Growth dynamics of gut microbiota in health and disease inferred from single metagenomic samples

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Metagenomic sequencing increased our understanding of the role of the microbiome in health and disease, yet it only provides a snapshot of a highly dynamic ecosystem. Here, we show that the pattern of metagenomic sequencing read coverage for different microbial genomes contains a single trough and a single peak, the latter coinciding with the bacterial origin of replication. Furthermore, the ratio of sequencing coverage between the peak and trough provides a quantitative measure of a species’ growth rate. We demonstrate this in vitro and in vivo, under different growth conditions, and in complex bacterial communities. For several bacterial species, peak-to-trough coverage ratios, but not relative abundances, correlated with the manifestation of inflammatory bowel disease and type II diabetes.

characterization of microbiome composition and function through shotgun sequencing has provided many insights into its roles in health and disease. Gene calling (1, 2), functional/pathway analysis (3–6), metagenomic-wide association studies (7, 8), genome assembly (9, 10), and metagenomic single-nucleotide polymorphism (SNP) detection (11) have all shown associations between microbiome configurations and susceptibility to several diseases, including obesity (4, 22), type II diabetes (7), auto-inflammatory disorders (1, 13), metabolic disease (12, 14), and cancer (15, 16). However, these approaches, which only examine a static snapshot of the microbiome at the point of collection, cannot be used to observe the highly dynamic nature of the microbiota and the differential activity of its microbial members.

Here, we asked whether microbiota growth dynamics could be probed from a single metagenomic sample by examining the pattern of sequencing read coverage across bacterial genomes. Apart from a few examples (17), most bacteria harbor a single circular chromosome, which replicates bidirectionally from a single fixed origin toward a single terminus (18) (Fig. 1A). Thus, during DNA replication, regions that have already been passed by the replication fork will have two copies, whereas the yet unreplicated regions will have a single copy.

This concept was previously used to detect the location of the replication origin in synchronized yeast colonies (19) but also holds true in an asynchronous bacterial population in which every cell may be at a different stage of replication. Summed across the population, the copy number of a DNA region will be higher the closer that region is to the replication origin and, conversely, lower the closer the region is to the terminus (20, 21). Hence, the ratio between DNA copy number near the replication origin and that near the terminus, which we term peak-to-trough ratio (PTR), should reflect the growth rate of the bacterial population. At higher growth rates, a larger fraction of cells undergo DNA replication and more active replication forks are present in each cell (22). This results in a ratio higher than 1:1 between near-origin DNA and near-terminus DNA, thereby providing a quantitative readout of the population growth rate (21).

We grew in vitro cultures of Escherichia coli (K-12 strain) and sequenced them at multiple time points during late lag phase, exponential phase, and early stationary phase (23). During stationary phase, when most of the cells in the culture are not growing and thus have a single copy of their genome, we found uniform cover- age across the genome (Fig. 1, A to C). In contrast, during exponential growth, when each bacterial cell may be at a different stage of DNA replication, the coverage pattern exhibited a single trough and a single peak, and the peak coincided with the known (24) replication origin (Fig. 1, A to C).

Similar patterns to those seen in vitro were also found for E. coli in 538 publicly available (3, 7, 9) human metagenomic fecal samples (Fig. 1B). PTRs extracted from these samples varied across individuals, in the range of 1 to 2.4, resembling the 1 to 2.6 range of ratios measured in vitro (Fig. 1B). Ratios higher than 2 are indicative of multifork replication, previously documented for E. coli (18, 22).

To examine whether PTRs provide a quantitative measure of growth rate, we calculated the temporal growth rate of E. coli at different times during its growth experiment as the derivative of its abundance across time (23). PTRs were correlated with the measured growth rate, peaking at 30 min (R = 0.95, P < 10⁻⁵) (Fig. 1D), indicating that PTR predicts the change in abundance. To determine whether PTRs accurately reflect steady-state growth rates (as opposed to temporal growth), we grew E. coli in an aerobic chemostat in which steady growth rates were controlled by changing the dilution rate of the system to induce a 16-fold range (23) and found excellent correlation (R = 0.996, P < 0.001) (fig. S1A) between the calculated PTRs and the measured growth rates. According to theoretical models (21), PTR = 2°C/G, where C is the replication time (C-period), and G is the generation time, and thus 1/log(PTR) is proportional to G/C. This transformation was correlated with measured bacterial generation time (R = 0.96, P < 0.01) (fig. S1B), confirming PTR as its proxy, even when the replication time is unknown.

The relationship between PTR and growth rate extends to other commensal strains, as we found that PTR and temporal measured growth rate were significantly correlated in similar cell growth experiments performed on Lactobacillus gasseri and Enterococcus faecalis under anaerobic conditions (L. gasseri, R = 0.74, P < 0.001; E. faecalis, R = 0.57, P < 0.05) (fig. S2, A and B). PTR also detects changes in growth rate mediated by changes in growth conditions, as PTRs and measured growth rate were correlated in additional cell growth experiments performed on E. faecalis in aerobic conditions and on E. coli in restricted growth conditions (E. coli, R = 0.92, P < 0.001; E. faecalis, R = 0.78, P < 10⁻⁵) (fig. S2, C and D). L. gasseri exhibited no growth in aerobic conditions, and accordingly we observed no change in PTRs (fig. S2E). PTRs for L. gasseri and E. faecalis were significantly different between aerobic and anaerobic conditions (E. faecalis, P < 0.05; L. gasseri, P < 0.001, Mann-Whitney U test) (fig. S2E).

To examine whether PTRs can be used to detect clinically relevant changes in culture conditions, we treated an in vitro culture of early log-phase naldixic acid-resistant Citrobacter rodentium with the bacteriostatic antibiotic ceftriaxone. Because bacteriostatic antibiotics halt bacterial growth, and thus indirectly inhibit replication, we postulated that ceftriaxone treatment would decrease PTRs. As a control, cultures were treated with naldixic acid, with the bactericidal antibiotic kanamycin, or left untreated. Indeed, ceftriaxone treatment lowered the PTR compared with controls (Mann-Whitney P < 0.001 and P < 10⁻⁴ for untreated control or naldixic acid treated control, respectively) (Fig. 2, A and B). PTR reduction
under erythromycin was evident within 30 min after administration and preceded the halt in exponential growth that was detected only 60 min after erythromycin treatment (Fig. 2, A to C). During antibiotic recovery, obtained by washing the cultures after 2.5 hours and removing the antibiotics (23), PTRs increased, consistent with the rise in abundance (Mann-Whitney P < 0.001) (Fig. 2, A and B).

Next, we grew L. gasseri within a mixture of six commensal bacterial strains (23) and observed a rise in its PTR corresponding with the rise in abundance, with PTR and temporal growth being correlated (R = 0.84, P < 0.001) (Fig. 2D).

Together, the in vitro experiments show that PTRs precede and predict changes in abundance even in the more complex setting of a mixed bacterial community, thereby establishing the link between PTRs and growth rate. To investigate whether PTRs remained accurate predictors of bacterial activity in a disease setting, we compared the proliferative behavior of virulent and nonvirulent (tir-mutant) strains of C. rodentium, which we used to infect C57BL/6 mice previously depleted of their native microbiota by wide-spectrum antibiotic treatment (23). We compared the in vivo abundance of both strains with PTRs and found that both showed similar behaviors 1 to 5 days post-infection (p.i.), with counts steadily rising from \(10^4\) to \(10^5\) CFU/ml at day 1 to \(10^8\) CFU/ml by day 5 (fig. S3). However, at 6 to 9 days p.i., the virulent strain displayed significantly higher counts (Mann-Whitney P < 0.05) (fig. S3) and PTRs (Mann-Whitney P < 0.001) (Fig. 3A) than the nonvirulent strain, likely reflecting preferential mucosal adhesion and proliferation (25). Whereas PTRs of the virulent strain were higher at days 6 to 9 p.i. compared with days 1 to 5 p.i. (Mann-Whitney P < 0.001) (Fig. 3A), PTRs of the nonvirulent strain 6 to 9 days p.i. were even lower than those of in vitro cultures of C. rodentium 1102.
in stationary phase (Mann-Whitney $P < 0.001$) (Fig. 3A).

To explore the utility of the PTR measure within the complex metagenomic setting, we devised a computational pipeline that extracts PTRs for multiple samples within large metagenomic cohorts (supplementary text and fig. S4). In devising this pipeline, we took care to address (i) genomic differences between strains of a certain species; (ii) copy number variation of different genomic regions; and (iii) variable coverage levels stemming from sequencing depth. We show that our method is robust to these drivers of noise (fig. S5 to S8 and supplementary text), attributed to our examination of coverage across the entire genome, as opposed to comparing the coverage of origin and terminus regions directly.

Examining the full length of the genome also allows us to predict the replication origin location in different bacteria. We verified that coverage peak locations coincided with known locations of origins of replication. To this end, we applied our pipeline to 759 metagenomic stool samples from Chinese and European cohorts (7, 9) and predicted the location of the origin and terminus of replication for 187 different microbial strains. Indeed, these predictions, computed solely based on our analysis of the bacterial genome coverage patterns, agreed with the known replication origins of 132 different strains ($24$ ($R^2 = 0.98, P < 10^{-30}$) (fig. S8), and for 55 strains whose replication origin location is unknown our method generated novel predictions (fig. S9).

To determine whether PTRs can uncover a possible interplay between host genetics and gut microbiome growth dynamics we collected fecal samples from three mouse strains (Swiss Webster, BALB/c, and C57BL/6) (23) grown under identical environmental conditions. Microbiota growth dynamics, as estimated by PTRs, differed significantly across the different mouse strains. In BALB/c mice, PTRs were lower overall compared with C57BL/6 and Swiss Webster mice ($P < 0.05$) (fig. S10A). The reduction in growth in the BALB/c mice was driven by Parabacteroides distasonis, which displayed consistently lower PTR than in other mouse strains (fig. S10, B to D), indicating that host genetics may affect the growth dynamics of this bacterium.

Another example of the utility of PTRs in assessing physiological microbiome growth patterns is provided in microbiome diurnal oscillation dynamics, as estimated by PTRs, which we recently linked to host susceptibility to obesity and glucose intolerance (26). Examining PTRs of fecal microbiomes collected from a human volunteer every 6 hours for four consecutive days, we identified two species, out of four that passed our PTR pipeline filters, that showed abundance levels cycling with a 24-hour periodicity (23). For both species, the PTRs also exhibited 24-hour oscillatory patterns ($P < 0.05$) (23) (Fig. 3B and fig. S11), suggesting that diurnal changes in the abundance of some bacteria were reflected in their PTRs.

To investigate the effect of an extreme dietary change on bacterial growth rates, two healthy human volunteers underwent an acute dietary change, in which they shifted their normal diet to one that contained only boiled white rice for 1 week, after which they reverted back to their regular dietary habits. In both participants, we observed a global change in gut bacterial growth dynamics between dietary regimens, as reflected in statistically significant differences in the PTRs of all bacteria between the days in which rice was consumed and the days in which the participant’s regular diet was followed ($P < 0.005$ for each participant) (Fig. 3, C and D).

We also examined body site–specific microbial growth rates in a metagenomic cohort (3) and found significantly higher PTRs in 229 tongue dorsum ($1.36 \pm 0.006$) and 193 buccal mucosa ($1.37 \pm 0.007$) samples, as compared with 325 stool samples ($1.16 \pm 0.002$; $P < 10^{-50}$ in both cases) (fig. S12). Six species were present in both the oral cavity and stool with sufficient coverage to calculate PTRs, with three featuring significantly different PTRs between sites [false discovery rate (FDR)–corrected Mann-Whitney $P < 0.1$] (fig. S12, B to G), indicating that intersite differences stem not only from distinct bacterial compositions but also from site-specific differences in growth dynamics.

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**Fig. 2. PTR accurately measures in vitro growth rates in multiple conditions.** (A to C) Absolute abundance levels [colony-forming units per ml (CFU/ml)] (top y axis, blue) and PTR (bottom y axis, red) as a function of time (minutes) of an in vitro culture of *C. rodentium* treated with erythromycin (bacteriostatic antibiotic in this setting; $N = 2$ repeats), compared to those of (A) an untreated control culture ($N = 3$ repeats); (B) a culture treated with nalidixic acid, a drug to which *C. rodentium* is resistant ($N = 3$ repeats); and (C) a culture treated with kanamycin, a bactericidal drug in this setting ($N = 3$ repeats). Background color indicates the treatment period (dark gray, left), recovery period (gray, middle), and early stationary phase (light gray, right). The black vertical line denotes antibiotic washout. PTR changes precede changes in growth. $P$ values are Mann-Whitney $U$ test between abundance (top) or PTR (bottom) of the two different cultures at times 30 to 150 min (left) or times 210 to 300 min (right). (D) Bacterial abundances (CFU/ml; top) and PTR (bottom) of *L. gasseri* and a mixture of six additional bacterial strains that inhabit the human gut. $N = 4$ repeats. Symbols, mean; error bars, mean $\pm$ SEM.
Overall, these results provide examples of functional insights that are not achievable using traditional metagenomics analysis methods and indicate that microbiome growth dynamics vary across diverse physiological conditions and locations.

To determine whether bacterial PTRs are associated with disease and different clinical parameters, we generated PTRs for every species in samples from European (N = 396) and Chinese (N = 363) cohorts. In both data sets, we found large variation in PTRs across samples (Fig. 4). Notably, we found statistically significant associations between the PTRs of 20 different bacteria and multiple clinical parameters, including significant correlations between the PTR of *Bifidobacterium longum* and occurrence of Crohn’s disease in the Spanish nationals of the European cohort (9) (FDR-corrected Mann-Whitney P < 0.005), and between the PTRs of 12 different bacteria and the occurrence of type II diabetes in the Chinese cohort (Fig. 4) (7). We also found significant correlations between PTRs and the occurrence of ulcerative colitis, body-mass index, the fraction of glycated hemoglobin (HbA1c%, a common marker of long-term glycemic control) (27), fasting serum insulin, and fasting blood glucose levels (Fig. 4).

These associations are independent of—and unobtainable by examining—bacterial abundances, because (i) in correlating PTRs with clinical parameters, we only used samples in which that bacteria was present, thereby withholding information about the presence or absence of the examined bacteria (23); (ii) in only 5 of the 38 statistically significant correlations were the abundance levels of the species also correlated with the same clinical parameter; and (iii) 36 of the 38 significant associations of PTR remained significant after correcting them for relative abundance levels. The PTRs of some species were correlated with clinical parameters only after correction for relative abundance, including *Eubacterium rectale* and the occurrence of Crohn’s disease (FDR-corrected Mann-Whitney P < 10^{-4}).

As a global measure of the growth dynamics of the entire microbiota, for every sample we...
calculated both the mean and the median of the PTRs of all the bacteria present. This global measure correlated with fasting blood glucose and HbA1c% levels and with the occurrence of Crohn’s disease and type II diabetes, indicating that global microbiome growth dynamics also associate with disease (Fig. 4).

A preliminary analysis of 40 samples from the Prospective Registry in Inflammatory Bowel Disease (IBD) Study at Massachusetts General Hospital (MGH) (PRISM) cohort (23) showed that only four bacteria passed our stringent pipeline filters for PTR calculation in more than half of the samples. Notwithstanding, *Eggerthella lenta* presented significantly different PTRs between patients with active Crohn’s disease and patients in remission (FDR-corrected Mann-Whitney *P* < 0.1). Neither the abundance of *E. lenta* nor of the other three species differed between active and quiescent Crohn’s patients, highlighting the fact that PTRs reflect an independent feature of the effect of the gut microbiome on its host.

Overall, we present a new type of metagenomic data analysis that provides an accurate quantitative estimate of the growth dynamics of the microbiota from a single snapshot sample. These estimates have clinical relevance and correspond to changes in absolute abundances, which are masked by and unobtainable through relative abundances.

Using PTRs to “fish out” microbial kinetic behavior in a complex microbiome population could extend our understanding of how flexibly the microbiota responds functionally to environmental signals. We may be able to identify active “driver” and “modulator” species, distinguish them from bystander commensal species, and pinpoint disease-causing or disease-modulating microbes that contribute to multifactorial diseases whose activities may be masked by other bacteria. Furthermore, our method may be able to detect, follow, and assess therapeutic responsiveness of pathogenic or probiotic species introduced into the microbiome.

**REFERENCES AND NOTES**

ALLERGY

Farm dust and endotoxin protect against allergy through A20 induction in lung epithelial cells

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Growing up on a dairy farm protects children from allergy, hay fever, and asthma. A mechanism linking exposure to this endotoxin (bacterial lipopolysaccharide)–rich environment with protection has remained elusive. Here we show that chronic exposure to low-dose endotoxin or farm dust protects mice from developing house dust mite (HDM)–induced asthma. Endotoxin reduced epithelial cell cytokines that activate dendritic cells (DCs), thus suppressing type 2 immunity to HDMs. Loss of the ubiquitin-modifying enzyme A20 in lung epithelium abolished the protective effect. A single-nucleotide polymorphism in the gene encoding A20 was associated with allergy and asthma risk in children growing up on farms. Thus, the farming environment protects from allergy by modifying the communication between barrier epithelial cells and DCs through A20 induction.

Alergic asthma is characterized by eosinophilic airway inflammation, goblet cell metaplasia, and bronchial hyperreactivity (BHR) and is controlled by innate and adaptive immune responses to inhaled allergens such as house dust mites (HDMs), pollen, and fungal spores that signal via pattern recognition receptors (PRRs) on barrier epithelial cells (ECs) and dendritic cells (DCs) (I, 2). In children, allergic sensitization and asthma are strongly influenced by genes and the environment. A dairy farm is one of the strongest protective environments (3–6). On farms, there is high-level exposure to endotoxin [lipopolysaccharide (LPS)], a cell wall component of Gram-negative bacteria. The protective effect that high levels of environmental endotoxin demonstrate against allergy has also been noted in nonfarming households, where exposure was measured in dust collected from mattresses or kitchen floors (7–9). Protection in these environments is influenced by genetic polymorphisms in key PRRs that recognize endotoxin (10). A clear mechanism encompassing the complex interactions between a protective environment, genes, and the immune response to allergens has been lacking.

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To address whether exposure to environmental endotoxin and protection from allergy are causally related, we exposed mice every other day for 2 weeks to a low dose (100 ng) of LPS or to control phosphate-buffered saline (PBS) before HDN sensitization and challenge (Fig. 1A) (see supplementary materials and methods). Sham-protected mice exhibited strong airway eosinophilia and lymphocytosis (Fig. 1B), T helper 2 (Th2)-dependent HDM allergen–specific immunoglobulin E (IgE) (Fig. 1C), and BHR to methacholine (Fig. 1D). However, mice pretreated with LPS failed to develop all of these canonical asthma features. Protective LPS led to reduced production of the type 2 cytokines interleukin (IL)-5 and IL-13 in mediastinal lymph node (MLN) cells (Fig. 1E), without a shift to Th1- or Th17-associated cytokines or to Th1-dependent serum immunoglobulin G2a (IgG2a) antibodies (Fig. 1F). All of the key asthma features were also suppressed when a single high dose (1 μg) of LPS was given as a preventive regimen 14 days before sensitization (Fig. 1G, A to E), as well as when chronic low-dose LPS was given before and throughout the entire HDN sensitization and challenge period (Fig. 1G, F to I).

Sensitization to HDMs depends on various DC subsets that migrate to the MLNs to prime CD4+ T cell responses (11, 12). When PBS-treated control mice were exposed to a single dose of HDN, CD11b+ conventional DCs (cDCs), CD103+ DCs, and monocyte-derived DCs (moDCs) were recollected to the lungs and MLNs (Fig. 2A). In mice receiving preventive LPS, there was less HDN-induced recruitment of both subsets of cDCs, whereas moDCs were unaffected (Fig. 2A). cDCs that migrate to the MLNs cells induce T<sub>2</sub> polarization in HDN-reactive naïve T cells (12). To study the primary immune response to HDMs, we adaptively transferred CD4<sup>+</sup> HDM-specific 1-DER T cells that express a transgenic T cell receptor...
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