Genetically related *Escherichia coli* strains associated with Crohn's disease

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Abstract

Escherichia coli strains isolated from patients with Crohn's disease (CD) with chronic ileal lesions (n=14), early endoscopic recurrent lesions (n=20), without endoscopic recurrence (n=7), and controls (n=21) were compared by ribotyping. The dendrogram generated by 50 ribotype profile analysis revealed a large cluster of genetically linked *E coli* strains isolated significantly more frequently from patients with chronic and recurrent CD (24/33 patients) than from controls (9/21) ($p<0.05$). Most patients operated on for chronic ileal lesions (78.5%) harboured *E coli* strains belonging to cluster A ($p<0.002$ v controls). The prevalence of patients with early recurrent lesions harbouring *E coli* strains belonging to this cluster was high but not significant, although 16 strains isolated from eight patients presented the same ribotype profile. In this cluster, 21 of 26 strains isolated from patients with active CD demonstrated adherent ability to differentiated Caco-2 cells, indicating that most of the genetically related strains share a common virulence trait. Comparison of *E coli* strains recovered from ulcerated and healthy mucosa of patients operated on for CD demonstrated in each patient that a single strain colonised the intestinal mucosa. Our results suggest that although a single *E coli* isolate was not found in Crohn's ileal mucosa, some genotypes were more likely than others to be associated with chronic or early recurrent ileal lesions.

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Keywords: *Escherichia coli*; Crohn's disease; chronic ileal lesion; early endoscopic recurrent ileal lesion; ribotyping

Crohn's disease (CD) is an inflammatory bowel disease of unknown aetiology. Various pathogenic mechanisms have been proposed, including an inflammatory response to a luminal pathogen or an abnormal luminal constituent, autoimmunity, or an abnormal immune response to normal ubiquitous intestinal bacteria or dietary antigens.¹ Experimental and clinical data incriminate intestinal bacteria or bacterial products in the initiation and perpetuation of chronic intestinal inflammation, characterised by a transmural granulomatous inflammation of discontinuously affected intestinal segments.^{2,3} Bacterial pathogens have

been suggested as aetiological agents in Crohn's disease, such as *Mycobacterium paratuberculosis* and *Listeria monocytogenes*, and recent studies have failed to resolve the contentious debate over their contribution in either the initiation or perpetuation of the disease.^{2,4}

The role of luminal bacteria in the pathogenesis of CD is strongly supported by observations that patients with CD clinically improve when luminal bacterial concentrations are decreased.^{5,6} *Escherichia coli* strains are among the bacteria of the luminal flora suspected of being involved in the pathogenesis of CD. It has been shown that *E coli* antibody titres are higher in patients with CD than in controls.⁷ In addition, immunocytochemistry revealed the presence of *E coli* antigens in most intestinal resection specimens from patients with CD.⁸ We previously assessed the prominence and virulence of *E coli* strains associated with the ileal mucosa of patients with CD.⁹ *E coli* was recovered from 65% of chronic lesions (resected ileum) and from 100% of biopsies of early lesions (postoperative endoscopic recurrence). The number of specimens colonised by *E coli* from early ileal lesions was higher than that from healthy ileal mucosa in patients without endoscopic recurrence of CD and in controls. Furthermore, no difference was observed between chronic ileal lesions and early ileal lesions. We also showed that *E coli* species were abnormally predominant (50-100% of the total number of aerobes and anaerobes) in early and chronic ileal lesions of CD. Most of these *E coli* strains adhered to cultured epithelial cells in vitro, a property that could enable them to colonise the intestinal mucosa. Further studies on the interactions between these strains and cultured human epithelial cells revealed an invasive potential, indicating that a new *E coli* pathovar might be associated with CD.¹⁰

The purpose of this study was to explore the genetic diversity among *E coli* isolates recovered from the ileal mucosa of 40 patients with CD and from 21 controls. We performed molecular typing using a DNA probe specifying genes encoding ribosomal ribonucleic acid (rRNA) that are conserved and are therefore good markers of phylogenetic lineage within bacteria and species.¹¹ Our aims were: (i) to assess if a particular *E coli* strain was associated

Abbreviations used in this paper: CD, Crohn's disease; rRNA, ribosomal ribonucleic acid; RT pattern, ribotype pattern; CIL, chronic ileal lesion; ER, early recurrent ileal lesion; CDH, CD patient without recurrent lesion; IC, ileal biopsy from control; SC, stool specimen from control.

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with CD; (ii) to examine if particular rRNA gene banding patterns (ribotype profiles or groups of ribotype profiles) were markers of colonising strains involved in either chronic or recurrent ileal lesions in patients with CD; and (iii) to explore the identity of isolates recovered from damaged or healthy mucosa in the same patient.

Materials and methods

BACTERIAL STRAINS

The *E. coli* strains studied were those which were isolated as dominating strains from ileal biopsy specimens from patients with chronic ileal lesions of CD (n=14), with early endoscopic recurrent ileal lesions (n=20), or without recurrence of the disease (n=7).⁹ One patient was counted twice because he was operated on for CD (chronic lesion) and presented endoscopic early recurrence after surgery. In seven patients operated on for CD, *E. coli* isolates were recovered from biopsy specimens from both healthy and ulcerated mucosa. The control group included *E. coli* strains isolated from ileal biopsy specimens from patients without inflammatory bowel disease (n=9) or from stool specimens of non-hospitalised healthy individuals belonging to the same age groups as the patients (n=12).

The *E. coli* strains were stored in Mueller-Hinton broth (Institut Pasteur Production, Marnes-la-Coquette, France) with glycerol (15% v/v) at -80°C. Unless otherwise stated, strains were cultured in Luria Bertani broth (Institut Pasteur Production) at 37°C for 18–24 hours.

RIBOTYPING

For each isolate grown in Luria Bertani broth, chromosomal DNA was extracted¹² and digested with *Hind*III endonuclease. After electrophoresis in 0.8% agarose gels (Gibco BRL, Life Technologies, Paisley, Scotland) at 100 V for five hours in 0.04 M Tris base-5 mM sodium acetate-2 mM EDTA (pH 7.9), DNA restriction fragments were Southern blotted onto a nylon membrane (Hybond-N+; Amersham Life Science, UK) and cross linked by exposure for three minutes to UV light.

The *rrnB7* probe was prepared from a 7.0 kb restriction fragment that carries the entire *rrnB* operon of *E. coli* K-12 and flanking sequences.^{13,14} The probe was radiolabelled with a random primer kit (Boehringer Mannheim GmbH, Mannheim, Germany) with [α -³²P]dCTP (Amersham Pharmacia Biotech, UK) and separated from unincorporated nucleotides through a Sephadex G-50 column (Pharmacia BioProcess Technology AB, Uppsala, Sweden). Hybridisation was performed five hours or overnight at 65°C in borosilicate tubes (hybridisation chamber; Techne Cambridge) using Rapid-hyb buffer (Amersham). Filters were then washed successively with 0.1% sodium dodecyl sulphate-0.3 M NaCl-0.03 M Tris sodium citrate dihydrate (one wash at 65°C for 20 minutes) followed by 0.1% sodium dodecyl sulphate-75 mM NaCl-7.5 mM Tris sodium citrate dihydrate (four washes at 65°C for 15 minutes). The membranes were

exposed to Hyperfilm MP film (Amersham) and processed in an automated film developer (Hyper processor; Amersham).

RIBOTYPE ANALYSIS

For computer analysis, all ribotypes were scanned on a light table using a COHU High Performance CCD camera and digitised by VisioL@b 200 software (Biocom SA). All patterns of restriction fragments were analysed with the Windows version of Phoretix 1D Advanced v4.00 computer program (Phoretix International, Newcastle upon Tyne, UK). The molecular size of each band was calculated from migration distances by "cubic spline" algorithms. The similarities between the patterns were calculated with the Jaccard coefficient (SJ). The proportion of bands common to two strains, A and B, was defined as $SJ = n_{AB} / (n_A + n_B - n_{AB})$, where n_{AB} is the number of bands for A and B, respectively. This Jaccard coefficient ranged from 0 to 1.0, where 1.0 represents 100% identity (presence and position) for all bands in the two fingerprints being compared. The clustering on a matrix of Jaccard coefficient was based on the unweighted pair group method using arithmetic averages (UPGMA) with the NTSYS-PC Numerical Taxonomy and Multivariate Analysis System (version 1.50- (C), Applied Biostatistics Inc.). This program performed a dendrogram and calculated a similarity coefficient between fingerprints on the basis of band position alone.

INDEX OF DISCRIMINATION

The single numerical index of discrimination (*D*) or Simpson's index of diversity described by Hunter and Gaston¹⁵ was used to evaluate the discriminatory power of the typing method. The probability of two unrelated strains being placed into different typing groups was calculated by the following equation: $D = 1 / (N - 1) \sum_{j=1}^S n_j / (n_j - 1)$, where *N* is the total number of strains in the sample population, Σ is the total number of types described, and n_j is the number of strains belonging to the *j*th type. This index can be used to evaluate the discriminatory power of ribotyping.

ADHESION TO HUMAN INTestinal CaCo-2 CELL LINE IN VITRO

Monolayers of differentiated CaCo-2 cells were prepared in 24 well Falcon tissue culture plates (ATGC Biotechnologie, Choisy le Grand, France), as previously described.⁹ The cells were seeded at 1×10^4 cells/cm² in Dulbecco modified Eagle's medium (Flow Laboratories, Inc., McLean, Virginia, USA) at 37°C containing 20% (vol/vol) fetal bovine serum (Seromed, Berlin, Germany), 1% L-glutamine (Boehringer Mannheim, Meylan, France), 1% non-essential amino acids (Boehringer), 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B (ATGC) at 37°C in an atmosphere of 10% CO₂. Monolayers of CaCo-2 cells were used at post-confluence after 15 days of culture. Before the adhesion test, cells were washed twice with phosphate buffered saline (pH 7.2).

Figure 1 Genetic relationships between *Escherichia coli* strains, as estimated by clustering analysis of rRNA gene restriction fragment length polymorphism data obtained after DNA digestion by *HindIII*. The 118 *E. coli* strains analysed were isolated as follows: 41 strains from patients with Crohn's disease (CD) with early recurrent lesions (ER), 39 strains from CD patients with chronic ileal lesions (CIL), 15 strains from CD patients without endoscopic recurrence (CDH), and 23 strains isolated from ileal biopsy (IC) or stool specimens (SC) from controls.

A suspension of 10^8 bacteria/ml in the cell line culture medium, containing 2% (wt/vol) D-mannose, was added to the tissue culture and incubated for three hours at 37°C. After three washes with phosphate buffered saline, the cells were fixed with methanol for 10 minutes, stained with 20% Giemsa for 20 minutes, and examined microscopically under oil immersion. Adhesion assays were performed three times and the mean number of bacteria per cell was determined by examining 200 cells. Adhesion was considered positive if the mean number of bacteria per cell was greater than one.

STATISTICAL ANALYSIS

Data were analysed using the χ^2 test unless the variables required a two tailed Fisher exact test. A p value ≤ 0.05 was considered statistically significant.

Results

The 118 *E. coli* strains analysed were isolated as follows: 41 strains from CD patients with early recurrent lesions (ER), 39 strains from CD patients with chronic ileal lesions (CIL), 15 strains from CD patients without endoscopic recurrence (CDH), and 23 strains from ileal biopsy (IC) or stool specimens (SC) from controls. Of the 27 patients or controls from whom several isolates were recovered, 19 harboured *E. coli* strains with the same ribotype profile. For a more accurate assessment of the results, we considered only one isolate for each of these 19 subjects. Thus the dendrogram in fig 1 indicating the genetic relationship between *E. coli* strains was made with 71 strains isolated from 40 patients and 21 controls.

DISTRIBUTION OF *E. COLI* STRAINS AMONG VARIOUS RIBOTYPE PROFILES

Ribotyping of genomic DNA from *E. coli* isolates generated multiple DNA fragments (5–12) in sizes ranging from 1.4 to 26.5 kb. An index of discrimination of greater than 0.95, the threshold value for interpreting typing results with confidence, was obtained with DNA digestion with the endonuclease *HindIII*, the *D* value being 0.978.

Fifty distinct ribotype profiles (RT patterns) were identified. The 118 strains diverged by up to 85%. At a level of 37% dissimilarity, 15 RT pattern groups (A–O) were identified. The strains were distributed into eight main clusters (A1, A2, B, C, F, H, I, and L) with 3–27 different isolates each and eight “outliers” (table 1).

The largest was cluster A, composed of two subgroups, A1 and A2, including *E. coli* strains isolated from 26 patients and nine controls, for which 21 ribotype patterns were identified. The subgroup A1 included *E. coli* strains isolated from 18 patients and eight controls, for which 15 ribotype patterns were identified. In this cluster, strains isolated from eight patients and one control presented the same ribotype profile. In cluster A2, which was the second major group, we found *E. coli* strains isolated from 10 patients, for which five ribotype profiles were identified. Twenty nine RT patterns were obtained with the remaining *E. coli* strains isolated from 16 patients and 12 controls and were distributed among clusters B–O (table 1).

RIBOTYPE PATTERNS OF *E. COLI* STRAINS ACCORDING TO THE ORIGIN OF THE ILEAL SPECIMENS

The distribution of RT patterns according to the origin of the *E. coli* isolates is given in table 2. In cluster A, *E. coli* strains were isolated from 9/21 controls (42.8%), 11/14 patients operated

Table 1 Distribution of *E. coli* strains isolated from patients and controls among various ribotype (RT) patterns

RT pattern	Total (50)	A		Group (No of RT patterns)														
		A1 (15)	A2 (6)	B (3)	C (5)	D (1)	E (1)	F (3)	G (1)	H (3)	I (3)	J (1)	K (1)	L (4)	M (1)	N (1)	O (1)	
No patients/ No isolates	40/95	18/34	10/26	1/4	3/8	1/2	1/1	3/6	1/2	—	4/6	1/1	1/1	4/4	—	—	—	
No controls/ No isolates	21/23	8/8	1/1	3/3	2/4	—	—	—	—	3/3	1/1	—	—	—	1/1	1/1	1/1	
Total of strains	118	42	27	7	12	2	1	6	2	3	7	1	1	4	1	1	1	

*Six patients harboured more than one *E. coli* isolate in several groups.

Table 2 Distribution of the 15 ribotype profile groups in relation to the origin of the *E. coli* strain (patients and controls)

	No patients and controls				
	Origin of <i>E. coli</i> strain				Controls ^d
	n	ER ^a	CIL ^b	CDH ^c	
A1	26	12 ^e	4	2	8
A2	11	2 ^e	7	1	1
B	4	—	1	—	3
C	5	1	1	1	2
D	1	1	—	—	—
E	1	—	—	1	—
F	3	—	2	1	—
G	1	1	—	—	—
H	3	—	—	—	3
I	5	3	—	1	1
J	1	1	—	—	—
K	1	1	—	—	—
L	4	3	—	1	—
M	1	—	—	—	1
N	1	—	—	—	1
O	1	—	—	—	1
No patients or controls ^f	61 ^f	20 ^g	14 ^g	7	21

^aER, early recurrent ileal lesion; ^bCIL, chronic ileal lesion; ^cCDH, healthy ileal mucosa from CD patients without recurrent lesion; ^dcontrols, biopsy specimens from patients with non-inflammatory bowel disease and stools from healthy non-hospitalised individuals; ^eone patient was considered twice because he presented early endoscopic recurrence at two and three years after surgery; ^fsome patients harboured more than one *E. coli* isolate distributed in several groups; ^ganother patient was also considered twice because he was operated on for CD (chronic lesion) and presented early endoscopic recurrence after surgery.

on for chronic ileal lesions (78.6%, $p < 0.002$), 13/20 patients with recurrent ileal lesion (65%, $p > 0.05$), and 3/7 patients without recurrence of CD (42.8%). A significant difference was observed between the total number of CD patients with chronic and early recurrent lesions and controls harbouring *E. coli* strains

clustered in group A ($p < 0.05$). A significant difference was also observed between the number of CD patients with chronic ileal lesions harbouring *E. coli* belonging to cluster A and controls ($p < 0.03$).

Patients with chronic ileal lesions harboured *E. coli* strains belonging to A2 (7/14) and A1 (4/14) subclusters. Only one of 21 controls harboured an *E. coli* strain clustered in the A2 group. A significant difference was observed between the number of patients with chronic lesions and the number of controls harbouring *E. coli* strains of cluster A2 ($p < 0.003$). In contrast, more than half of patients (12/20) with early recurrent lesions harboured *E. coli* strains belonging to A1. However, no significant difference was observed between the number of patients harbouring these *E. coli* strains and controls ($p > 0.05$).

The ability of *E. coli* from CD patients and controls to adhere to differentiated Caco-2 cells is shown in fig 1. For a more accurate assessment of the results, we considered only one adherent strain when different isolates had the same pattern. Adhesion ability was found for 17 (62.9%) of the 27 *E. coli* strains belonging to subcluster A1, and nine (75.0%) of the 12 *E. coli* strains belonging to subcluster A2. Only 14 (48.2%) of the 29 *E. coli* strains tested from all the other clusters were adherent. Of these 14 adherent strains, seven were distributed in two clusters: cluster I, in which four of the five strains were adherent, and cluster L, in which three of the four strains were also adherent. In cluster A, 10 (90.9%) of the 11 *E. coli* strains isolated from patients operated on for chronic ileal lesions, 11 (73.3%) of the 15 strains isolated from patients with recurrent ileal lesions, and three (33.3%) of the nine control strains were adherent to Caco-2 cells.

Table 3 Distribution of ribotype profile groups among *E. coli* strains isolated from healthy and ulcerated mucosa of chronic ileal lesions of seven patients

Patient No	No isolates	No RT pattern-groups (No isolates)	
		Healthy mucosa	Ulcerated mucosa
1	7	16-A2 (3), 34-F (1)	16-A2 (2), 34-F (1)
2	3	27-C (1)	27-C (2)
3	4	22-B (1)	22-B (3)
4	4	16-A2 (2)	16-A2 (2)
5	2	13-A1 (1)	13-A1 (1)
6	2	21-A2 (1)	21-A2 (1)
7	6	20-A2 (4)	20-A2 (2)

Figure 2 Ribotype profiles of *Escherichia coli* strains isolated from healthy and ulcerated mucosa. *E. coli* strains were isolated from healthy (H) and ulcerated (U) mucosa from three patients operated on for Crohn's disease.

COMPARISON OF THE RIBOTYPE PATTERN OF *E. COLI* STRAINS ISOLATED FROM HEALTHY OR ULCERATED MUCOSA

This comparison was performed in seven patients operated on for CD in whom *E. coli* strains were isolated from biopsy specimens performed on healthy and ulcerated mucosa. Results are presented in table 3 and ribotype patterns are shown in fig 2. In all but one patient, the *E. coli* strains isolated from healthy or ulcerated mucosa had the same ribotype, indicating that a single strain colonised the intestinal mucosa. In one patient (patient No 1), *E. coli* isolates belonging to two different ribotypes were isolated from both healthy and ulcerated mucosa.

Discussion

E. coli is the predominant aerobic Gram negative species of the normal intestinal flora where it plays an important role in promoting the stability of the intestinal microbial flora and maintaining the normal intestinal physiology. By acquisition of well known virulence factors, such as production of enterotoxins and cytotoxins, tissue invasion, and adherence to enterocytes, *E. coli* strains become pathogenic and consequently are involved in intestinal diseases (for a review see Nataro and Kaper¹⁶).

E. coli may also be involved in the pathogenesis of CD, suggesting that *E. coli* strains isolated from patients with inflammatory bowel disease are qualitatively different from those found in normal faeces.^{2 9 17-19}

In this study, ribotyping was used to discriminate *E. coli* strains isolated in patients with CD from those in controls. Ribotyping has been demonstrated to be a useful method for examining similarity in isolates that are separated in time or space.²⁰ Restriction endonuclease *HindIII* was used as recommended by many authors to perform *E. coli* ribotyping.²¹⁻²⁴ The dendrogram generated by ribotyping revealed a large A cluster of genetically linked *E. coli* strains isolated more frequently from patients with chronic or recurrent CD than from controls ($p < 0.05$). In this cluster, we found *E. coli* strains isolated from 11/14 patients operated on for chronic ileal lesions (78.5%) and from 13/20 patients with recurrent ileal lesions (65%). Most patients operated on for chronic ileal lesions harboured *E. coli* strains belonging to cluster A ($p < 0.002$). This distribution was significantly different from that of controls ($p < 0.03$). In contrast, the prevalence of patients with early recurrent lesions harbouring *E. coli* strains belonging to this cluster was high. However, the difference between patients and controls was not significant ($p > 0.05$) as *E. coli* strains from seven patients were distributed in seven other different ribotype clusters.

Cluster A can be divided into two subclusters, A1 and A2. Of the *E. coli* strains isolated from 13 patients with early recurrent lesions of CD, strains from 12 (85.2%) patients were distributed in subcluster A1. In addition, *E. coli* strains isolated from eight patients with early recurrent lesions presented the same ribotype profile. Thus although *E. coli* strains associated with early recurrent lesions were widely distributed in the dendrogram, some strains presented either the same or a very close ribotype profile belonging to the same cluster. This may indicate the existence of genetically linked *E. coli* strains.

Patients with chronic ileal lesions harboured *E. coli* strains belonging to both subcluster A1 (4/14) and subcluster A2 (7/14). Compared with controls, the distribution of *E. coli* strains isolated from chronic ileal lesions in subcluster A2 was significant ($p < 0.003$). Thus 50% of patients with chronic lesions of CD had *E. coli* that were genetically related. The main difficulty with bacterial analysis of chronic lesion biopsy specimens is that luminal bacteria may secondarily colonise the ulcerated mucosa. Bacteria may adhere to a damaged mucosal surface through increased exposure of receptors or through non-specific interactions between bacterial proteins and the mammalian extracellular matrix.²⁵ *E. coli* strains associated with chronic lesions that were not genetically related could correspond to second invaders belonging to normal endogenous flora. We have shown that some *E. coli* strains isolated from chronic lesions of patients with CD synthesised Pap and/or Sfa and/or Dr adhesins, also found in strains associated with urinary tract infections.⁹ The presence of these strains

in CD patients could therefore reflect the persistence of potential uropathogenic *E. coli* belonging to the endogenous flora.

E. coli strains were compared in seven patients operated on for CD. Analysis was performed on biopsy specimens corresponding to healthy and ulcerated mucosa. For each of the seven patients tested, the *E. coli* strains presented the same ribotype profile, regardless of the state of the mucosa, indicating uniform colonisation of the healthy and ulcerated mucosa by the same strain(s). A similar observation was reported by Giaffer and colleagues¹⁷ who found that adhesive *E. coli* strains were as frequently isolated during acute exacerbations of CD and ulcerative colitis as when the disease was quiescent. This is consistent with the notion that altered glycoproteins or glycolipids on the intestinal mucosa may represent receptors for specific adhesins harboured by *E. coli* associated with CD.^{26 27} If such a hypothesis is correct, these receptors are likely to be expressed uniformly on the intestinal mucosa.

The aim of our study was to determine if a particular *E. coli* strain was involved either in the initiation or perpetuation of CD. It was not possible to identify a unique CD associated *E. coli* strain. Some patients with early recurrence of CD harboured *E. coli* strains that were genetically linked as they presented an identical ribotype profile or belonged to a common cluster of ribotype patterns. In addition, 50% of patients with chronic lesions harboured genetically related *E. coli* strains. Thus some genotypes were more likely than others to be associated with chronic or early recurrent ileal lesions of CD.

A correlation between particular evolutionary lineages and putative virulence factors is possible in that these *E. coli* strains would be involved in the initiation of lesions by expressing common virulence factors such as adhesive properties to the intestinal mucosa. Analysis of the ability of the genetically related *E. coli* strains of cluster A to adhere to differentiated Caco-2 intestinal cells indicated that 90.9% of the *E. coli* strains isolated from patients operated on for chronic ileal lesions and 73.3% of strains isolated from patients with recurrent ileal lesions were adherent. In contrast, among the *E. coli* strains isolated from biopsy or stool specimens of controls also belonging to cluster A, only 33.3% exhibited adhesion ability. These results indicate that the ability to adhere to intestinal cells is a common virulence factor harboured by most of the genetically related *E. coli* strains isolated from patients with active CD.

Most studies have focused on the phenotypic characterisation of *E. coli* isolated from CD patients or on the characterisation of previously described virulence factors encoding genes.^{17 18 28 29} This study shows the existence of *E. coli* strains that may have evolved from the same ancestral strain, perhaps by acquisition of additional virulence factors via gene transfer or insertion of a pathogenicity island(s) in the bacterial chromosome. These changes would increase the pathogenicity of some *E. coli* strains and therefore lead to the colonisation of the

intestinal mucosa of patients with CD by particular *E coli* strains. Further work using molecular studies based on DNA mapping of insertional DNA sequences present in *E coli* strains associated with CD but absent in *E coli* strains isolated from healthy individuals is needed to identify potentially unknown virulence determinants.

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