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Culture of previously uncultured members of the human gut microbiota by culturomics

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Metagenomics revolutionized the understanding of the relations among the human microbiome, health and diseases, but generated a countless number of sequences that have not been assigned to a known microorganism[1]. The pure culture of prokaryotes, neglected in recent decades, remains essential to elucidating the role of these organisms[2]. We recently introduced microbial culturomics, a culturing approach that uses multiple culture conditions and matrix-assisted laser desorption/ionization-time of flight and 16S rRNA for identification[2]. Here, we have selected the best culture conditions to increase the number of studied samples and have applied new protocols (fresh-sample inoculation; detection of microcolonies and specific cultures of Proteobacteria and microaerophilic and halophilic prokaryotes) to address the weaknesses of the previous studies[3–5]. We identified 1,057 prokaryotic species, thereby adding 531 species to the human gut repertoire: 146 bacteria known in humans but not in the gut, 187 bacteria and 1 archaea not previously isolated in humans, and 197 potentially new species. Genome sequencing was performed on the new species. By comparing the results of the metagenomic and culturomic analyses, we show that the use of culturomics allows the culture of organisms corresponding to sequences previously not assigned. Altogether, culturomics doubles the number of species isolated at least once from the human gut.

The study of the human gut microbiota has been revived by metagenomic studies[6–8]. However, a growing problem is the gaps that remain in metagenomics, which correspond to unidentified sequences that may be correlated with an identified organism[9]. Moreover, the exploration of relations between the microbiota and human health require—both for an experimental model and therapeutic strategies—the growing of microorganisms in pure culture[10], as recently demonstrated in elucidations of the role of *Clostridium butyricum* in necrotizing enterocolitis and the influence of gut microbiota on cancer immunotherapy effects[11,12]. In recent years, microbial culture techniques have been neglected, which explains why the known microbial community of the human gut is extremely low[13]. Before we initiated microbial culturomics[13] of the approximately 13,410 known bacterial and archaea species, 2,152 had been identified in humans and 688 bacteria and 2 archaea had been identified in the human gut. Culturomics consists of the application of high-throughput culture conditions to the study of the human microbiota and uses matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF) or 16S rRNA amplification and sequencing for the identification of growing colonies, some of which have been previously unidentified[2]. With the prospect of identifying new genes of the human gut microbiota, we extend here the number of recognized bacterial species and evaluate the role of this strategy in resolving the gaps in metagenomics, detailing our strategy step by step (see Methods). To increase the diversity, we also obtained frozen samples from healthy individuals or patients with various diseases from different geographical origins. These frozen samples were collected as fresh samples (stool, small-bowel and colonic samples; Supplementary Table 1). Furthermore, to determine appropriate culture conditions, we first reduced the number of culture conditions used (Supplementary Table 2a–c) and then focused on specific strategies for some taxa that we had previously failed to isolate (Supplementary Table 3).

First, we standardized the microbial culturomics for application to the sample testing (Supplementary Table 1). A refined analysis of our first study, which had tested 212 culture conditions[4], showed that all identified bacteria were cultured at least once using one of the 70 best culture conditions (Supplementary Table 2a). We applied these 70 culture conditions (Supplementary Table 2a) to the study of 12 stool samples (Supplementary Table 1). Thanks to the implementation of the recently published repertoire of human bacteria[13] (see Methods), we determined that the isolated bacteria included 46 bacteria known from the gut but not recovered by culturomics before this work (new for culturomics), 38 that had

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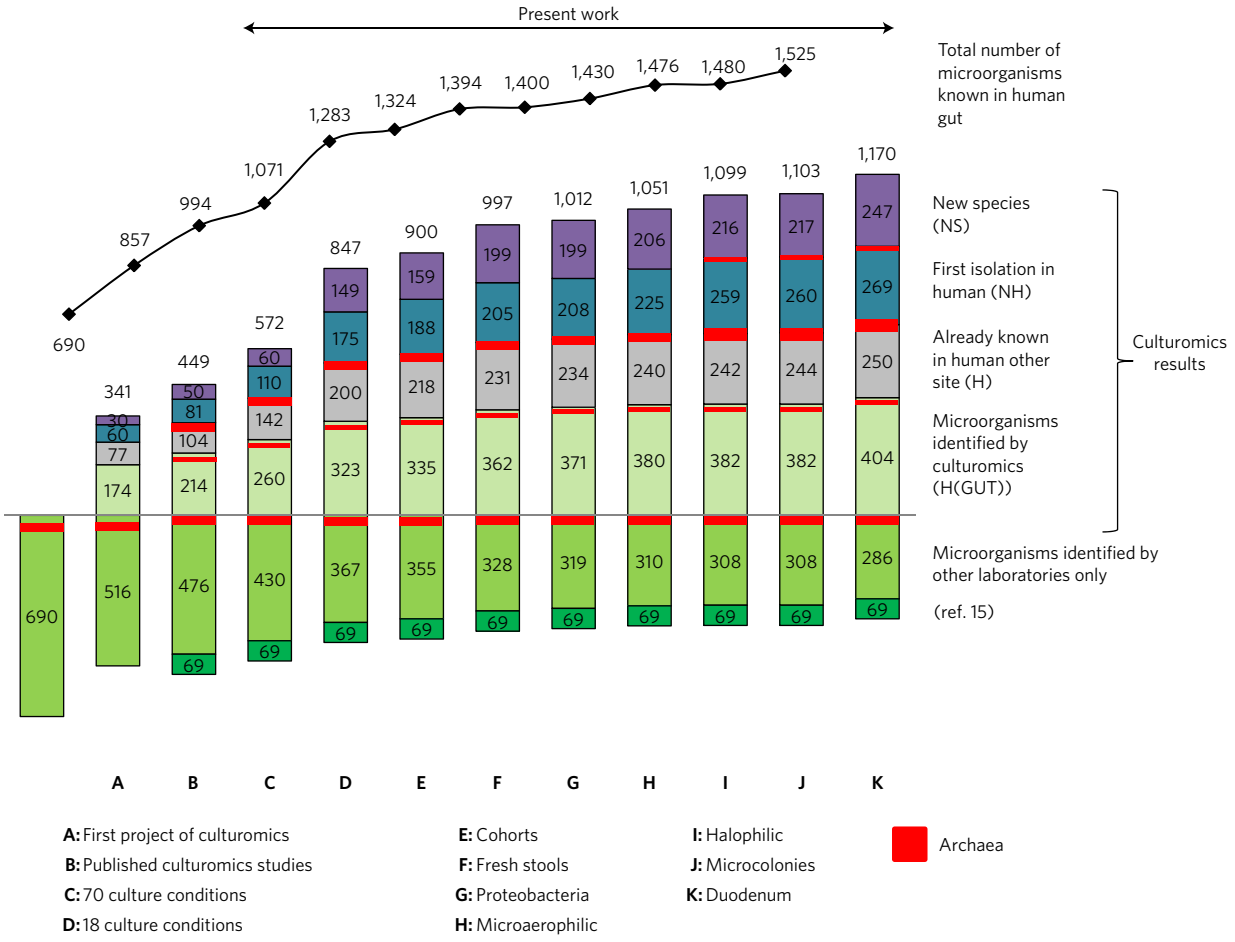

Figure 1 | Number of different bacteria and archaea isolated during the culturomics studies. Columns A and B represent the results from previously published studies, and columns C to K the different projects described herein. The bacterial species are represented in five categories: NS, new species; NH, prokaryotes first isolated in humans; H, prokaryotes already known in humans but never isolated from the human gut; H (GUT), prokaryotes known in the human gut but newly isolated by culturomics; and prokaryotes isolated by other laboratories but not by culturomics.

already been isolated in humans but not from the gut (non-gut bacteria), 29 that had been isolated in humans for the first time (non-human bacteria) and 10 that were completely new species (unknown bacteria) (Fig. 1 and Supplementary Tables 4a and 5).

Beginning in 2014, to reduce the culturomics workload and extend our stool-testing capabilities, we analysed previous studies and selected the 18 best culture conditions[2]. We performed cultures in liquid media in blood culture bottles, followed by subcultures on agar (Supplementary Table 2b). We designed these culture conditions by analysing our first studies. The results of those studies indicated that emphasizing three components was essential: pre-incubation in a blood culture bottle (56% of the new species isolated), the addition of rumen fluid (40% of the new species isolated) and the addition of sheep blood (25% of the new species isolated)[2–5]. We applied this strategy to 37 stool samples from healthy individuals with different geographic provenances and from patients with different diseases (Supplementary Table 1). This new strategy enabled the culture of 63 organisms new to culturomics, 58 non-gut bacteria, 65 non-human bacteria and 89 unknown bacteria (Fig. 1 and Supplementary Tables 4a and 5).

We also applied culturomic conditions (Supplementary Table 2c) to large cohorts of patients sampled for other purposes (premature infants with necrotizing enterocolitis, pilgrims returning from the Hajj and patients before or after bariatric surgery) (Supplementary Table 1). A total of 330 stool samples were analysed. This enabled the detection of 13 bacteria new to culturomics, 18 non-gut bacteria, 13 non-human bacteria and 10 unknown species (Fig. 1 and Supplementary Tables 4a and 5).

Among the gut species mentioned in the literature[13] and not previously recovered by culturomics, several were extremely oxygen-sensitive anaerobes, several were microaerophilic and several were Proteobacteria, and we focused on these bacteria (Supplementary Table 3). Because delay and storage may be critical with anaerobes, we inoculated 28 stools immediately upon collection. This enabled the culture of 27 new gut species for culturomics, 13 non-gut bacteria, 17 non-human bacteria and 40 unknown bacteria (Fig. 1 and Supplementary Tables 3a and 4). When we specifically tested 110 samples for Proteobacteria, we isolated 9 bacteria new to culturomics, 3 non-gut bacteria and 3 non-human bacteria (Fig. 1 and Supplementary Tables 4a and 5). By culturing 242 stool specimens exclusively under a microaerophilic atmosphere, we isolated 9 bacteria new to culturomics, 6 non-gut bacteria, 17 non-human bacteria and 7 unknown bacteria (Fig. 1 and Supplementary Tables 4a and 5). We also introduced the culture of halophilic prokaryotes from the gut and microcolony detection. The culture of halophilic bacteria was performed using culture media supplemented with salt for 215 stool samples, allowing the culture of 48 halophilic prokaryotic species, including one archaea (*Haloferax alexandrinus*), 2 new bacteria for culturomics, 2 non-gut bacteria, 34 non-human bacteria, 10 unknown bacteria and one new halophilic archaea (*Haloferax massiliensis* sp. nov.) (Fig. 1 and Supplementary Tables 4a and 5). Among these 48 halophilic prokaryotic species, 7 were

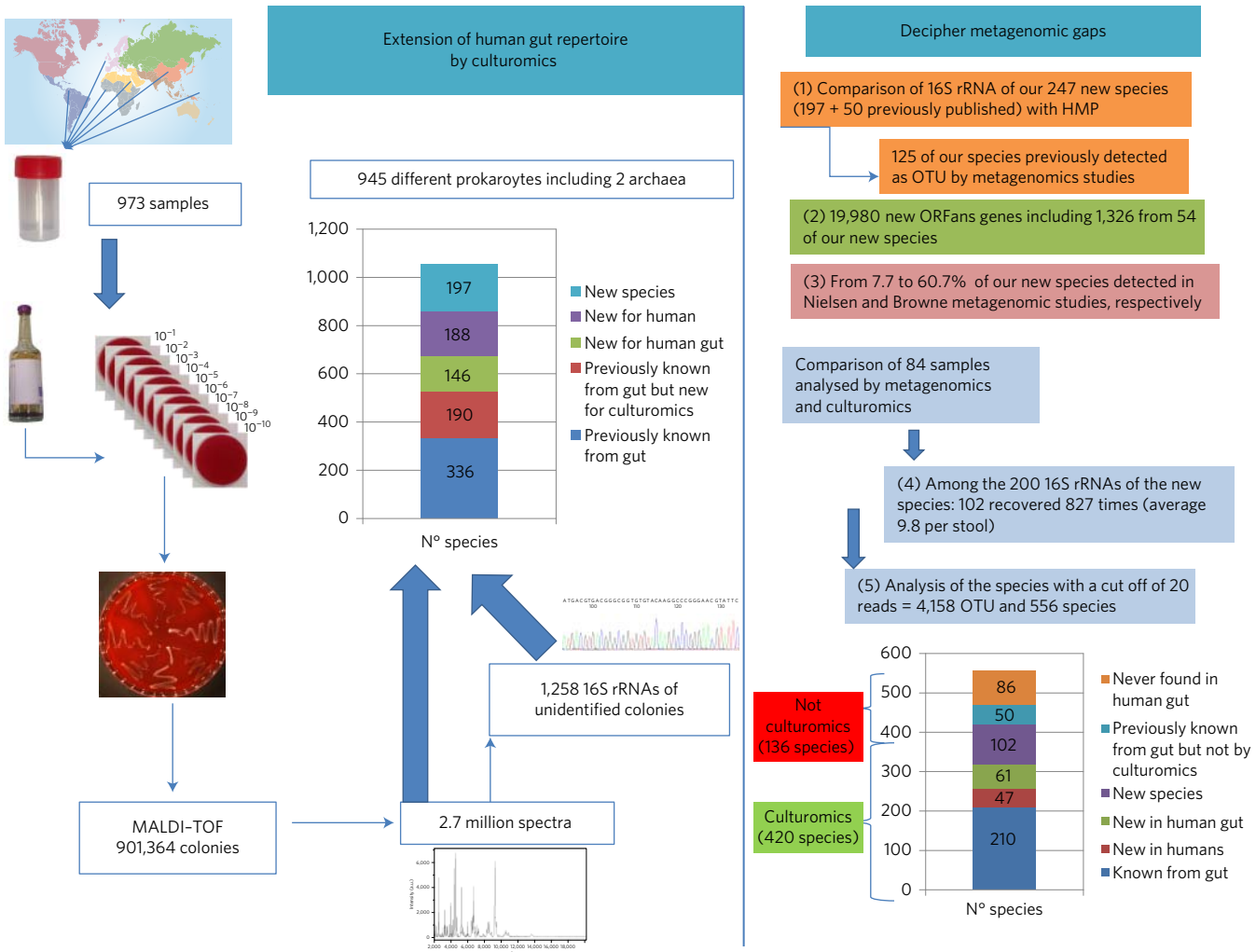

Figure 2 | Summary of the culturomics work that has extended the gut repertoire and filled some of the gaps in metagenomics.

slight halophiles (growing with 10–50 g l^{-1} of NaCl), 39 moderate halophiles (growing with 50–200 g l^{-1} of NaCl) and 2 extreme halophiles (growing with 200–300 g l^{-1} of NaCl).

We also introduced the detection of microcolonies that were barely visible to the naked eye (diameters ranging from 100 to 300 µm) and could only be viewed with magnifying glasses. These colonies were transferred into a liquid culture enrichment medium for identification by MALDI–TOF mass spectrometry (MS) or 16S rRNA amplification and sequencing. By testing ten stool samples, we detected two non-gut bacteria, one non-human bacterium and one unknown bacterium that only formed microcolonies (Fig. 1 and Supplementary Tables 4a and 5). Finally, by culturing 30 duodenal, small bowel intestine and colonic samples, we isolated 22 bacteria new to culturomics, 6 non-gut bacteria, 9 non-human bacteria and 30 unknown bacteria (Fig. 1 and Supplementary Tables 4a and 5). To continue the exploration of gut microbiota, future culturomics studies could also be applied to intestinal biopsies.

In addition, we performed five studies to evaluate the role of culturomics for deciphering the gaps in metagenomics[9]. First, we compared the 16S rRNA sequences of the 247 new species (the 197 new prokaryotic species isolated here in addition to the 50 new bacterial species isolated in previous culturomic studies[3–5]) to the 5,577,630 reads from the 16S rRNA metagenomic studies listed by the Human Microbiome Project (HMP) (http://www.hmpdacc.org/catalog). We found sequences, previously termed operational taxonomic units (OTUs), for 125 of our bacterial species (50.6%). These identified bacterial species included *Bacteroides bouchedurhonense*, which was recovered in 44,428 reads, showing that it is a common bacterium (Supplementary Table 6). Second, because the genome sequencing of 168 of these new species allowed the generation of 19,980 new genes that were previously unknown (ORFans genes) (Supplementary Table 7), we blasted these with 13,984,809 contigs/scaffolds from the assembly of whole metagenomic studies by HMP, enabling the detection of 1,326 ORFans (6.6%) from 54 of our new bacterial species (including 45 detected also from 16S) (Supplementary Table 8). Therefore, at least 102 new bacterial species were found but not identified in previous metagenomic studies from the HMP. Third, we searched for our 247 new species in the 239 human gut microbiome samples from healthy individuals described by Browne *et al.*, in which 137 bacterial species were isolated[15]. We captured 150 of our new species in these metagenomics data, representing 60.7% (Supplementary Table 9). Moreover, we also identified 19 of our species (7.7%) from 396 human stool individuals described by Nielsen *et al.*, from which 741 metagenomic species and 238 unique metagenomic genomes were identified[16] (Supplementary Table 9). Fourth, we analysed the 16S rRNA metagenomic sequences of 84 stools also tested by culturomics (Supplementary Table 10). We compared the OTUs identified by blast with a database including the 16S rRNA of all species isolated by culturomics. Among the 247 16S rRNA of the new species, 102 were recovered 827 times, with an average of

Figure 3 | Phylogenetic tree of the 247 new prokaryote species isolated by culturomics. Bacterial species from Firmicutes are highlighted in red, Actinobacteria (light green), Proteobacteria (blue), Bacteroidetes (purple), Synergistetes (green), Fusobacteria (dark green) and Archaea (grey), respectively. The sequences of 16 prokaryotic species belonging to six phyla previously known from the human gut and more frequently isolated by culture in human gut are highlighted in bold and by an asterisk. **Q6**

9.8 species per stool. Finally, analysis of these species using a cutoff threshold of 20 reads identified 4,158 OTUs and 556 (13.4%) species (Supplementary Table 11), among which 420 species (75.5%) were recovered by culturomics. Of these, 210 (50%) were previously found to be associated with the human gut, 47 were not previously found in humans (11.2%), 61 were found in humans but not in the gut (14.5%) and 102 (24.3%) were new species. Interestingly, among the 136 species not previously found by culturomics, 50 have been found in the gut and 86 have never previously been found in the human gut (Fig. 2 and Supplementary Table 11).

Overall, in this study, by testing 901,364 colonies using MALDI–TOF MS (Supplementary Table 1), we isolated 1,057 bacterial species, including 531 newly found in the human gut. Among them, 146 were non-gut bacteria, 187 were non-human bacteria, one was a non-human halophilic archaeon and 197 were unknown bacteria, including two new families (represented by *Neofamilia massiliensis* gen. nov., sp. nov. and *Beduinella massiliensis* gen. nov., sp. nov.) and one unknown halophilic archaeon (Fig. 1 and Supplementary Table 4a). Among these, 600 bacterial species belonged to Firmicutes, 181 to Actinobacteria, 173 to Proteobacteria (a phylum that we have under-cultured to date; Supplementary Table 5), 88 to Bacteroidetes, 9 to Fusobacteria, 3 to Synergistetes, 2 to Euryarchaeota, 1 to Lentisphaerae and 1 to Verrucomicrobia (Supplementary Table 4a). Among these 197 new prokaryotes

species, 106 (54%) were detected in at least two stool samples, including a species that was cultured in 13 different stools (*Anaerosalibacter massiliensis*) (Supplementary Table 4a). In comparison with our contribution, a recent work using a single culture medium was able to culture 120 bacterial species, including 51 species known from the gut, 1 non-gut bacterium, 1 non-human bacterium and 67 unknown bacteria, including two new families (Supplementary Table 12).

To obtain these significant results we tested more than 900,000 colonies, generating 2.7 million spectra, and performed 1,258 molecular identifications of bacteria not identified through MALDI–TOF, using 16S rRNA amplification and sequencing. The new prokaryote species are available in the Collection de Souches de l'Unité des Rickettsies (CSUR) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (Supplementary Tables 4a and 5). All 16S sequences of the new species and the species unidentified by MALDI–TOF, as well as the genome sequences of the new species, have been deposited in GenBank (Supplementary Tables 5 and 13). In addition, thanks in part to an innovative system using a simple culture for the archaea without an external source of hydrogen[17], among these prokaryotes we isolated eight archaeal species from the human gut, including two new ones for culturomics, one non-gut archaea, four non-human archaea and one new halophilic species.

We believe that this work is a key step in the rebirth of the use of culturing in human microbiology[2–5,16] and only the efforts of several teams around the world in identifying the gut microbiota repertoire will allow an understanding and analysis of the relations between the microbiota and human health, which could then participate in adapting Koch's postulates to include the microbiota[21]. The rebirth of culture, termed culturomics here, has enabled the culturing of 77% of the 1,525 prokaryotes now identified in the human gut (Fig. 1 and Supplementary Table 4b). In addition, 247 new species (197 cultured here plus 50 from previous studies) and their genomes are now available (Fig. 3). The relevance of the new species found by culturomics is emphasized because 12 of them were isolated in our routine microbiology laboratory from 57 diverse clinical samples (Supplementary Table 14). In 2016, 6 of the 374 (1.6%) different identifications performed in the routine laboratory were new species isolated from culturomics. As 519 of the species found by culturomics in the gut for the first time (Fig. 1) were not included in the HMP (Supplementary Table 15) and because hundreds of their genomes are not yet available, the results of this study should prompt further genome sequencing to obtain a better identification in gut metagenomic studies.

Methods

Samples. To obtain a larger diversity of gut microbiota, we analysed 943 different stool samples and 30 small intestine and colonic samples from healthy individuals living or travelling in different geographical regions (Europe, rural and urban Africa, Polynesia, India and so on) and from patients with diverse diseases (for example, anorexia nervosa, obesity, malnutrition and HIV). The main characteristics are summarized in Supplementary Table 1. Consent was obtained from each patient, and the study was approved by the local Ethics Committee of the IFR48 (Marseille, France; agreement no. 09–022). Except for the small intestine and stool samples that we directly inoculated without storage (see below), the faecal samples collected in France were immediately aliquoted and frozen at −80 °C. Those collected in other countries were sent to Marseille on dry ice, then aliquoted and frozen at −80 °C for between 7 days and 12 months before analysis.

Culturomics. Culturomics is a high-throughput method that multiplies culture conditions in order to detect higher bacterial diversity. The first culturomics study concerned three stool samples, 212 culture conditions (including direct inoculation in various culture media), and pre-incubation in blood culture bottles incubated aerobically and anaerobically[4]. Overall, 352 other stool samples, including stool samples from patients with anorexia nervosa[3], patients treated with antibiotics[5], or Senegalese children, both healthy and those with diarrhoea[22], were previously studied by culturomics, and these results have been comprehensively detailed in previous publications[3–5]. In this work, we only included the genome sequences of the 50 new bacterial species isolated in these previous works to contribute to our analysis of culturomics and to fill some of the gaps left by metagenomics (see below). In addition, these previously published data are clearly highlighted in Fig. 1, illustrating the overall contribution of culturomics in exploring the gut microbiota.

Bacterial species isolated from our new projects and described here were obtained using the strategy outlined in the following sections.

Standardization of culturomics for the extension of sample testing. A refined analysis allowed the selection of 70 culture conditions (Supplementary Table 2a) for the growth of all the bacteria[4]. We applied these culture conditions to 12 more stool samples and tested 160,265 colonies by MALDI–TOF (Supplementary Table 1). The 18 best culture conditions were selected using liquid media enrichment in a medium containing blood and rumen fluid and subculturing aerobically and anaerobically in a solid medium (Supplementary Table 2b)[2]. Subcultures were inoculated every three days on solid medium, and each medium was kept for 40 days. We applied these culture conditions to 40 stool samples, ultimately testing 565,242 colonies by MALDI–TOF (Supplementary Table 1).

Cohorts. In parallel to these main culturomics studies, we used fewer culture conditions to analyse a larger number of stool samples. We refer to these projects as cohorts. Four cohorts were analysed (pilgrims returning from the Hajj, premature infants with necrotizing enterocolitis, patients before and after bariatric surgery, and patients for acidophilic bacterial species detection). A total of 330 stool samples generated the 52,618 colonies tested by MALDI–TOF for this project (Supplementary Table 1).

Pilgrims from the Hajj. A cohort of 127 pilgrims was included and 254 rectal swabs were collected from the pilgrims: 127 samples were collected before the Hajj and 127 samples were collected after the Hajj. We inoculated 100 µl of liquid sample in an 8 ml bottle containing Trypticase Soy Broth (BD Diagnostics) and incubated the sample at 37 °C for 1 day. We inoculated 100 µl of the enriched sample into four culture media: Hektoen agar (BD Diagnostics), MacConkey agar+Cefotaxime (bioMérieux), Cepacia agar (AES Chemunex) and Columbia ANC agar (bioMérieux). The sample was diluted 10^{-3} before being plated on the MacConkey and Hektoen agars and 10^{-4} before being plated on the ANC agar. The sample was not diluted before being inoculated on the Cepacia agar. Subcultures were performed on Trypticase Soy Agar (BD Diagnostics) and 3,000 colonies were tested using MALDI–TOF.

Preterm neonates. Preterm neonates were recruited from four neonatal intensive care units (NICUs) in southern France from February 2009 to December 2012 (ref. 12). Only patients with definite or advanced necrotizing enterocolitis corresponding to Bell stages II and III were included. Fifteen controls were matched to 15 patients with necrotizing enterocolitis by sex, gestational age, birth weight, days of life, type of feeding, mode of delivery and duration of previous antibiotic therapy. The stool samples were inoculated into 54 preselected culture conditions (Supplementary Table 2c). The anaerobic cultures were performed in an anaerobic chamber (AES Chemunex). A total of 3,000 colonies were tested by MALDI–TOF for this project.

Stool analyses before and after bariatric surgery. We included 15 patients who had bariatric surgery (sleeve gastrectomy or Roux-en-Y gastric bypass) from 2009 to 2014. All stool samples were frozen before and after surgery. We used two different culture conditions for this project. Each stool sample was diluted in 2 ml of Dulbecco's phosphate-buffered saline, then pre-incubated in both anaerobic (BD Bactec Plus Lytic/10 Anaerobic) and aerobic (BD Bactec Plus Lytic/10 Aerobic) blood culture bottles, with 4 ml of sheep blood and 4 ml of sterile rumen fluid being added as previously described[4]. These cultures were subcultured on days 1, 3, 7, 10, 15, 21 and 30 in 5% sheep blood Columbia agar (bioMérieux), and 33,650 colonies were tested by MALDI–TOF.

Acidophilic bacteria. The pH of each stool sample was measured using a pH meter: 1 g of each stool specimen was diluted in 10 ml of neutral distilled water (pH 7) and centrifuged for 10 min at 13,000*g*; the pH values of the supernatants were then measured. Acidophilic bacteria were cultured after stool enrichment in a liquid medium consisting of Columbia Broth (Sigma-Aldrich) modified by the addition of (per litre) 5 g $MgSO_4$, 5 g $MgCl_2$, 2 g KCl, 2 g glucose and 1 g $CaCl_2$. The pH was adjusted to five different values: 4, 4.5, 5, 5.5 and 6, using HCl. The bacteria were then subcultured on solid medium containing the same nutritional components and pH as the culture enrichment. They were inoculated after 3, 7, 10 or 15 incubation days in liquid medium for each tested pH condition. Serial dilutions from 10^{-1} to 10^{-10} were then performed, and each dilution was plated on agar medium. Negative controls (no inoculation of the culture medium) were included for each condition.

Overall, 16 stool samples were inoculated, generating 12,968 colonies, which were tested by MALDI–TOF.

Optimization of the culturomics strategy. In parallel with this standardization period, we performed an interim analysis in order to detect gaps in our strategy. Analysing our previously published studies, we observed that 477 bacterial species

previously known from the human gut were not detected. Most of these species grew in strict anaerobic (209 species, 44%) or microaerophilic (25 species, 5%) conditions, and 161 of them (33%) belonged to the phylum Proteobacteria, whereas only 46 of them (9%) belonged to the phylum Bacteroidetes (Supplementary Table 3). The classification was performed using our own database: (http://www.mediterranee-infection.com/article.php?laref=374&titre=list-of-prokaryotes-according-to-their-aerotolerant-or-obligate-anaerobic-metabolism). Focusing on these bacterial species, we designed specific strategies with the aim of cultivating these missing bacteria.

Fresh stool samples

As the human gut includes extremely oxygen-sensitive bacterial species, and because frozen storage kills some bacteria[10], we tested 28 stool samples from healthy individuals and directly cultivated these samples on collection and without storage. Each sample was directly cultivated on agar plates, enriched in blood culture bottles (BD Bactec Plus Lytic/10 Anaerobic) and followed on days 2, 5, 10 and 15. Conditions tested were anaerobic Columbia with 5% sheep blood (bioMérieux) at 37 °C with or without thermic shock (20 min/80 °C), 28 °C, anaerobic Columbia with 5% sheep blood agar (bioMérieux) and 5% rumen fluid and R-medium (ascorbic acid 1 g l^{-1}, uric acid 0.4 g l^{-1}, and glutathione 1 g l^{-1}, pH adjusted to 7.2), as previously described[23]. For this project, 59,688 colonies were tested by MALDI–TOF.

Proteobacteria. We inoculated 110 stool samples using pre-incubation in blood culture bottles (BD Bactec Plus Lytic/10 Anaerobic) supplemented with vancomycin (100 µg l^{-1}; Sigma-Aldrich). The subcultures were performed on eight different selective solid media for the growth of Proteobacteria. We inoculated onto MacConkey agar (Biokar-Diagnostics), buffered charcoal yeast extract (BD Diagnostic), eosine-methylene blue agar (Biokar-Diagnostics), Salmonella–Shigella agar (Biokar-Diagnostics), Drigalski agar (Biokar-Diagnostics), Hektoen agar (Biokar-Diagnostics), thiosulfate-citrate-bile-sucrose (BioRad) and Yersinia agar (BD Diagnostic) and incubated at 37 °C, aerobically and anaerobically. For this project, 18,036 colonies were tested by MALDI–TOF.

Microaerophilic conditions. We inoculated 198 different stool samples directly onto agar or after pre-incubation in blood culture bottles (BD Bactec Plus Lytic/10 Anaerobic bottles, BD). Fifteen different culture conditions were tested using Pylori agar (bioMérieux), Campylobacter agar (BD), Gardnerella agar (bioMérieux), 5% sheep blood agar (bioMérieux) and our own R-medium as previously described[23]. We incubated Petri dishes only in microaerophilic conditions using GENbag microaer systems (bioMérieux) or CampyGen agar (bioMérieux), except the R-medium, which was incubated aerobically at 37 °C. These culture conditions generated 41,392 colonies, which were tested by MALDI–TOF.

Halophilic bacteria. In addition, we used new culture conditions to culture halophilic prokaryotes. The culture enrichment and isolation procedures for the culture of halophilic prokaryotes were performed in a Columbia broth medium (Sigma-Aldrich), modified by adding (per litre): $MgCl_2{\cdot}6H_2O$, 5 g; $MgSO_4{\cdot}7H_2O$, 5 g; KCl, 2 g; $CaCl_2{\cdot}2H_2O$, 1 g; NaBr, 0.5 g; $NaHCO_3$, 0.5 g and 2 g of glucose. The pH was adjusted to 7.5 with 10 M NaOH before autoclaving. All additives were purchased from Sigma-Aldrich. Four concentrations of NaCl were used (100 g l^{-1}, 150 g l^{-1}, 200 g l^{-1} and 250 g l^{-1}).

A total of 215 different stool samples were tested. One gram of each stool specimen was inoculated aerobically into 100 ml of liquid medium in flasks at 37 °C while stirring at 150 r.p.m. Subcultures were inoculated after 3, 10, 15 and 30 incubation days for each culture condition. Serial dilutions from 10^{-1} to 10^{-10} were then performed in the culture medium and then plated on agar medium. Negative controls (no inoculation of the culture medium) were included for each culture condition. After three days of incubation at 37 °C, different types of colonies appeared: yellow, cream, white and clear. Red and pink colonies began to appear after the 15th day. All colonies were picked and re-streaked several times to obtain pure cultures, which were subcultured on a solid medium consisting of Colombia agar medium (Sigma-Aldrich) NaCl. The negative controls remained sterile in all culture conditions, supporting the authenticity of our data.

Detection of microcolonies. Finally, we began to focus on microcolonies detected using a magnifying glass (Leica). These microcolonies, which were not visualized with the naked eye and ranged from 100 to 300 µm, did not allow direct identification by MALDI–TOF. We subcultured these bacteria in a liquid medium (Columbia broth, Sigma-Aldrich) to allow identification by MALDI–TOF after centrifugation. Ten stool samples were inoculated and then observed using this magnifying glass for this project, generating the 9,620 colonies tested.

Duodenum and other gut samples. Most of the study was designed to explore the gut microbiota using stool samples. Nevertheless, as the small intestine microbiota are located where the nutrients are digested[24], which means there are greater difficulties in accessing samples than when using stool specimens, we analysed different levels of sampling, including duodenum samples (Supplementary Table 1). First, we tested five duodenum samples previously frozen at −80 °C. A total of 25,000 colonies were tested by MALDI–TOF. In addition, we tested samples from the different gut levels (gastric, duodenum, ileum and left and right colon) of other patients. We tested 25,048 colonies by MALDI–TOF for this project. We tested 15 culture conditions, including pre-incubation in blood culture bottles with sterile rumen fluid and sheep blood (BD Bactec Plus Lytic/10 Anaerobic), 5% sheep blood agar (bioMérieux), and incubation in both microaerophilic and anaerobic conditions, R-medium[23] and Pylori agar (bioMérieux). Overall, we tested 50,048 colonies by MALDI–TOF for this project.

Archaea. The culture of methanogenic archaea is a fastidious process, and the necessary equipment for this purpose is expensive and reserved for specialized laboratories. With this technique, we isolated seven methanogenic archaea through culturomic studies as previously described[25–27]. In addition, we propose here an affordable alternative that does not require specific equipment[17]. Indeed, a simple double culture aerobic chamber separated by a microfilter (0.2 µm) was used to grow two types of microorganism that develop in perfect symbiosis. A pure culture of *Bacteroides thetaiotaomicron* was placed in the bottom chamber to produce the hydrogen necessary for the growth of the methanogenic archaea, which was trapped in the upper chamber. A culture of *Methanobrevibacter smithii* or other hydrogenotrophic methanogenic archaea had previously been placed in the chamber. In the case presented here, the methanogenic archaea were grown aerobically on an agar medium supplemented with three antioxidants (ascorbic acid, glutathione and uric acid) and without the addition of any external gas. We subsequently cultured four other methanogenic archaeal species for the first time aerobically, and successfully isolated 13 strains of *M. smithii* and 9 strains of *Methanobrevibacter oralis* from 100 stools and 45 oral samples. This medium allows aerobic isolation and antibiotic susceptibility testing. This change allows the routine study of methanogens, which have been neglected in clinical microbiology laboratories and may be useful for biogas production. Finally, to culture halophilic archaea, we designed specific culture conditions (described in the 'Halophilic bacteria' section).

Identification methods. The colonies were identified using MALDI–TOF MS. Each deposit was covered with 2 ml of a matrix solution (saturated α-cyano acid-4-hydroxycinnamic in 50% acetonitrile and 2.5% trifluoroacetic acid). This analysis was performed using a Microflex LT system (Bruker Daltonics). For each spectrum, a maximum of 100 peaks was used and these peaks were compared with those of previous samples in the computer database of the Bruker Base and our homemade database, including the spectra of the bacterial species identified in previous works[28,29]. An isolate was labelled as correctly identified at the species level when at least one of the colonies' spectra had a score ≥1.9 and another of the colonies' spectra had a score ≥1.7 (refs 28,29).

Protein profiles are regularly updated based on the results of clinical diagnoses and on new species providing new spectra. If, after three attempts, the species could not be accurately identified by MALDI–TOF, the isolate was identified by 16S rRNA sequencing as previously described. A threshold similarity value of >98.7% was chosen for identification at the species level. Below this value, a new species was suspected, and the isolate was described using taxonogenomics[30].

Classification of the prokaryotes species cultured. We used our own online prokaryotic repertoire[13] (http://hpr.mediterranee-infection.com/arkotheque/client/ihu_bacteries/recherche/index.php) to classify all isolated prokaryotes into four categories: new prokaryote species, previously known prokaryote species in the human gut, known species from the environment but first isolated in humans, and known species from humans but first isolated in the human gut. Briefly, to complete the recent work identifying all the prokaryotes isolated in humans[13], we examined methods by conducting a literature search, which included PubMed and books on infectious diseases. We examined the Medical Subject Headings (MeSH) indexing provided by Medline for bacteria isolated from the human gut and we then established two different queries to automatically obtain all articles indexed by Medline dealing with human gut isolation sites. These queries were applied to all bacterial species previously isolated from humans as previously described, and we obtained one or more articles for each species, confirming that the bacterium had been isolated from the human gut[13].

International deposition of the strains, 16S rRNA accession numbers and genome sequencing accession number. Most of the strains isolated in this study were deposited in CSUR (WDCM 875) and are easily available at http://www.mediterranee-infection.com/article.php?laref=14&titre=collection-de-souches&PHPSESSID=cncregk417fl97gheb8k7u7t07 (Supplementary Tables 4a and b). All the new prokaryote species were deposited into two international collections: CSUR and DSMZ (Supplementary Table 5). Importantly, among the 247 new prokaryotes species (197 in the present study and 50 in previous studies), we failed to subculture 9 species that were not deposited, of which 5 were nevertheless genome sequenced. Apart from these species, all CSUR accession numbers are available in Supplementary Table 5. Among these viable new species, 189 already have a DSMZ number. For the other 49 species, the accession number is not yet assigned but the strain is deposited. The 16S rRNA accession numbers of the 247 new prokaryotes species are available in Supplementary Table 5, along with the accession number of

the known species needing 16S rRNA amplification and sequencing for identification (Supplementary Table 14). Finally, the 168 draft genomes used for our analysis have already been deposited with an available GenBank accession number (Supplementary Table 5) and all other genome sequencing is still in progress, as the culturomics are still running in our laboratory.

New prokaryotes. All new prokaryote species have been or will be comprehensively described by taxonogenomics, including their metabolic properties, MALDI–TOF spectra and genome sequencing[30]. Among these 247 new prokaryote species, 95 have already been published (PMID available in Supplementary Table 5), including 70 full descriptions and 25 'new species announcements'. In addition, 20 are under review and the 132 others are ongoing (Supplementary Table 5). This includes 37 bacterial species already officially recognized (as detailed in Supplementary Table 5). All were sequenced successively with a paired-end strategy for high-throughput pyrosequencing on the 454-Titanium instrument from 2011 to 2013 and using MiSeq Technology (Illumina) with the mate pair strategy since 2013.

Metagenome sequencing. Total DNA was extracted from the samples using a method modified from the Qiagen stool procedure (QIAamp DNA Stool Mini Kit). For the first 24 metagenomes, we used GS FLX Titanium (Roche Applied Science). Primers were designed to produce an amplicon length (576 bp) that was approximately equivalent to the average length of reads produced by GS FLX Titanium (Roche Applied Science), as previously described. The primer pairs commonly used for gut microbiota were assessed *in silico* for sensitivity to sequences from all phyla of bacteria in the complete Ribosomal Database Project (RDP) database. Based on this assessment, the bacterial primers 917F and 1391R were selected. The V6 region of 16S rRNA was pyrosequenced with unidirectional sequencing from the forward primer with one-half of a GS FLX Titanium PicoTiterPlate Kit 70×75 per patient with the GS Titanium Sequencing Kit XLR70 after clonal amplification with the GS FLX Titanium LV emPCR Kit (Lib-L).

Sixty other metagenomes were sequenced for 16S rRNA sequencing using MiSeq technology. PCR-amplified templates of genomic DNA were produced using the surrounding conserved regions' V3–V4 primers with overhang adapters (FwOvAd_341F TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGG NGGCWGCAG; ReOvAd_785RGTCTCGTGGGCTCGGAGATG TGTATAAGA GACAGGACTACHVGGGTATCTAATCC). Samples were amplified individually for the 16S V3–V4 regions by Phusion High Fidelity DNA Polymerase (Thermo Fisher Scientific) and visualized on the Caliper Labchip II device (Illumina) by a DNA 1K LabChip at 561 bp. Phusion High Fidelity DNA Polymerase was chosen for PCR amplifications in this biodiversity approach and deep sequencing: a thermostable DNA polymerase characterized by the greatest accuracy, robust reactions and high tolerance for inhibitors, and finally by an error rate that is approximately 50-fold lower than that of DNA polymerase and sixfold lower than that of Pfu DNA polymerase. After purification on Ampure beads (Thermo Fisher Scientific), the concentrations were measured using high-sensitivity Qbit technology (Thermo Fisher Scientific). Using a subsequent limited-cycle PCR on 1 ng of each PCR product, Illumina sequencing adapters and dual-index barcodes were added to each amplicon. After purification on Ampure beads, the libraries were then normalized according to the Nextera XT (Illumina) protocol. The 96 multiplexed samples were pooled into a single library for sequencing on the MiSeq. The pooled library containing indexed amplicons was loaded onto the reagent cartridge and then onto the instrument along with the flow cell. Automated cluster generation and paired-end sequencing with dual index reads of 2 × 250 bp were performed in a single 39-hour run. On the instrument, the global cluster density and the global passed filter per flow cell were generated. The MiSeq Reporter software (Illumina) determined the percentage indexed and the clusters passing the filter for each amplicon or library. The raw data were configured in fasta files for R1 and R2 reads.

Genome sequencing. The genomes were sequenced using, successively, two high-throughput NGS technologies: Roche 454 and MiSeq Technology (Illumina) with paired-end application. Each project on the 454 sequencing technology was loaded on a quarter region of the GS Titanium PicoTiterPlate and sequenced with the GS FLX Titanium Sequencer (Roche). For the construction of the 454 library, 5 µg DNA was mechanically fragmented on the Covaris device (KBioScience-LGC Genomics) through miniTUBE-Red 5Kb. The DNA fragmentation was visualized through the Agilent 2100 BioAnalyser on a DNA LabChip7500. Circularization and fragmentation were performed on 100 ng. The library was then quantified on Quant-it Ribogreen kit (Invitrogen) using a Genios Tecan fluorometer. The library was clonally amplified at 0.5 and 1 cpb in 2 emPCR reactions according to the conditions for the GS Titanium SV emPCR Kit (Lib-L) v2 (Roche). These two enriched clonal amplifications were loaded onto the GS Titanium PicoTiterPlates and sequenced with the GS Titanium Sequencing Kit XLR70. The run was performed overnight and then analysed on the cluster through gsRunBrowser and gsAssembler_Roche. Sequences obtained with Roche were assembled on gsAssembler with 90% identity and 40 bp of overlap. The library for Illumina was prepared using the Mate Pair technology. To improve the assembly, the second application in was sometimes performed with paired ends. The paired-end and the mate-pair strategies were barcoded in order to be mixed, respectively, with 11 other genomic projects prepared with the Nextera XT DNA sample prep kit (Illumina) and 11 others projects with the Nextera Mate Pair sample prep kit (Illumina). The DNA was quantified by a Qbit assay with high-sensitivity kit (Life Technologies). In the first approach, the mate pair library was prepared with 1.5 µg genomic DNA using the Nextera mate pair Illumina guide. The genomic DNA sample was simultaneously fragmented and tagged with a mate-pair junction adapter. The profile of the fragmentation was validated on an Agilent 2100 Bioanalyzer (Agilent Technologies) with a DNA 7500 LabChip. The DNA fragments, which ranged in size, had an optimal size of 5 kb. No size selection was performed, and 600 ng of 'tagmented' fragments measured on the Qbit assay with the high-sensitivity kit were circularized. The circularized DNA was mechanically sheared to small fragments, with optimal fragments being 700 bp, on a Covaris S2 device in microtubes. The library profile was visualized on a High Sensitivity Bioanalyzer LabChip (Agilent Technologies). The libraries were normalized at 2 nM and pooled. After a denaturation step and dilution at 15 pM, the pool of libraries was loaded onto the reagent cartridge and then onto the instrument along with the flow cell. To prepare the paired-end library, 1 ng of genome as input was required. DNA was fragmented and tagged during the tagmentation step, with an optimal size distribution at 1 kb. Limited-cycle PCR amplification (12 cycles) completed the tag adapters and introduced dual-index barcodes. After purification on Ampure XP beads (Beckman Coulter), the library was normalized and loaded onto the reagent cartridge and then onto the instrument along with the flow cell. For the 2 Illumina applications, automated cluster generation and paired-end sequencing with index reads of 2 × 250 bp were performed in single 39-hour runs.

ORFans identification. Open reading frames (ORFs) were predicted using Prodigal with default parameters for each of the bacterial genomes. However, the predicted ORFs were excluded if they spanned a sequencing gap region. The predicted bacterial sequences were searched against the non-redundant protein sequence (NR) database (59,642,736 sequences, available from NCBI in 2015) using BLASTP. ORFans were identified if their BLASTP E-value was lower than 1e-03 for an alignment length greater than 80 amino acids. We used an E-value of 1e-05 if the alignment length was <80 amino acids. These threshold parameters have been used in previous studies to define ORFans (refs 12–14). The 168 genomes considered in this study are listed in Supplementary Table 7. These genomes represent 615.99 Mb and contain a total of 19,980 ORFans. Some of the ORFans from 30 genomes were calculated in a previous study[4] with the non-redundant protein sequence database containing 14,124,377 sequences available from NCBI in June 2011.

Metagenomic 16S sequences. We collected 325 runs of metagenomic 16S rRNA sequences available in the HMP data sets that correspond to stool samples from healthy human subjects. All samples were submitted to Illumina deep sequencing, resulting in 761,123 Mo per sample on average, and a total of 5,970,465 high-quality sequencing reads after trimming. These trimmed data sets were filtered using CLC Genomics Workbench 7.5, and reads shorter than 100 bp were discarded. We performed an alignment of 247 16S rRNA sequences against the 5,577,630 reads remaining using BLASTN. We used a 1e-03 e-value, 100% coverage and 98.7% cutoff, corresponding to the threshold for defining a species, as previously described. Finally, we reported the total number of aligned reads for each 16S rRNA sequence (Supplementary Table 8).

We collected the sequences of the 3,871,657 gene non-redundant gene catalogue from the 396 human gut microbiome samples (https://www.cbs.dtu.dk/projects/CAG/)[15]. We performed an alignment of 247 16S rRNA sequences against the 3,871,657 gene non-redundant gene catalogue using BLASTN with a threshold of 1e-03 e-value, 100% coverage and 98.7% cutoff. The new species identified in these data are reported in Supplementary Table 9. We collected the raw data sets of 239 runs deposited at EBI (ERP012217)[16]. We used the PEAR software (PMID 24142950) for merging raw Illumina paired-end reads using default parameters. We performed an alignment of 247 16S rRNA sequences against the 265,864,518 merged reads using BLASTN. We used a 1e-03 e-value, 100% coverage and 98.7% cutoff. The list of the new species identified in these data is included in Supplementary Table 9.

Whole metagenomic shotgun sequences. We collected the contigs/scaffolds from the assembly of 148 runs available in the HMP data sets. The initial reads of these samples were assembled using SOAPdenovo v.1.04 (PMID 23587118). These assemblies correspond to stool samples from healthy human subjects and generated 13,984,809 contigs/scaffolds with a minimum length of 200 bp and a maximum length of 371,412 bp. We aligned the 19,980 ORFans found previously against these data sets using BLASTN. We used a 1e-05 e-value, 80% coverage and 80% identity cutoff. Finally, we reported the total number of unique aligned ORFans for each species (Supplementary Table 8).

Study of the gaps in metagenomics. The raw fastq files of paired-end reads from an Illumina Miseq of 84 metagenomes analysed concomitantly by culturomics were filtered and analysed in the following steps (accession no. PRJEB13171).

Data processing: filtering the reads, dereplication and clustering. The paired-end reads of the corresponding raw fastq files were assembled into contigs using Pandaseq[31]. The high-quality sequences were then selected for the next steps of analysis by considering only those sequences that contained both primers (forward

and reverse). In the following filtering steps, the sequences containing N were removed. Sequences with length shorter than 200 nt were removed, and sequences longer than 500 nt were trimmed. Both forward and reverse primers were also removed from each of the sequences. An additional filtering step was applied to remove the chimaeric sequences using UCHIME (ref. 32) of USEARCH (ref. 33). The filtering steps were performed using the QIIME pipeline[34]. Strict dereplication (clustering of duplicate sequences) was performed on the filtered sequences, and they were then sorted by decreasing number of abundance[35–37]. For each metagenome, the clustering of OTUs was performed with 97% identity. Total OTUs from the 84 metagenomes (Supplementary Table 10) clustered with 93% identity.

Building reference databases. We downloaded the Silva SSU and LSU database1 and release 123 from the Silva website and, from this, a local database of predicted amplicon sequences was built by extracting the sequences containing both primers. Finally, we had our local reference database containing a total of 536,714 well-annotated sequences separated into two subdatabases according to their gut or non-gut origin. We created four other databases containing 16S rRNA of new species sequences and species isolated by culturomics separated into three groups (human gut, non-human gut, and human not reported in gut). The new species database contains 247 sequences, the human gut species database 374 sequences, the non-human gut species database 256 sequences and the human species not reported in gut database 237 sequences.

Taxonomic assignments. For taxonomic assignments, we applied at least 20 reads per OTU. The OTUs were then searched against each database using BLASTN (ref. 38). The best match of ≥97% identity and 100% coverage for each of the OTUs was extracted from the reference database, and taxonomy was assigned up to the species level. Finally, we counted the number of OTUs assigned to unique species.

Data availability and accession codes

The GenBank accession numbers for the sequences of the16SrRNA genes of the new bacterial species as well as their accession numbers in both Collection de Souches de l'Unité des Rickettsies (CSUR, WDCM 875) and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) are listed in Supplementary Table 5. Sequencing metagenomics data have been deposited in NCBI under Bioproject PRJEB13171 (https://www.ncbi.nlm.nih.gov/bioproject/PRJEB13171/).

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References

1. Lagier, J. C., Million, M., Hugon, P., Armougom, F. & Raoult, D. Human gut microbiota: repertoire and variations. *Front. Cell Infect. Microbiol.* **2,** 136 (2012).
2. Lagier, J. C. *et al.* The rebirth of culture in microbiology through the example of culturomics to study human gut microbiota. *Clin Microbiol Rev.* **28,** 237–264 (2015).
3. Pfleiderer, A. *et al.* Culturomics identified 11 new bacterial species from a single anorexia nervosa stool sample. *Eur. J. Clin. Microbiol. Infect. Dis.* **32,** 1471–1481 (2013).
4. Lagier, J. C. *et al.* Microbial culturomics: paradigm shift in the human gut microbiome study. *Clin. Microbiol. Infect.* **18,** 1185–1193 (2012).
5. Dubourg, G. *et al.* Culturomics and pyrosequencing evidence of the reduction in gut microbiota diversity in patients with broad-spectrum antibiotics. *Int. J. Antimicrob. Agents* **44,** 117–124 (2014).
6. Ley, R. E., Turnbaugh P. J., Klein S. & Gordon J. I. Microbial ecology: human gut microbes associated with obesity. *Nature* **444,** 1022–1023 (2006).
7. Ley, R. E. *et al.* Obesity alters gut microbial ecology. *Proc. Natl Acad. Sci. USA* **102,** 11070–11075 (2005).
8. Gill, S. R. *et al.* Metagenomic analysis of the human distal gut microbiome. *Science* **312,** 1355–1359 (2006).
9. Rinke, C. *et al.* Insights into the phylogeny and coding potential of microbial dark matter. *Nature* **499,** 431–437 (2013).
10. Lagier, J. C. *et al.* Current and past strategies for bacterial culture in clinical microbiology. *Clin. Microbiol. Rev.* **28,** 208–236 (2015).
11. Vetizou, M. *et al.* Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. *Science* **350,** 1079–1084 (2015).
12. Cassir, N. *et al. Clostridium butyricum* strains and dysbiosis linked to necrotizing enterocolitis in preterm neonates. *Clin. Infect. Dis.* **61,** 1107–1115.
13. Hugon, P. *et al.* A comprehensive repertoire of prokaryotic species identified in human beings. *Lancet Infect. Dis.* **15,** 1211–1219 (2015).
14. The Human Microbiome Project Consortium. A framework for human microbiome research. *Nature* **486,** 215–221 (2012).
15. Browne, H. P. *et al.* Culturing of 'unculturable' human microbiota reveals novel taxa and extensive sporulation. *Nature* **533,** 543–546 (2016).
16. Nielsen, H. B. *et al.* Identification and assembly of genomes and genetic elements in complex metagenomic samples without using reference genomes. *Nat. Biotechnol.* **32,** 822–828 (2014).
17. Khelaifia, S. *et al.* Aerobic culture of methanogenic archaea without an external source of hydrogen. *Eur. J. Clin. Microbiol. Infect. Dis.* **35,** 985–991 (2016).
18. Rettedal, E. A., Gumpert, H. & Sommer, M. O. Cultivation-based multiplex phenotyping of human gut microbiota allows targeted recovery of previously uncultured bacteria. *Nat. Commun.* **5,** 4714 (2014).
19. Hiergeist, A., Gläsner, J., Reischl, U. & Gessner, A. Analyses of intestinal microbiota: culture versus sequencing. *ILAR J.* **56,** 228–240 (2015).
20. Rajilic-Stojanovic, M. & de Vos, W. M. The first 1000 cultured species of the human gastrointestinal microbiota. *FEMS Microbiol. Rev.* **38,** 996–1047 (2014).
21. Byrd, A. L. & Segre, J. A. Infectious disease. Adapting Koch's postulates. *Science* **351,** 224–226 (2016).
22. Samb-Ba, B. *et al.* MALDI–TOF identification of the human gut microbiome in people with and without diarrhea in Senegal. *PLoS ONE* **9,** e87419 (2014).
23. Dione, N., Khelaifia, S., La Scola, B., Lagier, J.C. & Raoult D. A quasi-universal medium to break the aerobic/anaerobic bacterial culture dichotomy in clinical microbiology. *Clin. Microbiol. Infect.* **22,** 53–58 (2016).
24. Raoult, D. & Henrissat, B. Are stool samples suitable for studying the link between gut microbiota and obesity? *Eur. J. Epidemiol.* **29,** 307–309 (2014).
25. Khelaifia, S., Raoult, D. & Drancourt, M. A versatile medium for cultivating methanogenic archaea. *PLoS ONE* **8,** e61563 (2013).
26. Khelaifia, S. *et al.* Draft genome sequence of a human-associated isolate of methanobrevibacter arboriphilicus, the lowest-G+C-content archaeon. *Genome Announc.* **2,** e01181 (2014).
27. Dridi, B., Fardeau, M.-L., Ollivier, B., Raoult, D. & Drancourt, M. *Methanomassiliicoccus luminyensis* gen. nov., sp. nov., a methanogenic archaeon isolated from human faeces. *Int. J. Syst. Evol. Microbiol.* **62,** 1902–1907 (2012).
28. Seng, P. *et al.* Identification of rare pathogenic bacteria in a clinical microbiology laboratory: impact of matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* **51,** 2182–2194 (2013).
29. Seng, P. *et al.* Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clin. Infect. Dis.* **49,** 543–551 (2009).
30. Ramasamy, D. *et al.* A polyphasic strategy incorporating genomic data for the taxonomic description of novel bacterial species. *Int. J. Syst. Evol. Microbiol.* **64,** 384–391 (2014).
31. Masella, A. P., Bartram, A. K., Truszkowski, J. M., Brown, D. G. & Neufeld, J. D. PANDAseq: paired-end assembler for Illumina sequences. *BMC Bioinformatics* **13,** 31–13 (2012).
32. Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C. & Knight, R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27,** 2194–2200 (2011).
33. Edgar, R. C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26,** 2460–2461 (2010).
34. Caporaso, J. G. *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **7,** 335–336 (2010).
35. Stoeck, T. *et al.* Massively parallel tag sequencing reveals the complexity of anaerobic marine protistan communities. *BMC Biol.* **7,** 72–77 (2009).
36. Mondani, L. *et al. Microbacterium lemovicicum* sp. nov., a bacterium isolated from a natural uranium-rich soil. *Int. J. Syst. Evol. Microbiol.* **63,** 2600–2606 (2013).
37. Boissiere, A. *et al.* Midgut microbiota of the malaria mosquito vector *Anopheles gambiae* and interactions with *Plasmodium falciparum* infection. *PLoS Pathog.* **8,** e1002742 (2012).
38. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., Lipman, D. J. Basic local alignment search tool. *J. Mol. Biol.* **215,** 403–410 (1990).

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Author contributions

D.R. conceived and designed the experiments. J.-C.L., S.K., M.T.A., N.S., N.D., P.H., A.C., F.C., S.I.T., E.H.S., G.Dub., G.Dur., G.M., E.G. A.T., S.B., D.B., N.C., F.B., J.D., M.M., D.R., M.B., N.P.M.D.N., S.D.B., C.V., D.M., K.D., M.M., C.R., J.M.R., B.L.S., P.-E.F. and A.L. performed the experiments. D.M., J.A., E.I.A., F.B., M.Y., A.D., C.S., F.D. and V.V. contributed materials/analysis tools. J.-C.L., A.C., A.L. and D.R. analysed the data. J.-C.L., A.L. and D.R. wrote the manuscript. All authors read and approved the final manuscript. Q5

Additional information

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Competing interests

The authors declare no competing financial interest

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