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Functional Metagenomics using Stable Isotope Probing: a Review

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Abstract

The microbial eco-physiology has been the vital key of microbial ecological research. Unfortunately, available methods for direct identity of microorganisms and for the investigation of their activity in complicated community dynamics are limited. In this study, metagenomics was considered as a promising functional genomics tool for improving our understanding of microbial eco-physiology. Its potential applications and challenges were also reviewed. Because of tremendous diversity in microbial populations in environment, sequence analysis for whole metagenomic libraries from environmental samples seems to be unrealistic to most of environmental engineering researchers. When a target function is of interest, however, sequence analysis for whole metagenomic libraries would not be necessary. For this case, nucleic acids of active populations of interest can be selectively gained using another cutting-edge functional genomic tool, SIP (stable isotope probing) technique. If functional genomes isolated by SIP can be transferred into metagenomic library, sequence analysis for such selected functional genomes would be feasible because the reduced size of clone library may become adequate for sequencing analysis. Herein, integration of metagenomics with SIP was suggested as a novel functional genomics approach to study microbial eco-physiology in environment.

Keywords: Eco-physiology, Functional genomics, Microbial ecology, Systems biology

1. Introduction

A macroscopic global environment was created by multiple biotic and abiotic components interactions. The first form of life is microorganisms which have been evolving in response to changing environment conditions. The evolution of new species by selective pressures from the new conditions creates different species-represented environment. The structure of this environment can be figured as a pyramid in which the higher blocks are created and supported by the smaller structure.[1)] Therefore, researching on small ecological components, such as microorganisms, plays a pivotal role in deriving interaction between environmental components. Microorganisms have many of the same properties as more complex organisms such as amino acid biosynthesis. They also include novel properties such as the ability to recycle the huge masses of organic matter in form of waste products and to accelerate geochemical processes. Thus, it is necessary to understand what populations are present in a specific ecosystem and how populations interact with one another. As a result of requirement, the science of genetic and biological diversity of microorganisms, namely metagenomics, is an important area of environmental research.

Although the functional genomic tools are very powerful and useful in environmental studies, the concepts of such molecular tools have not been well introduced to Environmental Engineering researchers. Thus, we would like to review the principle and application of metagenomics. In addition, another cutting-edge functional community analysis tool, SIP (stable isotope probing) technique was introduced and reviewed herein because integration of metagenomics with SIP has potential advantages in making the fancy functional genomic techniques more feasible in environmental studies. In the third part, integration of metagenomics with SIP was suggested as a novel functional environmental genomics, which will be a future biotechnology tool for microbial ecology and environmental studies.

2. Metagenomics: Environmental Functional Genomics

The term “metagenomics” is derived from the statistical concept of *meta*-analysis (the process of statistically combining separate analyses) and genomics (the comprehensive analysis of an organism’s genetic material).[2)] Metagenomics is the culture-independent genomic analysis of microbial communities. Environmental DNA and RNA can be obtained directly from environ-

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mental samples such as water, sediment, soil, etc., and then functional and community analyses can be done for a variety of purposes by using of various molecular techniques. The limitation of cultivation, which is an essential prelude to characterization in laboratory, has being prevented scientists from capturing systematic biological information from the environment. It is generally accepted that less than 1% of bacteria present in most habitats have been cultured for studying in pure cultures.[3] In addition, it was shown that culturing did not capture the full spectrum of microbial diversity. For instance, Staley and Konopka[4] showed the discrepancy between the sizes of populations estimated by dilution plating and by microscopy, especially in some aquatic samples. Plate counts and viable cells, in aquatic samples, estimated by staining can differ by four to six orders of magnitude. In soil, only 0.1 to 1% of bacteria are able to culture readily on common media under a standard condition. To circumvent the limitations of cultivation-dependent methods, metagenomics is considered as a powerful technique to capture systems biology information from environmental samples of interest.

Metagenomic analyses of a variety of environmental samples, for example sea water,[5] cave water,[6] estuarine and brackish sediments,[7] freshwater sediments,[8] peat soil,[9] temperature forest soil[10] or acidic forest soil,[11] have revealed the enormous genetic diversity of complex microbial communities present in these environments. As seen in Figure 1, metagenomic analysis includes two main approaches: a function-driven analysis and a sequence-driven analysis.[12]

Function-driven analysis or target-driven analysis is an approach in which ecological or systems biology information from an environment is analyzed using known information on the target function or population. For example, metagenomic analysis of this class requires available biomarkers because function-driven analysis from a metagenomic library is generally performed using PCR-primers or hybridization-probes for target functional genes and/or phylogenetic biomarkers of populations having a specific function. If biomarkers for a function are available enough to capture the full range of genetic diversity of a functional group in environments, this approach is straightforward in getting the target information from environmental samples. Unfortunately, functional gene information is generally limited to capture full diversity of a function in environment, and functional analysis with limited biomarker information would provide biased conclusion on microbial diversity.

Meanwhile, sequence-driven analysis, an approach in which the full genomic sequences of microbial communities, is analyzed without having any pre-existing hypotheses or information on target functions or populations. The strength of this approach is determined in its capacity to harvest the whole information from an environmental sample. But there still remains limitation. The huge amount of sequencing and following bioinformatics analyses cause to high cost in this approach. Generally, the frequency of clones having desired functional information is relatively very low in metagenomic libraries. To solve this problem, the initial and essential step is enrichment of the genomes from metabolically active microbes (target populations). One of effective enrichment methods is stable isotope probing (SIP) which has potential for subdividing microbial communities into functional units.[12] SIP has been demonstrated to link taxonomic identity to metabolic function across a wide range of ecosystems, including soil, natural groundwater system,[13] soda lake sediment,[14] etc.

3. Principle and Applications of SIP Technique

SIP is one of cutting-edge environmental genomic methods used by environmental microbiologists. It is a molecular techni-

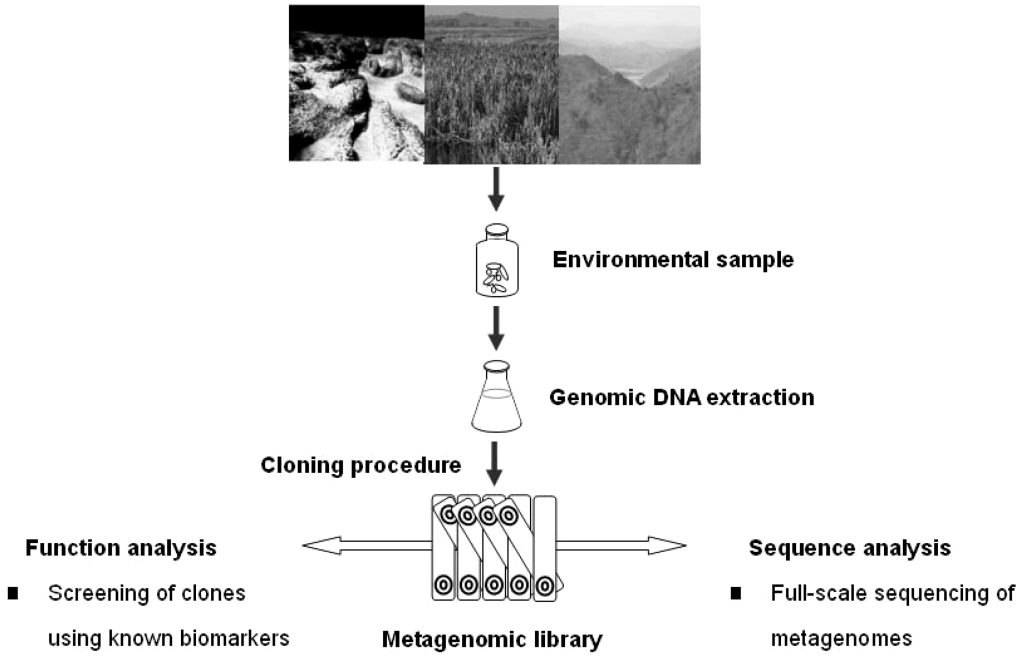

Fig. 1. Scheme of metagenomic analyses for a variety of environmental samples.

que allowing investigators to follow the flow of atoms in isotopically enriched molecules, especially respecting to carbon element, through complex microbial communities into metabolically active microorganisms.[15] Carbon element is the predominant constituent of all life forms. Its combining capabilities make it central to most biogeochemical reactions.[16] Understanding how carbon flow constituents activate key functional genes for interactions within and between microorganisms and macroscopic ecosystem is critical for sustainable ecological management and application, such as management of crops and biodegradation of organic pollutants. By using stable isotope probing, the majority of hosts responsible in situ for naphthalene degradation were derived phylogenetically.[17] Cell components of organisms that assimilated the substrate, which results in the incorporation of the stable-isotope, would be 'heavier' than those of microbes that used indigenous carbon sources. Separation, based on different weight of cell components, can then distinguish total and active members of the community.

SIP is based on the introduction of a labeled substrate into a system, allowing microbes to incorporate heavy isotopes into biomarkers such as phospholipids fatty acids (PLFAs), DNA, and ribosomal RNA (rRNA). In nature, most of carbon sources are ^{12}C-based. Therefore, the ^{13}C-labeled nucleic acids indicate the genomes or transcriptomes from microorganisms that actually mineralized and assimilated the stable-isotope substrate into their biomass. After extracting these biomaterials, it is possible to separate light and heavy fractions using ultracentrifugation, thus isolating the genomes (DNA), transcriptomes (RNA), and fatty acids of the microbial communities that have the target functions, i.e., mineralization of target carbon substrates in the environmental conditions.

To aid in explaining the principle of SIP, an example of SIP procedures for DNA/RNA isolation is given in Figure 2. First, environmental samples are co-incubated with a stable-isotope labeled compound, for example ^{13}C-biphenyl. Secondly, the ratio of ^{13}C-CO_2 in total CO_2 is monitored using GC-MS analysis, and the time point for sampling is determined based upon the measured ^{13}C incorporation ratio (i.e., the ratio of ^{13}C-CO_2 in total CO_2). Third, nucleic acids can be extracted from a variety of environmental samples using well-established extraction kits. Fourth, ^{13}C-labled nucleic acids (heavy portion) can be isolated from light portion of nucleic acids using ultracentrifugation and fractionation.

This approach was first demonstrated in PLFAs of active microorganisms from diverse community. PLFA-SIP methodology has been successfully applied in soils. However, uncultivated microorganisms cannot be identified because their PLFA composition is unknown.[18] Radajewski et al.[19] expanded this concept to nucleic acids, which are the most informative taxonomic biomarkers. SIP analysis of DNA (DNA-SIP), and recently also of rRNA (RNA-SIP), has shown very useful in linking the metabolic capabilities of microorganisms in natural environments to their taxonomy identity.[20]

4. DNA-SIP versus RNA-SIP

DNA (genomes) based SIP (DNA-SIP) is the incorporation of ^{13}C from a labeled substrate into DNA by the active populations of the microbial community. After selectively isolating the ^{13}C-labeled DNA from the total environmental DNA, these members of the ^{13}C-substarte utilizing communities are phylogenetically identified by performing PCR (polymerase chain reaction) of SSU (small subunit) rRNA genes from DNA templates (SSU rDNA), and cloning-sequencing analysis subsequently. By using tools for quantification (total nucleic acid, gene copy numbers) or PCR-based fingerprinting (T-RFLP, DGGE) or cloning and sequencing, structural SSU rRNA genes and functional genes can be targeted to identify microorganisms.[18] Based on the density difference, the 'heavy' isotopically labeled DNA and 'light' unlabeled DNA are separated by equilibrium density gradient centrifugation in a cesium chloride (CsCl) solution.

The principle of RNA (transcriptomes) based SIP (RNA-SIP)

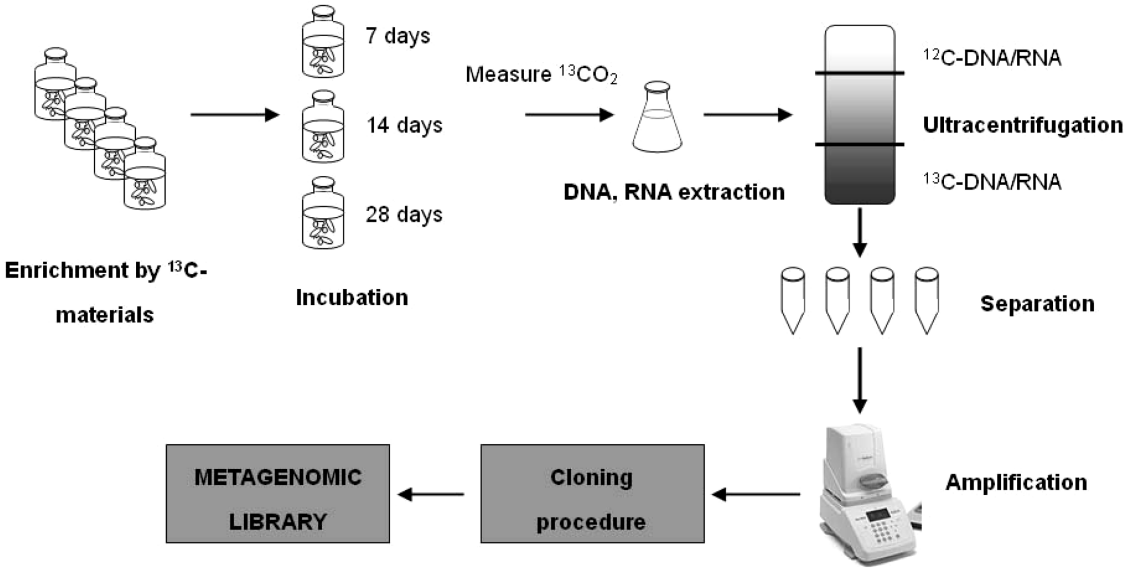

Fig. 2. An example procedure of stable-isotope probing method.

is similar to that of DNA-SIP. However, some technical aspects are different between RNA-SIP and DNA-SIP (Table 1). DNA-SIP is suitable for probing active populations from environmental samples but unsuitable for probing in situ community structure. Since a significant degree of cellular multiplication is required to detect ^{13}C-labeled DNA, the application of ^{13}C-substrate may cause to a shift in microbial community structure. Meanwhile, in response to a target substrate abundant rRNA copies can be expressed without letting active populations significantly grow. Because of this nature, RNA-SIP is advantageous in sensitively detecting active populations without shifting the in situ microbial community structure.

The easiness of nucleic acids is also an important factor influencing SIP performance. Environmental DNA is much more extractable and stable molecules than environmental RNA (Table 1). Because of this, when DNA is extracted from environmental samples, it is easy to gain both functional and structural DNA segments. Thus, normal DNA extraction methods including commercially-available simple kits allow us to easily get genomic DNA fragments from environmental samples. However, in the case of recovery of environmental RNA, functional RNA segments (messenger RNA [mRNA] produced from functional gene expressions) are not easily gained from environmental samples, because of their relatively-short half life-times i.e., couple minutes. Especially, it is known that mRNA recovery from soil samples is an extremely difficult molecular task in microbial ecology. Fortunately, recovery of structural RNA (rRNA) is more feasible because rRNA is abundant and relatively stable molecules. Thus, in literature, RNA-SIP generally means rRNA based SIP.

In SIP technique, isotope label sensitivity is affected by the rate of incorporation of isotope compounds into biomaterials, which is significantly related to the rate of synthesis of biomaterials in bacterial cells. In a bacterial cell, DNA is synthesized through DNA replication, which occurs together with cellular growth. Meanwhile, the synthesis of bacterial RNA occurs before cells are duplicated. This indicates that the synthesis of RNA is much faster than the synthesis of DNA in bacterial cells. Thus, the label sensitivity for RNA-SIP is greater than that for DNA-SIP (Table 1).

Isolating heavy nucleic acids is pivotal in successful SIP performance. For successful isolation, separation using equilibrium density gradient centrifugation and gradient evaluation are important steps. These steps are also somewhat different between DNA-SIP and RNA-SIP (Table 1). Separation-step in DNA-SIP can be processed in either CsCl or CsTFA (Cs-trifluouroacetate) gradient media. Only CsTFA gradient medium is preferred for RNA-SIP, because CsCl precipitates during centrifugation at the buoyant density required for rRNA banding.[21] After the significant growth of active populations on a ^{13}C-substrate, most of biomass in the active populations are incorporated with the isotope compound. This means that DNA-SIP generally results in almost complete labeling of the isotope compound into biomass (above 90%), which is relatively easy to separate from density gradient. In the case of RNA-SIP, partial labeling of an isotope compound into biomass occurs. This is probably because some active populations grow on not only indigenous carbon sources (^{12}C) but also the ^{13}C-labeled intermediates of the metabolism of the target substrate by other active populations. Ratios between ^{12}C-incorporation and ^{13}C-incorporation into biomass would significantly vary among active populations. The partial incorporation at relatively wide range of ^{12}C/^{13}C ratios causes to difficulties in separating the heavy portion of RNA. Because of these reasons, gradient evaluation for RNA-SIP is much stricter than that for DNA-SIP. For gradient evaluation in RNA-SIP, more labor-intensive molecular methods such as quantitative (real-time [RT]) PCR or DGGE (denaturing gradient gel electrophoresis) are required. Meanwhile, standard PCR is generally suitable for gradient evaluation for DNA-SIP.

In terms of detection resolution for 16S rRNA gene analysis, RNA-SIP has advantage against DNA-SIP because the abundance of RNA is much greater than that of DNA. In terms of accuracy in phylogenetic analysis, DNA-SIP provides a higher quality analysis than RNA-SIP (Table 1). For DNA-SIP, full-sized 16S rDNA sequences can be obtained. However, for rRNA-SIP, the size of reverse-transcribed PCR products is typically shorter than 800 bp (often shorter than 500 bp). Because of the short size in 16S rRNA sequences, rRNA-SIP cannot provide accurate phylogenetic analysis, i.e., it is impossible to provide species information. In addition, possible errors during the reverse-transcription of rRNA into rDNA may reduce the accuracy of sequence information in rRNA-SIP. Meanwhile, the natural amplification of RNA occurs in active cells while DNA amplification is done synthetically by PCR (Table 1). These facts imply that rRNA approach can probably reduce PCR bias artifacts, which could be a potential disadvantage of DNA-SIP.

5. Integration of Metagenomics with SIP

Table1. Comparison between DNA- and RNA-SIP

Comparison item	DNA-SIP	RNA-SIP	Ref.
Target microorganisms	Active populations	Active populations which have capacity to do work	24
Extraction from environmental samples	Easy	Difficult	25
Synthesis rate	Slow	Fast	26
Label sensitivity	Low	High	26
Centrifugation media	CsCl / CsTFA	CsTFA	21
Gradient evaluation	Easy (PCR)	Strict (RT-PCR, DGGE)	26
Phylogenetic resolution	High	Low	24
Amplification of signature molecule	PCR	Naturally occurring in active cells	20

Metagenomic analyses of environmental samples are able to reveal enormous genetic diversity of complex microbial communities present in environment. Technically speaking, it is possible to sequence all clones from environmental DNA or RNA. For example, Craig Venter[5] has been sequencing whole clones from marine metagenomic libraries, and had discovered novel genes and populations. However, this approach is known to be economically non-efficient. Most of laboratories cannot conduct that type of sequencing based metagenomics studies because of the extremely high cost. Instead of that, it is necessary to screen a large number of clones to find clusters of genes of interest. Unfortunately, this screening of metagenomic library is not that efficient either. The portion of microbes that possesses environmentally interesting functions is less than 1%. The percentage of environmentally-significant genes (biodegradative, antibiotic resistant, etc.) in a microbial genome is generally rare (0.1%). Considering these facts and assuming that the size of an average microbial genome is 40Mb, the screening efficiency to pick clones that contain environmentally interesting genes from a 40-kb cosmid cloning library is less than 0.1%, which is very rare.

Figure 3 indicates the effect of integration of SIP with metagenomics on metagenomic-library screening efficiency. The functional target genomes (i.e., biphenyl degrading microbes, antibiotic resistant microbes) can be isolated using SIP, which results in approximately 100 times higher concentration of the target populations' genomes than previously possible from total genomes in an environment. This could result in significant improvement of screening efficiency. The selectively isolated functional genome fragments can be inserted into metagenomic libraries. The clones have genomic information of active populations of interest. By parsing the sequences in the clone library, it is possible to gain novel and insightful information on environmentally important functions in ecological context. Furthermore, the clones can be expressed in standard organisms to exactly study the function, and then the clones also can be used as genetic resources for enhancing biodegradation of some persistent pollutants. Although sequencing analysis is still needed, integration of metagenomics with SIP will significantly reduce the amount and cost of sequencing.

To successfully integrate SIP with metagenomics, there are some technical challenges to overcome. For metagenomic library construction, it is necessary to extract high-molecular weight (HMW) DNA from environmental samples, which is very tedious procedure to perform. In addition, the amount of HMW DNA should be sufficient enough for SIP (at least a range of micro-grams), which is typically difficult to extract that much amount of HMW DNA from environmental samples, especially from soil and sediment samples. A progress in this step is needed in successfully integrating SIP with metagenomics. Secondly, it is important to estimate the coverage (size) of metagenomic clone library for capturing the whole environmental genomes. For this purpose, it is recommended to measure microbial population diversities in SIP-isolated environmental genomes or transcriptomes using 16S rDNA/RNA analyses, so that the number of present active populations can be estimated. Based upon the diversity of active populations, it will be possible to estimate the total size of microbial genomes in an environment. Based upon the total size of microbial genomes in the communities, the coverage of metagenomic clone library can be determined according to research goals. For example, at least 3X coverage of metagenomic clone library is recommended for gene pool screening in the SIP-selected active populations, and 8X coverage of metagenomic clone library is recommended for assembling cloned sequences into individual population genomes.

Because current sequencing technology has been improved, the cost for sequencing is rapidly decreasing. It would be feasible to fully sequence the SIP-based metagenomic library at a national laboratory level. However, at university or individual laboratory levels, the sequencing price for a metagenomic library may be still too expensive. In addition, if one is interested in utilizing functional metagenomic clones as genetic resources, fully sequencing of metagenomic libraries would be not necessary. For such circumstances, it is possible to "fish" out only the metagenomic clones that have a target gene or operon from the full scale of metagenomic library (Figure 3). An example method for this purpose is using magnetic bead probes to fish out target clones from library.[22]

6. Conclusion

In this study, we reviewed two cutting-edge functional genomic tools, metagenomics and SIP, respectively, and then we proposed a novel approach to combine metagenomics with SIP technique. Some of these concepts have been known to the lea-

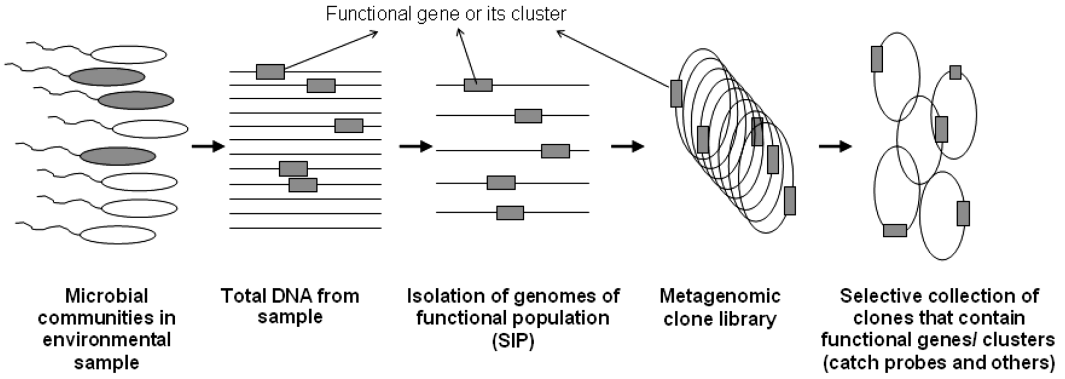

Fig. 3. Integration of metagenomics with SIP.

ding scientists of microbial ecology, especially who are members in International Symposium of Microbial Ecology. However, these concepts are still new to most of environmental engineering researchers. The outcomes from this review could provide convincing information that the functional genomics tools and its integrated technique are very powerful and useful in environmental studies. For example, wastewater treatment engineering researchers and U.S. Department of Energy Joint Genome Institute did great collaborative works of using metagenomics in studying biological phosphorus removal sludge communities.[23] The genomes of active populations of biological phosphorous removal can be selectively isolated from activated sludge communities by SIP. If the unique consortium genomes can be inserted into metagenomic library, the metagenomic clones could be very important resources for further wastewater treatment engineering. For making such SIP-metagenomics combined technology developed successfully, there are couple technical limitations to overcome. Possible ways to circumvent the limitations were also discussed in this review. Considered together, the integrated functional genomics tools have a huge potential in applying in many areas of environmental studies for biological treatment engineering, and are highly expected to provide more mechanistic and useful information on eco-physiology (systems biology) in bioprocesses in engineered and natural environmental systems.

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