Knockout of a labeled strain of *Escherichia coli* in the mouse gut using native phages

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Abstract

Background: There is a lack of methodological investigation of the in situ functions of a bacterial species in microecosystems such as the animal gut, although the microbiome has become a focus in the microbiology field worldwide. **Results:** We used native mixed phages containing Escherichia phages T1 and T4 as a microbial editing tool for eliminating Escherichia coli MG1655 labeled with green fluorescent protein in the mouse gut. The phages possessed rigorous host specificity at both the genus and species levels, resulting in an 8.8-log₁₀ decrease in the titer of viable bacteria after 12 h of phage treatment in an in vitro test. In vivo, they knocked out strain MG1655 not only at concentrations of 10⁶-10⁸ CFU g⁻¹ colonizing the mouse gut but also even in mice fed with feedstuff containing the bacterium. In addition, the impact of phage treatment on the microbial community structure of the mouse gut was not significantly (p > 0.05) based on a 16S rRNA amplicon gene sequencing analysis, although the richness of some bacteria changed significantly. **Conclusions:** We provide a feasible microbial editing technique for the animal gut. Native phages with strict host specificity can effectively knock out a target bacterium by single or continuous gastric perfusion, with limited perturbation of microbial diversity, which is beneficial for studies of the function of a specific bacterial species colonizing a complicated microecosystem.

Background

The microbiome, defined as the totality of microorganisms and their collective genetic materials in a well-recognized ecosystem, has become a current focus of scientists and governments. It contains information including the composition and structure of the microbiome, genetic and physiological functions, and even interactions between microbes and their hosts or environments. A series of national plans/initiatives have been launched to support research on the microbiome, with the aim to expand understanding about nature and humans themselves [1-3]. It is believed that the outcome of microbiome research will provide new resources and innovative technology for social and economic developments, as well as improve the quality of life of human beings. The human microbiome, including the gastrointestinal microbiome, respiratory microbiome, reproductive tract microbiome, oral microbiome and skin microbiome, is closely related to human health, as an integral part of human body [4]. The gut flora has particularly important roles in the nutritional, immunological, and physiologic processes of the host. The human gut harbors at least 100 trillion microbial cells, and the quantity is 10 times that of the human body's own cells. Approximately 1,000 to 1,150 species of bacteria colonize the human intestine, with an average of approximately 160 dominant species in each body [5-7]. The gut flora is correlated with intestinal inflammatory bowel disease, Crohn's disease, diabetes, asthma, liver disease, obesity, mental disease and cancer, making it an important metabolic organ [8-12].

There is currently a lack of knowledge of how to methodologically study the in situ function of a specific species in a complicated ecosystem. Generally, knowledge about microorganisms obtained by pure cultivation cannot accurately represent the role they play in the ecosystem. Current sequencing-based analyses of the microbiota, including DNA chips and metagenomic and 16S rRNA gene sequencing, only

permit evaluation of the correlation between a species, genus or core microbiota and the phenotype of the host by determining the variation in microbial abundance and diversity, and even further comparative analyses of relative microbiome data cannot provide information about the extent or directionality of changes in abundance or metabolic potential due to nonquantitative profiling [13-15]. Thus, these relative approaches are limited in revealing the interplay between the specific microorganism or microbiota constituent and host phenotypes or health [16, 17]. In addition, various gnotobiotic and germ-free (GF) animals, including zebrafish and mice, are also used to study the function of the gut flora [18, 19]. However, these animals can only be used to investigate the function of the specific microorganism or microbiota under a simple background setting, and how a species works in a complicated community cannot be uncovered in this manner. This methodological deficiency is a major bottleneck for studies of the function of a specific microorganism in the microbiota, although a few novel techniques are emerging, including isotope probes or quantitative microbiome profiling [17, 20]. Theoretically, similar to gene function revealed using gene knockout and complementation techniques, the function of a specific microorganism in a microbiota should be illuminated by microbial knockout and complementation. In fact, the identification of pathogenic bacteria in infectious diseases by Koch's law follows the principle of microbial complementation since it is technically easy and operable for a culturable microorganism. Conversely, strategies to knock out a microbial species in a complicated microsystem and ensure that its neighbors are not mistakenly removed due to off-target effects is a substantial technical challenge.

Phages, the viruses that infect bacteria, exist widely in the environment. As antibacterial agents, they have been applied in the prevention and control of bacterial infection or contamination in animals, crops and foods [21-23]. In the present work, we used isolated phages with high host specificity to knock out *Escherichia coli* MG1655 in the mouse gut, and the feasibility of phage as a bacterial knockout tool for the gut microbiota was evaluated. This study will contribute to the development of a simple and efficient technique for investigations of the function of a bacterial species in ecosystems.

Results

Morphology and sequencing analysis of phages

Two phages, W1 and W3, were isolated from mouse fecal samples and selected for further knockout testing due to their superior lysis ability against strain MG1655. Their plaques are shown in Additional file 1: Fig. S1a. Phage W1 had an icosahedral head (65 ± 2 nm in diameter) and a soft, but flexible curved tail with a length of approximately 100 ± 20 nm under TEM (Additional file 1: Fig. S1b). Compared with phage W1, phage W3 was larger, with a 100 ± 10 -nm-wide icosahedral head and 150 ± 10 -nm-long tail composed of the tail tube, base plate, short spines and long tail fibers (Additional file 1: Fig. S1c). Phage W1 contained a double-stranded DNA, and its complete genome consisted of 48,737 nucleotides with an average GC content of 46.13%. Gene annotation analysis showed that the phage contained 72 genes, and 8 genes encoded structural proteins of the capsid and tail (Fig. 1a). The genome of phage W1 showed the highest similarity to *Escherichia* virus T1 (GenBank accession numbers of MK213796 and AY216660), with 100% identity on the basis of the 100% query coverage [24], indicating that it is an *Escherichia* phage

T1 (Fig. 1b). The double-stranded [DNAgenome](#) of phage W3 is 168,040 bp with an average GC content of 35.54%, and it encodes 173 proteins, including structural proteins of the capsid and tail, as well as cytolysis, DNA repair, transcription and nucleic acid metabolism enzymes (Fig. 1c). The complete genome of phage W3 showed the highest similarity to *Escherichia* virus T4 (GenBank accession numbers of KJ477685, KJ477684 and KJ477686), indicating that the phage is an *Escherichia* phage T4 (Fig. 1d).

Host range of phages

Forty-two strains belonging to 21 genera were collected (Additional file 2: Table S1) for host-specific analysis of phages W1 and W3. No plaques appeared on LB plates, which indicated that neither phage infected these strains affiliated with twenty genera of *Enterobacteriaceae* (Fig. 2). Thus, the phages possessed high host specificity at the genus level. At the species level of *Escherichia*, phage W1 was not infectious to all six strains tested, while phage W3 could lyse *E. fergusonii*, an emerging species within the genus *Escherichia* proposed by Farmer et al [25], showing a wider host range than phage W1. In addition, phage W3 lysed all subspecies of *E. coli* tested. Interestingly, phage W1 was not suited for infecting strains DH5 α , Scarabxpress and zzy7 isolated. Therefore, the phages could distinguish the different genera under *Enterobacteriaceae* and species under *Escherichia* (except *E. fergusonii*), although they showed little host specificity at the subspecies level.

Transformation of pGFPuv and stability analysis

A strain of *E. coli* MG1655 tagged by green fluorescence protein (GFP) was prepared to differentiate the tested strain with the aboriginal *E. coli* strains in mouse gut. Plasmid extracted from the obtained transformants (T_{GFP}) was digested with the Mlu I enzyme, and electrophoresis showed that pGFPuv was successfully introduced into strain MG1655 (Additional file 3: Fig. S2). Compared with the untagged strain, T_{GFP} with green fluorescence is easily observed under fluorescence microscopy (Figs. 3a and 3b). Moreover, it also exhibits blue and green fluorescence on LB plates when exposed to UV light (Figs. 3c and 3d), facilitating enumeration due to the green fluorescent phenotype. Theoretically, plasmid loss for T_{GFP} occurs since the plasmid is not integrated into the bacterial genome [26], and the enumeration of T_{GFP} requires plasmid stability. Following passages 1 to 11 in the absence of ampicillin, less than 0.05% of plasmid loss was obtained, indicating that the plasmid was relatively stable even after 11 days of propagation (Fig. 3e). Thus, the low plasmid loss guarantees the reliability of bacterial enumeration based on fluorescence labeling.

Infection test in vitro

Infection of T_{GFP} with phages W1 and W3 at various multiplicities of infection (MOIs) was monitored for 48 hours, and efficiencies of the different phages in reducing the titer of viable T_{GFP} varied. In the phage-free control, there was a rapid increase in the T_{GFP} concentration at hour 12. Compared with the control, infection of phage W1 resulted in a 0.2- to 3.5 log₁₀ decrease at hour 24 at MOIs ranging from 10⁻³ to 10³ PFU/CFU. The infection test against W3 revealed that a high MOI is required for host lysis, and better

reduction of viable bacteria was observed at both hour 12 and hour 24 with an MOI of 10^3 PFU/CFU (Figs. 3f and g). The maximum decline in bacterial quantity reached 10^6 -fold less than that in the control at hour 12. However, it should be noted that neither phage was able to eliminate all bacteria, although W3 was more efficient than W1 in reducing viable bacteria.

During the infection of T_{GFP} with mixed phages (MP, W1 and W3 at a ratio of 1:1) at an MOI of 10^3 PFU/CFU, an 8.8-order of magnitude decrease in the titer of viable bacteria was obtained at hour 12 compared with that in the phage-free control, and the culture suspension in the test tube was clear. MP almost eliminated T_{GFP} at this point, although the culture finally recovered to a 10^8 CFU ml⁻¹ bacterial density at hour 48 [27, 28].

Knockout test in vivo

The sampling sites of the mouse gut in vivo test are shown in Fig. 4a. A 3-day low-concentration gastric perfusion (LCGP) with 10^6 CFU ml⁻¹ T_{GFP} suspension led to bacterial colonization in mouse intestine. The T_{GFP} quantity varied greatly in different intestinal sections. The caecum and colon are major sites of T_{GFP} colonization, and they harbor 9.0×10^6 CFU cm⁻¹ and 1.4×10^7 CFU cm⁻¹ T_{GFP} , respectively (Fig. 4b). A high-concentration gastric perfusion (HCGP) with 10^{11} CFU ml⁻¹ T_{GFP} suspension resulted in the appearance of more T_{GFP} in the mouse gut, and after 3 days of treatment, the bacteria in the caecum and colon reached up to 0.9×10^8 CFU cm⁻¹ and 1.38×10^8 CFU cm⁻¹, respectively (Fig. 4c). In both the LCGP and HCGP, T_{GFP} quantities in feces showed no significant difference (p values ≥ 0.05) with those in the caecum and colon (Fig. 4d), and these three sites were suitable to evaluate the knockout effect of T_{GFP} in the mouse gut in the following studies considering that they harbored more T_{GFP} than the other sites.

Subsequently, the results of the microbial knockout test showed that bacteria in the mouse gut were almost eliminated by MP in LCGP groups. After gastric perfusion of MP, the T_{GFP} quantity in feces was 6.16×10^2 CFU g⁻¹ at hour 24 (Fig. 4e), and at hour 48, T_{GFP} was undetectable in the caecum, colon and feces (Figs. 4f-4h). However, in the control gavaged with inactivated mixed phages (IMP), bacteria in the caecum, colon, and feces remained at 6 orders of magnitude in CFU cm⁻¹ or CFU g⁻¹. In addition, T4 showed better removal than T1, although neither of them could eliminate T_{GFP} . Therefore, only MP was selected for T_{GFP} knockout in subsequent experiments.

In the HCGP groups, after the first phage infusion (PI), T_{GFP} in feces still reached up to 0.77×10^4 CFU g⁻¹ at hour 24. Thus, the second PI was conducted at hour 48. At hour 72, the bacteria in feces decreased to 0.35×10^2 CFU g⁻¹, and until hour 120, T_{GFP} in the feces did not recover (Fig. 4i). Simultaneously, T_{GFP} quantities in the caecum and colon were 0.40×10^2 CFU cm⁻¹ and 0.20×10^2 CFU cm⁻¹, respectively (Fig. 4j), and the remnants were almost undetectable by the plate colony-counting method. Thus, T_{GFP} in the HCGP groups also decreased to a 10^2 CFU cm⁻¹ level after two gastric perfusions.

We investigated the knockout effect of MP when the mouse gut was constantly supplemented with T_{GFP} by feeding feedstuff containing T_{GFP} . In the control, the T_{GFP} quantity in feces was stable at the level of 10^9 - 10^{10} CFU g^{-1} (Fig. 4k), showing that feeding increased the T_{GFP} density in mouse feces. After MP treatment at an interval of 48 hours, the T_{GFP} quantity decreased by 4.5 to 6 orders of magnitudes compared with the initial content, indicating that MP could knock down the bacteria but could not knock out them when they were supplemented every day. Moreover, T_{GFP} enumeration in the caecum (6.30×10^4 CFU cm^{-1}) and colon (3.17×10^3 CFU cm^{-1}) also proved that T_{GFP} was not eliminated (Fig. 4l).

Discussion

Definition of microbial knockout

Microbial knockout is a novel issue in the microbiome field and is significantly different from gene knockout, which is used extensively for investigation of gene function. Generally, gene knockout is performed to remove one or several specific gene(s), while microbial knockout means that millions of microbial individuals are eliminated. Considering the insufficient contact between phages and host bacteria in the mouse gut, the resistance against phages developed by bacteria and other causes [29], it is very difficult to achieve complete elimination, especially regarding the high density of bacteria. Thus, we should consider how to define microbial knockout within a broader range. In the knockout experiments in both the LCGP and HCGP group, we obtained a microbial knockout effect considering that the T_{GFP} population in feces was 10^2 CFU g^{-1} , which is almost undetectable by the plate colony-counting method due to the inevitable dilution of solid samples. With constant T_{GFP} supplementation in the mouse gut, T_{GFP} could not be eliminated, although marked decreases of 4.5 to 6 orders of magnitude of viable bacteria were obtained. Considering that there were still thousands of surviving bacteria (10^4 CFU g^{-1}) in feces, the reduction in the bacteria titer was clearly not a knockout but a knockdown. Normally growing animals live in a poor-hygiene environment, and their intestines are open systems. They always have an opportunity to obtain bacteria from the environment, including the air, food and water, even if the bacteria have already been knocked out in the gut, which probably leads to the failure of microbial knockout due to bacterial population recovery. Therefore, it is much more difficult to completely knock out a specific microorganism in an open environment, and prevention of the continuous input of exogenous microorganisms is a key issue during microbial knockout.

Off-target analysis

The possibility of off-target effects should be evaluated during bacterial knockout by phages. Host specificity analysis of phages showed that although the two phages possessed strict host specificity, phage W3 might mistakenly knock out *E. fergusonii* at the species level. *E. fergusonii*, formerly known as enteric group 10, is an infrequently occurring but emerging animal and human pathogen [30], and it can be differentiated from *E. coli* by sorbitol and lactose fermentation negativity but adonitol, amygdalin and cellobiose fermentation positivity. *E. coli* and *E. fergusonii* are the closest relatives of one each other

under the genus *Escherichia*, and *E. fergusonii* isolates possess genotypic and phenotypic features found in known pathotypes of *E. coli*, which leads to the following argument: is *E. fergusonii* in fact another *E. coli* [31]? This question remains unanswered, and thus we retested the bacterial classification based on the 16S rRNA gene sequence. A 1421-bp gene sequence of *E. fergusonii* was determined, and the sequence comparison showed that the strain (GenBank accession number MK168572.1) shared the highest similarity (100%) with *E. coli* (CP020516.1). According to the current opinion of molecular identification based on the 16S rRNA gene, this strain is an *E. coli* in nature by phylogenetic analysis (Additional file 4: Fig. S3). Off-target effects did not occur at the species level if the taxonomic deviation was considered. However, this does not mean that off-target effects will never occur during future microbial knockouts because we only tested the reported strains under the *Escherichia* genus.

Impact of MP on the bacterial community

The design of experiments focused on the impact of MP on the bacterial community is shown in Fig. 5a. After 16S rRNA gene sequencing, trimmed sequences with an average sequence length of 398 bp were obtained, and statistical analysis of fecal samples, including the identified operational taxonomic units (OTUs) and alpha diversity indexes, are shown in Additional file 5: Table S2. Good's coverage indicated that 99.76–99.81% of the species in the samples were recovered at a cutoff of 97% sequence similarity. The Chao1 indexes of the PBS buffer (PBS), IMP, and MP groups were 399.2, 407.6, and 340.2, showing that the bacterial richness was significantly different after the phage treatments (Fig. 5b). The Shannon indexes of the three groups showed that phage treatment had no significant impact on bacterial diversity in the mouse gut (Fig. 5c). Dissimilarity comparison tests between the different groups at the genus level (Fig. 5d) also indicated that the addition of MP did not change the main microbial community structure (p values ≥ 0.05).

We analyzed the RA changes of bacteria in different treatments at the genus level because of the limited accuracy of molecular identification based on 16S rRNA gene sequencing at the species level. There were 42 and 43 genera with significant RA differences in the MP and IMP group (Fig. 5e) compared with the PBS group, indicating that the disturbance, defined as the quantity of bacterial genus with a significant RA change, caused by MP was not significantly different from that in the IMP treatment ($p \geq 0.05$). In addition, RAs of 34 genera changed significantly between the MP and IMP group. Co-occurrence network analysis showed that the three groups shared the great majority of the species. Twelve genera including *Faecalibaculum*, *Butyricimonas* and *Psychrobacter* appeared in both the PBS and IMP group but lost in the MP group (Fig. 5f). According to the previous phage host analysis, the disappearance of these bacteria was obviously not caused by off-target effect of phages. Therefore, treatment by live phages resulted in remodeling of the bacterial community in the mouse gut. However, whether in the IMP or MP group, the core top 30 genera (total RA $\geq 98.5\%$) did not disappear (Additional file 6: Fig. S4), and a similar conclusion could be drawn based on the OTU analysis. Among 483 OTUs in total, 58 OTUs present in the controls (MP and PBS groups) were absent in the MP group (Fig. 5g). However, the core 70 OTUs accounting for the total RA of 93.9% were not lost, although the RAs of 30 OTUs showed a significant difference (Fig. 5h). Therefore, it is feasible to knock out a core member of the gut flora with native

phages. However, the disappearance of noncore microorganisms or OTUs showed the reconstruction of the bacterial community structure. It is necessary to evaluate their interference during verification of the function of T_{GFP} knockout, and thus, further microbial supplementation experiments should be considered.

Microbial interactions upon knockout

RAs of the *Escherichia* genus in the MP group were significantly lower than in the PBS group (Fig. 5d), and enumeration results on day 8 showed that *E. coli* was reduced by approximately 2 orders of magnitudes (Fig. 6a) in the MP group. However, neither *Escherichia* nor *E. coli* was knocked out completely because in addition to *E. coli*, the genus might contain species that are not sensitive to phages T1 and T4, and the MP we used targeted strain MG1655 rather than the entirety of strains of *E. coli* in the mouse gut. We isolated 1000 strains of *E. coli* from the fecal samples, and 161 strains were insensitive to phage W1 or W3. The Spearman correlation network analysis revealed that *Escherichia* was negatively correlated with 5 genera and positively correlated with 12 genera (r values ≥ 0.6), indicating that it might affect the RA change of other genera through various cascade reactions (Fig. 6b). Moreover, it should be noted that knockout of a specific bacterium should not be attributed entirely to the phages used. In the mouse gut, T_{GFP} faces complicated ecological competition from various microorganisms. During coculture of *E. coli* and two common gut bacteria, *Proteus vulgaris* and *Sphingobacterium lactis* in visual biomimetic reactors (Fig. 6c), the bacterial presence significantly reduced the *E. coli* population in both the E+P (*E. coli* + *P. vulgaris*) and E+S (*E. coli* + *S. lactis*) groups (Fig. 6d). The addition of MP resulted in the clearance of T_{GFP} during bacterial coculture, while this phenomenon was not observed in the control group (E+MP) (Fig. 6e). This finding indicates that the interspecies competition improved phage control of the bacterial population [32]. Therefore, in addition to phages, bacterial interactions in the mouse gut contribute to the knockout of T_{GFP} .

Conclusion

Overall, the results presented herein demonstrate that it is feasible and effective to knock out a specific bacterium by its corresponding phages. The phages with rigorous host specificity not only knocked out *E. coli* MG1655 at a concentration of 10^6 - 10^8 CFU/g in the mouse gut but also knocked down the strain even in the mice fed with feedstuff containing strain MG1655. The changes in both the metabonomic and immune system caused by phage treatment were not evaluated in this work considering that the phages isolated from mouse feces are endogenous and the impact of MP on the bacterial community was not significant. However, these factors should be considered when assessing the function of a bacterial species in its host in future work.

Material And Methods

Microorganisms and chemicals

All chemicals used were spectral or analytical grade. Forty-two bacteria including *E. coli* subspecies, *Escherichia* species, and strains belonging to twenty different genera of *Enterobacteriaceae* were purchased from the China Center for Type Culture Collection (CCTCC), China General Microbiological Culture Collection Center (CGMCC), or China Center of Industrial Culture Collection (CICC) or provided by the Key Laboratory of Microbial Resources and Functional Molecules of Henan Province, China. The detailed information about these bacteria is listed in Additional files 2. Table S1.

Phage isolation and identification

Phages W1 and W3 were isolated from mouse feces collected from the Animal Breeding Base in Henan Normal University, China. Isolation, propagation, and titration measurement of phages were conducted as described by Adams [33]. After the staining of phage particles by 2.0% aqueous uranyl acetate (pH 4.5–5.5) on a carbon-coated grid, phages were observed using transmission electron microscopy (TEM, JEM-1400, JEOL Ltd., Japan) at an accelerating voltage of 80 kV. Phage DNA was extracted using a phage DNA extraction kit (Aidlab Biotech, China) [34]. DNA sequencing was conducted using the Illumina HiSeq (PE250) platform at Hangzhou Lianchuan Biological Information Co., Ltd., China. The paired-end reads were assembled using SOAP denovo v2.04 (<http://soap.genomics.org.cn/>), and the potential open reading frames (ORFs) were predicted using GeneMarkS 4.6b (<http://topaz.gatech.edu/GeneMark/>). Possible tRNAs in the genome were determined using tRNAscan-SE (<http://lowelab.ucsc.edu/tRNAscan-SE/>). Comparisons of nucleic acid and predicted protein sequences with other known sequences were performed by BLAST analysis (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Maps of a circular representation of phage genomes were generated using DNAMAN (version 6.0; Lynnon Biosoft). Neighbor-joining trees were drawn using MEGA 5.05 [35]. The complete genome sequences of phages W1 and W3 were deposited in GenBank under accession numbers PRJNA494624 and PRJNA494627, respectively.

Host range analysis of phages

Strains were cultivated in LB liquid medium (LB, 10 g l⁻¹ tryptone, 5.0 g l⁻¹ yeast extract, 10 g l⁻¹ NaCl) for 24 hours, and then bacterial lawns were prepared by pouring 3 ml of LB soft agar (LB_{SA}, LB liquid medium supplemented with 10 g l⁻¹ agar) containing 0.1 ml of broth culture onto LB agar plates (LB_A, 10 g l⁻¹ tryptone, 5.0 g l⁻¹ yeast extract, 10 g l⁻¹ NaCl and 15 g l⁻¹ agar). After solidification of the soft agar, the plates were spotted with a phage suspension (10 µl, 10⁵–10⁶ PFU µl⁻¹) on triplicate plates. The plates were dried for 15 minutes at room temperature (25±2°C) before incubation. After 18–22 hours of incubation at 37°C, the effects of phages on bacterial lawns were observed and recorded. LB broth culture without phage was used as a negative control, and a positive response was defined as a number of ≥20 plaques or full lysis on the plates.

1. *coli* MG1655 labeled with GFP

The plasmid pGFPuv, including a pUC origin of replication (ColE1-like), TEM β-lactamase as a selectable marker, and a cycle GFP reporter gene under control of the Plac promoter (Clontech, CA, USA), was used

to obtain a GFP-tagged transformant of *E. coli* MG1655. The plasmid was transformed into strain MG1655 by the heat-shock method [36]. Briefly, 100 μ l of chemically competent cells and 100 ng of plasmid DNA were mixed and incubated on ice for 30 min; then, they were heat-shocked in a water bath at 42°C for 90 seconds, allowed to recover for 1 hour, and plated in the presence of 100 μ g ml⁻¹ ampicillin. T_{GFP} was verified by PCR amplification after digestion with the Mlu I enzyme (Takara Biotechnology Dalian Co., Ltd.) and fluorescence microscopy observation.

To evaluate the stability of the transformant in the lab, T_{GFP} was picked from plates and incubated in a 250-ml flask containing 50 ml of nonselective LB medium at 37°C and 120 rpm. After 24 hours of cultivation, the obtained cultures were set as passage 1 (P1). Subsequently, 2 ml of culture broth from P1 was transferred to 50 ml of fresh LB medium every 24 hours, and this procedure was repeated ten times [37]. To determine plasmid loss, 2-ml cell cultures were collected at different passages, serially diluted, and plated on LB_A. Colonies with green fluorescence under UV light were counted, and plasmid loss was expressed as the percentage of viable cells with abolished GFP fluorescence to the total cells.

Preparation of phage stocks

Each phage suspension prepared using sterile salt-magnesium (SM) buffer was plated onto LB_A along with LB_{SA} containing 10⁷ CFU ml⁻¹ T_{GFP} overlay. After an overnight incubation at 37°C, SM buffer was added to the plates, and the top soft agar slurry was harvested and centrifuged twice at 12,000 $\times g$ for 20 minutes to collect phage-rich supernatant (lysate). The lysate was added with an equal volume of chloroform and then filtered using a 0.22- μ m filter to remove cells and debris. After titer measurement, the filtrate was serially diluted to obtain 10³-10⁶ PFU ml⁻¹ phage stocks.

High-titer phage stocks were prepared from the lysates by liquid infection. For each phage, the low-titer lysate (1.0 ml; 10⁶ PFU ml⁻¹) mixed with T_{GFP} (1.0 ml; 10⁷ CFU ml⁻¹) was added to 200 ml of LB and incubated for 24 hours at 37°C with aeration. The cultures were each treated with an equal volume of chloroform, and the lysates were harvested twice by centrifugation (12,000 $\times g$, 20 minutes) as well as by filtration with a 0.22- μ m filter. After titer measurement, the filtrate was serially diluted to obtain 10⁷-10¹¹ PFU ml⁻¹ phage stocks.

Knockout test in vitro

T_{GFP} was cultivated in LB for 24 hours, and 100 μ l of cultures with a concentration of 4.0 $\times$ 10⁷ CFU ml⁻¹ were transferred to a test tube containing 5 ml of LB. Then, phage suspensions (100 μ l) of W1, W3, and MP with different titers (10⁴-10¹⁰ PFU ml⁻¹) were seeded in different tubes. The tubes were incubated at 37°C, and T_{GFP} was enumerated every 12 hours by the plate colony-counting method. The reproducibility of the tests was confirmed in three independent continuous cultures. A phage-free culture containing only bacteria was used as a control to demonstrate the absence of contamination.

Knockout tests in vivo

Ten-week-old male Kunming species mice with an average body weight of 21 ± 2 g (Laboratory Animal Center of Henan Province, China) were used for knockout tests in vivo. All the mice were maintained in a 12-hour light/dark cycle, supplied with water and a standard diet (65% carbohydrate, 11% fat and 24% protein), and housed at 20–25°C. The mouse experiments were not performed in a blinded manner, and the experimental groups were randomly allocated.

We first investigated the colonization of T_{GFP} in the mouse gut. After 3 days of gastric perfusion with 5.0 g l⁻¹ ampicillin [38], 120 mice were divided into 2 groups, the LCGP and HCGP group, and gavaged with 200 µl of different concentrations of the T_{GFP} suspension (1.4×10^6 CFU ml⁻¹ and 4.3×10^{11} CFU ml⁻¹) once per day for six consecutive days. After the treatments, T_{GFP} in mouse feces was enumerated daily. On day 12 and 15, five mice from the LCGP and HCGP groups were dissected, and T_{GFP} in different sections of the gut was collected and enumerated to validate T_{GFP} colonization.

Forty-eight mice in the LCGP group were divided into four groups (n=12) and were gavaged with MP, phage T1, phage T4, or IMP. As a control, IMP was prepared by sterilization of MP at 121°C and 0.1-0.15 MPa of steam pressure for 30 min in an autoclave (Hiclave HVE-50, Hirayama, Japan). After gastric perfusion, T_{GFP} in mouse feces was enumerated every 24 hours.

Twenty mice in the HCGP group were divided into two groups (n=10) to quantitatively evaluate the knockout efficiency of MP against the high titers of T_{GFP} . The two groups were gavaged with MP or IMP. After gastric perfusion, T_{GFP} in mouse feces was enumerated every 24 hours, and T_{GFP} in the caecum and colon was evaluated at hour 120.

Thirty mice in the HCGP group were used to quantitatively evaluate the knockout effect of MP when T_{GFP} was constantly supplemented in the mouse gut. Mice used in this test received feedstuff with T_{GFP} (2.0×10^9 CFU g⁻¹) daily. They were divided into two groups (n=10) and gavaged with MP (10^{11} PFU ml⁻¹) or IMP. Moreover, ten mice without any treatment were used as a control to demonstrate the absence of T_{GFP} contamination between different groups, i.e., no naturally green-fluorescent bacteria in the mouse gut.

Phylogenetic analysis of *E. fergusonii*

Genomic DNA of *E. fergusonii* (Access number CICC24137) was extracted, and the 16S rRNA gene sequence was amplified using PCR with the primer pair 27F and 1492R [39]. The purified PCR product was cloned into the vector pMD19-T and sequenced. Sequences of related taxa were obtained from the GenBank and EzTaxon-e databases [40]. Phylogenetic analysis was performed using MEGA software version 5.05 after multiple alignment of data by DNAMAN. Evolutionary distances and clustering were constructed by the neighbor-joining method.

Experimental design and 16S rRNA gene sequencing

Forty-five Kunming species mice were used to test the impact of phage treatment on the microbial community structure. The detailed experimental design is described in Fig. 6a. Mice in the MP group were orally gavaged with MP once per day for 3 days, and as controls, the PBS and IMP groups were gavaged with PBS buffer and inactivated MP, respectively. During the tests, changes in movement, appetite, and dejecta were recorded. Fecal pellets of mice (20 mg per mouse) from the three groups were collected each day. Samples were shaken sufficiently for 30 min in a 50-ml sterile centrifuge tube. Subsequently, both sterile gauze and 5- μ m filter membranes were used to remove large particles, and the filter liquor was used for both enumeration of *E. coli* using CHROMagar™ *E. coli* chromogenic medium (CHROMagar, France) [41] and further genomic DNA extraction.

Bacterial genomic DNA in fecal samples was extracted using an Omega Bio-Tek, OMEGA-soil DNA Kit (Qiagen, Germany). After verification of the purity and concentration, PCR amplifications of the highly variable V3–V4 regions of the bacterial 16S rRNA gene were conducted based on the universal primer pair 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTA AT).

The thermocycling procedure consisted of an initial denaturation step at 95°C for 2 minutes, followed by 25 cycles each consisting of 94°C for 30 seconds (denaturation), 55°C for 30 seconds (annealing), and 72°C for 30 seconds (extension), and a final extension at 72°C for 5 minutes. Each reaction was conducted in a 20- μ l reaction mixture containing 10 ng of template DNA, 5 μ M each primer, 2.5 mM deoxynucleoside triphosphate mix, and 1 unit of FastPFU Polymerase (TransGen Biotech, China). PCR cycling reactions were performed in a GeneAmp® 9700DNA thermocycler (ABI, USA), and the amplified products were visualized on agarose gel containing EB and purified with a DNA gel extraction kit (Axygen Inc., USA).

Prior to sequencing, the DNA concentration of each PCR product was determined, and the amplicons from each PCR were pooled together in equimolar ratios to reduce biases of each individual reaction and subjected to emulsion PCR to generate amplicon libraries [42]. Deep sequencing was performed on the Illumina MiSeq platform at the Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China). Any sequence with more than two base mismatches was discarded by Seqcln software analysis. The low-quality sequences and redundant reads were further trimmed using Mothur software. The “dist.seqs” command was performed to identify OTUs by 97% similarity. The obtained sequences were subjected to Megablast and searched against SILVA, aligning to the 16S small subunit rRNA sequence database (version 111), to acquire high taxonomic resolution. The rarefaction curves, Chao1 richness, and Shannon diversity index were determined by Mothur analysis [43]. The 16S rRNA gene sequencing data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive under accession number PRJNA578836.

Coculture experiments

T_{GFP} was cocultivated with *Proteus vulgaris* and *Sphingobacterium lactis* in visual biomimetic reactors that can simulate peristalsis of the human intestinal tract [44], and a monoculture of T_{GFP} was used as

the control. In addition, MP was added in the reactors to validate the clearance effect of T_{GFP} during bacterial coculture. The medium used contained corn flour (20.0 g l^{-1}), protein powder (20 g l^{-1}), glucose (5.0 g l^{-1}), Ox-gall salt (10.0 g l^{-1}), Na_2HPO_4 (10.0 g l^{-1}), KH_2PO_4 (10.0 g l^{-1}), and NaCl (1.0 g l^{-1}), and the initial pH value was 7.2-7.5. During the cocultures, the compression frequency of the peristaltic pump was $10 \text{ times min}^{-1}$, the compression range was 1 cm, and the T_{GFP} quantity was measured every 12 hours.

Statistical methods

Statistical analyses to identify significant differences were performed using SPSS software. Unless otherwise specified, all data are presented as the mean $\pm$ SEM. When three or more means were compared for statistical significance, one or two-way ANOVA was conducted with treatments as independent factors. When two groups of measurements were examined for statistical significance, the two-sided Student's t-test was conducted, and a p-value <0.05 was considered statistically significant. A dissimilarity test of fecal samples was performed in R based on the Bray–Curtis dissimilarity index using analysis of similarities [45]. Co-occurrence and correlation network analysis were performed by Networkx software [46], and only Spearman correlations with an $r > 0.6$ ($p < 0.05$) were considered to indicate a valid interactive event.

Declarations

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Authors' contributions

WH and LG conceived of and designed experiments; CJ, ZZ and LL performed the Experiments; LP and Li Y contributed the reagents/materials/analysis tools. Chen W provided technical and scientific support on the experimental analysis; LP and WH wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated and analyzed during the current study including genome sequences of phages W1 and W3, and 16S rRNA gene sequencing data, are available in the NCBI sequence reads archive (SRA) under accession number PRJNA494624, PRJNA494627 and PRJNA578836.

Ethics approval and consent to participate

Animals were treated according to the guidelines of Regulations for the Administration of Laboratory Animals (Decree No. 2 of the State Science and Technology Commission of the People's Republic of China, 1988). The sampling procedure was validated by the Ethics Committee of the Henan Normal University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Genome characteristics and phylogenetic analysis of phages W1 and W3. a Genome characteristics of phage W1. b Phylogenetic tree of phage W1 based on the complete genome sequence. c Genome characteristics of phage W3. d Phylogenetic tree of phage W3 based on the complete genome sequence (confidence values above 50% obtained from 1000-replicate bootstraps are indicated at branch nodes. The scale bar indicates the number of base substitutions per site).

Figure 2

Host range analysis of phages W1 and W3.

Figure 3

pGFPuv transformation and phage infection tests in vitro. *E. coli* MG1655 (a) and TGFP (b) under fluorescence microscopy. Colonies of *E. coli* MG1655 (c) and TGFP (d) on an LB plate when exposed to UV light. e Variation in pGFPuv loss during 11-day propagation. The TGFP quantity varied with time during infection with phage W1 (f) and phage W3 (g) at various MOIs. h Variation in TGFP with time during infection with the mixed phages. Note: The significance analyses in f and g show the difference between the PFU/CFU=10³ group and the control. ns, nonsignificant; *p < 0.05; **p < 0.01.

Figure 4

TGFP Knockout test in vivo. a Sampling sites. TGFP quantity at different intestinal sections in LCGP (b) and HCGP (c). d TGFP quantity in fecal samples in LCGP and HCGP. e TGFP quantity in feces after phage treatments at hour 24 in LCGP. TGFP quantity in the caecum (f), colon (g), and feces (h) after phage treatment at 48 hours in LCGP. i Variation in TGFP quantity with time after two MP gastric perfusions in HCGP. j TGFP quantity in the caecum and colon at hour 120 after two MP treatments in HCGP. k Variation in TGFP quantity in feces with time after continuous MP treatment when TGFP was constantly supplemented in the mouse gut. l TGFP quantity in the caecum and colon at hour 168 after continuous MP treatment when TGFP was constantly supplemented in the mouse gut. ns, nonsignificant; * $p < 0.05$; ** $p < 0.01$.

Figure 5

Impact of MP on the microbial community structure. a Schematic illustration of the experimental design. The Chao1 index (b) and Shannon index (c) of the PBS, IMP, and MP groups after the treatments (on day 4). d Heatmap illustrating the RA change at the genus level, and the color scale indicating the magnitude of RA. e Number of genera with significant RA differences among the MP, IMP, and PBS groups. f Network analysis of the co-occurrence of bacteria at the genus level. g Venn diagram illustrating the OTU distribution in the MP, IMP and PBS groups. h One-way ANOVA bar plot illustrating the OTUs with significant RA differences among the top 70 OTUs. ns, nonsignificant; *p < 0.05; **p < 0.01.

Figure 6

Enumeration of *E. coli* as well as correlation network analysis of bacteria in knockout, and coculture tests. a Quantity of *E. coli* in the MP, IMP, and PBS groups on day 8. b Correlation network analysis reveals the bacterial interaction in fecal samples. The size of the nodes shows the abundance of OTUs (top 70), and the different colors indicate the corresponding taxonomic assignment at the genus level. The edge color represents positive (red) and negative (green) correlations. The edge thickness indicates the correlation values, and the black arrow points to *Escherichia*. c Design of biomimetic reactors simulating the human intestinal tract. d Variation in the quantity of TGFP with time during coculture with *Proteus vulgaris* and *Sphingobacterium lactis*. e Effect of MP on the quantity of TGFP during coculture. ns, nonsignificant; *p < 0.05; **p < 0.01.

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