E. coli diversity: low in colorectal cancer

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Abstract

Background: Escherichia coli are mostly commensals but also contain pathogenic lineages. It is largely unclear whether the commensal E. coli as the potential origins of pathogenic lineages may consist of monophyletic or polyphyletic populations, elucidation of which is expected to lead to novel insights into the associations of E. coli diversity with human health and diseases.

Methods: Using genomic sequencing and pulsed field gel electrophoresis (PFGE) techniques, we analyzed E. coli from the intestinal microbiota of three groups of healthy individuals, including preschool children, university students, and seniors of a longevity village, as well as colorectal cancer (CRC) patients, to probe the commensal E. coli populations for their diversity.

Results: We delineated the 2280 fresh E. coli isolates from 185 subjects into distinct genome types (genotypes) by PFGE. The genomic diversity of the sampled E. coli populations was so high that a given subject may have multiple genotypes of E. coli, with the general diversity within a host going up from preschool children through university students to seniors. Compared to the healthy subjects, the CRC patients had the lowest diversity level among their E. coli isolates. Notably, E. coli isolates from CRC patients could suppress the growth of E. coli bacteria isolated from healthy controls under nutrient-limited culture conditions.

Conclusions: The coexistence of multiple E. coli lineages in a host may help create and maintain a microbial environment that is beneficial to the host. As such, the low diversity of E. coli bacteria may be associated with unhealthy microenvironment in the intestine and hence facilitate the pathogenesis of diseases such as CRC.

Keywords: Escherichia coli, Genetic diversity, Intestinal microbiota, Colorectal cancer, Longevity

Background

Escherichia coli had been generally known as commensal bacterial components of the normal microbiota in the gastrointestinal tract of humans and animals until the 1940s, when a variety of pathogenic strains began to be reported [1]. Pathogenic E. coli have different types, causing intestinal (see a nice review in [2]) or extra-

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the commensals, as potential evolutionary origins of the emerged and emerging pathogens, consist of monophyletic or polyphyletic E. coli populations is unclear.

In this study, we collected 2280 E. coli isolates from fresh fecal specimens of healthy individuals in three age groups, who did not have intestinal or extra-intestinal illnesses, and of CRC patients for comparative studies. Based on their genomic differences, we delineated the bacteria into discrete genomic types (genotypes). In general, the analyzed E. coli isolates were highly diverse, with one genotype having from several hundred to over a thousand genes not found in any other types compared and a particular participant harboring from a single to multiple genotypes of E. coli. Remarkably, the diversity went up with age in the groups of healthy subjects, from preschool children through university students to seniors of a longevity village, but the CRC patients of all ages had the lowest diversity. These results suggest that the coexistence of multiple benign E. coli lineages in the same microbiota may help create a beneficial microbial environment for human health.

Methods

Bacterial strains

Fecal specimens were collected from 68 preschool children of Yifu Kindergarten, three to six years old; 87 university students of Harbin Medical University, 17 to 22 years old; 15 senior people of Bama Longevity Village, Guangxi Province, 90 to 106 years old; and 15 colorectal cancer (CRC) patients from the Second Affiliated Hospital of Harbin Medical University, 34 to 77 years old (Table 1). The healthy participants did not have intestinal or extra-intestinal illnesses, and the CRC patients were diagnosed by experts of the Second Affiliated Hospital of Harbin Medical University. The participants of all four groups did not use any antimicrobials in the past 6 months prior to specimen collection. We obtained a written informed consent from each participant or their guardian and the present work was approved by the Ethics Committee of Harbin Medical University. All experiments were performed in accordance with relevant guidelines and regulations, consistent with the 1975 Declaration of Helsinki. For isolating the bacteria, we spread 30 μl X-gal (20 mg/ml) on the LB plate [24] and purified the bacteria by streaking a single blue colony on fresh LB plates. Bacterial identification was performed at the Clinical Laboratory of the Second Affiliated Hospital, Harbin Medical University, and all bacterial strains were confirmed to be E. coli before analyses. The bacterial strains were stocked at -80 °C in 25% glycerol and streaked on an LB plate for another round of single colony isolation by incubation at 37 °C overnight prior to use.

Table 1 E.coli genotype profiles of participants

| No. | Participant ID | Sex | Age | Number of Genotypes |
|-----|----------------|-----|-----|---------------------|
| 1   | WZW            | Male| 5   | 1                   |
| 2   | ZYC            | Male| 5   | 2                   |
| 3   | WSC            | Male| 6   | 1                   |
| 4   | JZH            | Female| 5 | 1                  |
| 5   | XZM            | Male| 6   | 1                   |
| 6   | XHR            | Female| 6 | 1                  |
| 7   | SYF            | Male| 4   | 2                   |
| 8   | ZJH            | Female| 6 | 1                  |
| 9   | HJB            | Male| 5   | 2                   |
| 10  | CYF            | Female| 3 | 2                  |
| 11  | SRH            | Male| 3   | 2                   |
| 12  | LBW            | Male| 4   | 3                   |
| 13  | WYB            | Female| 3 | 1                  |
| 14  | WYH            | Female| 3 | 1                  |
| 15  | GB             | Male| 4   | 2                   |
| 16  | KK             | Male| 6   | 1                   |
| 17  | ZWH            | Female| 4 | 1                  |
| 18  | CEY            | Female| 4 | 2                  |
| 19  | HYX            | Male| 3   | 1                   |
| 20  | QMZ            | Male| 3   | 1                   |
| 21  | LMQ            | Male| 4   | 1                   |
| 22  | XWR            | Female| 6 | 1                  |
| 23  | YPY            | Male| 5   | 2                   |
| 24  | SBW            | Male| 4   | 2                   |
| 25  | ZAK            | Female| 4 | 3                  |
| 26  | LJY            | Female| 6 | 1                  |
| 27  | ZZJ            | Male| 5   | 3                   |
| 28  | XHS            | Male| 4   | 1                   |
| 29  | LZT            | Male| 5   | 1                   |
| 30  | HYH            | Female| 5 | 1                  |
| 31  | ZYJ            | Female| 5 | 1                  |
| 32  | JYH            | Female| 5 | 4                  |
| 33  | TEZ            | Male| 3   | 1                   |
| 34  | WYF            | Female| 5 | 1                  |
| 35  | XZH            | Male| 6   | 3                   |
| 36  | ZHL            | Male| 4   | 3                   |
| 37  | QJY            | Male| 6   | 1                   |
| 38  | ZXZ            | Female| 4 | 2                  |
| 39  | DYL            | Female| 4 | 2                  |
| 40  | YPY            | Female| 6 | 2                  |
| 41  | LTW            | Male| 5   | 3                   |
| 42  | CPY            | Male| 6   | 2                   |
### Table 1 E. coli genotype profiles of participants (Continued)

| No. | Participant ID | Sex | Age | Number of Genotypes<sup>a</sup> |
|-----|----------------|-----|-----|---------------------------------|
| 43  | WJS            | Male| 6   | 1                               |
| 44  | HBY            | Male| 5   | 3                               |
| 45  | GRY            | Female| 3 | 2                               |
| 46  | LEQ            | Female| 3 | 2                               |
| 47  | LZY            | Male| 4   | 1                               |
| 48  | XWQ            | Female| 4 | 2                               |
| 49  | WZH            | Male| 6   | 3                               |
| 50  | JYX            | Male| 5   | 2                               |
| 51  | YCX            | Female| 3 | 1                               |
| 52  | ZJH            | Female| 3 | 1                               |
| 53  | PJY            | Male| 6   | 1                               |
| 54  | LXZ            | Male| 5   | 1                               |
| 55  | SYZ            | Male| 3   | 1                               |
| 56  | FSK            | Female| 3 | 1                               |
| 57  | WC             | Male| 3   | 2                               |
| 58  | RHM            | Female| 6 | 1                               |
| 59  | CBC            | Female| 4 | 1                               |
| 60  | ZMX            | Female| 5 | 2                               |
| 61  | WX             | Female| 3 | 1                               |
| 62  | WJT            | Female| 3 | 3                               |
| 63  | LXY            | Female| 4 | 2                               |
| 64  | YQT            | Female| 3 | 1                               |
| 65  | LZR            | Male| 3   | 2                               |
| 66  | TZL            | Male| 5   | 1                               |
| 67  | YZY            | Female| 5 | 1                               |
| 68  | ZYJ            | Female| 4 | 1                               |

**Students**

| No. | Participant ID | Sex | Age | Number of Genotypes<sup>a</sup> |
|-----|----------------|-----|-----|---------------------------------|
| 1   | YZY            | Female| 18 | 2                               |
| 2   | WB             | Male| 17  | 5                               |
| 3   | YX             | Male| 17  | 2                               |
| 4   | DSJ            | Female| 21 | 1                               |
| 5   | AB             | Male| 19  | 5                               |
| 6   | TWX            | Male| 19  | 3                               |
| 7   | MP             | Male| 18  | 4                               |
| 8   | ZGQ            | Female| 20 | 6                               |
| 9   | YX             | Male| 20  | 7                               |
| 10  | WHY            | Female| 19 | 4                               |
| 11  | FCL            | Female| 20 | 3                               |
| 12  | HSJ            | Female| 19 | 2                               |
| 13  | ZJL            | Female| 20 | 4                               |
| 14  | GMQ            | Female| 22 | 6                               |
| 15  | ZSY            | Female| 21 | 1                               |
| 16  | TZL            | Male| 20  | 7                               |

<sup>a</sup> Number of genotypes refers to the number of different genotypes identified for each participant.

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**Students**

| No. | Participant ID | Sex | Age | Number of Genotypes<sup>a</sup> |
|-----|----------------|-----|-----|---------------------------------|
| 17  | LL             | Female| 20 | 5                               |
| 18  | SMN            | Female| 20 | 3                               |
| 19  | LY             | Female| 19 | 3                               |
| 20  | YZR            | Female| 20 | 4                               |
| 21  | CPP            | Female| 21 | 4                               |
| 22  | CH             | Male| 21  | 1                               |
| 23  | WQY            | Male| 19  | 2                               |
| 24  | HQ             | Female| 19 | 1                               |
| 25  | FC             | Male| 22  | 3                               |
| 26  | ZS             | Female| 21 | 6                               |
| 27  | MWH            | Female| 19 | 4                               |
| 28  | SQ             | Male| 22  | 4                               |
| 29  | LSY            | Female| 18 | 3                               |
| 30  | YXX            | Male| 20  | 3                               |
| 31  | ZYX            | Male| 20  | 3                               |
| 32  | FYJ            | Female| 22 | 2                               |
| 33  | MHY            | Female| 21 | 7                               |
| 34  | XXM            | Female| 19 | 2                               |
| 35  | BJ             | Female| 18 | 2                               |
| 36  | CS             | Male| 21  | 4                               |
| 37  | SX             | Female| 21 | 1                               |
| 38  | CYB            | Female| 21 | 5                               |
| 39  | LHM            | Female| 21 | 2                               |
| 40  | LYY            | Female| 20 | 2                               |
| 41  | WRM            | Female| 19 | 2                               |
| 42  | WY             | Male| 19  | 2                               |
| 43  | LXL            | Female| 22 | 1                               |
| 44  | WYJ            | Female| 19 | 2                               |
| 45  | LHC            | Male| 20  | 7                               |
| 46  | SR             | Female| 20 | 3                               |
| 47  | HL             | Female| 21 | 2                               |
| 48  | CYJ            | Female| 22 | 1                               |
| 49  | WYT            | Female| 21 | 3                               |
| 50  | ZQZ            | Female| 22 | 1                               |
| 51  | ZJB            | Male| 21  | 4                               |
| 52  | ZWL            | Male| 20  | 1                               |
| 53  | WHR            | Female| 21 | 4                               |
| 54  | ZYZ            | Male| 19  | 6                               |
| 55  | WJJ            | Female| 19 | 4                               |
| 56  | QJ             | Female| 19 | 2                               |
| 57  | WYY            | Female| 18 | 2                               |
| 58  | LJ             | Female| 19 | 3                               |
| 59  | SN             | Female| 18 | 2                               |
Genomic comparisons by pulsed field gel electrophoresis techniques

Methods for intact genomic DNA extraction from the bacteria and PFGE analyses were according to the protocols published previously [25–27]. The endonuclease I-CeuI recognizes phylogenetic diversity of the bacteria from the genus and up levels [27–29] and cleavage data from the CTAG-recognizing endonucleases reflect bacterial diversity at the species level, which are consistent with genomic sequence data [30–33].

Genomic sequencing and analysis

Genomic sequencing of the bacteria was conducted on the Illumina HiSeq 2000 platform, which produced 620 Mb data for each of the strains. Library construction and sequencing were carried out according to the manufacturer’s recommendation at the Illumina web site. The sequence data from Illumina HiSeq 2000 were assembled with SOAPdenovo 2.04 software and the sequence analysis was performed as previously described [34, 35]. The draft genome sequences can be accessed under accession numbers shown in Table 2. We predicted genes from the assembled sequences using Glimmer 3.02 [66, 67].

Phylogenetic analysis

Orthologs were determined by BLAST alignment with the criteria that identity was larger than 70% and alignment length was longer than 70% of the whole gene.

### Table 1 E.coli genotype profiles of participants (Continued)

| No. | Participant ID | Sex   | Age | Number of Genotypes<sup>a</sup> |
|-----|----------------|-------|-----|---------------------------------|
| 60  | WSL            | Female| 21  | 5                               |
| 61  | SH             | Male  | 20  | 4                               |
| 62  | LYY            | Female| 20  | 2                               |
| 63  | JHN            | Male  | 20  | 4                               |
| 64  | YMX            | Female| 20  | 2                               |
| 65  | XKL            | Female| 19  | 3                               |
| 66  | XX             | Female| 20  | 3                               |
| 67  | WY             | Male  | 20  | 2                               |
| 68  | YR             | Female| 19  | 3                               |
| 69  | MXL            | Female| 19  | 5                               |
| 70  | GS             | Female| 19  | 2                               |
| 71  | LZY            | Female| 19  | 2                               |
| 72  | TR             | Female| 19  | 2                               |
| 73  | SHY            | Female| 19  | 1                               |
| 74  | GYX            | Female| 21  | 3                               |
| 75  | WL             | Female| 19  | 2                               |
| 76  | LLJ            | Female| 19  | 1                               |
| 77  | SYH            | Female| 19  | 3                               |
| 78  | MZY            | Female| 19  | 1                               |
| 79  | BZ             | Male  | 18  | 2                               |
| 80  | FXF            | Female| 20  | 3                               |
| 81  | HJ             | Female| 19  | 1                               |
| 82  | HKP            | Male  | 20  | 1                               |
| 83  | FR             | Female| 18  | 2                               |
| 84  | JT             | Female| 18  | 1                               |
| 85  | WL             | Female| 19  | 4                               |
| 86  | PHM            | Female| 17  | 4                               |
| 87  | ZMM            | Female| 19  | 2                               |
|     | Bama Seniors   |       |     |                                 |
| 1   | BMS1           | Female| 106 | 5                               |
| 2   | BMS2           | Female| 90  | 5                               |
| 3   | BMS3           | Male  | 91  | 5                               |
| 4   | BMS4           | Female| 105 | 6                               |
| 5   | BMS5           | Female| 102 | 4                               |
| 6   | BMS6           | Female| 96  | 4                               |
| 7   | BMS9           | Male  | 93  | 4                               |
| 8   | BMS11          | Female| 100 | 5                               |
| 9   | BMS12          | Female| 101 | 5                               |
| 10  | BMS17          | Male  | 94  | 5                               |
| 11  | BMS18          | Female| 103 | 13                              |
| 12  | BMS21          | Female| 98  | 13                              |
| 13  | BMS28          | Male  | 92  | 5                               |
| 14  | BMS63          | Female| 93  | 3                               |

**CRC patients**

| No. | Participant ID | Sex   | Age | Number of Genotypes<sup>a</sup> |
|-----|----------------|-------|-----|---------------------------------|
| 15  | BMS67          | Female| 96  | 8                               |

| No. | Participant ID | Sex   | Age | Number of Genotypes<sup>a</sup> |
|-----|----------------|-------|-----|---------------------------------|
| 1   | LSJ            | Female| 68  | 2                               |
| 2   | ZFQ            | Female| 63  | 1                               |
| 3   | LJH            | Female| 45  | 2                               |
| 4   | SSR            | Male  | 75  | 1                               |
| 5   | CYZ            | Male  | 61  | 1                               |
| 6   | LDP            | Male  | 52  | 1                               |
| 7   | GJB            | Male  | 56  | 1                               |
| 8   | WWX            | Male  | 57  | 1                               |
| 9   | ZMD            | Female| 61  | 1                               |
| 10  | BZZ            | Female| 77  | 4                               |
| 11  | ZSH            | Female| 61  | 1                               |
| 12  | ZWF            | Male  | 61  | 1                               |
| 13  | ZYM            | Male  | 38  | 4                               |
| 14  | BZS            | Male  | 64  | 3                               |
| 15  | CYT            | Male  | 34  | 3                               |

<sup>a</sup> Number of genotypes over the number of strains analyzed: for seniors, 16 colonies were analyzed; for participants of the other three groups, 12 colonies were analyzed.
| Strain           | Accession number | Pathogenicity/Source          | Reference                                                                 |
|------------------|------------------|-------------------------------|---------------------------------------------------------------------------|
| E.coli MG1655    | NC_000913        | Non pathogenic                | [36]                                                                      |
| E.coli H10407    | NC_017633        | ETEC                          | [37]                                                                      |
| E.coli P12b      | NC_017663        | Non-pathogenic                | [38]                                                                      |
| E.coli UMNK88    | NC_017641        | Enterotoxigenic *E. coli* (ETEC) | https://www.ncbi.nlm.nih.gov/nuccore/NC_017641                            |
| E. coli REL606   | CP000819         | Non-pathogenic                | https://www.ncbi.nlm.nih.gov/nuccore/CP000819                             |
| E. coli ATCC 8739| NC_010468        |                               | https://www.ncbi.nlm.nih.gov/nuccore/NC_010468                             |
| E. coli O78      | NC_020163        | APEC                           | [39]                                                                      |
| E. coli E24377A  | NC_009801        | ETEC                          | https://www.ncbi.nlm.nih.gov/nuccore/NC_009801                             |
| E. coli 11,128   | NC_013364        | STEC/EHEC                     | [40]                                                                      |
| E. coli 12,009   | NC_013353        | STEC/EHEC                     | [40]                                                                      |
| E. coli SE11     | NC_011415        | Non pathogenic                | [41]                                                                      |
| E. coli IAI1     | NC_011741        | Non pathogenic                | https://www.ncbi.nlm.nih.gov/nuccore/NC_011741                             |
| E. coli 2099EL-2050 | NC_018650 | EAggEC-EHEC                   | [42]                                                                      |
| E. coli LYG180   | NC_022364        |                               | [43]                                                                      |
| E. coli NA114    | NC_017644        | ExPEC (multidrug-resistant UPEC) | [44]                                                                     |
| E. coli SE15     | NC_013654        | Non-pathogenic                | [45]                                                                      |
| E. coli E2348/69 | NC_011601        | EPEC                          | [46]                                                                      |
| E. coli S36      | NC_008253        | ExPEC (UPEC)                  | [47]                                                                      |
| E. coli UT189    | NC_007946        | ExPEC (UPEC)                  | [48]                                                                      |
| E. coli S88      | NC_011742        | ExPEC (neonatal meningitis)   | [49]                                                                      |
| E. coli LF82     | NC_011993        | AIEC                          | https://www.ncbi.nlm.nih.gov/nuccore/NC_011993                             |
| E. coli ED1a     | NC_011745        | Non-pathogenic                | https://www.ncbi.nlm.nih.gov/nuccore/NC_011745                             |
| E. coli CFT073   | NC_004431        | ExPEC (UPEC)                  | [50]                                                                      |
| E. coli UMN026   | NC_011751        | ExPEC (UPEC)                  | https://www.ncbi.nlm.nih.gov/nuccore/NC_011751                             |
| E. coli 042      | NC_017626        | EAEC                          | [51]                                                                      |
| E. coli CE10     | NC_017646        | ExPEC (neonatal meningitis)   | [52]                                                                      |
| E. coli SMS-3-5  | NC_010498        | Multi-resistant               | https://www.ncbi.nlm.nih.gov/nuccore/NC_010498                             |
| E. coli O55 RM12579 | NC_017656 | Atypical EPEC (aEPEC)         | https://www.ncbi.nlm.nih.gov/nuccore/NC_017656                             |
| E. coli O55 CB9615 | NC_013941 | aEPEC                         | [53]                                                                      |
| E. coli O157 Xuzhou21 | NC_017906 | STEC/EHEC                     | [54]                                                                      |
| E. coli O157 Sakai | NC_002695 | STEC/EHEC                     | [55]                                                                      |
| E. coli O157 EDL933 | NC_002655 | EHEC                          | [56]                                                                      |
| Sh. boydii Sb227 | CP000306         | Dysentery                     | [57]                                                                      |
| Sh. sonnei 53G   | NC_016822        | Dysentery                     | https://www.ncbi.nlm.nih.gov/nuccore/NC_016822                             |
| Sh. flexneri 58,401 | NC_008258 | Dysentery                     | [58]                                                                      |
| Sh. flexneri 2a 301 | AE005674 | Dysentery                     | https://www.ncbi.nlm.nih.gov/nuccore/AE005674                             |
| Sh. dysenteriae 5d197 | NC_007606 | Dysentery                     | [57]                                                                      |
| S. heidelberg B182 | NC_017623 | Salmonella food poisoning     | [59]                                                                      |
| S. typhimurium LT2 | NC_003197 | Gastroenteritis               | [60]                                                                      |
| S. paratyphi B SPB7 | NC_010102 | Paratyphoid fever             | https://www.ncbi.nlm.nih.gov/nuccore/NC_010102                             |
| S. paratyphi C RKS4594 | CP000857 | Paratyphoid fever             | [35]                                                                      |
| S. choleraesuis SC-B67 | NC_006905 | Systemic infections           | [61]                                                                      |
| S. dublin CT_02021853 | NC_011205 | Systemic infections           | [62]                                                                      |
| S. enteritidis P125109 | NC_011294 | Gastroenteritis               | [63]                                                                      |
Concatenation of conserved genes was done by using home-made Perl scripts. The phylogenetic tree was constructed by MEGA6.

Detection of genetic boundaries
The detection of genetic boundaries was performed as previously described [68].

Probing the pan-genomes
We determined the genes common to all compared strains and used the genes as the “core-genome” for the strains compared; we added all non-redundant genes of the bacterial strains in comparison to the core-genome to obtain the “pan-genome”. The analysis of pan-genomes and core-genomes was done by using home-made Perl scripts.

Growth competition assays among the E. coli strains in different nutrient conditions
For the growth competition assays, we mixed three strains in a set, including one from a CRC patient and two from two separate healthy subjects. We inoculated a single colony from a strain into 4 ml LB broth and incubated the bacteria at 37 °C overnight. On day 1 of the growth competition assays, we transferred 1 ml of each of the three overnight cultures in a set into one fresh 15 ml test tube with a water-tight cap (for genomic DNA extraction manipulations [25]). After brief vortex mixing, we transferred an aliquot of 30 μl into a 10 ml test
tube containing 3 ml LB broth and another aliquot of 30 μl into a 10 ml test tube containing 3 ml M9 medium [69] and incubated the 100-fold diluted cultures overnight at 37 °C with shaking (200 rpm); all ensuing cultures were conducted under such conditions. Then we extracted genomic DNA of the bacteria from each of the three single overnight cultures and the pooled cultures 1 ml each from the three single overnight cultures by procedures provided previously [25].

On day 2 of the assays, the overnight cultures initiated on day 1 were 100-fold diluted again (a 30 μl aliquot into a 10 ml test tube containing 3 ml LB broth and a another aliquot of 30 μl into a 10 ml test tube containing 3 ml M9 medium). This procedure was repeated daily until day 10, when a 30 μl aliquot from the day 10 LB culture was transferred into a 10 ml test tube containing 3 ml LB broth as usual and the 30 μl aliquot from the day 10 M9 culture was transferred into a 10 ml test tube also containing 3 ml LB broth, not M9 medium, to obtain sufficient bacterial cells for genomic DNA extraction on day 11.

The procedure of dilution and culture was terminated on day 11 and genomic DNA was extracted from the bacteria of the end cultures.

Statistical analysis
Statistical analysis was conducted by using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) and GraphPad Prism statistical software; as the data were not normally distributed, Wilcoxon rank-sum test was used.

Results
Genomic diversity of E. coli in healthy people and CRC patients
To probe the general diversity of commensal E. coli in the human microbiota and test for any possible associations between the diversity and health status, we made genomic comparisons on the 2280 E. coli isolates (see details in Methods and Table 1). To reveal the overall genomic differences among the E. coli strains, we profiled the endonuclease cleavage patterns of the bacteria using the pulsed field gel electrophoresis (PFGE) techniques, which reflect relatedness of the bacteria and can delineate very closely related bacteria into distinct phylogenetic lineages [28, 31].

We found that the E. coli strains had highly diverse cleavage patterns; particularly, the endonuclease I-CeuI delineated the E. coli strains into distinct genotypes (Fig. 1). I-CeuI cleaves a 26 bp sequence in the 23S rRNA genes [70, 71], which are evolutionarily conserved in both genomic location and nucleotide sequence, so its cleavage patterns reflect genomic distribution of the 23S rRNA genes and hence the overall physical structure of the bacterial genome [27, 28].

Although in general the E. coli isolates from the 185 participants had high genomic diversity, the level of diversity was different considerably among the groups: from very low to remarkably high among the individual participants (see Table 1). Notably, the magnitude of diversity went up with age in the healthy participants from preschool children through university students to senior individuals, whereas the colorectal cancer patients all had low diversity and the differences were statistically significant (Fig. 2). As shown in Table 1, as many as 13 genome types were resolved from the 16 randomly picked E. coli isolates of a senior individual, which suggests that higher diversity might be detected if more isolates from a subject were examined. To look into this, we extended the number of isolates from 12 to 16 per person to 60 in a subset of the subjects for the analysis; however, no further diversity was detected (data not shown).

Phylogenetic divergence of the E. coli strains
The high diversity of the analyzed E. coli strains as reflected by their I-CeuI profiles suggests different gene contents among the E. coli bacteria. To look into this, we sequenced 25 selected strains to make genomic comparisons at higher resolution and determine the phylogenetic relationships among them. We constructed a phylogenetic tree for the 25 E. coli strains along with 37 reference E. coli complex strains, representing phylogenetic groups A, B1, B2, D, E and Shigella lineages, as well as 12 reference Salmonella strains, representing the leading pathogens (Table 2), by concatenating and comparing 927 genes common to them. We included these reference E. coli and Salmonella strains in the phylogenetic analysis to estimate the evolutionary relationships as well as the genetic distances among the E. coli strains. On the constructed tree, the 25 E. coli strains were either mixed with strains of a phylogenetic group of E. coli (ccpm6201, ccpm5179, ccpm5064, ccpm5071, ccpm5063, ccpm5065, ccpm6207, ccpm6219, ccpm5177, ccpm5176, ccpm5174, ccpm6220, ccpm5175, BAMA0321 and BAMA0315 with Group A; ccpm5062, BAMA0361 and BAMA0374 with Group B1; and ccpm5069, ccpm6195 and BAMA0397 with Group D) or formed a branch between the groups (ccpm5172, ccpm3961 and ccpm3962 between Groups B1 and E). Additionally, ccpm5171 was clustered together with Shigella; none of the 25 strains was found to cluster with Group B2, which contains many extra-intestinal pathogens [72–74], or E, which contains O157:H7 and O55:H7 [56] (Fig. 3). Also as shown in Fig. 3, the six strains of subject ZGQ and the seven strains of subject TZL (see Table 1 for details about subjects ZGQ and TZL) were broadly distributed on the phylogenetic tree without a clear tendency of clustering toward their origin hosts.
On the other hand, some *E. coli* strains isolated from different hosts were closely related, such as ccpm3961 and ccpm3962 from WZW with ccpm5172 from TZL, and ccpm5065 from ZGQ with ccpm6207 from CRC-EC2, demonstrating the spreading of *E. coli* strains among the human populations (Fig. 3). Notably, genetic distances among the *E. coli* strains (including reference *E. coli* complex strains and the 25 *E. coli* strains isolated in this study) were similar to, or even greater than, those seen among the *Salmonella* lineages, indicating their phylogenetic divergence over evolutionary times.

**Genetic boundaries among the *E. coli* lineages**

Such a high genetic diversity of the commensal *E. coli* isolates and the remarkable evolutionary distances among them suggest the existence of genetic boundaries between them to separate them into discrete phylogenetic clusters. To detect such postulated genetic boundaries among the *E. coli* bacteria, we determined the ratios...
of homologous genes that have identical nucleotide sequences between pairs of strains by the method as reported previously for *Salmonella* [75]. The overall scales of differences among the profiled ratios (Supplementary Table 1) were consistent with the relative genetic distances revealed on the phylogenetic tree among the bacteria (see Fig. 3). Similar to the *Salmonella* lineages [68, 75], the *E. coli* strains well separated on the phylogenetic tree had low ratios of homologous genes with zero nucleotide sequence degeneracy between them, mostly below 10% like in *Salmonella* (Supplementary Table 1), demonstrating the existence of genetic
boundaries that circumscribe the bacteria into discrete clusters. Such remarkable genomic divergence among the *E. coli* strains suggests that they may also have large numbers of genes different from one another, analysis of which may lead to novel insights into the evolution of different *E. coli* lineages, especially regarding the emergence of nascent pathogens from their commensal ancestors.

Fig. 3 Phylogenetic tree for comparisons of relative genetic distances among the bacteria. On the left is a phylogenetic tree for strains of both *E. coli* and *Salmonella* and on the right is an enlarged part of the phylogenetic tree for *E. coli*. The tree was constructed with 1000 bootstrap replicates by the neighbor-joining (NJ) method.
Profiling novel genes and probing the potential pan-genome

We annotated the genomes of the 25 sequenced *E. coli* strains. We first identified genes that are also present in strain K12 MG1655 (Supplementary Table 2), and then profiled genes that are not present in MG1655 nor in one or more of the 25 *E. coli* isolates (Supplementary Table 3 and Supplementary Figure 1). We found that a given strain may have hundreds of genes not present in other *E. coli* strains, further demonstrating enormous diversity of the commensal *E. coli* populations. Such large numbers of specific genes will certainly make an *E. coli* strain biologically distinct from other *E. coli* lineages, especially in terms of their contributions to the human health, including their potentials to benefit the host or to rise as novel pathogens. In any case, a plastic genomic construction would be a prerequisite for the *E. coli* bacteria to readily accept foreign genes and become a unique lineage.

To validate the postulation that the *E. coli* genome is more amenable than those of some closely related bacteria to accept lateral genes and diverge, we probed the pan-genomes of *E. coli* and *Salmonella* and compared them. Comparison of 62 *E. coli* complex strains (including the 25 fresh *E. coli* isolates and the 37 reference *E. coli* complex strains) and 45 representative *Salmonella* strains revealed remarkable differences in their pan-genomes: whereas the 45 *Salmonella* strains had 2959 shared genes and 9338 total non-redundant genes, the 52 *E. coli* complex strains had only 1884 shared genes but as many as 17,335 total non-redundant genes (Fig. 4), giving an impression of an “open pan-genome” that may keep growing in size by accepting additional novel genes.

Low *E. coli* diversity in colorectal cancer patients

The apparent tendency of low *E. coli* diversity in CRC patients pointed to a possibility of the bacteria as potential pathogens, directly or indirectly contributing to CRC, such as by creating an unhealthy intestinal micro-environment through suppressing or even purging beneficial *E. coli* lineages. To test such a postulation, we set

![Fig. 4 Comparison of sampled pan-genomes between *E. coli* and *Salmonella*.](image-url)
up a series of growth competition assays and inspected the *E. coli* strains isolated from CRC patients for their competing growth abilities in rich (LB broth) or nutrient-limited (M9) medium with *E. coli* strains isolated from healthy subjects.

In such experiments, we co-cultured an *E. coli* strain isolated from a CRC patient with two *E. coli* strains isolated from two separate healthy subjects and detected the genomic cleavage sites by the CTAG tetra-nucleotide sequence recognizing endonuclease XbaI in the end co-cultures (See details in Methods). XbaI and other CTAG recognizing endonucleases, such as BlnI/AvrII, Spel and NheI, have rare cleavage sites in *E. coli* and the overall cleavage patterns are unique to bacteria of a particular phylogenetic cluster [26, 31, 76, 77]. In experiment set 1, for example, we included an *E. coli* strain from a CRC patient (ccpm6195) and one strain each from two university students (ccpm5172 and ccpm5602). After 10 days of diluted cultures by daily 100-fold dilution of 30 μl overnight culture into 3 ml fresh medium, we found that the three *E. coli* strains grew equally well in LB broth but the situation was dramatically different in M9 medium (Fig. 5). As shown in Fig. 5, the *E. coli* strain from a CRC patient (ccpm6195; lane 2) and the two *E. coli* strains from two university students (ccpm5172 and ccpm5602; lanes 3 and 4, respectively) had distinct XbaI cleavage patterns, so they could be distinguished unambiguously. The mixture of the three strains showed all bandings of lanes 2, 3 and 4 on day 0 (Fig. 5, lane 5) when they were mixed immediately prior to genomic extraction. After incubation for 10 days in LB broth, the end mixture culture showed a similar growth pattern (Fig. 5, lane 6) to that of the initial mixture (Fig. 5, lane 5), whereas the end mixture culture of the three strains in M9 medium showed only the XbaI cleavage pattern of ccpm6195 (the *E. coli* strain from a CRC patient; Fig. 5, lane 7), demonstrating that ccpm6195 may have greater capability of harnessing the hardly available nutrient in the M9 medium, although direct or indirect suppression of the *E. coli* strains from healthy individuals by the *E. coli* strain from a CRC patient cannot be ruled out.

**Discussion**

In this study, we demonstrated the associations of high phylogenetic diversity of *E. coli* with health, with the diversity going up with age from children through young adults to longevity seniors. We believe that the assumed commensal *E. coli* bacteria, i.e., those that are not associated with apparent intestinal or extra-intestinal infections, are the products of co-evolution with the human host, adapting to different microenvironments in the human intestine and providing a variety of beneficial functions to their host. Low diversity therefore may mean the absence of several such beneficial functions, making the host vulnerable to certain unhealthy factors and hence susceptible to some diseases such as CRC. Although the observed association between low *E. coli* diversity and CRC does not exclude the possibility that the conditions that lead to low *E. coli* diversity are conditions that lead to colorectal cancer as having been extensively discussed with a focus on changes in the intestinal microbiome [78, 79], we are inclined to believe low *E. coli* diversity, caused by the purging capabilities of certain non-benign *E. coli* lineages, to be a novel risk factor for CRC based on the results we obtained in this study, especially the phylogenetically distinct and biologically aggressive *E. coli* lineages.

These findings provoke a key question: what is the nature of the diversity? Are the diverse *E. coli* strains some randomly picked members of a wide spectrum of continual genetic variants collectively called a species of *E. coli* or do they represent discrete phylogenetic clusters? For a definite answer to this health-relevant question, we analyzed the collected *E. coli* isolates and dissected the diversity among them. The collected 2280 *E. coli* isolates were all assumed to be commensals, because they were not related to intestinal or extra-intestinal illnesses in the healthy participants. At least for this study, we also deemed the *E. coli* isolates from the CRC patients to be commensals, because etiological associations of *E. coli* with CRC had not been established.

Using a combination of genomic analysis methods, including PFGE to determine the global physical structure of the bacterial genome [25], CTAG tetra-nucleotide profiling by the use of CTAG sequence recognizing endonucleases to distinguish different phylogenetic clusters [80, 81], and whole genome sequence comparisons to identify genes common to the bacteria in comparison and those present only in a subset of the bacteria, we delineated these bacteria into discrete genotypes. It was the use of the combined methods that made this work possible. One of the core techniques used in this study was I-CeuI cleavage profiling by PFGE, which can categorize bacteria into genus or sub-genus phylogenetic levels unambiguously and intuitively [27, 28, 82]. Although the next generation sequencing techniques have been developing extremely rapidly, with continuously increasing capacity and radically dropping cost, sequencing of 2280 strains is still an enormous task, let alone the fact that all the thousands of bacterial genomes would have to be completely finished without a single gap or sequencing error for this kind of global genome comparisons. Additionally, distinguishing three co-cultured *E. coli* strains without labeling any of them by additional manipulations such as antimicrobial resistance markers can be conveniently and unambiguously achieved only by CTAG tetra-nucleotide sequence profiling; in fact, no
any other molecular methods currently available can fulfill this task.

All the results demonstrate that the great diversity revealed among the 2280 E. coli strains was due to the divergence of the bacteria into discrete phylogenetic clusters, not a continual spectrum of genetic variants, over evolutionary times, during which the bacteria in the phylogenetic clusters accumulated genomic variations independently and became genetically isolated from one another by genetic boundaries. The absence of genetic continuum, or rather continuum of genetic variations, among the highly related Salmonella subgroup I pathogens has been previously proven [32, 33, 68, 75], but it is the first time for the E. coli bacteria to be delineated into independent phylogenetic lineages that did not have detectable continuum of genetic variations among them. Therefore, we think it reasonable to treat different genotypes of E. coli as different bacteria or as bacteria of highly related but distinct species, especially considering the finding that a given E. coli strain may have hundreds of genes not found in any other E. coli strains analyzed in this study (see Supplementary Table 3). As such, the commonly assumed commensal E. coli bacteria with large numbers of genes specific to only one or a few genotypes may accomplish a wide range of different biological activities for the benefit of their host and lack of any of them may mean the lack of certain beneficial functions to contribute to the health of the host, leading to the vulnerability of the host to diseases, e.g., CRC.

In fact, recent discoveries on the genetic diversity and population structure of E. coli have encouraged investigators to associate certain phylogenetic groups or clonal complexes with human diseases [23, 83–85]. Such “intra-” E. coli diversity has mostly been treated as genetic differences within a “single” bacterial species. However, at least in views of bacterial genetics and pathogenicity, the evolutionary divergence between E. coli K12 and O157:H7 should have separated them into entirely different bacteria, which, with the former being

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**Fig. 5** Growth competition among E. coli isolates from CRC patients and healthy controls. Lanes: 1, λ ladder as molecular size marker; 2, ccpm6195; 3, ccpm5172; 4, ccpm5062; 5, a mixture of ccpm6195, 5172 and 5062 before the competition assay; 6, a mixture of ccpm6195, 5172 and 5062 cultured in LB broth at the end of the competition assay; 7, a mixture of ccpm6195, 5172 and 5062 cultured in M9 medium at the end of the competition assay. Remarkably, after culture for 10 days in M9 medium, only ccpm6195 (see lane 7, the E. coli strain from a CRC patient, in which the genomic cleavage pattern is indistinguishable with that in lane 2) survived. These growth competition assays demonstrate that when nutrient was ample (as in LB broth), the three E. coli strains did not interfere with one another for growth; but when the nutrient was limited, the E. coli strains from a CRC patient had greater capabilities to compete for nutrient to grow.
a harmless commensal and the latter a deadly pathogen [56, 86], may have over a thousand genes different between them. In the analysis of the fresh *E. coli* isolates in this study, we profiled the genetic variations among them and found that the levels of overall genetic divergence among them were similar to that of *E. coli* K12 and O157:H7. The large numbers of novel genes specific to the individual *E. coli* genotypes would render the bacteria different abilities or preference to adapt to a broad range of micro-niches in the gut environment. Additionally, the genetic separation of the *E. coli* bacteria by clear-cut boundaries strongly indicates their non-overlapping environmental settings in the intestinal lumen of a host. Indeed, the co-existence of genetically diverse *E. coli* lineages in healthy people would be best explained by their inability or unneeessity to purge each other, very possibly due to their difference in resource requirement. Therefore the very low diversity of the *E. coli* isolates from CRC patients in sharp contrast with the high diversity of the bacteria in the healthy individuals is indicative of the health significance of *E. coli* diversity to the host. Notably, the *E. coli* isolates from the CRC patients suppressed the growth of *E. coli* isolates from healthy controls when the nutrients were limited, suggesting their greater growth capabilities, which may in turn result in unhealthy microenvironments in the intestine and facilitate the carcinogenesis of the intestinal tissues.

It is a very interesting observation that the *E. coli* diversity increased with age, which may reflect a general scenario of dynamic *E. coli* diversification during the life time of the host. But how is the diversity reached?

Bacteria acquire genetic novelty and diverge by two major mechanisms, including incorporating large exogenous DNA segments, e.g., prophages, genomic islands, etc., and accumulating nucleotide substitutions, with the former being mostly acute to divert the evolutionary direction and the latter usually chronic to make gradual genomic ameliorations in a chronological way [31, 87, 88]. Comparisons between *E. coli* K12 and O157:H7 as well as among the genotypes indicate that the acquisition of large exogenous DNA segments is highly frequent in evolution and the events may happen almost instantly. However, the final acceptance of the acquired DNA segments may take time for genomic ameliorations toward eventual adaptation. If one assumes that bacterial genomic ameliorations take place at similar rates among different bacteria [89, 90], the different *E. coli* lineages, with genetic distances between them being similar to that as between *S. typhi* and *S. typhimurium* (see Fig. 3), would be estimated to have a divergence time of 35–50 thousand years [91]. Therefore, the striking diversity of commensal *E. coli* revealed in this study would be a result of gradual colonization of a host by pre-existing *E. coli* lineages, rather than de novo creation of nascent *E. coli* lineages through divergence. Indeed, the phylogenetic divergence would require evolutionary times that are much longer than the lifespan of a human host. The colonization process may take certain lengths of time, which may partly explain why *E. coli* in people with advanced ages may have higher genomic diversity than in junior people. Although high genomic diversity of the *E. coli* populations is positively correlated with health status, we currently have no direct evidence yet to show whether the low *E. coli* diversity in the CRC patients might be the consequence or an etiologic factor of the disease.

**Conclusions**

Commensal *E. coli* are diverse and the diversity increases in the healthy hosts with age from children through young adults to longevity seniors, suggesting that the co-existence of multiple *E. coli* lineages may help create and maintain a microbial environment that is beneficial to the host. The diversity is due to a high number of discrete phylogenetic clusters rather than continual genetic variations spanning a wide spectrum of the *E. coli* bacteria. *E. coli* isolates from CRC patients had growth advantages over those from healthy individuals, suggesting their potential pathogenic roles.

**Supplementary information**

Supplementary information accompanies this paper at https://doi.org/10.1186/s12920-020-0704-3.

**Additional file 1: Table S1.** Percentages of common genes with identical nucleotide sequences among the fresh *E. coli* isolates from healthy individuals.

**Additional file 2: Table S2.** Profile of genes in the 25 *E. coli* isolates in common with *E. coli* K12 MG1655.

**Additional file 3: Table S3.** Profiles of genes not present in K12 MG1655 but present in one or more of the 25 *E. coli* isolates.

**Additional file 4: Figure S1.** Genomic comparison of sequenced fresh *E. coli* isolates with strain K12 MG1655 to show the presence or absence of K12 MG1655 in the sequenced fresh *E. coli* isolates. Genes present in any of the sequenced fresh *E. coli* isolates but not in K12 MG1655 are listed in Supplementary Table 3.

**Abbreviations**

CRC: Colorectal cancer; Genotypes: Genome types; NJ method: Neighbor-joining method; PFGE: Pulsed field gel electrophoresis

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**Authors’ contributions**

LT carried out the experimental work and conducted genomic sequence analyses; YJZ and SLZ were involved in genomic sequence analyses; GDL, HZ, MFZ, ZL, XYC, HNL, ZL, ZRG, XQM and XL collected bacterial strains and were involved in experiments; XH, WQL, CXW, DDZ, JJL, BQY were involved in experiments; LBD and LW performed bacterial identification; YGL, JBZ, RNJ, JZ and GRL participated in data analysis and discussions; LT, YJZ and SLL.

**Supplementary**

(2020) 13:59
conceived and designed the study and wrote the manuscript. The authors read and approved the manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author.

Ethics approval and consent to participate
All experimental protocols were reviewed and approved by The Ethics Committee, Harbin Medical University, and all experiments were performed in accordance with relevant guidelines and regulations. We obtained a written informed consent from each participant or their guardian, consistent with the 1975 Declaration of Helsinki on Ethical Principles for Medical Research Involving Human Subjects.

Consent for publication
All authors have read the manuscript and agreed to its content. This work is original and is not currently under consideration by another journal.

Competing interests
The authors declare that they have no competing interests.

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