes has been tested in *in vitro* and *in vivo* models, little work has been done to confirm their role in avian colibacillosis. Marc and colleagues [26] studied the colonization of a *fim* mutant of an avian *E. coli* O2:K1 isolate in a chick model and concluded that type 1 fimbriae are but one factor involved in adhesion. In common with other pathogenic *E. coli*, avian colisepticaemic *E. coli* serotypes elaborate other surface appendages including curli fimbriae and flagella [27, 28]. Curli fimbriae, first described by Olsen and colleagues [29] bind fibronectin, plasminogen, human contact phase factors and Congo red [30]. Whilst nothing is known of the role curli in avian colibacillosis, flagella are considered to mediate penetration of the mucous layer over epithelial cells [31]. However, many avian *E. coli* isolates, even those associated with clinical disease, are aflagellate. In addition to conferring motility, expression of flagella has been associated with intracellular survival of certain pathogens [32–34] and has been demonstrated to enhance adherence to epithelial cells *in vitro* [28, 35].

In order to study the contribution of fimbriae and flagella to avian colibacillosis, we constructed a series of isogenic single and multiple insertionally inactivated mutants defective for the elaboration of type 1 and curli fimbriae and flagella in a well characterized clinical *E. coli* O78:K80 isolate [27, 28]. The mutants were less adherent than their progenitor wild type strain in *in vitro* adherence assays on HEp-2, HT29/16E and HT29/19A cell lines as well as chick gut and trachea explants [28]. Thus, the aim of the work reported here was to extend our understanding of the role of these surface antigens in pathogenesis by the study of the invasion, colonization and persistence of these mutants in a day-old SPF chick model.

MATERIALS AND METHODS

Bacterial isolates, culture and storage

Bacterial isolates and mutants are listed in Table 1 and were obtained from the *E. coli* reference laboratory, VLA (Weybridge) whilst the generation of mutants was described previously [28]. Storage was on Dorset egg slopes at room temperature whilst master stock cultures stored frozen at −80 °C in heart infusion broth (HIB) supplemented with glycerol (30% w/v). Isolates were cultured on blood or nutrient agar (Oxoid) and supplemented with antibiotics (Sigma) where appropriate. Planktonic culture was in nutrient broth. Wild-type *E. coli* O78:K80 isolate EC34195 was selected for resistance to nalidixic acid (15 μg ml^{-1}) by serial passage on antibiotic containing media.

Growth curves of the nalidixic acid resistant derivative of EC34195 and the isogenic afimbriate and aflagellate mutants were performed as follows. Single well isolated colonies were picked into nutrient broth and incubated overnight at 37 °C with gentle agitation. An aliquot containing 1×10^7 c.f.u. was transferred to 100 ml fresh pre-warmed nutrient broth to give a final density of 1×10^5 c.f.u. ml^{-1}. Incubation was at 37 °C with gentle agitation and samples taken at 0, 1–9 h were plated onto MacConkey agar supplemented as appropriate by antibiotics for selection to enumerate particular bacterial strains.

For inoculation of birds, bacteria were streaked to single colonies on LB agar and single well isolated colonies were picked into nutrient broth and incubated overnight at 37 °C. Bacterial cells were centrifuged at 3500 ***g*** for 5 min and resuspended in Phosphate Buffered Saline (PBS) to give the required dilution. To enumerate bacteria from chick organs and the environment, samples were homogenized in PBS and serial dilutions were plated onto MacConkey agar supplemented as appropriate by antibiotics for selection. Cloacal swabs were streaked directly onto LB agar supplemented with appropriate antibiotics and then placed in LB broth for enrichment by incubation at 37 °C for 24 h prior to plating again. Inoculated plates were incubated overnight at 37 °C.

Localization of *E. coli* O78:K80 in the chick

Day-old specific pathogen free (SPF) White Leghorn chicks (SPAFAS) were dosed orally with 1×10^5 c.f.u. of EC34195. Birds were clocally swabbed after 24 h to

Table 1. E. coli *O78 : K80 isolates and afimbriate and aflagellate mutants*

Identity*	Genotype	Antibiotic resistance	Phenotype†
Wild-type EC34195		none	Lacy$^+$, curli ELISA$^+$, Congo red$^+$, fibronectin$^+$ MSHA$^+$ & motile
EC34195 Nal^r	*gyrA*	Nalidixic acid 15 μg/ml	Lacy$^+$, curli ELISA$^+$, Congo red$^+$, fibronectin$^+$ MSHA$^+$ & motile
RML1 (Fim$^-$)	*fim*C::kan^R	Kanamycin at 25 μg/ml	MSHA$^-$
RML2 (Crl$^-$)	*csg*A::cam^R	Chloramphenicol at 10 μg/ml	Lacy$^-$, curli ELISA$^-$, Congo red$^-$ & fibronectin$^-$
RML3 (Fim$^-$, Crl$^-$)	*fim*C::kan^R	Kanamycin at 25 μg/ml	MSHA$^-$ & Lacy$^-$, curli ELISA$^-$,
	*csg*A::cam^R	Chloramphenicol at 10 μg/ml	Congo red$^-$ & fibronectin$^-$
RML6 (Fla$^-$)	*fli*C::str^R	Streptomycin at 25 μg/ml	Non-motile
RML7 (Fim$^-$, Fla$^-$)	*fim*C::kan^R	Kanamycin at 50 μg/ml	MSHA$^-$ & non-motile
	*fli*C::str^R	Streptomycin at 25 μg/ml	
RML8 (Crl$^-$, Fla$^-$)	*csg*A::cam^R	Chloramphenicol at 10 μg/ml	Lacy$^-$, curli ELISA$^-$,
	*fli*C::str^R	Streptomycin at 25 μg/ml	Congo red$^-$, fibronectin$^-$ & non-motile
RML9 (Fim$^-$, Crl$^-$, Fla$^-$)	*csg*A::cam^R	Kanamycin at 50 μg/ml	Lacy$^-$, curli ELISA$^-$,
	*fim*C::kan^R	Chloramphenicol at 10 μg/ml	Congo red$^-$, fibronectin$^-$
	*fli*C::str^R	Streptomycin at 25 μg/ml	MSHA$^-$ & non-motile

* Fim, type 1 fimbriae; Crl, curli fimbriae; Fla, flagella.

† MSHA, Mannose sensitive haemagglutination of guinea-pig erythrocytes, Lacy, colony morphology after culture on CFA agar at 25 °C for 72 h; motility, tested by straight wire stab into semi-solid agar and hanging drop method, Congo red binding after culture at 25 °C for 72 h on CFA agar supplemented with 0·05% Congo red dye, curli ELISA and fibronectin binding assay performed after culture on CFA agar at 25 °C for 72 h.

establish the presence of an infection. Groups of three birds were killed by cervical dislocation at 1, 7, 14, 21, 28 and 35 days post infection (p.i.). *Post mortem* examinations were performed and the heart, liver, spleen, caeca, oesophagus, proximal gut, mid gut, distal gut, trachea and lungs were removed aseptically and homogenized in PBS. Tenfold serial dilutions were then plated on LB agar plates and these were incubated aerobically at 37 °C overnight. Organ homogenate (1 ml) was enriched in LB broth for 24 h at 37° C prior to plating again.

Colonization and invasion of day-old SPF chicks

Groups of about 40 one-day-old SPF White Leghorn chicks (SPAFAS) were divided randomly and equally into a test and a control group. All birds in the test group received 1×10^5 c.f.u. of one mutant by oral gavage whilst all birds in the control group received 1×10^5 c.f.u. of the wild-type. Dosed birds of the two groups were housed in separate heated isolators with food and water *ad libitum*. At 24, 48 and 120 h p.i. between 5 and 8 birds from both test and control groups were killed by cervical dislocation and *post mortem* examinations were performed. Bacteria in livers, spleens and caeca were enumerated as described above. Each experiment was repeated at least twice with the data for identical experiments pooled such that each bacterial count by time point by each organ was derived from a minimum of 11 birds.

In vivo persistence study

Groups of 10 one-day-old SPF White Leghorn chicks (SPAFAS) were dosed orally with 1×10^2 c.f.u. of either a single strain (persistence study) or with 5×10^2 c.f.u. wild-type nalidixic acid resistant derivative of EC34195 and 5×10^2 c.f.u. of one mutant strain (competition persistence study). Each group of birds was maintained in a single isolator and provided feed and water *ad libitum*. Cloacal swabs from each bird were taken at weekly intervals for 5 weeks and plated onto LB agar supplemented with appropriate antibiotics. At the end of 5 weeks, all birds were sacrificed by cervical dislocation and *post mortem* examinations performed. Bacteria in entire caeca were enumerated as described above.

Table 2. *Mean bacterial counts* (log_{10}) *of* in vivo *localization of* E. coli *O78:K80 EC34195 wild type after oral inoculation of day-old chicks with* 1×10^5 *c.f.u.*

Tissue	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35
Liver	4·40	1·11	0·63	1·94	0	0
Spleen	1·79	0	0	1·46	0	0
Trachea	2·54	1·35	0	2·52	2·39	0·766
Crop	4·39	4·55	3·49	4·68	4·71	3·63
Heart	3·12	0·96	0	1·04	0	0
Lungs	3·97	3·04	1·88	1·84	2·97	1·44
Proximal gut	4·36	4·45	1·63	4·00	3·87	3·89
Middle gut	4·64	6·28	0	3·89	5·17	4·95
Distal gut	5·32	6·38	4·01	4·99	4·52	4·77
Caecum	7·87	8·78	8·62	8·76	8·73	8·64
Oesophagus	—*	—*	—*	—*	1·18	1·54

* No tissue examined.

Environmental infection of chicks with wild-type and afimbriate and aflagellate *E. coli* O78:K80 mutants

Eighteen day-old SPF White Leghorn chicks were randomly divided into 3 groups of 6 birds. Each group was introduced into one isolator which 2 weeks previously had been cleared of chicks inoculated with both wild-type and one mutant (RML1, RML2 or RML6). All birds were cloacally swabbed twice at 1, 7 and 14 days and swabs were plated separately one onto LB agar supplemented with nalidixic acid to select for wild-type and the other onto LB agar supplemented with appropriate antibiotics to select for the mutant. *Post mortem* examinations and caecal counts were done on these birds at day 14.

Statistical analyses

For the analysis of bacterial numbers from colonization and invasion experiments, a generalized linear model was applied and means, standard errors and *P* values calculated. To analyse organism counts analyses of variance were done for each experiment and organ. Counts were transformed to $\log_{10}$ to test for the effects of time, type and their interaction. Means, standard errors and *P* values were calculated.

For the analysis of bacterial numbers from the persistence studies, bacterial counts for the wild-type and the mutants were transformed to their logarithms to base 10 and compared by one-way analyses of variance. For the analysis of swab plates, growth was assigned a numerical value [no growth = 0, < 200 colonies = 1, > 200 colonies = 2 and confluent growth = 3] and analysed as continuous variables in multivariate repeated measures analyses of variance with time as the repeated factor. The overall differences between the wild-type and the mutant strains were tested by *F*-tests and the differences over time and the strain by time interactions by Wilk's λ.

RESULTS

Growth characteristics of afimbriate and aflagellate derivatives of EC34195

Prior to inoculating birds with mutants of EC34195 defective for the elaboration of fimbriae and flagella, it was necessary to establish that there was no overt growth disadvantage of the mutants compared with the progenitor wild-type strain. Growth curves were established for each mutant as described in Methods and no differences were observed.

In vivo localization of wild-type *E. coli* O78:K80 strain EC34194 in the SPF chick

Day-old SPF chicks were dosed orally with 1×10^5 c.f.u. wild-type *E. coli* O78:K80 isolate EC34195 and serial *post mortem* examinations were performed on individual birds over a 5-week period post inoculation. Bacterial counts recovered from tissues are given in Table 2. No morbidity or mortality was noted throughout the experiment. All birds were colonized within 24 h after oral inoculation, with bacteria invading the liver, spleen and heart and colonizing the proximal and distal gut, caeca, trachea and lungs. Infection was cleared from the liver, spleen and heart

Table 3. *Mean bacterial counts (log_{10}) for liver and spleen invasion by wild-type* E. coli *O78 : K80 and isogenic mutant derivatives*

Organ/ days p.i.	Wild-type*	RML1 Fim$^-$	RML2 Crl$^-$	RML3 Fim$^-$ Crl$^-$	RML6 Fla$^-$	RML7 Fim$^-$ Fla$^-$	RML8 Crl$^-$ Fla$^-$	RML9 Fim$^-$ Crl$^-$ Fla$^-$
Liver								
1	4·59 (0·11)	1·40 (0·30)	3·20 (0·35)	2·29 (0·44)	2·40 (0·32)	3·42 (0·33)	4·22 (0·53)	3·03 (0·39)
	83/83	13/13	11/11	9/11	12/12	12/12	12/12	10/12
	—	$P < 0·01$	$P < 0·01$	$P < 0·01$	$P < 0·01$	$P < 0·05$	$P < 0·05$	$P < 0·05$
2	4·79 (0·09)	3·79 (0·33)	3·75 (0·37)	2·72 (0·46)	2·52 (0·32)	3·85 (0·33)	3·41 (0·52)	4·19 (0·39)
	81/81	11/11	11/11	8/11	12/12	12/12	12/12	12/12
	—	$P < 0·01$	$P < 0·01$	$P < 0·01$	$P < 0·01$	$P < 0·05$	$P < 0·05$	$P < 0·05$
5	4·09 (0·08)	2·95 (0·29)	3·31 (0·35)	2·93 (0·44)	2·49 (0·32)	3·25 (0·33)	3·07 (0·52)	3·16 (0·29)
	86/86	14/14	12/12	12/12	11/12	12/12	12/12	12/12
	—	$P < 0·01$	$P < 0·01$	$P < 0·01$	$P < 0·01$	$P < 0·05$	$P < 0·05$	$P < 0·05$
Spleen								
1	2·20 (0·21)	0·96 (0·19)	0·61 (0·27)	1·35 (0·39)	0·52 (0·22)	1·03 (0·32)	1·54 (0·36)	0·69 (0·24)
	84/84	12/13	7/12	8/11	6/12	10/12	12/12	7/12
	—	$P < 0·001$	$P < 0·001$	$P < 0·001$	$P < 0·001$	$P < 0·001$	$P < 0·001$	$P < 0·001$
2	2·50 (0·26)	0·78 (0·22)	1·04 (0·28)	0·81 (0·39)	1·01 (0·22)	1·40 (0·32)	1·43 (0·36)	1·05 (0·24)
	81/81	8/11	8/11	6/11	10/12	12/12	12/12	9/12
	—	$P < 0·001$	$P < 0·001$	$P < 0·001$	$P < 0·001$	$P < 0·001$	$P < 0·01$	$P < 0·001$
5	1·82 (0·17)	1·27 (0·20)	0·61 (0·27)	0·61 (0·37)	0·61 (0·22)	1·04 (0·32)	0·59 (0·36)	1·14 (0·24)
	86/86	10/14	7/12	6/12	7/12	12/12	4/12	10/12
	—	$P < 0·001$	$P < 0·001$	$P < 0·001$	$P < 0·001$	$P < 0·001$	$P < 0·001$	$P < 0·001$

* The wild-type figure represents the mean of seven separate experiments. No significant differences were observed between the seven wild-type control experiments.

Table 4. *Mean caecal bacterial counts (log_{10}) for persistence experiments and mean level of shedding in persistence studies and statistical analysis*

Bacterial counts				
Isolates	Number of birds	Mean log_{10}	S.D.	Significance probability (P)
Control	10	7·41	0·35	—
RML1 (Fim$^-$)	8	6·62	0·36	$P < 0·009$
Control	10	7·41	0·35	—
RML2 (Crl$^-$)	10	5·88	0·86	$P < 0·001$
Control	9	6·41	0·36	—
RML3 (Fim$^-$, Crl$^-$)	10	6·41	0·41	$P = 0·992$
Control	9	6·41	0·36	—
RML6 (Fla$^-$)	10	5·32	0·60	$P < 0·001$
Control	10	7·23	0·37	—
RML7 (Fim$^-$, Fla$^-$)	10	5·90	0·09	$P < 0·001$
Control	10	6·92	0·16	—
RML8 (Crl$^-$, Fla$^-$)	10	5·90	0·41	$P < 0·001$
Control	10	7·61	0·32	—
RML9 (Fim$^-$, Crl$^-$, Fla$^-$)	9	6·74	0·35	$P < 0·001$
Shedding				
Isolates	Number of birds	Mean*		Significance (P) between wild-type and mutant
Control	10	2·9		—
RML1 (Fim$^-$)	8	2·1		$P < 0·001$
Control	10	2·9		—
RML2 (Crl$^-$)	10	1·7		$P < 0·001$
Control	9	2·9		—
RML3 (Fim$^-$, Crl$^-$)	10	2·0		$P < 0·001$
Control	9	2·9		—
RML6 (Fla$^-$)	10	2·0		$P < 0·001$
Control	10	2·9		—
RML7 (Fim$^-$, Fla$^-$)	10	1·6		$P < 0·001$
Control	10	2·5		—
RML8 (Crl$^-$, Fla$^-$)	10	2·0		$P = 0·004$
Control	10	2·6		—
RML9 (Fim$^-$, Crl$^-$, Fla$^-$)	9	1·9		$P < 0·001$

Separate groups of birds were dosed with one mutant or the wild-type as a control in a series of seven pair-wise experiments.
* Mean bacterial shedding score for swabbing results accumulated over a 5-week period.

within 4 weeks. However, the trachea, crop and the entire alimentary tract remained infected throughout the study.

In vivo colonization and invasion of isogenic afimbriate and aflagellate mutants

To investigate the role of fimbriae and flagella in pathogenesis, mutants defective for the elaboration of these surface structures were dosed orally into day-old SPF chicks. Based on data from the localization experiment (Table 2), bacteria in the whole caeca from dosed birds were enumerated to confirm the dose had taken whilst invasion and colonization of internal organs was assessed by enumeration of bacteria in livers and spleens over the first five days post inoculation. The findings are summarized in Table 3.

In these experiments, pairwise experiments were set up in which a single batch of chicks was divided into two groups, one dosed with wild-type as a control and one dosed with one mutant. For all wild-type control experiments, the livers and spleens were colonized in all birds at all sampling times but no morbidity or deaths occurred. Statistical analysis between controls

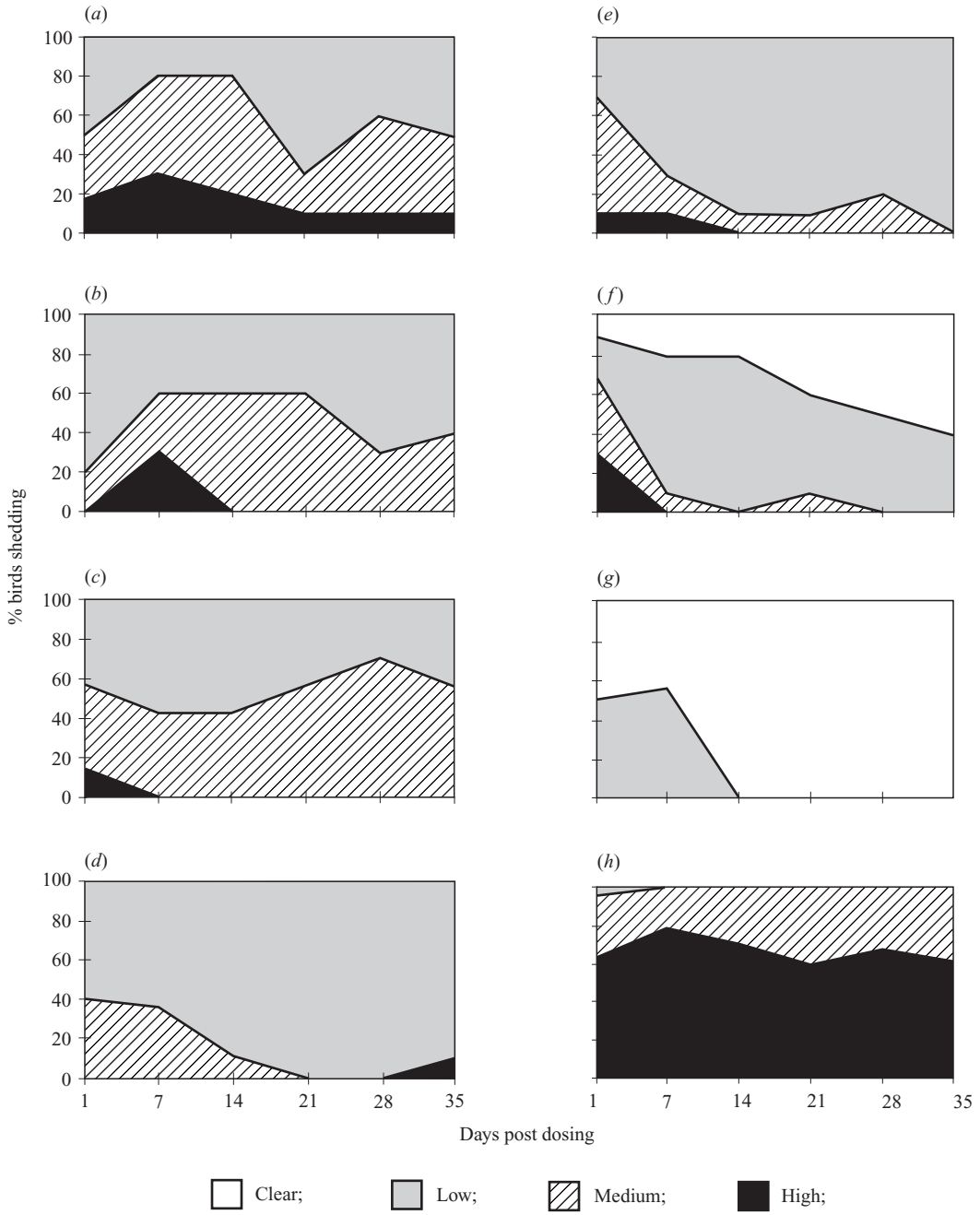

Fig. 1. (*a*) Persistence competition of RML1. (*b*) Persistence competition of RML2. (*c*) Persistence competition of RML3. (*d*) Persistence competition of RML6. (*e*) Persistence competition of RML7. (*f*) Persistence competition of RML8. (*g*) Persistence competition of RML9. (*h*) Persistence competition wild-type*. Graphs represent the mean shedding as determined by cloacal swabbing. Shedding was scored as 0 for clear, 1 for low, 2 for medium and 3 for high. * Wild-type results represent the mean of seven separate experiments. No significant differences were observed between wild-type results. □, Clear; ▨, low; ▨, medium; ■, high.

for each pairwise experiment showed no significant differences, which indicated that the model was consistent, and for convenience the data for the wild-type is summarized collectively (Table 3, col. 2).

With the notable exceptions of RML3 (Fim$^-$ Crl$^-$) and RML9 (Fim$^-$ Crl$^-$ Fla$^-$), the livers of all birds were colonized at all time points. However, the number of mutant bacteria in the livers were in the order of $1 \times \log_{10}$ lower than for the wild type which was a statistically significant finding ($P = \text{or} < 0{\cdot}05$) in each case. Between 50 and 80% of spleens were colonized by all mutants by 24 h post infection and for

Table 5. *Mean bacterial counts (log_{10}) and mean level of shedding in persistence competition experiments*

Bacterial counts Isolates	Number of birds	Mean log_{10}	S.D.	*t*	Significance probability (*P*)
Wild-type		6·24	0·43	3·17	$P = 0·011$
& RML1 (Fim$^-$)	10	5·74	0·44		
Wild-type		6·24	0·43	5·68	$P < 0·001$
& RML2 (Crl$^-$)	10	5·05	0·54		
Wild-type		7·94	0·07	4·34	$P < 0·005$
& RML3 (Fim$^-$, Crl$^-$)	7	7·08	0·49		
Wild-type		6·66	0·21	16·9	$P < 0·001$
& RML6 (Fla$^-$)	9	3·42	0·49		
Wild-type		6·71	0·28	12·5	$P < 0·001$
& RML7 (Fim$^-$, Fla$^-$)	10	4·46	0·71		
Wild-type		7·46	0·45	45·6	$P < 0·001$
& RML8 (Crl$^-$, Fla$^-$)	10	2·57	0·41		
Wild-type		7·27	0·41	52·8	$P < 0·001$
& RML9 (Fim$^-$, Crl$^-$, Fla$^-$)	9	0·00	0·00		
Shedding Isolates	Number of birds	Mean*			Significance (*P*) between strains
Wild-type		2·6			$P < 0·001$
& RML1 (Fim$^-$)	10	2·0			
Wild-type		2·6			$P < 0·001$
& RML2 (Crl$^-$)	10	1·5			
Wild-type		2·6			$P < 0·001$
& RML3 (Fim$^-$, Crl$^-$)	7	1·6			
Wild-type		2·7			$P < 0·001$
& RML6 (Fla$^-$)	9	1·2			
Wild-type		2·7			$P < 0·001$
& RML7 (Fim$^-$, Fla$^-$)	10	1·3			
Wild-type		2·8			$P < 0·001$
& RML8 (Crl$^-$, Fla$^-$)	10	0·9			
Wild-type		2·3			—
& RML9 (Fim$^-$, Crl$^-$, Fla$^-$)	9	0·2			

Separate groups of birds were dosed with wild-type and mutant together.
* Mean bacterial shedding score for swabbing results accumulated over a 5-week period.

subsequent time points. At 24 h post infection the number of mutant bacteria found in colonized spleens were in excess of $1 \times \log_{10}$ lower than for the wild-type and in the order of $1 \times \log_{10}$ lower than for the wild-type at other times. These findings were highly significant ($P < 0·001$) in each case. Interestingly, the notable exception to the overall trends was RML7 (Fim$^-$ Fla$^-$) for which 34 of 36 spleens examined were colonized.

Caeca in all animals were readily colonized in all experiments by the wild-type and by each mutant. The mean number of wild type bacteria per whole caecum was $\log_{10}$ 8·43 at 24 h, $\log_{10}$ 8·66 at 48 h and $\log_{10}$ 8·69 at 120 h. Each mutant colonized the caecum to a similar extent although the numbers of bacteria at each time point were generally less than for the wild-type (data not shown) but by way of an example the mean number of mutant RML9 (Fim$^-$ Crl$^-$ Fla$^-$) per whole caecum was $\log_{10}$ 8·24 at 24 h, $\log_{10}$ 8·39 at 48 h and $\log_{10}$ 8·41 at 120 h ($P = 0·012$).

In vivo persistence of isogenic afimbriate and aflagellate mutants

Data from the localization experiment described above indicated that the caecum was readily colonized and that the bacteria persisted in this organ for the duration of that experiment (35 days). Thus, to investigate the role of fimbriae and flagella in persistence of *E. coli* in the chick day-old SPF chicks were dosed orally in separate experiments with 1×10^2

Table 6. *Mean bacterial counts (log_{10}) and mean level of shedding and statistical analyses of environmental infection.*

Bacterial counts Isolates	Number of birds	Mean $\log_{10}$	S.D.	Significance probability (P)
Wild-type		7·91	0·32	$P = 0·079$
& RML1 (Fim$^-$)	6	7·60	0·20	
Wild-type		7·38	0·56	$P = 0·014$
& RML2 (Crl$^-$)	6	6·31	0·54	
Wild-type		8·88	0·36	$P = 0·002$
& RML6 (Fla$^-$)	6	4·81	1·22	
Shedding Isolates	Number of birds	Mean*		Significance (P) between strains
Wild-type		2·1		$P = 0·065$
& RML1 (Fim$^-$)	6	1·8		
Wild-type		2·7		$P = 0·027$
& RML2 (Crl$^-$)	6	1·7		
Wild-type		2·8		$P < 0·001$
& RML6 (Fla$^-$)	6	1·4		

* Mean bacterial shedding score for swabbing results accumulated over a 2-week period.

c.f.u. of either the nalidixic acid marked wild-type *E. coli* O78:K80 or single or multiple antibiotic marked isogenic afimbriate or aflagellate mutant. A semi-quantitative measure of bacterial load in the caecum was determined indirectly by plating cloacal swabs over a 5-week period and caecal counts at the end of the study (Table 4). The mean shedding scores over the 5-week study period were significantly lower for all mutants than for the wild-type (Table 4). All mutants, except RML3 (Fim$^-$ Crl$^-$), gave caecal counts in the order of $\log_{10}$ 6·0 at 5 weeks post inoculation, whereas the wild-type gave in the order of $\log_{10}$ 7·0.

In vivo persistence of isogenic afimbriate and aflagellate mutants in the presence of competing wild-type (EC 34195)

Experiments to study the persistence of wild-type and mutants in competition one with another were performed. Day-old SPF chicks were dosed orally with a mixed inoculum of 5×10^2 of each nalidixic acid resistant wild-type and one mutant. Shedding over a 5-week period post inoculation was assessed by plating cloacal swabs onto selective media to enable differentiation between wild-type and mutant. Shedding scores for each time point during the persistence competition experiments are presented graphically (Fig. 1*a–h*). In all experiments, the wild-type persisted for the duration of the experiment (mean shedding score 2·6) with no significant differences between the wild-types in individual experiments and, for convenience, these shedding data are presented collectively (Fig. 1*h*). Notably, the multiple mutant RML9 (Fim$^-$ Crl$^-$ Fla$^-$) did not persist beyond 2 weeks whilst other aflagellate mutants, RML6 (Fla$^-$), RML7 (Fim$^-$ Fla$^-$) and RML8 (Crl$^-$ Fla$^-$), showed a markedly reduced capacity to persist. Mutants defective for type 1 and curli fimbriae alone also showed a reduced capacity to persist also. Caecal counts were assessed at the end of week 5 (Table 5). Counts for the wild-type were in the range between $6·24 \times \log_{10}$ and $7·94 \times \log_{10}$ and each experiment were significantly higher than for the co-inoculated mutant ($P < 0·001 - P = 0·01$).

Lateral and environmental transfer of afimbriate and aflagellate mutants

To investigate whether fimbriae and flagella contribute to transmissibility, day-old SPF chicks were introduced into isolators which had been left uninhabited for 2 weeks after removal of chicks dosed in previous experiments and monitored for colonization. All birds were infected within 24 h of introduction to

the contaminated isolators (Table 6). The mean shedding scores over a 2-week period were reduced significantly for the mutants defective for the elaboration of curli fimbriae or flagella ($P = 0{\cdot}027$, $P < 0{\cdot}001$ respectively), but not the type 1 mutant ($P = 0{\cdot}065$). Caecal counts were determined 2 weeks post challenge and were significantly reduced for the non-curliated and non-flagellated mutants ($P = 0{\cdot}014$, $P = 0{\cdot}002$), but not the type 1 fimbriae mutant ($P = 0{\cdot}079$).

DISCUSSION

In this paper we have described the use of isogenic afimbriate and aflagellate mutants of *E. coli* O78 : K80 strain EC34195 to study the role of these organelles in colonization, invasion and persistence in a day-old chick model.

Each mutant showed a statistically significant reduction in the capacity to colonize and invade compared to the wild-type which supports the hypothesis that type 1 fimbriae, curli fimbriae and flagella are virulence factors. Whilst all mutants colonized the liver, albeit with lower numbers of bacteria compared to wild-type, they were consistently less able to colonize the spleen. Collectively these data indicated a reduced ability to invade across the gut barrier or to multiply/survive within macrophages or a combination of both. Surprisingly, RML9 defective for the elaboration of three surface antigens was apparently no more attenuated than any individual single mutant. It was possible that the additive effects of each mutation were sufficiently modest that it was not possible to detect them in the day-old chick model. Clearly, the fact that RML9 colonized and invaded the chick suggested that other virulence determinants were involved in the early events of infection although P fimbriae may be discounted because EC34195, the progenitor strain, did not elaborate these [28]. Alternatively, the early events of infection such as adhesion and invasion which are regarded to be dependent at least in part upon fimbriae and flagella [12, 14–17, 28, 31] may have been bypassed possibly due to the relatively high inoculum administered, the lack of competitive flora, immune naivety or permeability of the gut of day-old SPF chicks.

The data for colonization and invasion were accumulated from eight individual experiments in which the wild-type infected 100% of livers and spleens within 24 h post inoculation and broadly similar numbers of bacteria were recovered from each organ at each time point. The day-old SPF chick model was highly reproducible. However, other models for experimental *E. coli* infections in poultry have described lethality and pathological changes after predisposition with a viral challenge of infectious bronchitis virus (IBV) or *Mycoplasma* spp. prior to oral, subcutaneous, intravenous or aerosol challenge at 3–10 weeks of age [36–38]. However, colisepticaemia has been reproduced by administration of *E. coli* alone to day-old chicks [39, 40] and this model was used in these studies successfully to assess colonisation of internal organs quantitatively. Although it may be argued that this model was inappropriate for avian colibacillosis which is regarded generally as a respiratory pathogen in poultry, the observation of significant differences between mutants and wild-type in this model indicated that this model differentiated between the pathogenic potential of the strains tested. Of alternative methods, the aerosol challenge for *E. coli* infection in very young poultry has been described [39]. The question arises whether the mode of delivery of the challenge bacterium would have changed the overall conclusion that fimbriae and flagella are important in colonization and invasion.

Mutants defective for the elaboration of flagella, curli fimbriae and type 1 fimbriae were reduced in their capacity to persist in the chick gut compared to the wild-type. In addition, the trends of persistence gave an indication of the relative contribution of each surface appendage to the persistence of *E. coli* O78 : K80 in the chick with flagella the most important with additive contributions made by curli and type 1 fimbriae.

Type 1 fimbriae have been cited as important in colonization of the caeca of mice [41] and have been shown to be important in colonization and invasion of the chick in this work. Type 1 fimbriae may play a role in persistence by mediating adhesion to mucus [42, 43] and allowing intimate adhesion to epithelial cell surfaces enabling bacteria to occupy available niches and subsequently prevent removal by host physiological processes such as peristalsis.

In *in vitro* studies curli fimbriae were shown to be of particular importance in mediating adhesion to avian gut tissue whereas flagella were shown to only be of importance in adhesion in the presence of mucus [28] and, in these studies, mutants defective for the elaboration of curli were significantly reduced in their capacity to persist. It is possible that curli fimbriae

mediate aggregation of curliated bacterial cells [44] at the epithelial cell surface and so enhance the number of bacteria colonizing, whereas flagella may be involved in bacterial penetration of the mucus sheet to maintain their position within the gut against peristaltic flow [28, 31].

In the absence of the progenitor wild-type as a competitor, mutants defective for the elaboration of two or more surface appendages were reduced in their capabilities to persist and were found to be less persistent than their single mutant counterparts. The sole exception to this trend was RML3 which lacked type 1 and curli fimbriae but this may be due to an experimental anomaly or this mutant expressed a compensatory factor enabling persistence despite a lack of two fimbriae. This is worthy of further investigation. Clearly, the mutants persisted whilst the natural flora developed during the 5-week duration of the experiment.

Multiple afimbriate and aflagellate mutants were considerably less persistent in the presence of the wild-type and in particular RML9 (Fim^-, Fla^-, Crl^-) was cleared from all birds within 2 weeks of infection. *Post mortem* examination revealed that all tissues including the caecal tonsils were clear of RML9 (Fim^-, Fla^-, Crl^-), whereas the wild-type was present in high numbers in both organs. Whether persistence may depend upon additional factors such as lipopolysaccharide (LPS) and non-fimbrial adhesions is questionable in the light of these results.

Mutants defective for the elaboration of type 1 fimbriae, curli fimbriae or flagella survived in the environment for at least 2 weeks and readily infected naïve birds placed in these infected environments. However, the afimbriate and aflagellate mutants (RML1, RML2 & RML6) were significantly less able to infect birds compared to the wild-type and in infected birds the wild-type reached significantly higher levels in the caeca. Given that low doses (1×10^2 c.f.u.) given by oral inoculation resulted in rapid and high level colonization of day-old SPF chicks, it is reasonable to assume that the reduced colonization and shedding in this model reflected a reduced competence of the mutants to colonize rather than their ability to survive in the environment. Collectively these data point to a lesser role for type 1 fimbriae in gut persistence than curli fimbriae.

Cloacal swabbing appeared to be an acceptable semi-quantitative measure of persistence as judged by comparison between mean shedding scores and bacterial counts of the caeca. The caeca were chosen for assessment of bacterial numbers because this organ was readily and stably colonized to high numbers by the wild-type. Interestingly, the detailed bacteriological analysis of the localization of the wild-type in the chick also showed what appeared to be cyclical colonization. By day 14, the numbers of bacteria colonizing was reduced but by day 21, the number of bacteria from all sites had increased. These results may be anomalies associated with the techniques used or more profound factors such as recolonization from the environment prior to maturation of the immune system. This is worthy of further study.

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