Microbes and Infection xxx (xxxx) xxx

Contents lists available at ScienceDirect

Microbes and Infection

journal homepage: www.elsevier.com/locate/micinf

Original article

Colonic adenoma-associated *Escherichia coli* express specific phenotypes

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ARTICLE INFO

Article history:
Received 3 December 2018
Accepted 2 February 2019
Available online xxx

Keywords:
Escherichia coli
ExPEC
Colonic adenomatous polyp
Virulence factors
Biofilm
Galleria mellonella

ABSTRACT

Specific *Escherichia coli* strains have been associated to colorectal cancer, while no data are available on genotypic and phenotypic features of *E. coli* colonizing premalignant adenomatous polyps and their pathogenic potential. This study was aimed at characterizing isolates collected from polyps and adjacent tissue in comparison with those from normal mucosa.

From colonoscopy biopsies, 1500 *E. coli* isolates were retrieved and genotyped; 272 were characterized for phylogroup and major phenotypic traits (i.e., biofilm formation, motility, hemolysins, and proteases). Selected isolates were analyzed for extraintestinal pathogenic *E. coli* (ExPEC)-associated virulence genes and in vivo pathogenicity using *Galleria mellonella*.

The majority of isolates collected from polyps were strong biofilm and poor protease producers, whereas those isolates from normal mucosa were highly motile, proteolytic and weak biofilm formers. Isolates from adjacent tissues shared features with those from both polyps and normal mucosa. Among selected *E. coli* isolates, ExPEC gene content/profile was variable and uncorrelated with the tissue of collection and larval mortality.

Despite the heterogeneous virulence-gene carriage of the *E. coli* intestinal population, *E. coli* colonizing colonic adenomatous polyps express specific phenotypic traits that could represent an initial pathoadaptation to local environmental changes characterizing these lesions.

Escherichia coli is the most well adapted bacterium within the human gut. Over 100 years of studies focused on *E. coli* but their classification is still challenging. The identification of specific genetic and/or phenotypic traits among all the different types of *E. coli* is useful to study the effects on human health [1,2]. The first strains to be classified in six distinct pathogenic categories were the intestinal pathogenic *E. coli* (InPEC), being mainly obligate pathogens [2,3]. The other two main groups include commensal and extraintestinal pathogenic strains (ExPEC). This latter group can cause most extraintestinal infections, due to the acquisition of several virulence traits responsible for their capability to colonize extraintestinal tissues [2]. Commensal strains, possessing few ExPEC-genes, represent the majority of the fecal flora of healthy subjects [4]. However, there is not a clear dividing line between commensals

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https://doi.org/10.1016/j.micinf.2019.02.001

and ExPEC being both part of the normal gut microbiota in healthy population [4,5].

According to the genome structure, *E. coli* were grouped into eight phylogenetic groups (A, B1, B2, C, D, E, F and clade I). Commensal strains usually belong to A and B1, whereas ExPEC derive predominantly from B2 and D [2]. Several studies indicated that the human gut is the reservoir for B2 and virulence should be considered as a by-product of commensalism [6,7].

Being part of this complex ecosystem, gut *E. coli* have been implicated in colorectal cancer (CRC) [8,9]. In CRC there is an overgrowth of *E. coli*, mainly B2, showing a high carriage of virulence genes, including those encoding toxins and effectors that trigger eukaryotic cell transformation [10]. This class of toxins includes: cycle-inhibiting factor, cytolethal distending toxins, cytotoxic necrotizing factors and colibactin, encoded by the polyketide synthase (*pks*) island [11,12]. Several data support *E. coli*-mediated carcinogenesis, although the direct link in CRC has not been proven yet [13,14]. We have previously demonstrated that *pks*-positive *E. coli* colonize adenomatous polyps at low frequency [15]. The aim of this study was to extend our knowledge on adenoma-associated *E. coli* by comparing genotypic/phenotypic traits and in vivo pathogenicity of isolates from polyp lesions and adjacent tissues with those from normal mucosa.

1. Material and methods

1.1. Patients' characteristics

The study was approved by the Ethics Committee of the University hospital "Umberto I" (protocol number 308/17). All participants provided written informed consent about the purpose of the research. Biopsies were collected from 20 patients presenting adenomatous polyp; 20 from polyps and 20 from adjacent tissue close to polyps (5–7 cm); 10 biopsies from normal mucosa of healthy subjects. The average age and the M/F ratio were 65/1.6 for patients, and 55/1.0 for control subjects, respectively. Polyps were in ascending colon (66.8%), transverse colon (22.2%), descending colon and cecum (5.5% each). Lesions (0.5–3 cm) were classified as tubular adenomas (83.0%), with low grade of dysplasia (94.4%). Exclusion criteria: familial polyposis, previous colon resection or CRC and inflammatory bowel disease (IBD).

1.2. *E. coli* colony isolation, genotyping and phylogrouping

Each lyzed biopsy was streaked onto MacConkey agar [16]. Thirty *E. coli* colonies per biopsy were chosen and identified by 16S rDNA gene sequencing [17]. Isolates were genotyped using RAPD and ERIC PCR combined profiles as previously described [15]. Phylogenetic grouping was performed by quadruplex PCR [18]. ExPEC virulence genes presence was assessed by single PCR assays [11,19]. Each isolate was assigned a virulence factor (VF) score [20].

1.3. Phenotypic assays

Production of hemolysin(s) was assessed using blood agar plates (bioMérieux), whereas proteolysis and swimming motility using skim milk agar (SMA) plates and 0.3% agar plates, respectively [21]. Swim zones (cm^2) were evaluated using ImageJ software [22]. Biofilm formation was measured as previously reported [23,24].

1.4. Measurement of mortality rates in larval infection

The putative virulence of selected isolates was evaluated using the *Galleria mellonella* infection model [25]. No ethical approval was required because there was no use of a mammalian model of infection and animal house. Three doses (10^4–10^6 CFU) were administrated and the number of dead caterpillars was scored 24 h post-infection. The dose (CFU/larva) needed to kill 50% of larvae (LD_{50}) was calculated using nonlinear regression model.

1.5. Hierarchical clustering and statistical analyses

Data from *E. coli* phenotypic assays were combined in a matrix for calculation of samples' distance similarity according to the Jaccard index using the "vegdist" function (vegan R package) [26]. Samples were clustered hierarchically according to unweighted pair group method with arithmetic mean (UPGMA) method by "hclust" function (stats R package).

Permutational MANOVA (PERMANOVA) test was performed using the "adonis" function (vegan R package) with 999 permutations. *P*-values were calculated using the Wilcoxon rank-sum test and corrected for multiple hypothesis controlling the False Discovery Rate (FDR) (stats R package) [27]. Occurrence of VF genes among phylogroups was compared by Fisher's exact test. Differences among groups were measured by the Mann–Whitney U test or by ANOVA followed by the Tukey's multiple comparisons post-test. Relationship between LD_{50} and VF score was assessed calculating the Spearman's r coefficient. A *P*-value <0.05 was considered as statistically significant.

2. Results

2.1. Phylogroups A and B2 are prevalent among *E. coli* isolates

A total of 1500 *E. coli* isolates were obtained from biopsies of patients who underwent colonoscopy at the University hospital "Umberto I" of Rome: 600 from adenomatous polyp lesions (polyps), 600 from adjacent non-adenomatous tissues (adjacent tissue), and 300 from healthy normal mucosa (normal mucosa). According to RAPD and ERIC PCR combined profiles, the 1500 *E. coli* isolates represented 272 different clones, with an approximate mean of 5 clones per tissue. The analysis of phylogroup distribution among the 272 isolates showed that phylogroup A was the most prevalent (Table 1), in agreement with previous data on human intestinal *E. coli* [1,28,29]. The second most prevalent phylogroup was B2, followed by D, typically observed in industrialized countries [28]. Vice versa, the less prevalent phylogroups were B1 and F.

2.2. Biofilm-forming activity is associated with isolates collected from polyps

Biofilm represents a strategy to establish persistent infections [30] therefore, the biofilm-forming ability of each isolate was measured. Isolates collected from both polyps and adjacent tissue produced higher amounts of biofilm than those from normal mucosa which showed no or low biofilm-forming activity (median values: 0.18, 0.25 and 0.11, respectively; FDR-corrected $P = 0.0018$ and $P = 0.0004$, respectively), regardless of the phylogroup analyzed (Fig. 1A). These results suggest that polyps exert a positive selection for *E. coli* biofilm-producers.

2.3. *E. coli* colonizing polyps show a low degree of motility

Bacterial motility is commonly considered a virulence factor in *E. coli* where it mediates both bacterial adhesion and deep tissue colonization [31]. Swimming motility assays showed that isolates colonizing polyps and adjacent tissue were less motile in comparison to those collected from normal mucosa (median values: 1, 1 and 3, respectively; FDR-corrected $P = 0.0399$ and $P = 0.0008$, respectively) and that this phenotype was mainly associated with

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Table 1
Distribution of *E. coli* isolates displaying unique genotypic profiles according to phylogroup assignments.

E. coli phylogroup [N (%)]						
Site of collection	A	B1	B2	D	F	Total
Normal mucosa	22 (35,5)	11(17,7)	12 (19,4)	8 (12,9)	9 (14,5)	62 (100)
Adjacent tissue	46 (47,4)	4 (4,1)	30 (30,9)	12 (12,4)	5 (5,2)	97 (100)
Polyps	43 (38,1)	11 (9,7)	35 (31,0)	16 (14,2)	8 (7,0)	113 (100)
Total	111 (40,8)	26 (9,6)	77 (28,3)	36 (13,2)	22 (8,1)	272 (100)

isolates belonging to A and D phylogroups (median values for phylogroup A: 1.5, 2 and 2, FDR-corrected $P = 0.0186$ and $P = 0.0564$ [raw $p = 0.0376$], respectively; median values for phylogroup D: 0, 0 and 2, respectively; FDR-corrected $P = 1{,}24e\text{-}05$ and $P = 0.0004$, respectively) (Fig. 1B).

Isolates belonging to phylogroup B1 collected from normal mucosa and polyps showed the highest degree of motility in comparison to isolates belonging to the other phylogroups (median values: 3 and 3; FDR-corrected $P < 0.01$ and $P < 0.05$, respectively). B1 isolates collected from adjacent tissue showed a significantly higher motility in comparison to isolates belonging only to phylogroups D and F (median values: 2.5, 0 and 0, respectively; FDR-corrected $P = 0.0074$ and $P = 0.0226$, respectively) (Fig. 1B). Overall these results indicate a tight link between phylogroup B1 and the ability to move.

2.4. B2 *E. coli* colonizing polyps exhibit hemolytic activity but poor proteolytic one

The presence of toxins and proteolytic enzymes is a common feature among pathogenic *E. coli* [12,14]. A higher number of proteolytic isolates were collected from normal mucosa and adjacent tissue in comparison to those from polyps (normal mucosa = 22/62, 35.5%; adjacent tissue = 28/97, 28.9%; polyps = 17/113, 15%; FDR-corrected $P = 0.0089$ and $P = 0.0331$, respectively). Among phylogroups, proteolytic B2 *E. coli* were retrieved exclusively from normal mucosa (normal mucosa = 8/12, 66%; adjacent tissue = 0/30, 0%; polyps = 0/35, 0%; FDR-corrected $P = 5e\text{-}07$, respectively), while proteolytic A and F isolates were collected also from adjacent tissue (Fig. 1C).

The screening for the production of toxins revealed a total of 6.6% (18/272) of hemolysin(s)-producing isolates, mainly belonging to phylogroup B2 collected from both polyps and adjacent tissue (9/18, 50.0% and 8/18, 44.4%, respectively) (Fig. 1D). This result corroborates previous data describing the hemolytic activity of B2 *E. coli* [32]. Altogether, these results led us to suggest that proteolytic activity is a feature of *E. coli* isolates residing in the healthy human mucosa while hemolytic activity represents a characteristic of *E. coli* colonizing colon polyps.

2.5. Specific genotypic and phenotypic *E. coli* traits are associated with adenomatous polyps

Based on *E. coli* phenotypes, a hierarchical clustering analysis grouped the isolates into two main clades, I (84 isolates) and II (40 isolates) according to the site of collection ($P < 0.0001$, PERMANOVA) (Fig. 2). Clade I included principally motile to hyper-motile isolates (70/84, 83.3%) and no to low biofilm-forming *E. coli* (69/84, 82.2%). Within this clade, isolates collected from normal mucosa grouped principally into two closely related subclusters, I-2.1.2 and I-2.2 (16 out of a total of 25 isolates, 64%). Vice versa, the more homogeneous clade II clustered isolates collected from adjacent tissue (19/40) and polyps (21/40) were characterized by the lack of motility (40/40, 100%) and by moderate to strong biofilm forming activity (18/40, 45%). Proteolytic *E. coli* were mainly isolated from normal mucosa (10/28, 36%) and adjacent tissue (10/28, 36%) in comparison to those collected from polyps (7/28, 25%), within subclusters I-2.2 and II-1 (Fig. 2).

2.6. *E. coli* possessing high VF scores are not correlated with increased larval mortality

To determine the intrinsic pathogenicity potential of our *E. coli* collection, 2–4 isolates representative for each main cluster of the dendrogram were selected and analyzed for the presence of ExPEC virulence genes (N = 22) (Fig. 2 and Table 2). Twenty major VFs were chosen and a VF score was calculated for each isolate. Results showed that 45.5% (10/22) of isolates possess a VF score $\geq$10 (Table 2) distributed into 50.0% (5/10) from polyps, 30% (3/10) from adjacent tissue and 20% (2/10) from normal mucosa. Phylogroup B2 accounted for the highest number of isolates with a VF score $\geq$10 (8/10, 80%, $P = 0.0034$).

The *E. coli* in vivo pathogenicity was investigated using the *G. mellonella* infection model. Strain MG1655 was selected as negative control, whereas strain IHE3034 was included as colibactin-producer control. A range of inoculum doses of each *E. coli* isolate was used to infect larvae and mortality rates (LD_{50} values) were evaluated at 24 h post-infection. No reproducible results were obtained for isolate A1C and, therefore, it was excluded from further analyses. Despite some degree of heterogeneity in LD_{50}, 15/21 isolates (71.4%) showed a $LD_{50} < 10^6$ CFU/larva. Strain MG1655 exhibited a LD_{50} of $10^{14.3}$ CFU/larva confirming poor virulence [33], while, unexpectedly, strain IHE3034 exhibited poor larval killing activity (data not shown). No statistically significant difference was observed according to the site of collection or phylogroup and LD_{50} values (Fig. 3A and data not shown). Moreover, no relationship was found between VF score and LD_{50} values (Fig. 3B), indicating that a high VF score does not necessarily correlate with larval mortality. Based on the presence/absence of the analyzed VF genes, we identified 14 different gene profiles, including 8 unique and 6 sharing from 3 to 15 common genes (Fig. 3C and Table 2). Four pairs of isolates, each possessing the same profile, displayed similar LD_{50} values. Differently, isolates H21C, H20P, H8C and H30P sharing the same profile showed heterogeneous LD_{50} values as isolate P5 sharing the same profile with MG1655 (Fig. 3C). These findings indicate that larval mortality depends on an array of virulence genes set in a specific genetic background.

3. Discussion

There is a growing body of evidence supporting the involvement of microorganisms in gut pathological disorders, including cancer and IBD [34]. Several studies support the association between *E. coli* and CRC, emphasizing a reconfiguration of the *E. coli* population in response to colonic environmental changes [14,32,35]. Therefore, the aim of the present study was to provide insights into *E. coli* colonizing adenomatous polyps for which there is a paucity of the current literature.

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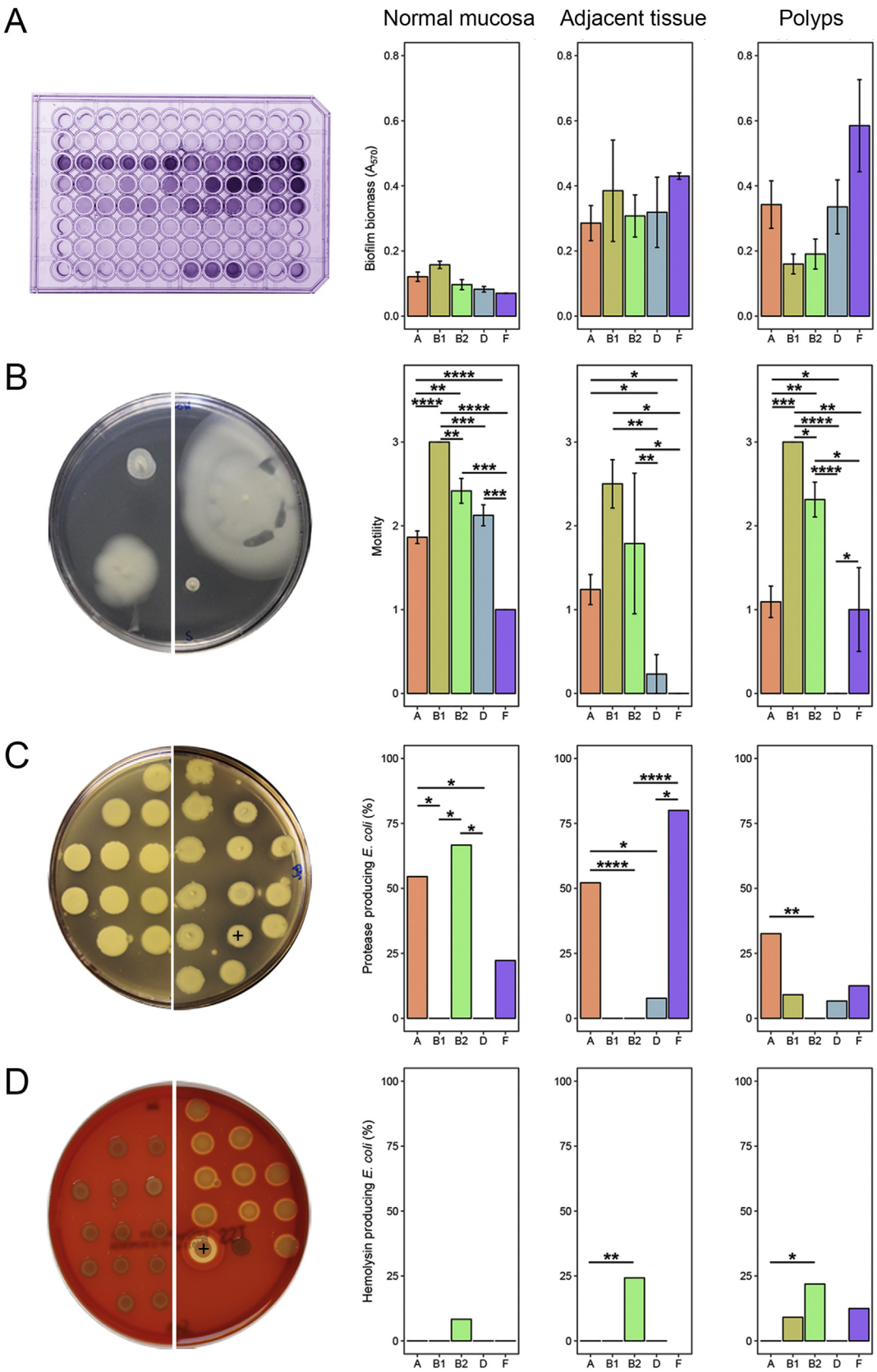

Fig. 1. Phenotypic traits of *E. coli* isolates. (**A**) *E. coli* biofilm producers. Representative image of the microtiter plate method showing biofilm formation differentiated by crystal violet stain in 96-well tissue culture plate (left) and relative distribution of isolates according to phylogroup, reported in the x-axis of the histogram, and site of collection (right). Isolates were arbitrarily grouped according to A_{570} values: no biofilm producer ($\leq$0.06), weak biofilm producer (0.06–0.12), moderate biofilm producer (0.12–0.24), strong biofilm producer (>0.24). (**B**) *E. coli* swimming motility. Representative image of motility phenotype on soft agar plate (left) and relative distribution of isolates according to phylogroup, reported in the x-axis of the histogram, and site of collection (right). Isolates were arbitrarily grouped according to the size of swim zones: 0, non-motile (<0.2 cm^2, lower right), 1,

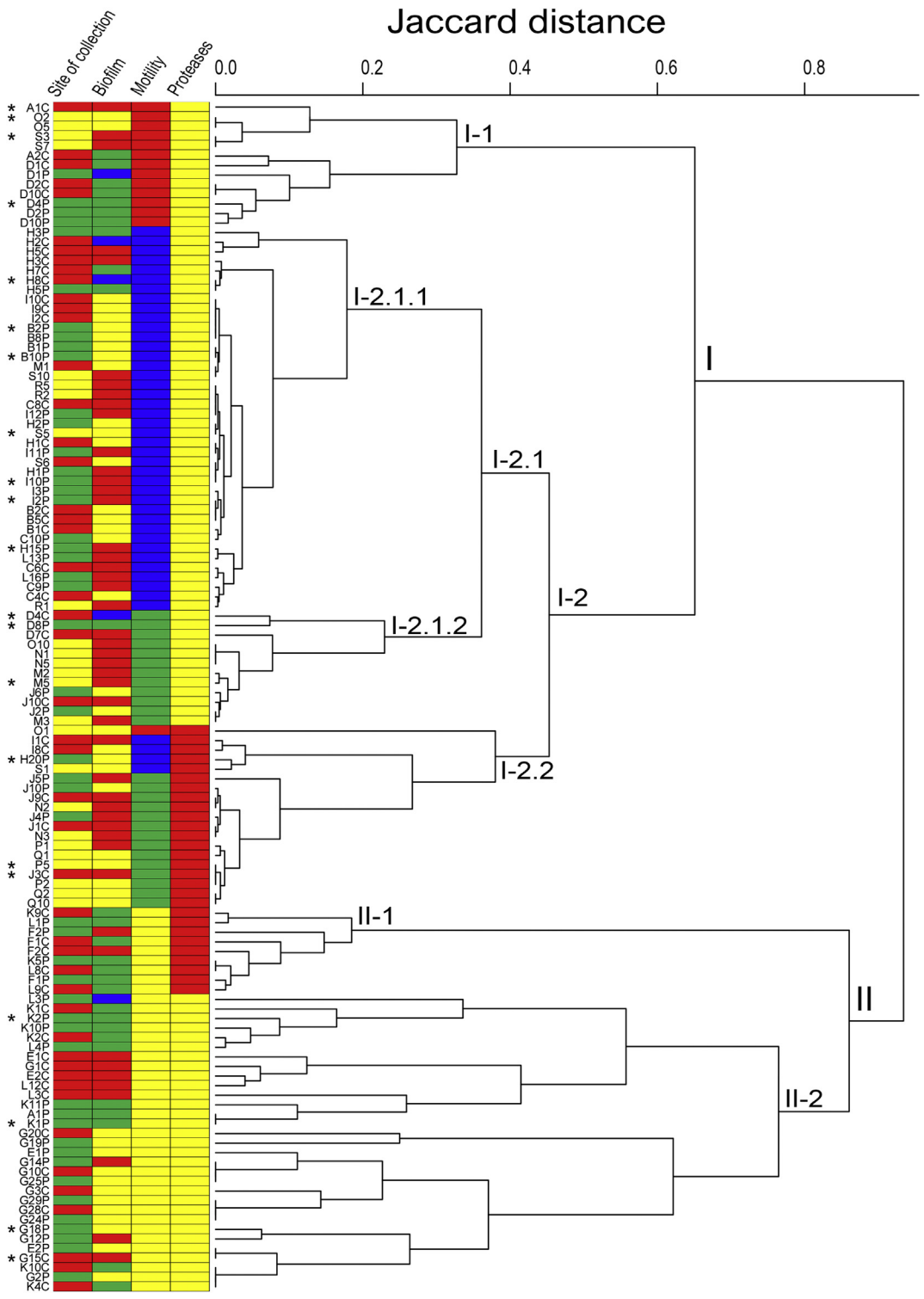

Fig. 2. Hierarchical clustering analysis of phenotypic traits of *E. coli* isolates. At least two representative isolates for each group (N from 272 to 124) were included. The dendrogram is divided into two main clades (I and II), with relevant subclusters indicated by numbers. The different phenotypes are box colored as following: yellow, absence or lowest; red, presence or low; green, medium and blue, high. *E. coli* isolate identifications are listed on the left. Asterisks indicate isolates selected for virulence gene screening and in vivo testing.

low motile (0.2−0.8 cm^2, upper left), 2, motile (0.8−5 cm^2, lower left) and 3, hyper-motile (>15 cm^2, upper right). (**C**) *E. coli* protease producers. Representative image of protease producing *E. coli* isolates spotted onto SMA plates (left) and relative distribution of isolates according to phylogroup, reported in the x-axis of the histogram, and site of collection (right). (**D**) *E. coli* hemolysin producers. Representative image of hemolysin producing *E. coli* isolates spotted onto blood agar plates (left) and relative distribution of isolates according to phylogroup, reported in the x-axis of the histogram, and site of collection (right). Positive controls (+): *E. coli* strain MG1655 (ATCC 47076) for proteolysis, *E. coli* strain J96 (ATCC 700336) for hemolysis. For each assay, three independent experiments, at least in duplicate, were performed. *FDR-corrected $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, Wilcoxon rank-sum test.

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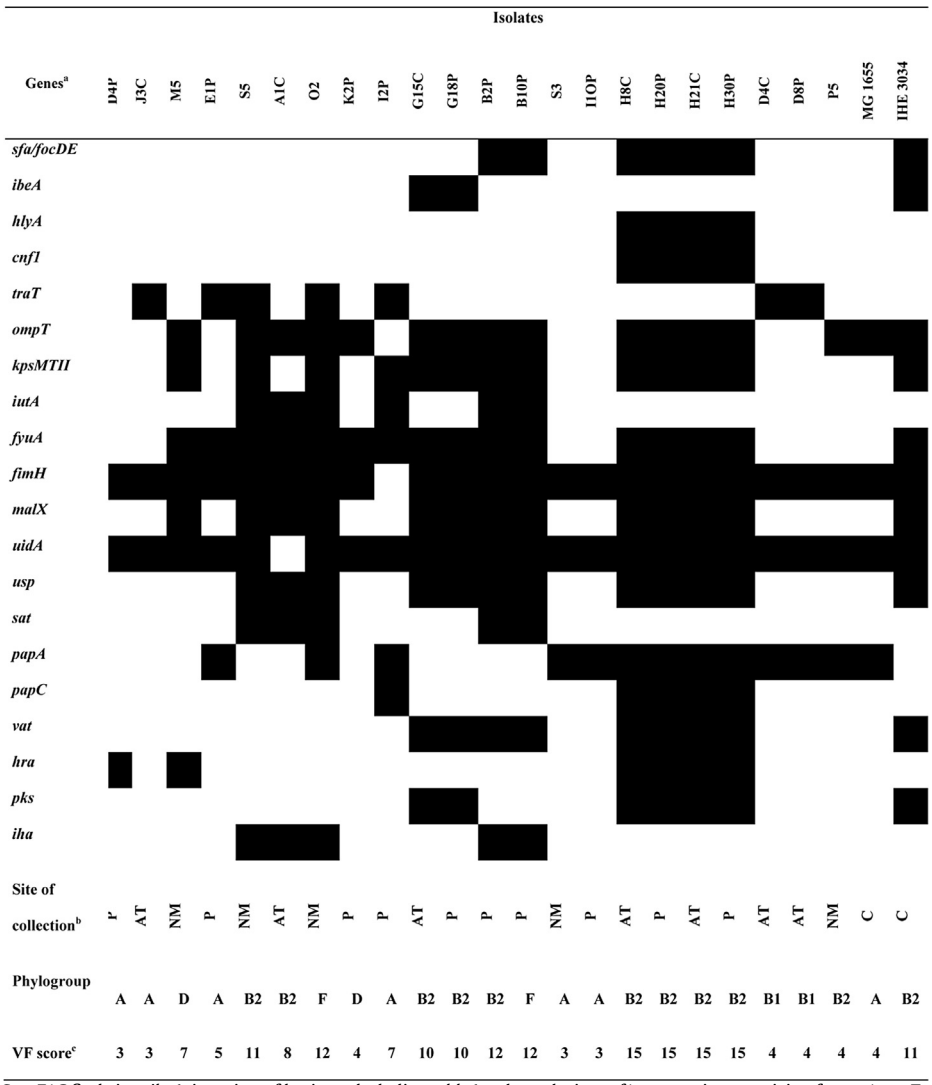
Table 2
Distribution of virulence factor (VF) genes among selected *E. coli* isolates. The absence and the presence of a gene is indicated by empty and filled boxes, respectively.



[a]Gene definitions: *sfa/focDE*, S or F1C fimbriae; *ibeA*, invasion of brain endothelium; *hlyA*, α-hemolysin; *cnf1*, cytotoxic necrotizing factor 1; *traT*, serum resistance-associated; *ompT*, outer membrane protease T; *kpsMII*, group 2 capsule; *iutA*, aerobactin (siderophore) receptor; *fyuA*, yersiniabactin (siderophore) receptor; *fimH*, type 1 fimbriae; *malX*, pathogenicity island marker; *uidA*, β-glucoronidase; *usp*, uropathogenic-specific protein; *sat*, secreted autotransporter toxin; *papA*, P fimbrial structural subunit; *papC*, P fimbrial assembly subunit; *vat*, vacuolating toxin; *hra*, heat-resistant agglutinin; *pks*, polyketide synthase pathogenicity island; *iha*, adhesin-siderophore.
[b]Site of collection: NM, normal mucosa; AT, adjacent tissue; P, polyps; C, control strains.
[c]Virulence factor (VF) score is calculated as the total number of VF genes detected in each isolate.

Large-scale epidemiological studies on commensal *E. coli* populations in industrialized countries revealed the prevalence of groups A and B2, including colibactin-producing strains. *E. coli* phylogroup distribution depends on several variables such as lifestyle, diet and hygiene status, with fluctuations in the ratio between A and B2, while others are more rare [29]. Our data indicate a prevalence of phylogroup A, followed by B2 in all the tissues analyzed. Since the distribution of phylogroups was not statistical significant different among tissues, we decided to analyze isolates for distinctive phenotypic properties. Indeed, we found that isolates collected from normal mucosa were motile and characterized by proteolytic activity, at significantly higher levels than isolates retrieved from other sites of collection (Figs. 1 and 2). Vice versa, isolates recovered from both polyps and adjacent tissue were strong biofilm producers, poorly proteolytic and less motile when compared with those from normal mucosa, although more proteolytic isolates were collected from adjacent tissue than those from polyps. Adenomatous polyps are gland-like growths that develop on the mucous membrane in which the intestinal epithelium is replaced by a low-grade dysplastic epithelium, leading to changes affecting glands and cells and decreasing mucin production [36]. Based on the phenotypic differences observed in isolates from polyps, it can be speculated a progressive adaptation of *E. coli* population as a consequence of the environmental changes of the mucosa or a selection of more adapted strains within this new environment. This hypothesis is in line with previous observations on selective colonization of cancer lesions by *E. coli* strains that might benefit from the changed conditions within these lesions, such as the decreased oxygen level [37]. Additionally, it has been reported that CRC-associated polymicrobial bacterial biofilms contribute metabolically to epithelial changes relevant to oncogenic progression [38]. Therefore, the establishment of biofilm-forming *E. coli* in polyps and adjacent tissue could represent a step for tumor-promoting conditions. The dramatic decrease in

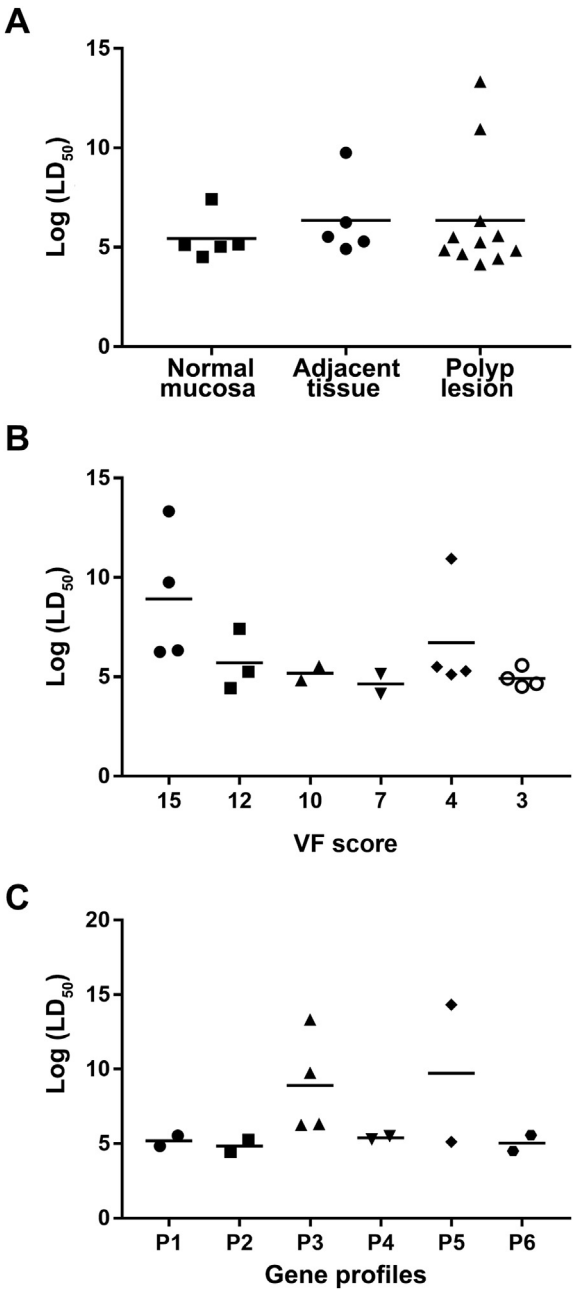


Fig. 3. Relationship between in vivo virulence and site of collection, VF score or gene profiles. Twenty-one *E. coli* isolates were collected from normal mucosa (n = 5), adjacent tissue (n = 5), or polyps (n = 11). (**A**) In vivo virulence was assessed in *G. mellonella* wax moth larvae and expressed as lethal dose (CFU/larva) needed to kill 50% of the infected larvae (LD_{50}). All killing assays were performed at least twice in duplicate using larvae (N = 20/group) from different batches. (**B**) Each isolate was assigned a virulence factor (VF) score, calculated as the number of virulence factors detected [20]. (**C**) Six shared virulence gene profiles were found: profile 1 (P1), strains G15C and G18C; profile 2 (P2), strains B2P and B10P; profile 3 (P3), strains H21, H20P, H8C and H30P; profile 4 (P4), strains D8P and D4C; profile 5 (P5), strains P5 and MG1655; and profile 6 (P6), strains S3 and I10P. No statistically significant difference was found at ANOVA followed by Tukey's multiple comparisons post-test ($P > 0.05$).

swimming motility in those biofilm-forming isolates could be due to the functional and structural adaptation of flagella as previously described for other bacterial pathogens [39]. Therefore, these opposite phenotypes shown by *E. coli* from both polyps and adjacent tissue could allow them to reside through the development of a productive biofilm.

The high number of proteolytic *E. coli* retrieved from normal mucosa and adjacent tissue could be related to the production of beneficial short chain fatty acids (SCFAs) from protein breakdown in the large intestine [40,41]. SCFAs have several effects on epithelial growth, differentiation and metabolism [42]. Therefore, it seems that the proteolytic phenotype of *E. coli* is associated with its role as a commensal inhabitant of the human gut. Vice versa, the low number of proteolytic *E. coli* observed in the polyps suggests that this metabolic characteristic is no longer required for isolates living within this modified environment (i.e., modification of the intestinal epithelium and reduction of mucus layer).

To further characterize the *E. coli* population associated with adenomatous polyps, selected isolates were analyzed for the carriage of ExPEC genes and in vivo virulence. B2 isolates showed the highest number of virulence genes, confirming their ability to tailor the genome, by gene loss and acquisition, to match the most favorable conditions for colonization and persistence in the human gut [2,43]. Although the majority of isolates displayed in vivo pathogenicity, results failed to highlight the correlations between LD_{50} values and the site of collection, phylogroup, VF score or gene profile. The lack of relationship between VF and in vivo pathogenicity was previously reported for both commensal and clinical *E. coli* isolated from healthy animals or tested using the larval model [44,45]. Since isolates with the most heterogeneous genetic backgrounds were included in this analysis, it cannot be ruled out that the presence of other uninvestigated/unknown gene(s), alone or in combination, transcriptional regulators or two-component systems might be required for full expression of virulence genes.

Overall, this study provides new data regarding the genotypic and phenotypic characterization of *E. coli*-associated with adenomatous polyps. Herein, we propose that polyps and, to a lesser extent, adjacent tissue contribute to new local environmental conditions that induce an adaptation of associated *E. coli* or a selection of isolates carrying specific traits required to best fit within these lesions. These specific traits include improved biofilm formation and poor proteolytic activity possibly representing a shift from commensalism to pathogenicity, a typical adapting behavior of *E. coli* [2,46]. Although specimens were retrieved from different subject cohorts, it should be considered that all subjects enrolled for this study live in the same area, share the Mediterranean diet and lifestyle. Regardless of this limitation, we believe that the phenotypic differences observed among isolates colonizing the different tissues reflect a change in the *E. coli* population associated with adenomatous polyps. Additional studies will strengthen the role of *E. coli* specific phenotypic traits as potent predictors for *E. coli* pathoadaptation within the adenomatous polyp environments.

Conflict of interest statement

The authors declare no conflict of interest.

Author contributions statement

CA, MS and DS conceived and designed the work. CA, MS, MRA, ArP, GDB, AnP, FS and DS performed the experiments. MS, CA, ArP, GDB, FS and DS analyzed the data. CZ and MN contributed to reagents/materials/analysis tools. CA, MS, DS, GDB and ATP wrote the manuscript. All authors contributed to manuscript revision and approved the submitted version.

Financial disclosure statement

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial

interest in or financial conflict with the subject matter or materials discussed in the manuscript.

Acknowledgments

We would like to thank GL Scoarughi for his indispensable help for bacterial plate photos and figure revision. We thank JP Nougayrède for the kind gift of strain IHE3034. This study was supported by Ministero della Salute DGFDM (Direzione generale dei dispositivi medici e del servizio farmaceutico) to ATP. Salaries of CA and DS were supported by the Dani Di Giò Foundation-Onlus, Rome, Italy.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2019.02.001.

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