Abundance determines the functional role of bacterial phylotypes in complex communities

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Bacterial communities are essential for the functioning of the Earth’s ecosystems1. A key challenge is to quantify the functional roles of bacterial taxa in nature to understand how the properties of ecosystems change over time or under different environmental conditions2. Such knowledge could be used, for example, to understand how bacteria modulate biogeochemical cycles3, and to engineer bacterial communities to optimize desirable functional processes4. Communities of bacteria are, however, extraordinarily complex with hundreds of interacting taxa in every gram of soil and every millilitre of pond water5. Little is known about how the tangled interactions within natural bacterial communities mediate ecosystem functioning, but high levels of bacterial diversity have led to the assumption that many taxa are functionally redundant6. Here, we pinpoint the bacterial taxa associated with keystone functional roles, and show that rare and common bacteria are implicated in fundamentally different types of ecosystem functioning. By growing hundreds of bacterial communities collected from a natural aquatic environment (rainwater-filled tree holes) under the same environmental conditions, we show that negative statistical interactions among abundant phylotypes drive variation in broad functional measures (respiration, metabolic potential, cell yield), whereas positive interactions between rare phylotypes influence narrow functional measures (the capacity of the communities to degrade specific substrates). The results alter our understanding of bacterial ecology by demonstrating that unique components of complex communities are associated with different types of ecosystem functioning.

The functional roles of bacterial taxa within communities can be estimated using manipulative experiments that build communities from pure cultures or that remove taxa from intact communities6. There are many difficulties with experiments using pure cultures: most bacteria cannot be isolated in pure culture, synthetic communities constructed from pure cultures might not represent any natural community and there are no general methods for knocking out specific taxa from natural communities. The alternative has been to use observational methods to look for correlated changes in taxon abundance and ecosystem functioning in nature, or to infer which functional processes are important from metagenomic or metatranscriptomic data7–10. Observational approaches also have noteworthy weaknesses because abiotic conditions (for example, pH, temperature) can impact both ecosystem functioning and community structure, making it difficult to infer causal relationships. We developed an alternative experimental approach that exploits the natural variation in bacterial community composition, comparable to the microbiome association studies that have been proposed for inferring causal relationships between human health and microbiome community composition11 (Fig. 1, steps 1–6).

We sampled 753 aquatic microbial communities, which were taken from a natural micro-ecosystem (rainwater pools in the buttressing of beech trees)12,13. The bacterial cells were separated from the surrounding environmental matrix by filtration and then stored (frozen) so that they could be revived (thawed) for repeatable experiments. Each bacterial community was revived and placed in laboratory microcosms containing a sterile beech leaf medium, which simulated some of the environmental conditions in the natural system. We quantified seven measures of ecosystem functioning associated with leaf litter degradation. Since the communities were assayed in a common environment, variation in ecosystem functioning was due to the initial differences in community composition. Correlations between the initial absolute abundance of each phylotype and ecosystem functioning allowed us to obtain community-wide estimates of the phylotypes that were associated with changes in functioning14, which could reflect their impact on functioning in nature. Although the approach requires growing the communities in simplified microcosms, there is no need to isolate individual taxa. The approach therefore lies between the complexity of natural ecosystems and the artificiality of synthetic communities.

We searched for associations between the abundance of each phylotype and the functional measurements, analogous to a genome-wide association scan in genetics15. We found 182 significant associations between phylotype abundance and the functional measurements, involving 112 of the 522 phylotypes used in the analysis. The associations were approximately equally balanced between negative (96 significant associations) and positive (86 significant associations), but differed substantially among the functional measurements (Supplementary Fig. 1). We therefore divided the functional measurements into two categories16,17. First, respiration, cell yield and metabolic potential (adenosine tri-phosphate (ATP) concentration) of the community were categorized as ‘broad’ ecosystem functions because they amalgamate many activities and are therefore performed by most community members. We expected widespread functional redundancy of taxa within the broad functional measures, which should result in few significant associations. Second, the cleavage rates of four substrates added to the microcosms were categorized as ‘narrow’ ecosystem functions because they encompass fewer activities and were therefore likely to be impacted by a relatively limited set of phylotypes. In contrast with the broad functional measures, we expected that the rise and fall in abundance of particular niche specialists would be associated with the rise and fall of the narrow functional measurements18.

Contrasting strongly with our predictions, significant associations were largely confined to the broad functional measures. For the broad functional measures, 90% of the 174 significant
associations were ‘common’ phylotypes (phylotypes that exceeded the median phylotype abundance) (Fig. 2, top panel). For example, the two phylotypes with the highest overall abundance across all communities (Serratia fonticola and Klebsiella pneumonia) also had among the strongest positive associations with respiration and cell yield. By contrast, only eight significant associations were found between phylotype abundances and the narrow functional measurements (Fig. 2, right panel). The associations imply that the abundance of individual phylotypes plays a role in the broad functional measures in ways that are not compensated by fully redundant phylotypes. There were linear increases in the number of significant associations with increasing sampling effort (number of communities) (Supplementary Fig. 2), showing that there are opportunities to uncover many more significant associations.

We expected the impact of each bacterial phylotype on the functional measurements to be mediated by the hundreds of phylotypes that surrounded them. We used the same experiment to characterize ‘functional interactions’: whether the associations between the abundance of each phylotype and the functional measurements were altered by the abundance of any of the other phylotypes in the community (Fig. 1, steps 5–6). A positive functional interaction indicated that ecosystem functioning tended to be elevated when both phylotypes had high abundance, whereas a negative functional interaction indicated that ecosystem functioning was lower when both phylotypes had high abundance. Functional interactions might result from direct (that is, biological) interactions among taxa, but such direct interaction would need to be verified (see Validation experiment). The method allowed us to reconstruct a complete portrait of functional interactions within a diverse assemblage (Fig. 2, central panel).

Theoretical studies have predicted that direct interspecific interactions (for example, competition, mutualism) should be negative and weak, but it is unclear whether these predictions extend to functional interactions. We found that negative and positive functional interactions tended to occur between common phylotypes for the broad functional measurements. In principle, all of the phylotypes contributed to the broad functional measurements, so it was unsurprising that interactions among the most common phylotypes had the largest influence. For the narrow functional measurements, functional interactions were between rare phylotypes, consistent with the idea that narrow functional measurements were driven by niche specialists (Fig. 2, central panel). The approach therefore generated predictions of which phylotypes in the ‘rare biosphere’ facilitate specific pathways.

The large number of significant functional interactions among phylotypes (364 of 368 significant interactions) suggested that specific metabolic pathways were maintained by collaborations among rare phylotypes. Positive interactions (for example, cross-feeding) have been known to emerge over short evolutionary timescales in simplified communities. The narrow functions used here measure
the capacity of the communities to produce secreted exo-enzymes that are publicly available, and which might provide a similar mechanism for positive interactions\(^2\). Positive functional interactions were less common for the broad functional measurements (131 of 265 significant interactions), with negative interactions concentrated among the most common phylotypes (Fig. 2, central panel). This result is compatible with culture-based studies using isolated bacteria: isolates obtained from the same study system showed a strong tendency towards negative functional interactions, and were also common phylotypes in our 16S gene libraries\(^{24,25}\). Within the limitations of the approach, the large number of positive interactions among rare phylotypes have not previously been documented, and could play a key role in generating hypotheses about how rare taxa impact functional processes.

The functional interactions uncovered here are correlations, and should be viewed as hypotheses that require independent validation using experiments that isolate each pairwise interaction. In practice, validation experiments face significant challenges because many of
The functional interactions are between phylotypes that have not previously been isolated in pure culture, and because the functional interactions might be contingent on the surrounding community. An advantage of using frozen, archived communities is that it is possible to perform post hoc ‘community mixture’ experiments to validate the correlational results. We mixed communities in microcosms to place potentially interacting phylotypes in contact. We determined whether the functional interactions we observed (Fig. 2) had a biological basis by mixing communities that each contained one member of the interacting pair. Mixtures of communities that placed interacting phylotypes together should increase (positive interaction) or decrease (negative interaction) the functional measurement relative to the mean functional measurement associated with each of the individual communities.

We revisited 12 communities to validate the positive functional interactions that we observed in the production of hemicellulase. We measured hemicellulase production in microcosms containing each of the communities on its own and all pairwise mixtures of the communities. Under a scenario of no interactions, the hemicellulase production would simply be the average of the constituent communities. Under a scenario of no interactions, the hemicellulase activity would be the mean activity of the two communities in the mixture. The y axis is the deviation from this expectation, with positive values indicating that hemicellulase activity in the mixed communities exceeded the mean of the two constituent communities. Hemicellulase activity was elevated in pairwise mixtures of communities that placed interacting phylotypes together (interactions, \( n = 9 \) mixtures) but not in communities that did not place interacting phylotypes together (no interactions, \( n = 56 \) mixtures) (analysis of variance, \( F_{1,8} = 5.1, P = 0.027 \)). The boxes are the interquartile range (IQR), the dark grey line is the median, and the whiskers extend to the most extreme datapoint that is no more than 1.5x IQR. Data points are individual mixtures average across \( n = 4 \) replicates. Mixtures were created from 12 communities.

The results here document strong relationships between structure and function in complex, non-synthetic communities under controlled conditions. Natural bacterial communities have previously been painted as worlds of vast functional redundancy, where high levels of niche overlap among phylotypes buffer ecosystem functioning against extinction\(^{36}\). It is clear from our results that the abundance of phylotypes is significantly associated with a range of functional measurements, both through their direct effects and through their interactions with other phylotypes. In both environmental microbiology and medical microbiology, there is a recognition that even those diseases or phenotypes that are caused by individual bacterial strains are mediated by complex interactions with many other taxa\(^{36}\). The common garden method offers a window into the functional role of phylotypes, and could in the future provide a method for unravelling the complex interactions among the thousands of phylotypes that inhabit natural environments.

**Methods**

**Microbiome collection.** We sampled 753 beech (Fagus sp.) rainwater-filled tree holes during August 2013 to April 2014 from locations across the south of England. Trees were primarily located in the Chilterns, west of London. These miniatures of aquatic habitats have been used extensively as ‘natural microcosms’\(^{38}\) that house diverse and accessible microbial communities. The water from each tree hole was homogenized by stirring, after which we collected a 1 ml sample, which was kept at ambient temperature until the samples were returned to the laboratory \((\leq 24\) hours). Each sample was diluted 1:4 in sterile PBS (pH 7.0, Sigma-Aldrich) using illumination (pore size 20–22 µm, Whatman 4 filter paper) to remove debris and large organisms. The filtrate containing the communities was used to inoculate 5 ml into a sterile beech leaf medium supplemented with 200 µg ml\(^{-1}\) cyclohexamide (Sigma-Aldrich) to inhibit fungi. Although fungi were excluded here to simplify the communities, fungi are likely to be important decomposers in this ecosystem, and would therefore be useful additions to future studies. Beech leaf medium was created by autoclaving 50 g dried beech leaves in 500 ml PBS\(^{15}\), which gave a concentrated stock after filtration of coarse particles. Beech leaf medium is composed of a complex array of carbon sources, which are exploited to differing degrees by bacteria isolated from the tree holes\(^{36}\). Each microcosm (polypropylene centrifuge tube) was incubated at 22°C under static conditions for 1 week to allow communities to reach stationary phase (Supplementary Fig. 4). Each regrown community was stored at –80°C after addition of freezing solution (final concentration 30% v/v glycerol and 0.85% w/v NaCl). Communities were stored frozen so as to allow repeatable experiments using the same starting community compositions. Community composition of the frozen communities was assessed using Illumina MiSeq (250-bp paired end) sequencing performed by Molecular Research DNA (www.mrdnalab.com). The V4 region of the 16S ribosomal RNA gene was amplified, using primers 515f/806r with the forward primer barcoded. Sequences were curated using a proprietary analysis pipeline by Molecular Research DNA; any sequences <150 bp and those with ambiguous base calls were removed, before denoising and editing for chimeras. Operational taxonomic units were specified at a 97% similarity cut-off, which we refer to as ‘phylotypes’ in the text\(^{36}\). We randomly sampled 15,000 sequences per sample to normalize sequencing effort. We used the number of reads per phylotype as a measure of their relative abundance in the community. Although many biases are introduced during the DNA extraction and PCR steps, these biases would be applied equally across the experiment. Rarefaction curves indicated that we had sampled most of the diversity going into the microcosms (Supplementary Fig. 5), and extrapolating to 20,000 sequencing reads\(^{36}\) (number of cells inoculated into each microcosm) indicated that we captured on average 95% of the total phylotype richness in the samples. We used a microbial community standard (Zymo Research) as a positive control to test the degree to which our DNA gene amplification step biased estimates of abundance. We found good correspondence between observed and expected abundances for most classes of bacteria, but Enterococcaceae and

**Fig. 3** Validation of the functional interactions using community mixture experiments. When communities were mixed together, hemicellulase activity was observed to be the mean activity of the two communities in the mixture. The y axis is the deviation from this expectation, with positive values indicating that hemicellulase activity in the mixed communities exceeded the mean of the two constituent communities. Hemicellulase activity was elevated in pairwise mixtures of communities that placed interacting phylotypes together (interactions, \( n = 9 \) mixtures) but not in communities that did not place interacting phylotypes together (no interactions, \( n = 56 \) mixtures) (analysis of variance, \( F_{1,8} = 5.1, P = 0.027 \)). The boxes are the interquartile range (IQR), the dark grey line is the median, and the whiskers extend to the most extreme datapoint that is no more than 1.5x IQR. Data points are individual mixtures average across \( n = 4 \) replicates. Mixtures were created from 12 communities.
Letters

Common garden experiment. Microcosms were established in 1.2-ml-deep 96-well plates containing 840 µl sterile beechn leaf medium and inoculated with 40 µl of each revived community (4 replicates per community, 3,172 microcosms in total). Cell densities within the communities were consistent across samples (mean 4.9 ± 10^6 cells ml^-1 ± 2.1 × 10^5 s.e.m.); thus, each microcosm was initiated with an average of 19,680 cells per µl. The sequencing effort (15,000 reads per sample) was similar to the number of cells used to initiate the microcosms, so we assumed the communities were almost fully characterized. We multiplied the relative abundances obtained from the sequencing by the initial cell numbers to obtain an estimate of absolute abundance of each phylotype at the beginning of the experiment. Although there are known biases in using amplicon sequencing data to measure relative abundance (for example, due to PCR conditions or DNA extraction methods), we expected those biases to be the same across the microcosms. The microcosms were incubated under static conditions at 22 °C for 7 days, after which we quantified each of the measurements of ecosystem functioning. Our intention was not to mimic the precise conditions of their native environment since those conditions differed among the communities. However, the microcosms successfully re-created an environment that produced both a standing density (mean 1.7 ± 10^6 cells ml^-1 ± 6.9 × 10^5 s.e.m.) and communities (Supplementary Fig. 4) that were similar to the native environment. We also tracked cell densities over 12 days in 32 randomly chosen communities (Supplementary Fig. 5). The data showed that all of the communities had reached carrying capacity well before seven days, implying that there would have been competition both for labile substrates (typically used during growth phase) and for more recalcitrant substrates (typically important later in succession).

We categorized community respiration, cell yield and metabolic potential as ‘broad’ functional measurements. Bacterial respiration was measured using the MicroResp CO2 detection system (www.microresp.com) according to the manufacturer instructions, with absorbance readings converted to weight of CO2 using a linear log–log relationship (R^2 = 0.965; Supplementary Fig. 7). Respiration measurements were taken as the cumulative respiration of the whole community over the 7-day incubation period. Yield was the final abundance of all bacterial cells in the community, which was quantified by staining the cells with thiazole orange (42 nM, Sigma-Aldrich) followed by obtaining absolute counts using a C6 Accuri flow cytometer (size threshold of 8,000 forward scatter height (FSC-H)), orange (42 nM, Sigma-Aldrich) followed by obtaining absolute counts using a C6 Accuri flow cytometer (size threshold of 8,000 forward scatter height (FSC-H)), and fungal cell walls), β-chitinase enzymes associated with organic matter degradation, including xylosidase (cleaves the labile substrate xylene, a monomer of cellulose and hemicellulose), β-chitinase (breaks down chitin, which is the main component of arthropod exoskeletons and fungal cell walls), β-glucosidase (breaks down cellulose, the structural component of plants) and phosphatase (breaks down organic monooesters for the mineralization and acquisition of phosphorus).

Common garden analysis. We used linear regressions to relate the functional measurements to phylotype abundance across the sites. For simplicity, we averaged the functional measurements across the four replicates, which yielded functional measurements from 753 communities. We excluded phylotypes from the analysis that were rare (<100 individuals across all samples) or that occurred (abundance >10) only <10 samples, which reduced the number of phylotypes for analysis from 1,314 to 522. These rare phylotypes were excluded because individual data points frequently had high leverage in the regressions, and because pairwise interactions were undefined because there was no covariation in abundances between rare phylotypes.

One-way associations. We performed linear regressions between the initial absolute abundance of every phylotype and every functional measurement:

\[ y = b_0 + b_1 \times \log(y+1), \]

where \( y \) is the functional measurement, \( b_0 \) is the intercept, \( b_1 \) is the slope associated with phylotype 1 and \( x \) is the absolute abundance (number of cells per microcosm) of phylotype 1 at the start of the experiment. We used the significance of the slope as an indication of whether the phylotype was associated with the functional measurement. \( P \) values were corrected for multiple testing across all of the one-way analyses using a Bonferroni correction, yielding a threshold \( P \) value of 1.4 × 10^{-5}.

Two-way functional interactions. We performed linear regressions that related the functional measurements to every pair of phylotypes:

\[ y = b_0 + b_1 \times \log(y+1), \]

where \( y \) is the functional measurement, \( b_0 \) is the intercept, \( b_1 \) and \( b_2 \) are the slopes (coefficients) associated with phylotype 1 and phylotype 2 (\( x_1 \) and \( x_2 \) respectively), \( y \) is the functional measurement associated with the interaction between phylotype 1 and phylotype 2 (\( x_1 \times x_2 \)), and \( b_2 \) is the coefficient associated with the interaction between phylotype 1 and phylotype 2 (\( x_1 \times x_2 \)). We used \( b_2 \) as an estimate of the ‘functional interaction’ between each pair of phylotypes. \( P \) values associated with multiple testing using a Bonferroni correction, yielding a significance threshold of 5.3 × 10^{-8}. Significant functional interactions imply that correlated changes in pairs of phylotype abundance are directly linked to changes in functioning, but such causal links would need to be verified with experiments.

Validation experiment. We revived 12 communities to validate the functional interactions. We replicated the common garden experiment (above) by dispensing 40 µl community into 840 µl sterile beechn leaf medium. The 40 µl of community contained either a single community or a 50:50 mixture of two communities. We measured the communities to degrade the substrates within the microcosms rather than the cells directly. This was particularly problematic for the broad functions, since our results indicated that community mixtures would bring together both positively and negatively interacting phylotypes. We therefore focused on hemicellulase production because our analysis indicated that all of the significant functional interactions were positive, making qualitative predictions straightforward.

Communities were incubated at 22 °C for 7 days, after which the hemicellulase activity was recorded as described above. We categorized the communities according to whether they contained none, one or both interacting phylotypes from among any of the significantly interacting phylotype pairs (Fig. 2, central panel). Mixed communities that placed two interacting phylotypes together that were not found together in either of the constituent communities would be expected to ‘realise’ their functional interaction. For the communities we examined, the following significantly interacting phylotypes pairs were combined: Deinococcus holkataensis, Acidimicrobium spp., Solibacter spp. × Aurantimonas manganoxydans, Solibacter spp. × Legionella spp., Deinococcus holkataensis, Leptospirillum spp., Anaerorhabdus spp. × Psychrobacter spp. Epilithonimonas lactis × Psychrobacter spp. The phylotype pairs have not previously been implicated in hemicellulase degradation so far as we are aware.

There were 9 of 65 community mixtures that placed interacting phylotypes into contact (Interactions), whereas the remainder did not place interacting phylotypes into contact (No interactions). We tested the hypothesis that hemicellulase production exceeded what would be expected under a null model. We assumed that, in the absence of any new interactions, mixed communities should be simply the mean of the communities in the mixture. For all of the community mixtures, we therefore subtracted the mean hemicellulase production in each constituent single phylotype community from the observed hemicellulase production in the mixture. We conducted a t-test to determine whether the deviation of hemicellulase production from this null expectation was higher in mixed communities with Interactions than communities with No interactions.

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The data used in the analysis are available in the FigShare repository with digital object identifiers 10.6084/m9.figshare.6100181 (phytotype table) and 10.6084/m9.figshare.6100340 (functional data). Sequence data that support the findings of this study have been deposited in the NCBI Short Read Archive (project number PRJNA453972, accession numbers SRRT136127–SRRT136873).

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Author contributions
The research was conceived by T.B. Experimental procedures were undertaken by D.W.R. Analysis and writing was done by T.B. and D.W.R.

Competing interests
The authors declare no competing interests.

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Software and code

Policy information about availability of computer code

Data collection

All data were collected using publicly available software; flow cytometry data was collected using the BD C6 Accuri software, all fluorescence/ luminescence data was collected using BioTek Gen5 Data Analysis software. Sequencing data were generated using the Illumina MiSeq platform and related software.

Data analysis

All data were analysed using R and related packages as cited in the text. DNA sequence data were initially processed using QIIME before being passed to R. All software used is publicly available.

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| Study description | Bacterial communities collected from the field were assayed in a common laboratory environment. |
|-------------------|--------------------------------------------------------------------------------------------------|
| Research sample   | Each bacterial community was a research sample.                                                  |
| Sampling strategy | Sample sizes were selected based on time and cost constraints.                                   |
| Data collection   | Data were collected by coauthor DWR using automated plate readers and flow cytometer.          |
| Timing and spatial scale | Timing of data collection was based on growth curves of the communities (see supplementary information). |
| Data exclusions   | The only data to be excluded from the analysis were from samples that were unable to reach the threshold for read coverage during 16S rRNA gene sequencing as described in the text. All other data were included in the analysis. |
| Reproducibility  | Communities were independently revived and assayed 4 times.                                    |
| Randomization     | Communities were randomised across the microcosms.                                              |
| Blinding          | No formal blinding was possible, but data were collected by automated readers.                   |

Did the study involve field work?  ☒ Yes  ☐ No

Field work, collection and transport

| Field conditions | Study locations and timing are described in the text. |
|------------------|-----------------------------------------------------|
| Location         | Study locations were in southern England. Specific locations are described in the text.          |
| Access and import/export | Local collection sites. No permits necessary.         |
| Disturbance      | Any disturbance would be very ephemeral.             |

Reporting for specific materials, systems and methods
Materials & experimental systems

- Involved in the study
- Unique biological materials
- Antibodies
- Eukaryotic cell lines
- Palaeontology
- Animals and other organisms
- Human research participants

Methods

- Involved in the study
- ChIP-seq
- Flow cytometry
- MRI-based neuroimaging

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

All unique bacterial communities used in this experiment are available from the corresponding author upon request.

Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation: Samples were extracted from the microcosms and immediately diluted in water containing 42 nM thiazole orange to stain “live” cells. Cells were then incubated at room temperature in the dark for at least 5 minutes prior to analysis.

Instrument: BD Biosciences C6 Accuri (C6 SN 4408)

Software: Flow cytometry data were collected using the BD Accuri™ C6 Software provided with the C6 Accuri flow cytometer.

Cell population abundance: Cell abundances were total absolute counts. There was no counting of subpopulations except to exclude background noise.

Gating strategy: Gates were created to exclude background fluorescence relative to negative controls (sterile media).

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.