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Research Paper

Host age increased conjugal plasmid transfer in gut microbiota of the soil invertebrate *Caenorhabditis elegans*

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ABSTRACT

Plasmid conjugation contributes greatly to the spread of antibiotic resistance genes (ARGs) in soils. However, the spread potential in the gut of soil fauna remains poorly studied, and little was known about the impact of host age on ARGs dissemination in the gut microbiota of soil animals. Here, the typical nematode-*Caenorhabditis elegans* was employed as the model soil animal, aiming to investigate transfer of broad-host-range IncP-1ε from *Escherichia coli* MG1655 to gut microbiota within 6 days under varied temperature gradients (15, 20 and 25 °C) using qPCR combined with plate screening. Results showed that conjugation rates increased with incubation time and rising temperature in the gut of *C. elegans*, sharing a similar trend with abundances of plasmid conjugation relevant genes such as *trbBp* (mating pair formation) and *trfAp* (plasmid replication). Incubation time and temperature significantly shaped the gut microbial community of *C. elegans*. Core microbiota in the gut of *C. elegans*, including Enterobacteriaceae, Lactobacillaceae and Leuconostocaceae, constituted a large part of transconjugal pool for plasmid IncP-1ε. Our results highlight an important sink of gut microbiota for ARGs dissemination and upregulation of ARGs transfer in the gut microbiota with host age, further potentially stimulating evolution of ARGs in terrestrial environments.

1. Introduction

Antibiotic-resistant infections have emerged as a major worldwide health issue (Zhu et al., 2017). Extensive use of antibiotics in medicine, animal husbandry and agriculture is the key driving force for the development of resistance genes in the host-bacterial community (Huddleston, 2014). Bacteria susceptible to antibiotics become resistant via genetic mutation or horizontal transfer of resistance genes from other strains (Huddleston, 2014). Conjugation is one of the most common and efficient route of horizontal gene transfer (HGT) (Lerner et al., 2017). The transfer of DNA, through a conjugative element such as plasmid from a donor to a recipient microorganism via cell-to-cell touch, is an important force in the evolution of bacteria and promotes the adaptation of microorganisms under antibiotic pressures (Lerner et al., 2017).

Animal intestine serves as a reservoir for ARGs available for transferring to other resident intestinal bacteria (Huddleston, 2014; Wang et al., 2017; Ding et al., 2019). Gut is colonized with high density bacteria supporting the cell-to-cell contact required for conjugation (Huddleston, 2014). In addition to forming intercellular mating bridge, bacterial pilus promotes cell adhesion, which aids cells in colonization of the gastrointestinal alimentary and further increases the opportunities for mating events between cells (Modi et al., 2014). Recent work indicated that gene uptake via plasmid conjugation is common in human gut environment and exhibits significantly higher frequency in human gut-associated bacteria than those from other environmental microbiota (Huddleston, 2014), suggesting that animal gut environment is a melting pot for HGT. Substantial work performed in mammalian guts indicates that age facilitates plasmid conjugation due to increased persistence of the colonizing bacteria (Cabreiro and Gems, 2013), however, limit information is known about the impact of age on the horizontal transfer of ARGs in guts of soil animals. Besides, most of work

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has used specific donor (broad host range plasmids) and single recipient bacterial strain under specific stress (Cabreiro and Gems, 2013). In *Streptococcus pneumonia*, antibiotic exposure enhances competence and promiscuity toward foreign DNA (Slager et al., 2014). Similarly, *Pseudomonas putida* increases the acceptance and maintenance of plasmids in the host cells after sodium dodecyl sulfate treatment (Pinedo and Smets, 2005). Although the broad-host-range plasmids are able to transfer across bacterial phyla, diverse communities and strains are not equally permissive toward these plasmids (Klumper et al., 2015). The microbial community that resides within the gut is not a static state, which can be shaped by several factors, such as age of host and temperature (Portal-Celhay and Blaser, 2012; Berg et al., 2016). To date, HGT pattern across complex gut microbiota remains unrevealed, especially in soil animals.

Caenorhabditis elegans N2 (wild type) is a free-living soil nematode that feeds mainly on bacteria, permitting it to establish nature-like gut microbiota in the lab (Portal-Celhay and Blaser, 2012). The simplified invertebrate model system assists the understanding of gene flow occurring in the gut microbiota under the natural environment. Its ease propagation and rapid generation time offer *C. elegans* a model to characterize the contribution of host age to gut microbiota composition. Temperature and incubation time largely determined the aging of *C. elegans* (Riddle et al., 1997). In this study, in order to address the role of host age in the HGT pattern in the gut of soil animals, *C. elegans* N2 was served as the model soil animals, and *E. coli* harboring a broad-host-range plasmid tagged with green fluorescent protein (GFP) gene was utilized as ARG-carrying plasmid donor colonized in the gut of *C. elegans* N2. Quantitative PCR combined with culture-based plate screening were employed to track the abundances of both donor strains and ARG- carrying plasmids in the *C. elegans* intestine over a 6-d cultivation at 15, 20 and 25 °C, respectively, aiming to explore 1) whether ARG- carrying plasmids conjugation occurred in the gut of *C. elegans*, 2) the effect of temperature and incubation time on the HGT pattern in the gut of *C. elegans*.

2. Materials and methods

2.1. C. elegans, donor strain and plasmid

C. elegans N2 was provided by the Canorhabditis Genetic Center and maintained on nematode growth medium (NGM) (Brenner, 1974). In brief, *C. elegans* strain (Bristol) N_2 was maintained on nematode growth media (NGM) seeded with *E. coli* OP50 at 15 °C (Brenner, 1974). Cells of *E. coli* MG1655::lacIq-pLpp-mCherry-KmR served as donor strain, carrying the IncP-1ε broad-host-range plasmid pKJK5::*gfpmut3b* (Klumper et al., 2017). This plasmid was tagged with an entranceposon-derived gene that carries a $lacI^q$ repressible promoter upstream of the conditionally expressed *gfpmut3* gene, which encoding the green fluorescent protein (GFP) (Klumper et al., 2017). This donor strain was chromosomally marked with gene cassette encoding constitutive $lacI^q$ production and red fluorescence protein expressed by *mCherry* gene (Klumper et al., 2017). Therefore, *gfp* expression is repressed in the donor strain, while it would de-repress after the plasmid is successfully transferred to a recipient cell (Klumper et al., 2017). As a result, green fluorescent cells or colonies can be detected by fluorescence microscopy (Klumper et al., 2017). Agar plates grew with colonies were directly placed under the objective lens, and the work distance was about 5 cm. The donor strain was cultured overnight in LB agar supplemented with 30 μg mL^{-1} tetracycline. The viability of donor cells and transconjugants was checked using fluorescence microscopy (Zeiss, Germany). *C. elegans* N2, plasmids and donor strains used in this study were detailed in Table S1.

2.2. Soil sampling

Soil samples were collected in Ningbo, China (29°49′38.3808″ N, 121°16′39.6372″ E). Aliquots (5 g) of fresh soil were transferred into 3 cm-diameter plates and was supplemented with chopped and partially rotting produce at a ratio of 1:2 (w/w) and left to decay for a week, which aimed to increase the microbial diversity of soil and provide adequate "nutrition" for *C. elegans* (Berg et al., 2016). The produce contained bananas (high fiber and simple sugars), potatoes (rich in starch and iron), and apples (mainly simple sugars) (Berg et al., 2016).

2.3. Worm growth and collection, and plasmid conjugation in the gut of C. elegans

Eggs of *C. elegans* N2 were collected from gravid worms by bleaching, and they were synchronized by hatching on NGM plates without any food, finally, arrested as L1 larvae (Berg et al., 2016). Bleaching gravid worms was in order to harvest sterile eggs, which was following with obtaining sterile larvae and then nematode gut colonized with target strains harboring ARGs. To ensure bacterial conjugation in the gut microbiota, L1 larvae were pre-grown on NGM agar plates seeded with *E. coli* MG1655 at 15 °C until the L4 stage (fourth instar larvae), aiming to colonize the ARGs-carrying plasmid donor strains in the gut of *C. elegans*. After 3–6 days of cultivation, hundreds of L4 *C. elegans* were collected from NGM plates, washed six times with M9 medium (Fujita et al., 1981), transferred into plates containing soil and cultured in the dark at 15 °C, 20 °C and 25 °C, respectively. Firstly, it was in order to synchronize test worms because the crescent-shaped immature vulva would form on the 2/3 worm bodies, which is easy to be recognized under the microscope (Barriere and Felix, 2006). Then, it was to ensure harvest as many worms as possible owing to the high survival rate of adult worms which were developed from 4th-stage larva only within several hours at 15–25 °C (Riddle et al., 1997; Barriere and Felix, 2006). Additionally, the transferring of worms from NGM plate to soils can introduce soil microbiota into the intestine of *C. elegans* as the potential gut microbes and also the ARGs- carrying plasmid recipient cells. A total of 110 adult gravid *C. elegans* and 0.5 g of surrounding soil were collected from the plates on the 2nd, 4th and 6th day, respectively. *C. elegans* N2 were harvested within 1 h from soil using a Baermann funnel lined with filter (Barriere and Felix, 2006). All the setups were prepared in triplicate. *C. elegans* N2 were paralyzed and washed extensively with 2 mM levamisole in M9 medium, surface sterilized using 100 μg mL^{-1} gentamycin for 1 h, washed three times with M9 medium for further analysis. The presence of bacteria on *C. elegans* N2 surface was checked by spreading the last wash medium on LB agar plates without any antibiotics.

To investigate the conjugation in the gut microbiota, 10 of *C. elegans* worms harvested in the last step were ground using a motorized pestle in 200 μL M9 medium. Gut homogenate was plated onto PYG (for 16–22 h), R2A (for 48 h) and LB (for 16–22 h) plates with 30 μg mL^{-1} tetracycline at 37 °C (Samuel et al., 2016). Plates grown with colonies were kept at − 4 °C for 3 days to ensure GFP maturation. Colonies with green fluorescence were counted using fluorescence microscopy (Zeiss, Germany). A maximum of 50 GFP-expressed colonies were randomly picked up from each plate and subjected to 16S *rRNA* gene sequencing using 27F/1492R primers (Wang et al., 2009).

2.4. DNA extraction and qPCR

The remaining 100 worms were ground in 200 μL of M9 medium for DNA extraction. DNA was extracted from *C. elegans* and the surrounding soil according to the manufacturer's instructions (MP Biomedical, Santa Ana, CA, United States) and kept at − 20 °C until use. The concentrations of donor strains and plasmids in the gut and surrounding soil were measured by quantifying *mCherry* and *gfp* genes, respectively. All quantification was carried out in triplicate using Roche 480 (Roche Molecular Systems Inc., United states). qPCR was performed in a 20 μL reaction consisting of 10 μL 2 × SYBR Premix ExTaq II (TaKaRa, Japan), 0.8 μL of each primer, 2 μL of template DNA, 0.6 μL of bovine serum albumin (20 mg mL^{-1}) and 5.4 μL of nuclease-free water. A 202-bp

fragment of *mCherry* gene was amplified using primers 1F (AGCAAGGGCGAGGAGGATAAC) and 202R (ACTGAGGGGACAGGATGTCC), and a 623 bp fragment of *gfp* gene was targeted using the primers 6F (TAGCAGTAAAGGAGAAGAAC) and 629R (CGAAAGGGCAGATTGTG) (Fan et al., 2019). The V4 regions in 16S *rRNA* gene were quantified as described before (Wang et al., 2017; Zhou et al., 2017). Gene copies of *trfAp* (74 bp) and *trbBp* (137 bp) in IncP-1ε plasmids were determined using primers trfAε941f (ACGAAGAAATGGTTGTCCTGTTC), trfAε1014r (CGTCAGCTTGCGGTACTTCTC) (Heuer et al., 2012), trbBf (GCGCACAAAAACATCCTGGT) and trbBr (CATTGGATTTCGCCGGTGTC), respectively.

PCR thermal cycle for *mCherry*, *gfp*, *trfAp* and *trbBp* were 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 10 min. Negative controls without DNA template were prepared in each amplification reaction. Standard plasmids bearing 16S *rRNA*, *gfp* and *mCherry* genes from soil microbiota and *E. coli* MG1655 were generated and extracted using mini plasmid kit (Tiangen Biotech, Beijing, China), respectively. Standard curve was produced using 10-fold serial dilutions of standard plasmid. Only one peak at the melting temperature was detected, and reaction efficiencies ranged from 90% to 110% were accepted.

Copies are the concentrations of genes *gfp* and *mCherry* quantified by qPCR. Copies of gene *gfp* reflected number of *gfp*-contained in the plasmid, which was harbored by both plasmid donor and recipient strains. Whereas, copies of gene *mCherry* represented number of *mCherry* carried by the plasmid donor strains. Therefore, $Copies_{gfp}$ - $Copies_{mCherry}$ presents the number of transconjugants with assumption that every donor strain carried plasmid. Conjugation ratio was roughly estimated using the formula: TR = ($Copies_{gfp}$ - $Copies_{mCherry}$)/($Copies_{16S\ rRNA}$/4). One bacterial cell contains around four 16S *rRNA*-encoding genes based on the Ribosomal RNA database (Klappenbach et al., 2001). $Copies_{16S\ rRNA}$/4 represents the number of total bacteria in *C. elegans* gut and soil.

2.5. 16S rRNA gene amplification, sequencing and data processing

The V4 regions of 16S *rRNA* gene were amplified from the genomic DNA of soil and *C. elegans* gut microbiota using primers 515F and 806R with 6-bp barcode as described previously (Wang et al., 2017). PCR products were then purified, quantified, pooled, and sequenced on the Illumina Miseq PE 250 platform (Novogene, China). Sequence were processed using Quantitative Insights Into Microbial Ecology (QIIME 1.9.1) (Caporaso et al., 2010). Low-quality and ambiguous reads were removed and the filtered sequences were clustered into operational taxonomic units (OTUs) at 97% similarity using UCLUST clustering (Wang et al., 2007). The most abundant sequence of each OTU was selected and the taxonomy was classified by RDP classifier (Wang et al., 2007).

2.6. Statistical analysis

Data were displayed as the mean value ± standard deviations (SD) in Excel 2016 (Microsoft Office 2016, Microsoft, USA). The relationship between the abundances of families for the gut microbiota of *C. elegans*, temperature and incubation time was determined by redundancy analysis (RDA) using RStudio (version 1.2.1335) with the vegan package. Statistical analysis was performed through single factor analysis of variance (ANOVA), and Duncan's multiple range test ($P < 0.05$) was utilized for statistical significance analysis. Heatmap and phylogenetic tree were draw in the R version 4.0.2 with vegan 2.5–6.

2.7. Data accessibility

The 16S *rRNA* gene sequences have been deposited in NCBI (Sequence Read Archive) with accession numbers SRR10418914-SRR10418969.

2. Results

2.1. Plasmid conjugation in the C. elegans gut

In order to investigate the pattern of plasmid conjugation among gut microbiota, qPCR was used to quantify the abundances of *mCherry* and *gfp*, which reflected the numbers of plasmid donors and recipients (Fig. 1A). Conjugation ratios in gut of *C. elegans* were at least two orders of magnitude higher than those in the surrounding soil (Fig. 1B and S1). In addition to the setup incubated on day 4 at 15 °C, plasmid conjugation ratios almost increased with incubation time and temperature in the gut microbiota of *C. elegans* (Fig. 1B). For the surrounding soils, the highest conjugation ratio was 1.2×10^{-7} transconjugants per recipient, and most of the setups were detected with no transconjugant during 6-day incubation (Fig. S1). Higher ratio (*mCherry*/16S *rRNA*) of both donor and potential recipient bacteria were detected in the gut of *C. elegans* than those in the surrounding soil using qPCR (Fig. S2).

To further characterize the plasmid conjugation in the *C. elegans* gut, colonies of transconjugants were detected based on plate screening via using fluorescence microscope. The number of *gfp*-expressing colonies,

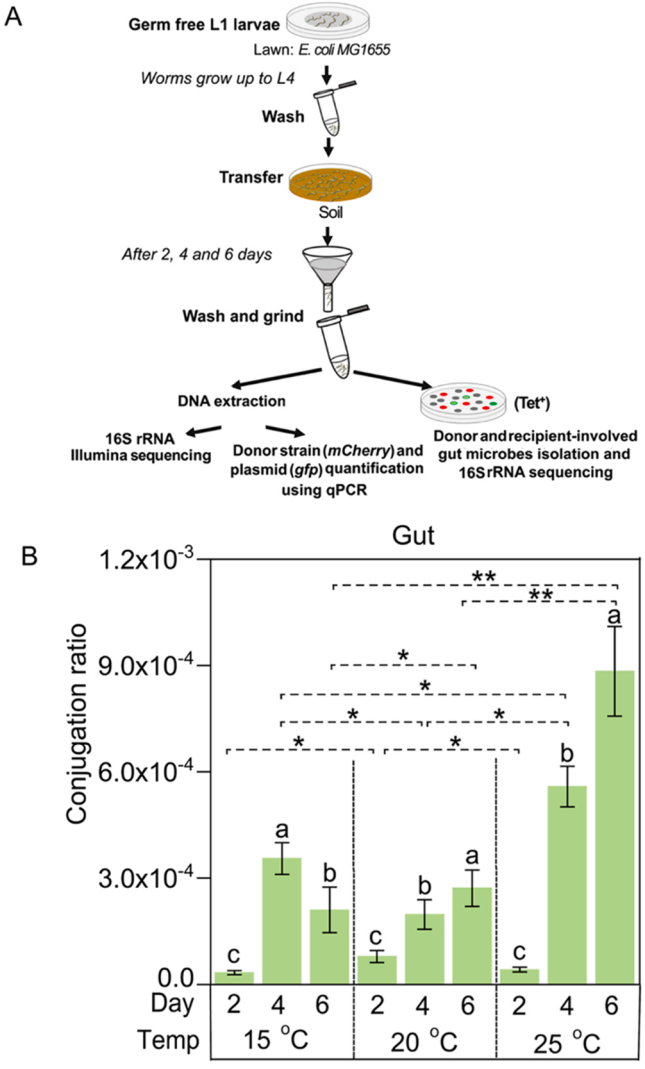

Fig. 1. Plasmid conjugation in the gut microbiota of *C. elegans*. (A) Flow chart of plasmid conjugation in this study. (B) Conjugation ratios in the gut microbiota of *C. elegans* N2 during 6-d cultivation at 15, 20 and 25 °C. The error bars indicate the standard deviations of three replications. Statistical significance between setups was denoted by asterisks ("*": $P < 0.05$; "**": $P \leq 0.01$) or different lowercase letters.

indicating as transconjugants, was impacted by the incubation time and temperature during the incubation (Fig. S3). Totally, nine strains of recipients for ARG-harboring plasmids were detected from the *C. elegans* gut (Fig. 2A). Of all, *Escherichia fergusonii* was the most abundant transconjugants detected from the gut of *C. elegans*, followed by *Salmonella Typhimurium, Shigella flexneri*, *S. sonnei*, *S. enterica* subsp. *arizonae*, *Lactobacillus brevis*, *Burkholderia contaminans*, *Microbacterium paraoxydans* and *Achromobacter insuavis* (Fig. 2B). Moreover, the abundances of transconjugants containing *E. fergusonii*, *S. Typhimurium, S. flexneri* and *S. sonnei* presented incubation time and temperature-dependent trend during 6-day incubation (Fig. 2B).

2.2. Microbial community composition of the C. elegans gut

Gram-negative families (e.g. Enterobacteriaceae, Nocardiaceae and Moraxellaceae) were prevalent in the *C. elegans* intestinal tract (Fig. 3A). In comparison, the surrounding soil was dominated by Lactobacillus, Leuconostocaceae, and Rhodosirillaceae (Fig. S4). Incubation time and temperature were identified to significantly ($P < 0.05$) shape the gut microbial community structures of *C. elegans* (Fig. 3B). The identified members shared among all setups in the nematode intestine were defined as the worm core gut microbiome (Berg et al., 2016), which accounted for 58.7–91.4% of the gut microbiota (Fig. 3C), among which, Enterobacteriaceae, Lactobacillaceae, Leuconostocaceae, Moraxelaceae and Nocardiaceae were the dominant families (Fig. 3C). Families of Nocardiaceae, Burkholderiaceae, Micrococcaceae and Pseudomonadaceae in the *C. elegans* gut displayed relatively lower abundances which increased with cultivation time, while the abundances of other families such as Xanthomonadaceae, Oxalobacteraceae and Comamonadaceae presented the opposite trend (Fig. 3C). In addition,

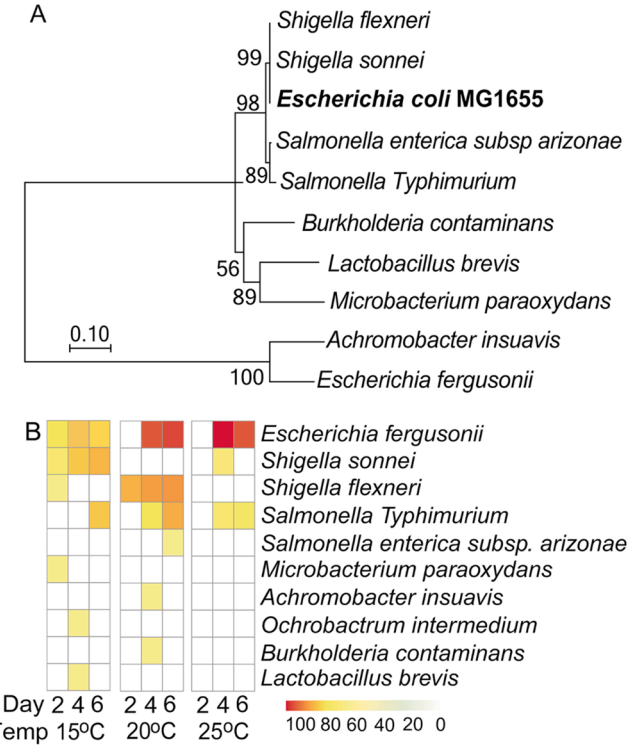

Fig. 2. Characterization of transconjugants detected in the gut of *C. elegans*. (A) Neighbor-Joining (bootstrap: 1000 replicates) phylogenetic trees based on 16S *rRNA* gene sequences showing the phylogenetic position of the donor strain *E. coli* MG1655 among transconjugants. Bar represents 0.1 substitution per 100 nucleotide positions, respectively. Bootstrap values above 50% given at the node. (B) Pattern of transconjugal pool. The transconjugants were collected from LB, R2A and PYG medium based on plate screening.

transconjugants-associated genus, including *Escherichia* (8.6–33.0%), *Lactobacillus* (0.7–6.4%), *Ochrobactrum* (0.2–6.3%), *Achromobacter* (0.02–0.5%), *Burkholderia* (0.008–0.6%), *Salmonella* (0.03–4.1%) and *Microbacterium* (0.004–0.02%), were detected with high abundances in the gut microbiota of *C. elegans* during the incubation (Fig. 3D).

2.3. The copies numbers of plasmid conjugation-relevant genes in the gut microbiota of C. elegans

To further investigate the frequency of plasmid conjugation, the relevant genes containing *trfAp* and *trfBp* were quantified in the gut of *C. elegans*. The values of *trfAp*/16S *rRNA* were 1.5×10^{-4} in the gut microbiota of *C. elegans* at 15 °C on day 2, which increased with incubation time and temperature during 6-day incubation at 15, 20 and 25 °C (Fig. 4A). Furthermore, ratios of *trfAp*/16S *rRNA* (gene copy numbers) shared a similar trend with those of *trbBp*/16S *rRNA* in the gut microbiota of *C. elegans* during 6-day incubation at 15, 20 and 25 °C (Fig. 4A and B).

4. Discussion

4.1. Core gut microbiota contributed greatly to the transconjugal pool

Significantly higher ratio of ARG- carrying plasmid conjugation was found in the gut of adult *C. elegans* than those in the surrounding soil (Fig. 1 and S1), indicating a huge potential of ARGs dissemination via gut microbiota of soil animals. Furthermore, plasmid conjugation ratio (ranged from 3.4×10^{-5} to 8.8×10^{-4} per recipient) in the gut of *C. elegans* was comparative with that in the soil amended with heavy metal and manure (8.2×10^{-5}-4.6×10^{-4} per recipient bacteria cell) (Klumper et al., 2017), further suggesting that the gut might be an important hotspot for conjugative plasmids.

Higher density of microbes inhabited in the gut of *C. elegans* than those in the surrounding soil was the main reason for high conjugation ratios in gut microbiota (Fig. S2). Physical contact is a prerequisite for occurrence of plasmid conjugation, and high concentration of gut microbes and donor cells ensured a higher possibility of cell-to-cell contact between recipient and donor cells in the gut, probably leading to higher conjugation ratios in the nematode gut than that in the soil. The diversity of transcojugants indicated that gut core microbiota contributed largely to the transconjugal pool. Especially, the identified recipients represented close evolutionary relationships with the donor strain *E. coli* MG1655 (Fig. 2A and Table S2). *E. coli* MG1655 has 99% similarity to *S. flexneri* and *S. sonnei*, and 96% similarity to *E. fergusonii* and *B. contaminans*, providing evidence that transconjugation were more likely to occur between phylogenetically close species. Most transconjugants (including *E. fergusonii*, *S. flexneri*, *S. enterica* subsp. *enerica*, *S. Typhimurium*, *A. insuavis*) have pili or flagella (Korhonen et al., 1980; Buchanan et al., 1985; Utsunomiya et al., 1992; Eto et al., 2013; Gaastra et al., 2014; Ridderberg et al., 2015), not only promoting cell adhesion and aiding in gut colonization but also forming a channel for the plasmid passage (Huddleston, 2014). Transconjugants including L. *brevis* and *M. paraoxydans* are gram-positive bacteria, which agreed with that the IncP-1 plasmid group can be maintained in almost all gram-negative bacteria and also transfer between gram-negative and gram-positive bacteria and even eukaryotic cells (Norberg et al., 2011; Greene, 2014; Ricaboni et al., 2016). However, the abundances of the gram-positive transconjugants were considerably lower than those of gram-negative strains in *C. elegans* gut (Fig. 2). Transconjugants including *E. fergusonii*, *S. flexneri*, *S. typhimurium* and *S. enterica* subsp. *arizonae* were found in the gut of *C. elegans* throughout the 6-day cultivation at 15, 20 and 25 °C, while L. *brevis*, *M. paraoxydans*, *A. insuavis*, *O. intermedium*, and *B. contaminans* transiently existed in the gut of *C. elegans* (Fig. 2). For the gut of *C. elegans*, *Lactobacillus* was detected with the comparable abundances with other gram-negative genera including *Escherichia*, *Achromobacter*, *Burkholderia* and

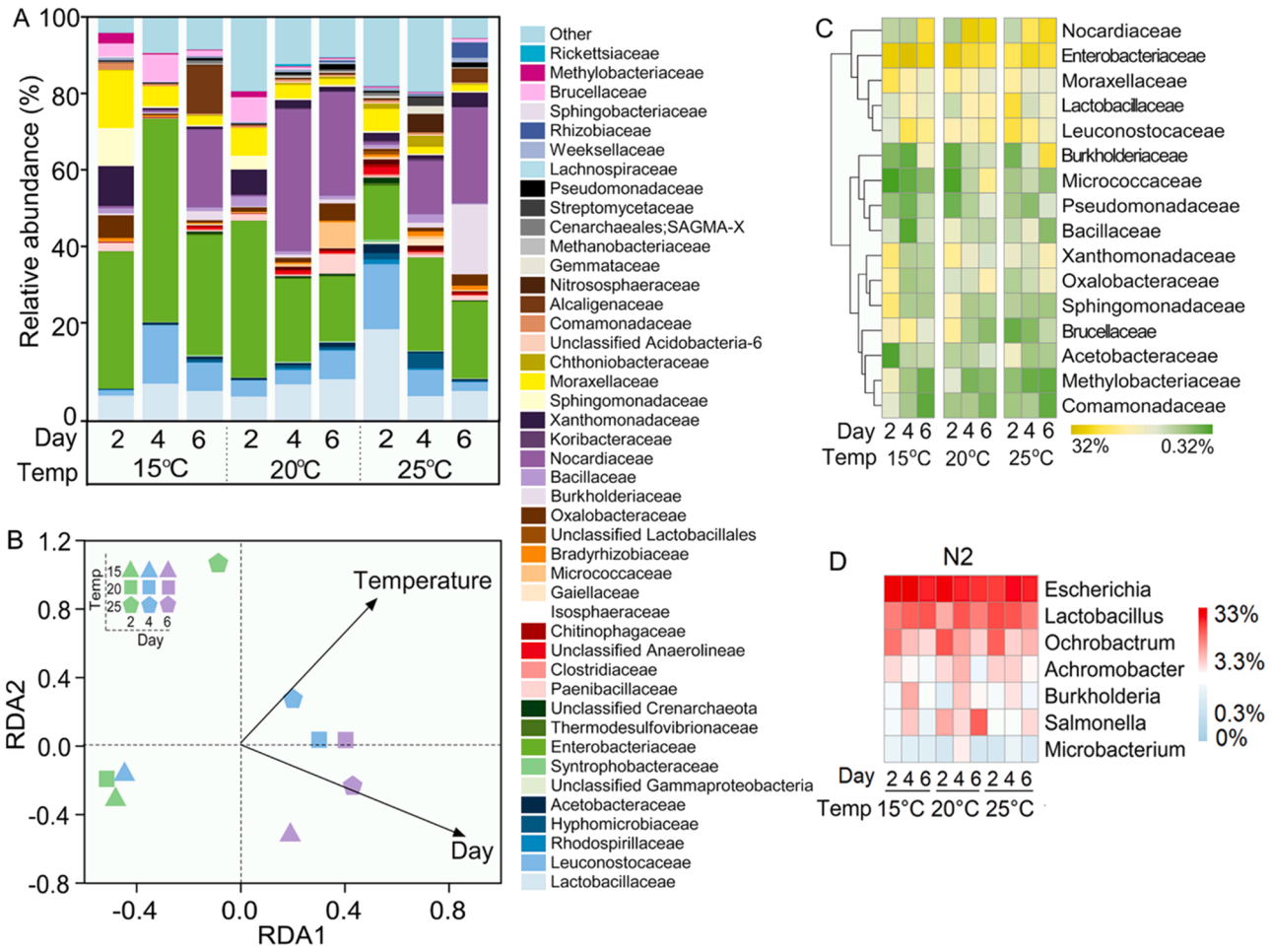

Fig. 3. Bacterial community composition at family level in the gut of *C. elegans* during 6-d cultivation at 15, 20 and 25 °C. (A) The 44 most-abundant families in all the setups. (B) RDA of gut microbial composition of *C. elegans* N2 at family level. "Temperature" ($P = 0.049$) and "Day" ($P = 0.024$) represented temperature and incubation time significantly impacted the gut microbial composition pattern of *C. elegans*. (C) The relative abundances of core microbiota at the family level in the gut of *C. elegans*. (D) The relative abundances of transconjugant-linked genera in the gut of *C. elegans*.

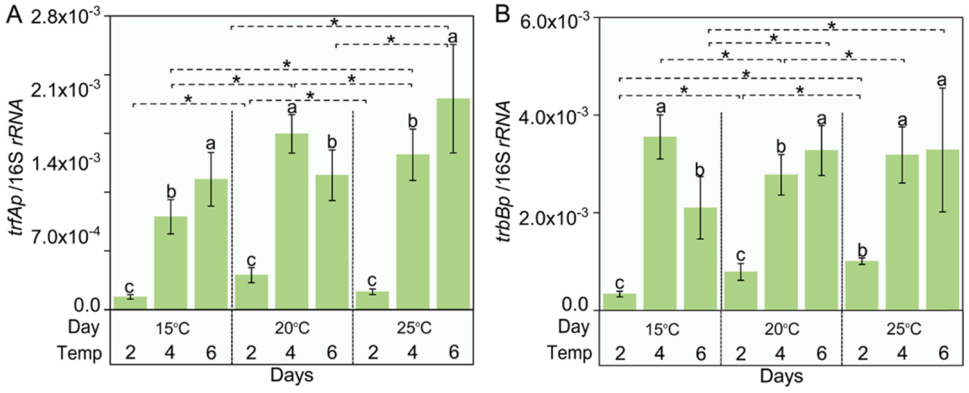

Fig. 4. The values of *trfAp*/16S *rRNA* (A) and *trbBp*/16S *rRNA* (B) in the gut microbiota of *C. elegans* during 6-d cultivation at 15, 20 and 25 °C. The error bars indicate the standard deviations of three replications. Statistical significance between setups was denoted by asterisks ("*": $0.01 < P < 0.05$; "**": $P \leq 0.01$) or different lowercase letters.

Samonella (Fig. 3D), which indicated that maintenance of IncP-1ε was more stable in the phylogenetically related gram-negative bacteria than in the gram-positive bacteria after plasmid conjugation in the gut microbiota of nematode. The difference of IncP-1ε plasmids transfer rates from donor *E. coli* to recipients between gram-negative and gram-positive strains was likely due to the inability of IncP-1ε to replicate in the gram-positive cells where the plasmids may be degraded by restriction enzymes derived from their hosts (Musovic et al., 2006). Transconjugants distribute over an extremely diverse phylogenetic range of 12 phyla (e.g. Acidiobacteria, Nitrospirae, Fusobacteria, Planctomycetes, Verrucomicrobia, Firmicutes) in soil microbiota using IncP-1ε as donor plasmid (Klumper et al., 2015, 2017). However, only 9

species clustered into several families were detected among all the transconjugants in our study (Fig. 3). This can be probable due to the limited culturability of the plate screening, leading to partial identification of transconjugants in the *C. elegans* gut.

4.2. Host age shaped conjugation ratios in gut microbiota of nematode

Temperature range of 15, 20 and 25 °C is considered physiological for *C. elegans* maintenance (Gómez-Orte et al., 2018). Laboratory maintained wild type *C. elegans* completes its life cycle in 2.5 days at 25 °C, whereas development of worms is slow down at 15 °C and life cycle extends up to 5 days (Gómez-Orte et al., 2018). Incubation time and temperature therefore markedly impacted the age of *C. elegans* (Gómez-Orte et al., 2018). Plasmid conjugation ratios of gut microbiota showed an upward trend with age of *C. elegans* except the setup incubated on day 4 at 15 °C (Fig. 1). For one thing, it can be explained by higher ability of colonization by potential recipients and donors in the gut on the day 6 and 4 than that on day 2 (Fig. S2). Gut microbiota of elder worms are found to be more permissive for bacterial cells in *C. elegans* (Portal-Celhay et al., 2012). The increase in relative abundances of the potential recipients including Enterobacteriaceae, Lactobacillaceae, Burkholderiaceae, Microbacteriaceae and Alcaligenaceae with incubation time was well corresponded with the elevated ratios of plasmid conjugation (Figs. 1 and 3). Although family Enterobacteriaceae dominated in the gut of *C. elegans*, the colonization ability within nematode gut of its members indicates a complex relationship with host age. For example, *Salmonella* resilience in the gut is independent of the host age, whereas *Escherichia* is capable of establishing persistent gut colonization depended on age of *C. elegans* (Ilka et al., 2011). It may result from cellular and structural damage of the host gut produced by other bacteria such as *Salmonella* in the gut which increased with incubation time and temperature of *C. elegans* (Portal-Celhay et al., 2012). Moreover, the ability of genera such as *Escherichia* and *Latobacillusm* persist in the *C. elegans* intestine became stronger over time (Fig. 3). Therefore, there was an increase in the abundance of potential plasmid recipient *Escherichia* with age of *C. elegans*, which would likely contribute to accumulation in conjugation events occurred in *Escherichia* in the gut of *C. elegans* during 6-day incubation at 15, 20 and 25 °C. Additionally, the gram-positive transconjugants, including L. *brevis, M. paraoxydans* and *R. timonensis* only can be detected on day 2 (15 °C), day 4 (15 and 20 °C) (Fig. 2). It was likely to contribute to the "unexpectedly" higher conjugation ratios during the incubation on day 4 than those on day 6 at 15 °C owing to instability of plasmid IncP-1ε kept in these gram-positive bacteria (Musovic et al., 2006).

Genes of *trfAp* and *trbBp* are responsible for plasmid replication and development of conjugation bridge between plasmid donors and recipients, respectively (Schreiner et al., 1985; Zatyka et al., 1997). The ratios of *trfAp*/16S *rRNA* and *trbBp*/16S *rRNA* indicated that the total gene copies of *trfAp* and *trbBp* in each cell increased with incubation time and temperature (Fig. 4). *gfp* is the reporter gene for plasmid pKJK5, which can be used to track the expression of *trfAp* or *trbBp* harbored in the plasmid (Klumper et al., 2017). Although it was hard to know whether the genes *trfAp* and *trbBp* expressed based on the DNA-level quantification, the detection of transconjugants with GFP fluorescence provided a direct evidence for their expression during the incubation (Fig. S2). Furthermore, conjugation ratios were consistent with the upregulation of abundances of *trfAp* and *trbBp* genes in the gut microbiota during the incubation (Figs. 1 and 4). All of these results indicated the expression level of genes *trfAp* or *trbBp* upregulated with incubation time and temperature in the gut microbiota of nematode. Therefore, plasmid replication and conjugation bridge development in transconjugant were stimulated with increase of incubation time and temperature. This might be another reason for accumulative frequency of plasmid horizontal transfer during 6-day incubation at 15–25 °C. In addition, the growth of F pili, which is the conjugative pili, is regulated by growth temperature, and both the number and length of F pili increase with the temperature (20–37 °C) for *E. coli* (Novotny and Lavin, 1971). As a result, it suggested that the acceleration of F pili growth can create more chance for the formation of conjugation bridge between plasmid donors and recipients over a long distance. This may be another explanation for the up-forward shift in conjugation ratio with increased host age in the gut microbiota of *C. elegans*.

4.3. Mobile transconjugal pool of animal gut microbiota for ARGs transfer in soils

In this study, donor strain *E. coli* MG1655 harboring IncP-1ε plasmid can persist in the nematode intestine, which was the basis for conjugation. We here showed that ARGs-carrying plasmid can be immediately transferred into recipients and maintained in the new host for a long time even in absence of antibiotic selection. Although there was only an uncomplete atlas of transfer range for IncP plasmids, family Enterobacteraceae (including *E. fergusonii*, *S. sonnei*, *S. flexneri*, *S. enterica* subsp. *enterica typhimurium*, *S. enterica* subsp. *arizonae*) were identified to compose an important part of transconjugal pool in the gut of soil nematode. Members of Enterobacteriaceae, Burkholderiaceae and Alcaligenaceae are common gut microflora in soil animals such as earthworm, termite, springtail (Bahrndorff et al., 2018; Zhou et al., 2019). Moreover, comparative analysis indicates that IncP-1 plasmids have adapted to different environments or hosts experienced over evolutionary time scales (Norberg et al., 2011). The low abundant gram-positive transconjugants such as L. *brevis, M. paraoxydans* and *R. timonensis* indicated that transfer and maintenance of IncP-1 plasmids is possible across the gram border, as least, in the short term. It was suggested that a few species from gut microbiota can be the core nodes of the connected network of horizontal ARGs acquisition in soils.

The presence of antibiotics or common antibacterial biocides not only fosters horizontal transfer of ARGs even at low antibiotic concentrations but also potentially stimulates the transfer of ARGs from donor strains *E. coli* to pathogens (Jutkina et al., 2018). Antibiotic-induced disruption of the well-organized microbial composition structure in the gut environment can weaken host defenses against bacterial infections and then lead to shaping new niches for pathogens occupation (Modi et al., 2014). The super permissiveness of pathogens for plasmids would boost them evolve into more tenacious and virulent environments.

5. Conclusion

Conjugation ratios were significantly higher in the gut of *C. elegans* than those in the surrounding soil. Incubation time and temperature were important deterministic factors affecting the age of *C. elegans* and significantly shaped the gut microbial community. Conjugation ratios and the relevant genes including *trfAp* and *trbBp* increased with the incubation time and temperature in the *C. elegans* gut. Gut core microbiota Enterobacteriaceae, including *E. fergusonii*, *S. Typhimurium*, *S. flexneri* and *S. sonnei*, contributed a lot to the transconjugal pool. This finding highlights that host age markedly influences the dissemination of ARGs via gut microbiota of soil animals in the terrestrial ecosystem.

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CRediT authorship contribution statement

Guo-Wei Zhou: Conceived and designed the project, Did the experiments, Analyzed the data, Wrote the manuscript, Revised the manuscript. **Xiao-Ru Yang:** Conceived and designed the project, Wrote

the manuscript, Revised the manuscript. **Yong-Guan Zhu:** Conceived and designed the project, Revised the manuscript. **Fei Zheng:** Did the experiments, Analyzed the data, Wrote the manuscript, Revised the manuscript. **Xiao-Ting Fan:** Gave assistance in lab work and laboratory analyses, Analyzed the data, Revised the manuscript. **Ming-Jun Li:** Gave assistance in lab work and laboratory analyses, Revised the manuscript. **Qing-Ye Sun:** Revised the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.127525.

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