Culturing of ‘unculturableView’ human microbiota reveals novel taxa and extensive sporulation

Hilary P. Browne1,2*, Samuel C. Forster1,2,3,4, Blessing O. Anonye1, Nitin Kumar1, B. Anne Neville1, Mark D. Stares1, David Goulding4 & Trevor D. Lawley1

Our intestinal microbiota harbours a diverse bacterial community required for our health, sustenance and wellbeing1,2. Intestinal colonization begins at birth and climaxes with the acquisition of two dominant groups of strict anaerobic bacteria belonging to the Firmicutes and Bacteroidetes phyla2. Culture-independent, genomic approaches have transformed our understanding of the role of the human microbiome in health and many diseases1. However, owing to the prevailing perception that our indigenous bacteria are largely recalcitrant to culture, many of their functions and phenotypes remain unknown3. Here we describe a novel workflow based on targeted phenotypic culturing linked to large-scale whole-genome sequencing, phylogenetic analysis and computational modelling that demonstrates that a substantial proportion of the intestinal bacteria are culturable. Applying this approach to healthy individuals, we isolated 137 bacterial species from characterized and candidate novel families, genera and species that were archived as pure cultures. Whole-genome and metagenomic sequencing, combined with computational and phenotypic analysis, suggests that at least 50–60% of the bacterial genera from the intestinal microbiota of a healthy individual produce resilient spores, specialized for host-to-host transmission. Our approach unlocks the human intestinal microbiota for phenotypic analysis and reveals how a marked proportion of oxygen-sensitive intestinal bacteria can be transmitted between individuals, affecting microbiota heritability.

A typical human intestinal microbiota contains 100–1,000 bacterial species with tremendous compositional diversity between individuals, such that each individual’s microbiota is as unique as a fingerprint1,4. Despite the taxonomic diversity, metagenomic sequencing has highlighted that a health-associated intestinal microbiome codes for highly conserved gene families and pathways associated with basic bacterial physiology and growth2. However, many basic microbiota functions related to homeostasis, immune system development, digestion, pathogen resistance and microbiota inheritance have yet to be discovered5. This formidable challenge to validate and decipher the functional attributes of the microbiota has been hindered because the majority of intestinal bacteria are widely considered to be ‘unculturable’ and have never been isolated in the laboratory5,6.

We sought to establish a genomic-based workflow that could be used as a platform for targeted culturing of specific bacterial phenotypes (Extended Data Fig. 1). Accordingly, we collected fresh faecal samples from six healthy humans and defined the resident bacterial communities with a combined metagenomic sequencing and bacterial culturing approach. Applying shotgun metagenomic sequencing, we profiled and compared the bacterial species present in the original faecal samples to those that grew as distinct colonies on agar plates containing the complex, broad-range bacteriological medium, YCFA7. Importantly, we observed a strong correlation between the two samples at the species level (Spearman’s ρ = 0.75, P < 0.001) (Fig. 1a). When sequenced, the original faecal sample and the cultured bacterial community shared an average of 93% of raw reads across the six donors. This overlap was 72% after de novo assembly (Extended Data Fig. 2). Comparison to a comprehensive gene catalogue that was derived by culture-independent means from the intestinal microbiota of 318 individuals4 found that 39.4% of the genes in the larger database were represented in our cohort and 73.5% of the 741 computationally derived metagenomic species identified through this analysis were also detectable in the cultured samples.

Together, these results demonstrate that a considerable proportion of the bacteria within the faecal microbiota can be cultured with a single growth medium. However, more than 8 × 106 distinct colonies would need to be picked from YCFA agar plates to match the species detection sensitivity of metagenomic sequencing. Thus, we established a broad-ranging culturing method that, when combined with high-throughput archiving or specific phenotypic selection, can be used to isolate and identify novel bacteria from the gastrointestinal tract.

The human intestinal microbiota is dominated by strict anaerobic bacteria that are extremely sensitive to ambient oxygen, so it is not known how these bacteria survive environmental exposure to be transmitted between individuals. Certain members of the Firmicutes phylum, including the diarrhoeal pathogen Clostridium difficile, produce metabolically dormant and highly resistant spores during colonization that facilitate both persistence within the host and environmental transmission6–10. Relatively few intestinal spore-forming bacteria have been cultured to date, and while metagenomic studies suggest that other unexpected members of the intestinal microbiota possess potential sporulation genes, these bacteria remain poorly characterized11–14. We hypothesized that sporulation is an unappreciated basic phenotype of the human intestinal microbiota that may have a profound impact on microbiota persistence and spread between humans. Spores from C. difficile are resistant to ethanol and this phenotype can be used to select for spores from a mixed population of spores and ethanol-sensitive vegetative cells15. Faecal samples with or without ethanol treatment were processed using our combined culture and metagenomics workflow (Extended Data Fig. 1). Principle component analysis demonstrated that ethanol treatment profoundly altered the culturable bacterial composition and, when compared to the original profile, efficiently enriched for ethanol-resistant bacteria, facilitating their isolation (Fig. 1b). We picked ~2,000 individual bacterial colonies from both ethanol-treated and non-ethanol-treated conditions, re-streaked them to purity, and performed full-length 16S ribosomal RNA gene sequencing to enable taxonomic characterization. Unique taxa were then archived as frozen stocks for future phenotypic analysis. In total, we archived bacteria representing 96% of the bacterial abundance at the genus level and 90% of the bacterial abundance at the species level based on average relative abundance across the six donors (Extended Data Fig. 3a, b). Even genera that were present at low average

1Host-Microbiota Interactions Laboratory, Wellcome Trust Sanger Institute, Hinxton CB10 1SA, UK. 2Centre for Innate Immunity and Infectious Diseases, Hudson Institute of Medical Research, Clayton, Victoria 3168, Australia. 3Department of Molecular and Translational Sciences, Monash University, Clayton, Victoria 3800, Australia. 4Microbial Pathogenesis Laboratory, Wellcome Trust Sanger Institute, Hinxton CB10 1SA, UK.

*These authors contributed equally to this work.

© 2016 Macmillan Publishers Limited. All rights reserved
Relative abundance (<0.1%) were isolated (Extended Data Fig. 3c). Overall, we archived 137 distinct bacterial species including 45 candidate novel species (Fig. 1c, Extended Data Fig. 3d and Supplementary Table 1), and isolates representing 20 candidate novel genera and 2 candidate novel families. Our collection contains 90 species from the Human Microbiome Project’s ‘most wanted’ list of previously uncultured and unsequenced microbes16 (Supplementary Table 1). Thus, our broad-range YCFA-based culturing approach led to massive bacterial discovery, and challenges the notion that the majority of the intestinal microbiota is unculturable.

We isolated and purified bacteria representing 66 distinct ethanol-resistant species that are distributed across 5 known families and 2 newly identified candidate families (Extended Data Fig. 3d and Extended Data Fig. 4). The identification of these new and unexpected spore-formers highlights the broad taxonomic distribution of this phenotype among the enteric species of the Firmicutes. To define the conserved genetic pathways underlying sporulation and germination within the intestinal microbiota, we sequenced, assembled and annotated the whole genomes of 234 archived ethanol-resistant and ethanol-sensitive bacteria. Previously, the gene markers used to identify spore-forming bacterial species have been based on underlying genetic assumptions13,17,18; here we applied an unbiased computational approach to define 66 conserved genes linked to an ethanol-resistance phenotype (Extended Data Fig. 5 and Supplementary Table 1). This gene set allows for the prediction of the sporulation capabilities of bacterial species isolated from diverse environments with a high degree of accuracy (Extended Data Fig. 6a and Supplementary Table 1) and consists of genes from a wide range of functional classes (Extended Data Fig. 6b and Supplementary Table 1).

To test whether commensal spore formation facilitates long-term environmental survival, we exposed a phylogenetically diverse selection of commensal spore-forming and non-spore-forming bacteria and C. difficile to ambient oxygen for increasing periods of time. Under these conditions, non-spore-forming bacteria remained viable for 2–6 days (48–144 h) (Fig. 2a). In contrast, commensal spore-forming bacteria, C. difficile and the facultative anaerobe Escherichia coli were able to survive stably to the end of the experiment on day 21 (504 h). In addition, spore-forming commensals and C. difficile, but not non-spore-forming commensals, survived prolonged exposure to the common disinfectant ethanol (Extended Data Fig. 7). These results demonstrate that commensal spore-formers and C. difficile share a core set of sporulation genes that confer a highly resistant phenotype that is associated with environmental spread between humans. C. difficile spores have evolved mechanisms to resume metabolism and vegetative growth after intestinal colonization by germinating in response to digestive bile acids released into the small intestine from the gall bladder9. We exposed enteric spore-formers and non-spore-formers to common bile acids (taurocholate, glycocholate and cholate) to assess their response to germinants after ethanol-shock treatment (Fig. 2b). Taurocholate was a potent germinant for all spore-formers, increasing the culturability of spores from commensal bacteria by between 8- and 70,000-fold (P < 0.05 for all spore-formers tested), whereas the other cholate derivatives had varying efficacy in germinating commensal spore-formers (Fig. 2b). Taurocholate and the other bile acids had no impact on the culturability of non-spore-formers, demonstrating that the effect is specific to spore-formers (Extended Data Fig. 8). We propose that this bile-acid-triggered ‘colonizing germination’ mechanism serves as a conserved in vivo cue to promote colonization by intestinal spore-forming bacteria. Thus, a duality of purpose exists in the modus operandi of intestinal spore-forming bacteria; spore formation ensures their survival and transmission while germination in response to in vivo cues ensures their persistence in the human population.

We next sought to estimate the proportions of spore-forming bacteria within the intestinal microbiota. Interrogation of the metagenomic data sets with the gene signature predicted that, on average, 60% of the genera contained spore-forming bacteria (Fig. 3a). These genera represented 30% of the total intestinal microbiota (Fig. 3b). We independently validated these observations with 16S rRNA gene amplicon sequencing (Extended Data Fig. 9). Importantly, these proportions of spore-forming bacteria were also observed in 1,351 publicly available faecal metagenomic data sets generated from healthy individuals19 (Fig. 3a, b). We also found the same proportion of spore-formers (61.3%) within the human population.

While the intestinal microbiota is considered to be relatively stable over time20, evidence suggests that close contact of family members promotes sharing of Ruminococcaceae and Lachnospiraceae bacteria21, families that we describe as spore-formers (Extended Data Fig. 4).
bacteria compared with non-spore-forming bacteria over this period.

We noted that in our cohort, the spore-forming bacteria of the micro-
biota were significantly more diverse than the non-spore-forming
bacteria (Fig. 3c). To test the dynamics of the spore-forming
and non-spore-forming bacteria over time, we analysed the metagenomic
profiles of faecal samples collected from the same healthy subjects
one year after the original sampling. Interestingly, we noted a sig-
nificantly increased variability in the proportion of spore-forming
bacteria compared with non-spore-forming bacteria over this period.
Received 25 September 2015; accepted 8 March 2016. Published online 4 May 2016.

1. Qin, J. et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464, 59–65 (2010).
2. Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K. & Knight, R. Diversity, stability and resilience of the human gut microbiota. Nature 489, 220–230 (2012).
3. Walker, A. W., Duncan, S. H., Louis, P. & Flint, H. J. Phylogeny, culturing, and metagenomics of the human gut microbiota. Trends Microbiol. 22, 267–274 (2014).
4. Nielsen, H. B. et al. Identification and assembly of genomes and genetic elements in complex metagenomic samples without using reference genomes. Nature Biotechnol. 32, 822–828 (2014).
5. Nelson, K. E. et al. A catalog of reference genomes from the human microbiome. Science 328, 994–999 (2010).
6. Stewart, E. J. Growing unculturable bacteria. J. Bacteriol. 194, 4151–4160 (2012).
7. Duncan, S. H., Hold, G. L., Harmsen, H. J., Stewart, C. S. & Flint, H. J. Growth requirements and fermentation products of Fusobacterium prausnitzii, and a proposal to reclassify it as Faecalibacterium prausnitzii gen. nov., comb. nov. Int. J. Syst. Evol. Microbiol. 52, 2141–2146 (2002).
8. Lawley, T. D. et al. Antibiotic treatment of Clostridium difficile carrier mice triggers a supershedder state, spore-mediated transmission, and severe disease in immunocompromised hosts. Infect. Immun. 77, 3661–3669 (2009).
9. Francis, M. B., Allen, C. A., Shrestha, R. & Sorg, J. A. Bile acid recognition by the Clostridium difficile germinant receptor, CspC, is important for establishing infection. PLoS Pathog. 9, e1003356 (2013).
10. Janoir, C. et al. Adaptive strategies and pathogenesis of Clostridium difficile from in vivo transcriptomics. Infect. Immun. 81, 3757–3769 (2013).
11. Rajilčić-Stojanović, M. & de Vos, W. M. The first 1000 cultured species of the human gastrointestinal microbiota. FEMS Microbiol. Rev. 38, 996–1047 (2014).
12. Galperin, M. Y. et al. Genomeic determinants of sporulation in Bacilli and Clostridia: towards the minimal set of sporulation-specific genes. Environ. Microbiol. 14, 2870–2890 (2012).
13. Abecasis, A. B. et al. A genomic signature and the identification of new sporulation genes. J. Bacteriol. 195, 2101–2115 (2013).
14. Meehan, C. J. & Beiko, R. G. A phylogenomic view of ecological specialization in the Lachnospiraceae, a family of digestive tract-associated bacteria. Genome Biol. Evol. 6, 703–713 (2014).
15. Riley, T. V., Brazier, J. S., Hassan, H., Williams, K. & Phillips, K. D. Comparison of alcohol shock enrichment and selective enrichment for the isolation of Clostridium difficile. Epidemiol. Infect. 95, 355–359 (1987).
16. Fodor, A. A. et al. The “most wanted” taxa from the human microbiome for whole genome sequencing. PLoS One 7, e41294 (2012).
17. de Hoon, M. J., Eichenberger, P. & Vitkup, D. Hierarchical evolution of the bacterial sporulation network. Curr. Biol. 20, R735–R745 (2010).
18. Onyenwoke, R. U., Brill, J. A., Farahi, K. & Wiegert, J. Sporulation genes in the bacterial sporulation, germination, and spore structural proteins. Trends Microbiol. 22, 406–416 (2014).
19. He, M. et al. Emergence and global spread of epidemic healthcare-associated Clostridium difficile. Nature Genet. 45, 109–113 (2013).
20. Faith, J. J. et al. The long-term stability of the human gut microbiota. Science 341, 1237439 (2013).
21. Schloss, P. D., Verson, K. D., Petrosino, J. F. & Schloss, S. J. The dynamics of a family’s gut microbiota reveal variations on a theme. Microbiome 2, 25 (2014).
22. Paredes-Sabja, D., Shen, A. & Sorg, J. A. Clostridium difficile spore biology: sporulation, germination, and spore structural proteins. Trends Microbiol. 22, 406–416 (2014).
23. Goodman, A. L. et al. Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice. Proc. Natl Acad. Sci. USA 108, 6252–6257 (2011).
24. Lager, J. C. et al. Microbial culturomics: paradigm shift in the human gut microbiome study. Clin. Microbiol. Infect. 18, 1185–1193 (2012).

Supplementary Information is available in the online version of the paper.

Acknowledgements This work was supported by the Wellcome Trust (098051); the United Kingdom Medical Research Council (PF451 to T.D.L.); the Australian National Health and Medical Research Council (1091097 to S.C.F.) and the Victorian Government’s Operational Infrastructure Support Program (S.C.F.). We are grateful to G. Dougan and A. Walker for their input. We would also like to acknowledge funding from the Wellcome Trust Sanger Institute Technology Translation team, and sequencing and bioinformatics support from the Pathogen Informatics team.

Author Contributions H.P.B., B.A.N. and H.P.B. performed bioinformatics analyses; H.P.B., S.C.F. and T.D.L. designed the study. H.P.B., B.A.N. and T.D.L. carried out anaerobic culturing and bacterial isolation; D.G. prepared TEM images; S.C.F., N.K. and H.P.B. performed bioinformatics analyses; H.P.B., S.C.F. and T.D.L. designed the study; H.P.B., S.C.F., B.A.N. and T.D.L. analysed data and wrote the paper.

Author Information Assembled and annotated genome sequence data have been deposited in the European Nucleotide Archive under accession number ERP012217. Bacterial isolates have been deposited at the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (http://www.dsmz.de), the CCUG-Culture Collection, University of Gothenburg, Sweden (http://www.ccug.se), the Belgian Co-ordinated Collection of Micro-organisms hosted by the Laboratory of Microbiology (BCCM/LMG) at Ghent University (http://www.bccm.belspo.be/) and at the Japan Collection of Microorganisms (JCM; http://jcm.br.nkan.riken.jp/en/). Isolate accession numbers are listed in Supplementary Table 1. Any isolates without accession numbers are available upon request. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to T.D.L. (t2@sanger.ac.uk).

This work is licensed under a Creative Commons Attribution 4.0 International (CC BY 4.0) licence. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons licence, users will need to obtain permission from the licence holder to reproduce the material. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© 2016 Macmillan Publishers Limited. All rights reserved
METHODS

Culturing. Fresh faecal samples were obtained from six consenting healthy adult human donors (1 faecal sample per donor: minimum 0.5 g) and were placed in anaerobic conditions within 1 h of passing to preserve the viability of anaerobic bacteria. All sample processing and culturing took place under anaerobic conditions in a Whitley DG250 workstation at 37 °C. Culture media, PBS and all other materials that were used for culturing were placed in the anaerobic cabinet 24 h before use to reduce to anaerobic conditions. The faecal samples were divided in two and each part was homogenized in reduced PBS (0.1 g of each per ml PBS) and was serially diluted and plated directly onto YCFA agar supplemented with 0.002 g ml⁻¹ each of glucose, maltose and cellobiose in large (13.5 cm diameter) Petri dishes. This sample was also subjected to metagenomic sequencing to profile the entire community. The other part was treated with an equal volume of 70% (v/v) ethanol for 4 h at room temperature under ambient aerobic conditions to kill vegetative cells. Then, the solid material was washed three times with PBS and it was eventually resuspended in PBS. Plating was performed as described earlier.

For the ethanol-treated samples, the medium was supplemented with 0.1% sodium taurocholate to stimulate spore germination. Colonies were picked 72 h after plating from Petri dishes of both ethanol-treated and non-ethanol-treated conditions harbouring non-confluent growth, (that is, plates on which the colonies were distinct and not touching). The colonies that were picked were re-streaked to confirm purity. No statistical methods were used to predetermined sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Microbiota profiling and sequencing. Identification of each isolate was performed by PCR amplification of the full-length 16S rRNA gene (using 7F (5′-AGAGTTTGATCCTGGCTCAG-3′) forward primer and 1510R (5′-ACGGYTACCTTGTTACGACTT-3′) reverse primer followed by capillary sequencing) and the remaining DNA sequence reads were aligned in the Ribosomal Database Project (RDP), manually curated in ARB26 and mothur27 was then used to classify reads to operational taxonomic units (OTUs). The R package seqinr version 3.1 was used to determine sequence similarity between OTUs and 98.7% was used as a species-level cut-off28,29. The full-length 16S rRNA gene sequence of each species-level OTU was compared to the RDP reference database to assign taxonomic designations to the genus level30 and a BLASTn search defined either a characterized or candidate novel species31.

Comparisons with the Human Microbiome Project (HMP) were carried out using 97% sequence similarity of the 16S rRNA gene sequenced from the cultured bacteria to define a species because only partial 16S rRNA gene sequences were available. HMP data regarding the most wanted taxa and the completed sequencings of the HMP catalogued in publically available metagenomic data sets from healthy individuals20 after taxonomic assignment using the Kraken35 taxonomic classification approach based on a custom database comprising communities of bacteria to define a species because only partial 16S rRNA gene sequences were available. HMP data regarding the most wanted taxa and the completed sequencings of the HMP catalogued in publically available metagenomic data sets from healthy individuals20 after taxonomic assignment using the Kraken database.

Oxygen sensitivity assay. Pure cultures were grown overnight in YCFA broth under anaerobic culture conditions as described earlier and the cultures were spotted in a dilution series onto YCFA agar containing 0.1% sodium taurocholate. Plates were incubated under ambient (aerobic) conditions at room temperature for specified time periods before being returned to the anaerobic cabinet. Colony-forming units (c.f.u.) were counted 72 h later. Cultures that were incubated anaerobically, and which were therefore not exposed to oxygen, acted as controls. Prior to the assay, all species were subjected to ethanol shock and were cultured anaerobically, and which were therefore not exposed to oxygen, acted as controls. Prior to the assay, all species were subjected to ethanol shock and were cultured anaerobically to determine their ability to sporulate. The viability of the oxygen-exposed cultures was expressed as a percentage of the viability of the anaerobic control cultures.

Germination response to intestinal bile acids assay. Pure cultures were grown overnight in YCFA broth under anaerobic conditions and were then washed by repeatedly centrifuging to a pellet and re-suspending in PBS. Vegetative cells were killed using an ethanol shock treatment as previously described and the cultures were then serially diluted and plated on YCFA agar with and without 0.1% intestinal bile salts (taurocholate, cholate and glycocholate). Colony-forming units (c.f.u.) were counted 72 h later. Cultures that were incubated anaerobically, and which were therefore not exposed to oxygen, acted as controls. Prior to the assay, all species were subjected to ethanol shock and were cultured anaerobically to determine their ability to sporulate. The viability of the oxygen-exposed cultures was expressed as a percentage of the viability of the anaerobic control cultures.

Oxidation of 16S rRNA gene sequence reads was applied to determine metagenomic species detection. Where appropriate, Spearman's rank correlation coefficient was applied for correlation analysis. Inverse Simpson's diversity index was calculated from Kraken output in R version 3.2.1 using the vegan: Community Ecology Package version 2.3-0.

Gene sporulation signature. Heuristic based bidirectional best hit analysis was performed to identify 21,342 conserved genes within the 694,300 genes annotated across the 234 sequenced genomes. Support vector, machine-based, contrast set association mining was applied to identify the optimal, weighted gene signature consisting of 66 genes. Species classification was performed using BLAST-based gene detection with percentage detection weighted by gene signature contribution and scaled to generate a total score between 0 and 1. Scores greater than 0.5 were considered true spore-formers based on comparison to known spore-formers. Signature-based abundance was assessed against 1,351 publically available metagenomic data sets from healthy individuals29 after taxonomic assignment using the Kraken database. Genera were considered spore-formers when all known species within that genus had a spore forming score greater than 0.5.

Transmission electron microscopy. Spore images were generated using transmission electron microscopy (TEM) as previously described30. Bacterial isolates for imaging were prepared by streaking pure cultures from frozen glycerol stocks and confirming purity by full-length 16S rRNA gene sequencing after one round of sub-culture to obtain visible and isolated single colonies. TEM images were prepared from culture plates 72 h after inoculation. The number of spore bodies visible in the TEM images was expressed as a percentage of the number of vegetative cells present and this ranged from 1% for Ruminococcus flavefaciens, 93% to 4% for Turicibacter sanguinis.

© 2016 Macmillan Publishers Limited. All rights reserved
30. Wang, Q., Garrity, G. M., Tiedje, J. M. & Cole, J. R. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* **73**, 5261–5267 (2007).
31. 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).
32. Price, M. N., Dehal, P. S. & Arkin, A. P. FastTree 2—approximately maximum-likelihood trees for large alignments. *PLoS One* **5**, e9490 (2010).
33. Letunic, I. & Bork, P. Interactive Tree Of Life v2: online annotation and display of phylogenetic trees made easy. *Nucleic Acids Res.* **39**, W475–W478 (2011).
34. Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K. & Schloss, P. D. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl. Environ. Microbiol.* **79**, 5112–5120 (2013).
35. Wood, D. E. & Salzberg, S. L. Kraken: ultrafast metagenomic sequence classification using exact alignments. *Genome Biol.* **15**, R46 (2014).
36. Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. *Nature Methods* **9**, 357–359 (2012).
37. Lawley, T. D. et al. Proteomic and genomic characterization of highly infectious *Clostridium difficile* 630 spores. *J. Bacteriol.* **191**, 5377–5386 (2009).
38. Bosshard, P. P., Zbinden, R. & Altwegg, M. *Turicibacter sanguinis* gen. nov., sp. nov., a novel anaerobic, Gram-positive bacterium. *Int. J. Syst. Evol. Microbiol.* **52**, 1263–1266 (2002).
39. Duncan, S. H., Hold, G. L., Barcenilla, A., Stewart, C. S. & Flint, H. J. *Roseburia intestinalis* sp. nov., a novel saccharolytic, butyrate-producing bacterium from human faeces. *Int. J. Syst. Evol. Microbiol.* **52**, 1615–1620 (2002).
40. Iino, T., Mori, K., Tanaka, K., Suzuki, K. & Harayama, S. *Oscillibacter valericigenes* gen. nov., sp. nov., a valerate-producing anaerobic bacterium isolated from the alimentary canal of a Japanese corbicula clam. *Int. J. Syst. Evol. Microbiol.* **57**, 1840–1845 (2007).
41. Paredes-Sabja, D., Setlow, P. & Sarker, M. R. Germination of spores of *Bacillales* and *Clostridiales* species: mechanisms and proteins involved. *Trends Microbiol.* **19**, 85–94 (2011).
**Extended Data Figure 1 | A workflow for culturing, archiving and characterization of the intestinal microbiota.** a–d. Schematic diagram of the workflow, encompassing bacterial culturing and genomics to isolate and characterize bacterial species from the human intestinal microbiota. The process incorporates several steps, which are culture, re-streak, archive and phenotype. a. Fresh faecal samples are left untreated or are treated to select for bacteria with a desired phenotype (such as sporulation). The stool is homogenized and then serially diluted and then aliquots of the homogenate are inoculated on YCFA agar to culture bacteria. b. Isolates are identified by selecting single colonies that are streaked to purity and full-length 16S rRNA genes are amplified and sequenced. c. Each unique, novel and desired isolate is archived frozen in a culture collection and a whole-genome sequence is generated for each. d. Phenotypic characterization and functional validation of metagenomics studies can be performed in vitro and in vivo.
Extended Data Figure 2 | Comparison of sequence read content of faecal samples and cultured samples for six donors. The majority of sequence reads from the original donor faecal samples (n = 6) are present in culture samples both as raw reads (93% shared on average across the six donors) and after de novo assembly (72% shared on average across the six donors).
Extended Data Figure 3 | Archiving of bacterial diversity and novelty through anaerobic culturing. a, b. Representative species from 21 of the 25 most abundant bacterial genera (a) and 23 of the 24 most abundant species (b) were isolated and archived (abundance was determined by metagenomic sequencing and based on average relative abundance across the six donors (n = 6)). This represents 96% of the average relative abundance at the genus level and 90% of the average relative abundance at the species level across the six donors. A red dot in a indicates the number of species archived from each genus. Lachnospiraceae incertae sedis, unclassified Lachnospiraceae, Clostridium IV and Clostridium XI are not strict genera and represent currently unclassified species. Odoribacter splanchnicus in b was the only species not archived. c, Lowly represented intestinal microbiota members were also cultured. At least one representative species from each of the genera presented were cultured. Median and range is presented for the above with taxa ranked by median value. d, The number of bacterial species cultured in this study. At least 40% from each category were previously unknown.
Extended Data Figure 4 | Phylogeny of intestinal spore-forming bacteria. Full length 16S rRNA gene phylogeny illustrating the taxonomic relationship of ethanol-resistant bacteria within the Firmicutes cultured from the donor faecal samples. Branch colours indicate distinct families. Shaded text indicates species cultured from an ethanol-treated faecal sample and unshaded text indicates species cultured from a non-ethanol-treated faecal sample. Percentage values represent closest identity to a characterized species. Transmission electron micrographs (TEMs) of spore ultrastructures for a phylogenetically diverse selection of cultured bacteria are shown with an arrow in images and include a candidate novel family with 86% identity to the 16S rRNA gene sequence from *Clostridium thermocellum*. Typical spore structures are defined and illustrated in the same image. TEMs are ordered according to boxes next to the species name. Scale bars are shown at the bottom of each image. *C. difficile* is included for context. Bacteria displaying an ethanol-resistant phenotype represent species previously classified as non-spore-formers (*Turibacter sanguinis* and closely related candidate novel species), species closely related to non-spore-formers (*Roseburia intestinalis* and *Oscillibacter valericigenes*) and closely related candidate novel species or species suspected of forming spores but which, to our knowledge, have never been demonstrated to do so until now (*Eubacterium eligens, Eubacterium rectale, Coprococcus comes*) and related candidate novel species.
Extended Data Figure 5 | Genomic signature of sporulation within the human intestinal microbiome. A genomic signature for identifying spore-forming bacterial species contains sporulation- and germination-associated genes and genes not previously associated with sporulation. Characterized sporulation genes are on the outer circle, genes not associated with a specific sporulation cycle or uncharacterized genes are in the inside rectangle. *Clostridium difficile* strain 630 gene names are used when possible, otherwise locus tag identifiers are shown. *Bacillus subtilis* gene names are used when no *C. difficile* homologue is available. The signature is enriched with known sporulation-associated genes from stages I–V of the spore formation and germination cycles (significant at $q < 3.0 \times 10^{-37}$, Fisher’s exact test). Genes associated with regulation are present with at least 10 genes coding for regulatory or DNA-binding roles ($q < 1.4 \times 10^{-35}$, Fisher’s exact test). Genes not previously associated with sporulation are also present and these have putative roles as heat shock, membrane-associated proteins and DNA-polymerase-associated proteins.
**Extended Data Figure 6** | Validation and characterization of the sporulation signature. **a**, The signature accurately distinguishes spore-forming and non-spore-forming bacteria cultured from this study and from across different environments (known spore-formers $n = 57$, known non-spore-formers $n = 50$, cultured after ethanol treatment $n = 69$, cultured after no ethanol treatment $n = 149$). Refer to Supplementary Table 1 for signature scores of the bacteria tested. Mean ± s.d. **b**, Assignment of functional classes to the signature reveals a wide range of functional processes with sporulation- and regulation-associated genes dominating.
Extended Data Figure 7 | Spore-forming bacteria are more resilient than non-spore-forming bacteria to environmental stresses such as disinfectants. Pure bacterial cultures were immersed in ethanol for 4 h before being washed and inoculated onto YCFA growth medium with sodium taurocholate as a germinant. Only spore-forming bacteria survived. Taxonomic family names are shown in brackets. The dashed line indicates the culture detection limit of 50 c.f.u. ml⁻¹. Mean ± s.d., n = 3 biological replicates for each species tested.
Extended Data Figure 8 | Growth response of non-spore-forming bacteria to intestinal germinants. The number of c.f.u. present on plates in the presence of a particular germinant expressed as a fold change with respect to the number of c.f.u. present on plates in the absence of a germinant. No ethanol shock treatment was performed beforehand. A fold change of one (dashed line) would indicate that a germinant had no effect on the number of c.f.u. recovered from the bacteria. There was no statistically significant difference based on an unpaired t-test of each germinant condition against the no germinant condition. Mean and range, \( n = 3 \) biological replicates for both species.
Extended Data Figure 9 | Validation of the estimation of the proportion of spore-formers in the intestinal microbiota. Full-length 16S rRNA gene amplicon sequencing was used to determine the taxonomic proportions of bacteria from the six donor faecal samples. Spore-forming bacteria were cultured from each donor and a taxonomic classification was assigned as described in the main text. The genus (circle) and family (square) taxonomic ranks were designated as the lower and upper limits for calculating the proportion of spore-formers at a taxonomic level. Specific genera and families were included if they contained a species that was cultured after ethanol shock treatment. Mean ± s.d.