

P fimbriae, capsule and aerobactin characterize colonic resident *Escherichia coli*

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SUMMARY

Resident and transient *Escherichia coli* strains from the colonic microflora of 13 Swedish schoolgirls were analysed for carriage of genes encoding a range of adhesins (P, type 1 and S fimbriae, Dr haemagglutinin and three varieties of the P fimbrial papG adhesin) and other virulence traits (K1 and K5 capsule, haemolysin and aerobactin) using multiplex PCR. Forty-four percent of the resident clones carried genes for P fimbriae, K1 or K5 capsule, and aerobactin, compared with only 3% of transient clones ($P < 0.0001$). The P-fimbriated clones most often had the class II variety of the P-fimbrial adhesin gene *papG* and this adhesin was significantly associated with persistence of a strain. S fimbriae and type 1 fimbriae were equally common in resident and transient strains. The results indicate that not only P fimbriae, but also, certain capsules and the ability to produce the siderophore aerobactin might contribute to persistence of *E. coli* in the large intestine.

INTRODUCTION

Escherichia coli is a member of the normal intestinal microflora of man and other mammals [1]. Certain *E. coli* strains may spread from their normal niche in the colon and cause extra-intestinal infections such as urinary tract infection and septicaemia [2, 3]. Such pathogenic *E. coli* isolates often express a range of virulence factors, including adhesins which confer binding to host cell structures, certain O and K antigens which contribute to protection against phagocytes and complement-mediated lysis, production of the iron-trapping compound aerobactin, and secretion of the cytolytic toxin haemolysin [4].

E. coli strains differ in their capacity to persist in the normal colonic microflora. Resident strains may colonize an individual for months or years [5, 6] while

transient strains are found only once, or on a few occasions closely spaced in time in an individual's intestine [5, 6]. We and others have shown that resident *E. coli* strains more often than transient strains express P fimbriae [7–9], previously recognized as the most important virulence factor for pyelonephritis [10]. P fimbriae, which bind to the Gal α 1 \rightarrow 4Gal β disaccharide, confer binding not only to urinary tract epithelial cells [11] but also to colonic epithelium [12, 13].

The Gal α 1 \rightarrow 4Gal β binding adhesin of P fimbriae, *papG*, occurs in three recognized variants, termed class I–III. All three *papG* classes bind to Gal α 1 \rightarrow 4Gal β containing glycolipids in the globo-series but have slightly different receptor specificities [14, 15]. The class I adhesin primarily binds globotriaosylceramide (Gb0₃), while class II recognizes globoside (Gb0₄) and class III the Forssman glycolipid (Gb0₅). No information is available concerning the role of the different P-fimbrial adhesins for intestinal

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persistence. In addition, a range of other *E. coli* adhesins, such as S fimbriae and Dr haemagglutinin, confer binding to human intestinal epithelial cells [13]. They could therefore contribute to intestinal persistence.

In a gnotobiotic rat model, in which two isogenic *E. coli* strains differing only in expression of the K5 capsule were allowed to compete for establishment in the normal intestinal microflora, the encapsulated strain had an advantage [16]. Similarly, an *E. coli* strain with smooth LPS colonized better in antibiotic-treated mice than its rough mutant [17]. This implies that other virulence factors than adherence may play a role in the competition between *E. coli* strains in the intestinal microflora.

In the present study, we examined a collection of resident and transient colonic *E. coli* strains from the large intestinal microflora of Swedish schoolgirls [7] for their carriage of a range of virulence genes. To this end, a set of multiplex PCR analyses were developed for identification of the genes for P, S and type 1 fimbriae, Dr haemagglutinin, the K1 and K5 capsular phenotypes, haemolysin and aerobactin. We also determined the *papG* adhesin class of the P-fimbriated isolates. The presence of each of these virulence-associated genes was compared between resident and transient strains.

MATERIALS AND METHODS

Bacterial strains

Twenty-five strains of *E. coli* isolated consistently and 38 strains isolated transiently in a longitudinal study of the rectal flora of 13 Swedish schoolgirls were studied [7]. The girls were identified as having asymptomatic bacteriuria during a school screening programme in the early 1970s and culturing of their rectal flora was performed at every visit to the outpatient clinic, usually every third month [18]. Three colonies were selected at random from each culture and identified as *E. coli* by limited biotyping. Each isolate was subjected to multilocus enzyme electrophoresis and assigned an electromorphic type (ET) based on the electromorphs for each of 12 loci tested [7]. Isolates from the same host that shared the same ET were defined as belonging to a single strain. Strains found more than once in the same host were designated resident while strains isolated on a single sample occasion were defined as transient. The resident strains had a mean persistence time in the

rectal flora of at least 120 days. Nineteen percent of the resident strains expressed P fimbriae, as shown by agglutination of Gal α 1 \rightarrow 4Gal β coated latex beads, whereas this was true for only 2.6% of the transient strains [7].

For development of multiplex PCR to identify a range of virulence genes, the following *E. coli* strains which had previously been genotypically and/or phenotypically characterized with respect to their virulence factors served as positive controls. For P fimbriae: strain 506 MR (class I adhesin) of serotype O19,22:K1:H⁻ [19], strain HU734 (class II adhesin) of serotype O75:K5:H⁻ [19] and strain HB101/pPAP⁶⁰¹ (class III adhesin) of serotype OR:K12 [15]; for S fimbriae: HB101 (PAZZ 50) (*sfaII*) [20] and HB101 (PANN 801-13) (*sfaI*), both K12 transformant strains of serotype OR:K12 [21]; for Dr haemagglutinin: C33 [22] and IH 11033 [23] of serotype O75:K5:H1 and strain C64 of serotype O75:K5:H⁻ [22] and for type 1 fimbriae: strain HU734 [19]. As positive controls for K1 capsule we used the strains 2822 (B2b) of serotype O16:K1 and 2806 (A3I) of serotype O21:K1 isolated from the intestinal microflora of Pakistani infants [24] and for K5 capsule the strains RZ513 of serotype O6:K5 [25] and 2822 (C1b1) of serotype O6:K5 [24]. For aerobactin the strains RZ513 [25] and 29423 (CCUG) were used and for haemolysin the strains RZ513 [25] and 2806 (A3I) [24].

Primers for multiplex PCR

Virulence factor genes were identified by PCR using the primers listed in Table 1. The primers for the P fimbrial *papC* gene and for S fimbriae have previously been published [26], as well as the primer pairs specific for the three *papG* adhesin varieties class I, class II and class III [27]. For aerobactin and haemolysin, we used previously published primers amplifying DNA fragments of the *iutA* [28] and *hlyA* genes [29], respectively.

Primers for type 1 fimbriae, Dr haemagglutinin, and the capsular antigens K1 and K5 were designed by us using the computer Oligo Primer Analysis Software (version 5.0). These primer pairs amplified sequences of the *fimA* gene in the type 1 fimbrial operon, the *draA* gene in the Dr operon, the *neuB* gene in the K1 operon and the *kfiC* gene in the K5 operon, respectively. Primers were designed and selected to avoid formation of dimers and to give PCR products sufficiently varying in size to allow separation of PCR products in gel electrophoresis (Table 1).

Table 1. Primers used for detection of *E. coli* virulence factors genes

Virulence factor	Gene	Primer sequence (5'-3')	Designation	Size of the PCR products	Reference
P fimbriae	<i>papC</i>	GACGGCTGTACTGCAGGGTGTGGCG ATATCCTTTCTGCAGGGATGCAATA	pap 1 pap 2	328 bp	[26]
<i>papG</i> adhesin 'Class I'	<i>papG</i>	TCGTGCTCAGGTCCGGAATTT TGGCATCCCCAACATTATCG	j96-193f j96-653r	461 bp	[27]
<i>papG</i> adhesin 'Class II'	<i>papG</i>	GGGATGAGCGGGCCTTTGAT CGGGCCCCAAGTAACTCG	ia2-383f ia2-572r	196 bp	[27]
<i>papG</i> adhesin 'Class III'	<i>papG</i>	GGCCTGCAATGGATTTACCTGG CCACCAATGACCATGCCAGAC	prs-198f prs-455r	258 bp	[27]
S fimbriae	<i>sfaD</i> <i>sfaE</i>	CTCCGGAGAACTGGGTGCATCTTAC CGGAGGAGTAATTACAAACCTGGCA	sfa 1 sfa 2	410 bp	[26]
Type 1 fimbriae	<i>fimA</i>	CGACGCATCTTCTCATTCTTCT ATTGGTTCCGTTATTCAGGGTTGTT	type1-331f type1-1052r	721 bp	This study
Dr haemagglutinin	<i>draA</i>	GCCAACTGACGGACGCAGCAC CCCCAGCTCCCGACATCGTTTTT	Dr-321f Dr-550r	229 bp	This study
Capsule K1	<i>neuB</i>	CTACCCCTTTTGACGAAGAC ACACACCTGACCCCAATAC	K1-1011f K1-1504r	493 bp	This study
Capsule K5	<i>kfiC</i>	GCCACCAACTGTGCGAAAA TGTCGCCCAAAACAAAAAGATT	K5-1756f K5-2564r	809 bp	This study
Aerobactin	<i>iutA</i>	GGCTGGACATCATGGAACTGG CGTCGGGAACGGGTAGAATCG	aer-851f aer-1152r	301 bp	[28]
Haemolysin	<i>hlyA</i>	AACAAGGATAAGCACTGTTCTGGCT ACCATATAAGCGGTCATTCCCGTCA	hly1 hly2	1177 bp	[29]

* f, forward; r, reverse.

Three separate multiplex PCRs were carried out as described below. The first PCR included primer pairs for type 1, P and S fimbriae as well as Dr haemagglutinin. Another PCR included primer pairs for the three *papG* alleles. A third multiplex PCR detected the genes for haemolysin, aerobactin and the capsular antigens K1 and K5.

DNA preparation and amplification procedure

A small amount of bacteria was picked with the tip of a sterile syringe directly from an agar-grown colony and suspended in 50 μ l PCR reaction mixture in an autoclaved thin-wall reaction tube (Perkin-Elmer, Foster City, CA). The mixture contained 1.5 or 2.0 mM MgCl₂ (Perkin-Elmer), the higher concentration being used in the PCR identifying the genes for haemolysin, aerobactin and the capsular antigens K1 and K5, 0.1 mM each of the four deoxynucleoside triphosphates (Perkin-Elmer) and 0.45 μ M of each primer pair. A drop of mineral oil (Perkin-Elmer) was overlaid and the mixture was heated to 94 °C for 10 min (Cetus Model 480 Thermocycler, Perkin-Elmer) to disrupt the bacteria and release their DNA.

After cooling to 80 °C, 2 U/ μ l Taq-polymerase (Perkin-Elmer) was added and the PCR reaction was run using the following temperature profile: an initial denaturation at 94 °C for 4 min was followed by 25 cycles of denaturation at 94 °C for 2 min, annealing at 65 °C for 1 min and extension at 72 °C for 2 min and a final extension at 72 °C for 3 min. The PCR products were separated by electrophoresis in a 2% agarose gel (No. A-9539, Sigma, St Louis, MO). The electrophoresis was run in Tris-Borate-EDTA buffer, pH 8.3 (0.445 M Tris borate, 0.01 M EDTA) at a constant voltage of 120 V for 2.5 h. A DNA marker (VIII 0.5 μ g, Boehringer-Mannheim, Bromma, Sweden) was used as molecular size standard. The gel was stained with 0.5 μ g/ml ethidium bromide (E-1510, Sigma) and photographed under UV light (Polaroid MP4⁺ instant camera system, Polaroid).

Phenotypic expression of virulence factors in *E. coli*

Phenotypic expression of *E. coli* adhesins was tested by hemagglutination using 3% suspensions in PBS of human, ox and horse erythrocytes with and without 2.5% methyl- α -D-mannoside. A loopful of bacteria

was mixed with one drop of an erythrocyte suspension and haemagglutination (HA) was read by the naked eye after gentle tilting of the slide for 3 min. Agglutination of human or ox erythrocytes in the absence and presence of methyl- α -D-mannoside was designated mannose-resistant HA, while agglutination of horse erythrocytes in the absence, but not in the presence of methyl- α -D-mannoside was defined as mannose-sensitive HA.

Capsule serotyping was performed against 67 capsule antigens at the Statens Serum Institut, Copenhagen, Denmark (personal communication).

Production of α -haemolysin was detected by the presence of a α -haemolytic zone around the bacterial colony on nutrient agar containing 5% horse erythrocytes [18].

Secretion of aerobactin was assessed by the ability to promote growth of an aerobactin-requiring *E. coli* strain LG 1522 [30].

Statistical methods

Comparisons of proportions were performed using Fisher's exact test.

RESULTS

Genes for adhesins in resident and transient *E. coli* strains

Figure 1 shows the results of a multiplex PCR using primer pairs detecting genes for P, S, and type 1 fimbriae and Dr haemagglutinin in *E. coli*.

The frequency in 25 resident and 38 transient *E. coli* strains of different fimbrial or adhesin genes is shown in Table 2. The *papC* gene in the operon encoding P fimbriae was seven times more frequent among resident than transient colonic *E. coli* strains (14/25 *vs.* 3/38, $P < 0.0001$). The 17 *papC*-positive strains were analysed for their adhesin variety. Eleven strains (65%) had the class II type of the *papG* adhesin and one (6%) had the class III type. None had the class I variety and five *papC*-positive strains (29%) failed to amplify any DNA fragment specific for the *papG* gene with the primers used. Carriage of the gene for the class II variety of the Gal α 1 \rightarrow 4Gal β specific adhesin was significantly associated with intestinal persistence (Table 2).

Genes for S fimbriae were carried by approximately 20% of both resident and transient strains and type 1

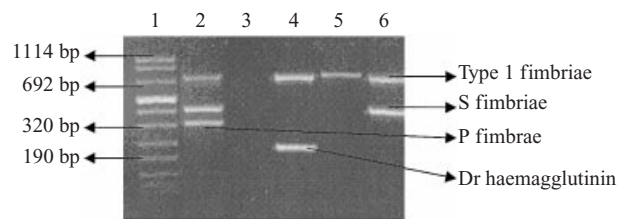


Fig. 1. Detection of genes for type 1, S, and P fimbriae and Dr haemagglutinin in intestinal *E. coli* strains using multiplex PCR. Lane 1, DNA size marker; lanes 2–6, intestinal *E. coli* strains. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide.

Table 2. Rates of carriage of adhesin genes in resident and transient colonic *E. coli* strains

Adhesins	% positive strains		<i>P</i> value
	Resident	Transient	
P fimbriae	56	8	< 0.0001
Class I	0	0	
Class II	40	3	0.0002
Class III	4	0	0.39
None of the above	12	5	0.37
S fimbriae	24	16	0.51
Type 1 fimbriae	92	74	0.10
Dr haemagglutinin	0	0	

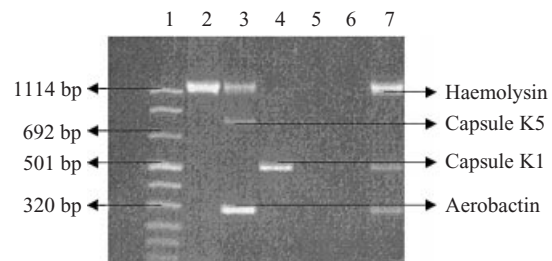


Fig. 2. Detection of genes for the capsular antigens K1 and K5, haemolysin and aerobactin in intestinal *E. coli* strains using multiplex PCR. Lane 1, DNA size marker; lanes 2–7, intestinal *E. coli* strains. PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide.

fimbriae by the great majority of all strains (Table 2). The gene encoding the Dr haemagglutinin was not detected in any of the isolates.

Genes for other pathogenicity factors in resident and transient *E. coli* strains

Figure 2 depicts a multiplex PCR detecting the genes for haemolysin, aerobactin and the capsular types K1

Table 3. Carriage of genes encoding capsules, aerobactin and haemolysin in resident and transient colonic *E. coli* strains

Virulence factor	% positive strains		<i>P</i> value
	Resident	Transient	
Capsular antigens			
K1	36	5.2	0.0045
K5	20	0	0.0076
Other			
Aerobactin	64	16	0.0001
Haemolysin	32	13	0.11

and K5. Table 3 shows the frequencies of these factors in resident and transient colonic *E. coli* isolates. The *neuB* gene encoding K1 capsule formation was seven times more common in resident compared with transient strains ($P = 0.0045$). The *kfiC* gene encoding K5 capsule was only detected in resident *E. coli* ($P = 0.0076$) and the *iutA* gene of the aerobactin gene operon was four times more common in resident than in transient strains ($P = 0.0001$). There was no significant difference in carriage of the *hlyA* gene coding for haemolysin between resident and transient strains.

Capsular types other than K1 and K5

As K1 and K5 appeared to be associated with persistence, capsular typing was performed on all strains using antisera to a large variety of capsular phenotypes. K1 and K5 were the most frequent capsular types, found together in 11/63 strains (17%), while other phenotypes such as K7 (2 strains), K51 (2 strains), K2, K13, K29, K43 (1 strain each), and K positive but non-typable comprised 19 of 63 strains (30%). There was no apparent association between capsules other than K1 and K5 and persistence of a strain. Thus, 38% of resident strains and 60% of transient strains had capsules other than K1 or K5, including those that were non-typable.

Combination of pathogenicity traits in resident and transient *E. coli*

Pathogenicity factors tend to appear in combinations. Indeed, 71% of *papC*-positive clones had the aerobactin gene compared with only 17% of the *papC*-negative clones ($P = 0.0001$). Similarly, 47% and 24% of *papC*-positive clones had the K1 or K5 genes, respectively, whereas this was true for 7 and 2% of

the *papC*-negative clones ($P = 0.0006$ for K1 and $P = 0.016$ for K5).

The combinations of the putative colonization factors P fimbriae, K1 or K5 capsule and aerobactin in resident and transient clones are shown in Table 4. Eleven (44%) of the 25 resident clones carried a combination of all 3 factors and 13 resident strains (52%) had at least 2 of them. This was true for only 1 of 38 (2.6%) transient strains ($P < 0.0001$).

K1 capsule was associated with O1, O2, O7 or rough LPS phenotypes while K5 capsule occurred in combination with serogroups O6, O10, O18 and O25. A large variety of different O antigens was found among the mainly transient strains which had neither P fimbriae, K1 or K5, or aerobactin genes (Table 4).

We analysed whether K1 or K5 capsule or aerobactin would be associated with persistence independent of P fimbriae. Eleven resident and 35 transient strains lacked the *papC* gene. The K1 or K5 capsular genes were carried by 27% of resident versus 3% of transient strains in this subgroup ($P = 0.06$). Aerobactin genes were found in 36% of resident compared with 11% transient strains devoid of P fimbrial genes ($P = 0.08$). All resident strains lacking the *papC* gene carried the *fimA* type 1 fimbrial gene ($P = 0.08$ compared to transient strains).

Phenotypic expression of pathogenicity factors in *E. coli* strains

The phenotypic expression of pathogenicity traits was examined. Fifty-five percent of *papC*-positive isolates expressed mannose-resistant agglutination of human erythrocytes, 55% of the strains carrying the *neuB* gene were serologically positive for K1 and 100% of *kfiC* positive strains reacted with anti-K5 antisera. Aerobactin production was noted in 95% of clones carrying the *iutA* gene and production of α -haemolysin in 46% of those carrying the *hlyA* gene. No differences was observed in phenotypic expression of traits in genotypically positive isolates related to whether the strains were resident or transient.

DISCUSSION

In the present study we investigated whether various bacterial traits, previously characterized as pathogenic properties required for extra-intestinal disease, were associated with persistence of *E. coli* in its natural ecological niche, the large intestine. Resident and transient *E. coli* strains from the large bowel microflora of Swedish schoolgirls were analysed for the

Table 4. Carriage of different combinations of virulence genes in resident and transient *E. coli* strains

Virulence factor combination	No. of strains		O serogroups (no. of strains)
	Resident (<i>n</i> = 25)	Transient (<i>n</i> = 38)	
P + K1 + aerobactin	7	1	O1 (3), O7 (2), OR* (2), O2
P + K5 + aerobactin	4	0	O18 (2), O10, O6
K1 + aerobactin	1	0	O1
K5 + aerobactin	1	0	O25
P fimbriae	3	2	O1, O8, O75, ONT†, OR
K1	1	1	OR (2)
Aerobactin	2	4	O9 (2), O27(2), O4, O8
Neither P fimbriae, nor K1, K5 or aerobactin	6	30	OR (5), O20 (4), O79 (3), ONT (3), O3 (2), O98 (2), O1, O4, O5, O6, O9, O13, O22, O28, O40, O44, O53, O77, O112, O136, O146, O147, O160

* OR, rough strain; † ONT, O antigen positive, but non-typable.

presence of virulence-associated genes using multiplex PCR with specific primers.

Genes encoding P fimbriae were detected in 56% of resident strains, which was seven times more common than in transient *E. coli* strains. When the set of strains examined here were previously assessed for phenotypic expression of Gal α 1 \rightarrow 4Gal β binding, resident strains were positive six times more often than transient, but still only 19% expressed P fimbriae [7]. Thus, phenotypic expression considerably underestimated gene carriage rates. As both expression of type 1 [31] and S fimbriae (unpublished observations) have been shown to increase in the intestinal environment, this could also be true of P fimbriae. The high carriage rate among resident strains of genes for P fimbriae indicate their major importance for intestinal colonization and persistence. P fimbriae have also been shown to facilitate intestinal colonization in a gnotobiotic rat model in which germ free rats were colonized with isogenic strains, differing in fimbrial expression [32].

The class II variety of the *papG* adhesin was most frequent among P-fimbriated strains and was also associated with persistence, while the class III variety, previously called *prs*, was rare and the class I variety was not found. A similar distribution of *papG* varieties among intestinal *E. coli* has previously been reported [33]. Almost one third of *papC*-positive strains did not yield any PCR product from the *papG* gene. Such strains could either have previously undefined adhesin variants or completely lack *papG* gene.

S fimbriae which are virulence factors for neonatal sepsis and meningitis also adhere to human colonic

epithelium [13]. Despite this, genes encoding S fimbriae were no more prevalent among resident than transient *E. coli* strains. It therefore seems as if mere capacity to adhere to intestinal epithelial cells is insufficient to enable long-term persistence in the gut but rather that the adhesin specificity is of major importance [34]. In accordance, two isogenic strains differing only in carriage of the *sfaII* gene colonized equally well in a gnotobiotic rat model (unpublished observations). No strains were found that carried the genes for Dr haemagglutinin which also confers adherence to human intestinal epithelium [13].

Type 1-fimbrial genes were carried by almost all resident *E. coli* strains and by the vast majority of transient strains. The mannose-specific adhesin of type 1 fimbriae mediates binding both to colonic epithelial cells [12, 13] and to the carbohydrate chains of secretory IgA, a molecule which is abundant in intestinal secretions [35]. The fact that persons lacking secretory IgA are less often colonized by type 1-fimbriated *E. coli* [36], suggests that type 1 fimbriae are of ecological importance in the colonic niche in immunocompetent individuals, but phenotypic expression of type 1 fimbriae has previously not been shown to be associated with persistence [7–9]. However, the genes for type 1 fimbriae might be important for persistence of strains devoid of P fimbriae. Thus, all resident strains which did not have P fimbriae carried the genes for type 1 fimbriae.

The clones which seemed especially apt to colonize the human large intestine did not only carry P-fimbrial genes, but also more often the genes for capsules of the K1 and K5 types as well as the siderophore

aerobactin. A combination of these three traits occurred in almost half of the resident *E. coli*, but only in a single transient isolate of 38 tested. In strains lacking genes for P fimbriae, there was an over-representation of genes for capsule and aerobactin among resident, as compared to transient strains, although the difference did not reach statistical significance. In a gnotobiotic rat model in which germ-free rats were colonized simultaneously by two isogenic strains both having type 1 as well as P fimbriae but differing in K5 expression, the K5-positive strains colonized at much higher levels than the capsule-negative mutant [16]. Capsules render the bacterial surface hydrophilic and negatively charged [37, 38] which makes the bacterium resistant to entrapment in mucus [39]. An efficient sequestration of iron could confer a competitive advantage to aerobactin producing strains in the colonic microflora. In accordance, genes for aerobactin were also significantly more often carried by resident as compared with transient strains in a set of colonic *E. coli* derived from a different age group and geographical region, i.e. Pakistani infants 0–6 months old [40].

We have previously suggested that P fimbriae may have evolved to increase the persistence of *E. coli* in its normal habitat, the large intestine [7, 9]. Other virulence-associated traits may as well primarily contribute to persistence of *E. coli* clones in the intestine. Secondarily, clones persisting in the large intestine for extended periods of time and reaching high population numbers may more easily spread to the urinary tract. In this case, virulence might be ‘coincidental’ to an adaptation to the colonic niche as has previously been suggested [41].

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