The ISME Journal (2017), 1–5

www.nature.com/ismej

SHORT COMMUNICATION

Microbial functional trait of rRNA operon copy numbers increases with organic levels in anaerobic digesters

Linwei Wu[1,2], Yunfeng Yang[1], Si Chen[3], Zhou Jason Shi[2], Mengxin Zhao[1], Zhenwei Zhu[3], Sihang Yang[1], Yuanyuan Qu[4], Qiao Ma[4], Zhili He[2], Jizhong Zhou[1,2,5,6] and Qiang He[3,7]

[1]*State Key Joint Laboratory of Environment Simulation and Pollution Control, School of Environment, Tsinghua University, Beijing, China;* [2]*Institute for Environmental Genomics and Department of Microbiology and Plant Biology, University of Oklahoma, Norman, OK, USA;* [3]*Department of Civil and Environmental Engineering, The University of Tennessee, Knoxville, TN, USA;* [4]*Key Laboratory of Industrial Ecology and Environmental Engineering (Ministry of Education), School of Environmental Science and Technology, Dalian University of Technology, Dalian, China;* [5]*School of Civil Engineering and Environmental Sciences, University of Oklahoma, Norman, OK, USA;* [6]*Earth Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA and* [7]*Institute for a Secure and Sustainable Environment, The University of Tennessee, Knoxville, TN, USA*

The ecological concept of the r-K life history strategy is widely applied in macro-ecology to characterize functional traits of taxa. However, its adoption in microbial communities is limited, owing to the lack of a measureable, convenient functional trait for classification. In this study, we performed an experiment of stepwise organic amendments in triplicate anaerobic digesters. We found that high resource availability significantly favored microbial r-strategists such as *Bacillus* spp. Incremental resource availability heightened average rRNA operon copy number of microbial community, resulting in a strong, positive correlation ($r>0.74$, $P<0.008$). This study quantifies how resource availability manipulations influence microbial community composition and supports the idea that rRNA operon copy number is an ecologically meaningful trait which reflects resource availability.
The ISME Journal advance online publication, 25 August 2017; doi:10.1038/ismej.2017.135

Understanding ecological strategies is fundamental in plant and animal ecology for predicting biodiversity patterns and community responses to disturbances. The concept of r- and K-life history strategies describes the early colonization of populations which is beneficial when resources are abundant (that is, r-strategy), and their subsequent replacement by populations which is beneficial when resources become limited (that is, K-strategy) (MacArthur and Wilson, 2015). Despite criticism to be an oversimplification, it provides a convenient framework to characterize the ecological trait across different taxa (Reznick *et al.*, 2002). The ecological characteristics of many microorganisms have been documented by studying laboratory isolates, leading to a proposition that microbial rRNA operon copy number can potentially serve as a unifying life strategy trait for tracking ecosystem response to environmental change (Green *et al.*, 2008). A large-scale survey of bacteria strains indicates that the rRNA operon copy number is positively correlated with their maximum reproductive rates and negatively correlated with the carbon use efficiency (Roller *et al.*, 2016). Cultivation experiments on microbial isolates also revealed a positive correlation between the rRNA operon copy number and the capacity for that microorganism to respond to resource availability (Klappenbach *et al.*, 2000). That is, organisms with few *rrn* operons tend to be K-strategy; those with multiple *rrn* operons tend to be r-strategy, which are dominant when resources are abundant (Lee *et al.*, 2009). Nevertheless, despite recent efforts to analyze the average rRNA copy number of bacterial community (DeAngelis *et al.*, 2015, Nemergut *et al.*, 2016), it remains elusive to what magnitude the mean rRNA copy number of microbial community responds to resource availability.

In this study, we increased resource availability through addition of poultry waste into triplicate digesters with dairy manure as the substrate, and used modern molecular methods to quantify changes

Correspondence: Y Yang or Q He, School of Environment, Tsinghua University, 1 Tsinghua Garden Road, Haidian District, Beijing 100084, China.
E-mail: yangyf@tsinghua.edu.cn or qianghe@utk.edu
Received 7 September 2016; revised 5 June 2017; accepted 15 June 2017

in bacterial community. We maintained the organic loading rate (OLR) at 1.0 g volatile solid (VS) l^{-1} day^{-1} (Day 1–44), then increased to 1.3 g VS l^{-1} day^{-1} (Day 45–76), and 1.5 g VS l day^{-1} (Day 77–98) by adding poultry waste to co-digesters (Supplementary Figure S1; see Supplementary Materials for experimental details). The VS level, an indicator index of organic matter (Federation and Association, 2005, Larney *et al*., 2005), is used as a measure of resource availability in this study. Following organic amendments, the VS level rose gradually from 14.15 ± 0.17 g l^{-1} to 17.80 ± 0.26 g l^{-1}. The increase in methane production was proportional to the increase in OLR in co-digesters, suggesting that the function of microbial community was not inhibited under our experimental conditions (Chen *et al*., 2012). In contrast, methane production, biogas production and VS removal remained largely stable in the control digesters fed with dairy manure as the only substrate (Supplementary Figure S1).

Principal coordinate analysis (PCoA) suggested that microbial taxonomic composition, as represented by the 16 S rRNA sequence data, changed over time in co-digesters (Figure 1a), which significantly differed from that of controls at both the first OLR increase (permutational MANOVA, $F = 5.30$, $P = 0.001$) and the second OLR increase (permutational MANOVA, $F = 11.01$, $P = 0.001$). We generated heatmaps with OTUs to compare the difference in microbial community compositions between control and co-digesters (Supplementary Figure S2). Three groups of OTUs were identified by selecting branches presenting specific patterns of dynamics in the relative abundance, which were then subjected to post hoc statistical tests (see Supplementary Methods for details). Group 1 represented abundant OTUs in both control and co-digesters. Group 2 represented OTUs significantly increased in the co-digesters, while OTUs in Group 3 were abundant in control but substantially decreased in the co-digesters. By comparing Group 2 and 3, we showed that phylogenetic clades were different (Figure 1b). For example, most of the OTUs (71.8%) in Group 2 belonged to the phylum *Firmicutes*, which was remarkably higher than that in Group 3 (39.1%) and in all of the detected OTUs (52.7%). Among them, there was a 24.4% enrichment of class *Bacilli* with many species known to be efficient in fermentation and fast-growing under favorable conditions, such as *Bacillus subtilis* (Karlin *et al*., 2001) and amylolytic lactic acid bacteria from the order *Lactobacillales* (Diaz-Ruiz *et al*., 2003). A total of 9.4% of OTUs belonged to the phylum *Actinobacteria* in Group 2, while none in Group 3 was from *Actinobacteria*.

The rRNA operon copy number for each OTU was estimated through the rrnDB database, based on its

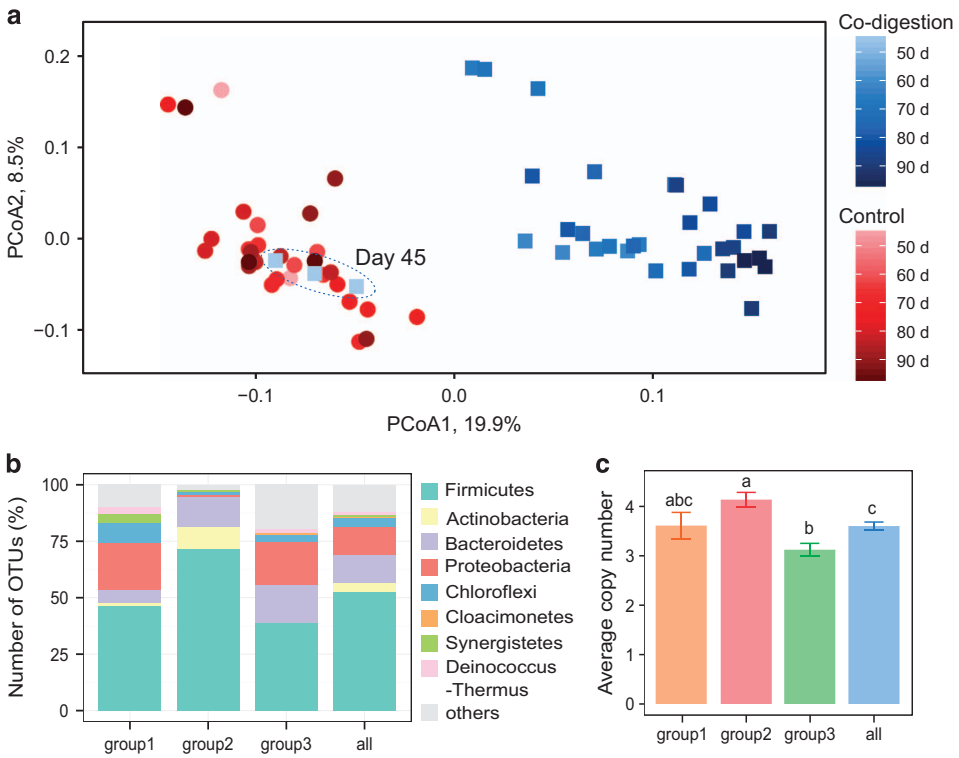

Figure 1 Dynamics of bacterial taxonomic compositions in response to OLR manipulation. (**a**) principal coordinate analysis (PCoA) of control and co-digestion samples. Control and co-digestion samples are indicated by red circles and blue squares, respectively, with the gradient of color representing sampling time. (**b**) Taxa classification at the phylum level of the four groups of OTUs: abundant OTUs across all samples (Group 1); OTUs enriched under co-digestion (Group 2); OTUs declined under co-digestion (Group 3); and the 'all' group representing all the OTUs detected. (**c**) The mean of estimated rRNA operon copy number of OTUs for each group. The error bar represents standard error. Values with different superscript letters at each group are significantly ($P < 0.05$) different from each other.

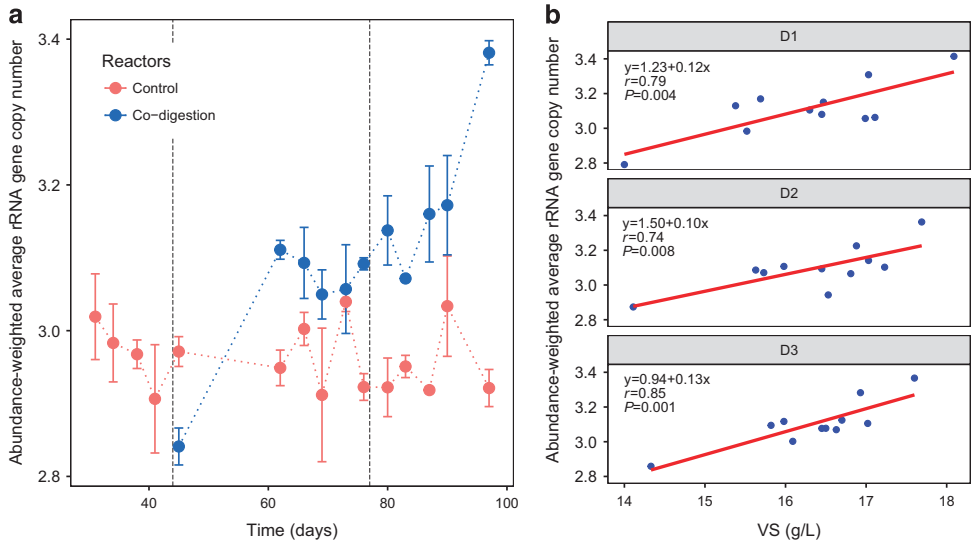

Figure 2 Effects of OLR manipulation on the abundance-weighted average rRNA operon copy number of microbial communities. (**a**) The abundance-weighted average rRNA operon copy number of communities across time. The error bar represents the standard error of three biological replicates. (**b**) linear regression showing the correlation between the abundance-weighted average rRNA operon copy number of microbial community and VS level in each co-digester.

closest relatives with known rRNA operon copy number (Stoddard *et al.*, 2014). ANOVA indicated significant differences in the average copy number between groups ($P<0.001$). The average copy number of OTUs from Group 2 was significantly higher ($P=0.02$, Tukey's test), while that of Group 3 was lower ($P=0.03$, Tukey's test) than the average copy number of all OTUs (Figure 1c), demonstrating that OTUs with higher rRNA operon copy numbers were enriched by incremental OLR. In particular, the most notable change was observed in OTU_333 belonging to the genus *Virgibacillus* with the estimated copy number of 6.67, which was undetected in control samples but presented in 31 out of 33 co-digestion samples.

We calculated the abundance-weighted average rRNA operon copy number of OTUs for each sample (Figure 2a). The average copy number of microbial communities in control digesters were relatively stable across the study period, with an average of 2.96. When supplied with additional poultry waste, the average copy number in co-digesters increased to 3.09 at the end of the first OLR increase and further increased to 3.38 at the end of the second OLR increase. Linear regression analysis indicated that the abundance-weighted average rRNA operon copy number of microbial community strongly correlated ($r>0.74$, $P<0.008$) with the VS level in each co-digester (Figure 2b), which was incremental over time. This finding appeared to be reliable, since results of the autocorrelation function (ACF) analysis (Supplementary Figure S3) and the Wald–Wolfowitz runs test revealed no autocorrelation ($P>0.4$) of residuals from the linear regression models. This finding suggested that microorganisms with higher rRNA operon copy number were favored when VS levels were higher.

The shift of microbial communities was unlikely to arise from microbes introduced by poultry waste because microbial community compositions of dairy manure and poultry waste were remarkably distinct from those of sludge (Supplementary Figure S4). For example, the relative abundance of *Firmicutes* in dairy manure and poultry waste was less than 4% but was more than 40% in sludge. In addition, the average rRNA operon copy number was similar between dairy manure (1.97 ± 0.01) and poultry waste (1.96 ± 0.01). The total ammonia was also correlated with the average rRNA operon copy number in co-digesters, which was not surprising since VS and ammonia were strongly correlated ($r>0.9$, $P<0.001$). In fact, the total ammonia (mg N per g VS) were similar between the two substrates (Supplementary Table S1). Nevertheless, VS remains to be the strongest predictor for rRNA copy number, as the standardized regression coefficient of VS is larger than that of ammonia in the multiple regression analysis (Supplementary Table S2). Therefore, the increase in average rRNA operon copy number were most likely to arise from the elevated VS level in co-digesters.

Cultivation studies have demonstrated that bacteria exhibit various ecological strategies (Gottschal, 1985,Koch, 2001). It has been shown that genomic signatures, such as codon usage bias indices and the rRNA operon copy number, could be used as proxies for ecological strategies (Lauro *et al.*, 2009,Vieira-Silva and Rocha, 2010). The rRNA operon copy number correlates to the pace with which microbes synthesize ribosomes and respond to resource availability (Klappenbach *et al.*, 2000,Roller *et al.*, 2016). However, the utility of using rRNA operon copy number as a readout of life history strategies may be overridden by the complex interactions present in

natural communities. It has been shown that the average rRNA operon copy number of bacteria communities decreased under warming (DeAngelis *et al.*, 2015). The average rRNA operon copy number also decreased over time in ecological succession, which was indirectly associated with changes in resource availability (Nemergut *et al.*, 2016). Here we showed that the average ribosomal copy number was lower under low-organic nutrient levels (Figure 2a), and established a causal relationship that incremental organic nutrient level in anaerobic digesters causes increase in the average ribosomal copy number of microbial community (Figure 2b), supporting the use of rRNA operon copy number as a community-level functional trait associated with ecological strategies. Given that the rRNA operon copy number can be conveniently estimated by environmental genomics sequences in DNA database because the rRNA operon copy number is conserved with phylogeny (Lee *et al.*, 2009), the rRNA operon copy number could be used in routine analyses of microbial communities.

We used functional gene array (GeoChip) to examine microbial functional gene composition, which remained unchanged (permutational MANOVA, $F = 0.66$, $P = 0.49$) at the first OLR increase but changed significantly (permutational MANOVA, $F = 4.65$, $P < 0.005$) at the second OLR increase (Supplementary Figure S5). In contrast, microbial taxonomic compositions changed more substantially as F values of taxonomic compositions (permutational MANOVA) were larger at both OLR increase intervals, suggesting that microbial functional trait might be more resistant to environmental perturbation than taxonomic trait, which had been observed in macro-ecology (Fukami *et al.*, 2005). While Chao-1 indices based on OTU sequences showed that microbial taxonomic diversities in co-digesters were not different from those of controls, Shannon's indices of GeoChip data suggested an increase in microbial functional gene diversity during the second OLR increase (Supplementary Table S3), which might be due to the increased resource diversity from additional poultry waste.

GeoChip probes carry taxonomic information of microbial community (Tu *et al.*, 2014). We found that taxonomic patterns revealed by GeoChip were consistent with the 16 S rRNA amplicon sequencing data. For example, 36.4% of the *cellobiase* gene sequences increased under co-digestion were derived from the phylum *Firmicutes*, which was higher than average (21.3%, Supplementary Figure S6). Among them, 66.7% of those *Firmicutes* sequences belonged to the class *Bacilli* (for example, *Bacillus clausii* KSM-K16). These results were consistent with sequencing data showing significant increases in the relative abundance of *Firmicutes*, especially *Bacilli* in co-digesters (Figure 1). Similar results were observed in the acetogenesis genes *codh* (Supplementary Figure S7) and *fthfs* (Supplementary Figure S8) involved in the reductive acetyl-CoA pathway, which encoded enzymes for carbon monoxide dehydrogenase and formyltetrahydrofolate synthetase, respectively.

Methanosaeta and *Methanosarcina* are the only two genera recognized as acetoclastic methanogens. *Methanosaeta* is strictly acetoclastic, that is, using acetate as the only substrate for methane production (Smith and Ingram-Smith, 2007), while *Methanosarcina* is more flexible, capable of all three pathways of methane production, that is, hydrogenotrophic, acetoclastic and methylotrophic methanogenesis (De Vrieze *et al.*, 2012). When the dynamics of *mcrA* gene sequences derived from *Methanosaeta* and *Methanosarcina* across time points was examined, average signal intensities of *Methanosaeta* sequences were higher than *Methanosarcina* throughout the study period, in consistency with qPCR data (Supplementary Figure S9; see Supplementary Table S4 for primers used in qPCR). Nonetheless, sequences from *Methanosarcina*, which could be considered as r-strategists (copiotrophic) since *Methanosarcina* populations are known to have higher maximum specific growth rates (μ_{max}) and half saturation concentrations (K_S) for growth on acetate (Conklin *et al.*, 2006) than *Methanosaeta*, were enriched during the second OLR increase as compared to controls, as indicated by both qPCR ($P < 0.05$, two-tailed t test) and GeoChip data ($P < 0.10$, two-tailed t test). Although the archaeal result alone was not strong evidence, it was consistent with the bacterial data showing the enrichment of r-strategists by OLR increase.

In summary, this study showed that both taxonomic and functional traits of microbial community were sensitive to organic amendments in anaerobic digesters, with r-strategists enriched under higher OLR. The positive linear correlation between the average rRNA operon copy number of microbial community and organic nutrient level raises a possibility to manipulate the microbial community on the r- to K- strategy continuum by organic management, which has important implications on the trade-off of microbial community stability because r-strategists might be less resistant but more resilient than K-strategists (De Vries and Shade, 2013).

Data Accessibility

Sequence data are accessible in the GenBank database under the accession number SRP070491. GeoChip data are accessible in the GenBank database under the accession number GSE93419.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

We wish to thank three anonymous reviewers and the editor for constructive comments and suggestions to make this manuscript greatly improved. This work was partly supported by Major Science and Technology Program for Water Pollution Control and Treatment (2013ZX07315-001-03), a U.S. Environmental Protection Agency Grant XA-83539201, and the Science Alliance—Tennessee Center of Excellence. SC was partly supported by the Institute for a Secure and Sustainable Environment at the University of Tennessee, Knoxville. YY was partly supported from National Science Foundation of China (41471202). JZ was partly supported from the National Science Foundation of China (41430856), Collaborative Innovation Center for Regional Environmental Quality, and the Office of the Vice President for Research at the University of Oklahoma.

References

Chen S, Zamudio Canas EM, Zhang Y, Zhu Z, He Q. (2012). Impact of substrate overloading on archaeal populations in anaerobic digestion of animal waste. *J Appl Microbiol* **113**: 1371–1379.

Conklin A, Stensel HD, Ferguson J. (2006). Growth kinetics and competition between Methanosarcina and Methanosaeta in mesophilic anaerobic digestion. *Water Environ Res* **78**: 486–496.

De Vries F, Shade A. (2013). Controls on soil microbial community stability under climate change. *Front Microbiol* **4**: 265.

De Vrieze J, Hennebel T, Boon N, Verstraete W. (2012). Methanosarcina: the rediscovered methanogen for heavy duty biomethanation. *Bioresource Technol* **112**: 1–9.

DeAngelis KM, Pold G, Topçuoğlu BD, van Diepen LT, Varney RM, Blanchard JL *et al.* (2015). Long-term forest soil warming alters microbial communities in temperate forest soils. *Front Microbiol* **6**: 104.

Diaz-Ruiz G, Guyot J-P, Ruiz-Teran F, Morlon-Guyot J, Wacher C. (2003). Microbial and physiological characterization of weakly amylolytic but fast-growing lactic acid bacteria: a functional role in supporting microbial diversity in pozol, a Mexican fermented maize beverage. *Appl Environ Microbiol* **69**: 4367–4374.

Federation WE, Association A (2005). *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association (APHA): Washington, DC, USA.

Fukami T, Martijn Bezemer T, Mortimer SR, Putten WH. (2005). Species divergence and trait convergence in experimental plant community assembly. *Ecol Lett* **8**: 1283–1290.

Gottschal JC. (1985). Some reflections on microbial competitiveness among heterotrophic bacteria. *Antonie Van Leeuwenhoek* **51**: 473–494.

Green JL, Bohannan BJ, Whitaker RJ. (2008). Microbial biogeography: from taxonomy to traits. *Science* **320**: 1039–1043.

Karlin S, Mrázek J, Campbell A, Kaiser D. (2001). Characterizations of highly expressed genes of four fast-growing bacteria. *J Bacteriol* **183**: 5025–5040.

Klappenbach JA, Dunbar JM, Schmidt TM. (2000). rRNA operon copy number reflects ecological strategies of bacteria. *Appl Environ Microbiol* **66**: 1328–1333.

Koch AL. (2001). Oligotrophs versus copiotrophs. *Bioessays* **23**: 657–661.

Larney FJ, Ellert BH, Olson AF. (2005). Carbon, ash and organic matter relationships for feedlot manures and composts. *Can J Soil Sci* **85**: 261–264.

Lauro FM, McDougald D, Thomas T, Williams TJ, Egan S, Rice S *et al.* (2009). The genomic basis of trophic strategy in marine bacteria. *Proc Natl Acad Sci* **106**: 15527–15533.

Lee ZM-P, Bussema C, Schmidt TM. (2009). rrnDB: documenting the number of rRNA and tRNA genes in bacteria and archaea. *Nucleic Acids Res* **37**: D489–D493.

MacArthur RH, Wilson EO. (2015). *Theory of Island Biogeography* vol. 1. Princeton University Press: Princeton, NJ, USA.

Nemergut DR, Knelman JE, Ferrenberg S, Bilinski T, Melbourne B, Jiang L *et al.* (2016). Decreases in average bacterial community rRNA operon copy number during succession. *ISME J* **10**: 1147–1156.

Reznick D, Bryant MJ, Bashey F. (2002). r-and K-selection revisited: the role of population regulation in life-history evolution. *Ecology* **83**: 1509–1520.

Roller BR, Stoddard SF, Schmidt TM. (2016). Exploiting rRNA operon copy number to investigate bacterial reproductive strategies. *Nat Microbiol* **1**: 16160.

Smith KS, Ingram-Smith C. (2007). Methanosaeta, the forgotten methanogen? *Trends Microbiol* **15**: 150–155.

Stoddard SF, Smith BJ, Hein R, Roller BR, Schmidt TM. (2014). rrnDB: improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. *Nucleic Acids Res* **43**: D593–D598.

Tu Q, Yu H, He Z, Deng Y, Wu L, Van Nostrand JD *et al.* (2014). GeoChip 4: a functional gene-array-based high-throughput environmental technology for microbial community analysis. *Mol Ecol Resources* **14**: 914–928.

Vieira-Silva S, Rocha EP. (2010). The systemic imprint of growth and its uses in ecological (meta) genomics. *PLoS Genet* **6**: e1000808.

Supplementary Information accompanies this paper on The ISME Journal website (http://www.nature.com/ismej)