Appl Microbiol Biotechnol (2016) 100:5313–5321
DOI 10.1007/s00253-016-7533-5

MINI-REVIEW

Microbial sequencing methods for monitoring of anaerobic treatment of antibiotics to optimize performance and prevent system failure

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Received: 26 February 2016 / Revised: 22 March 2016 / Accepted: 5 April 2016 / Published online: 3 May 2016

Abstract As a result of developments in molecular technologies and the use of sequencing technologies, the analyses of the anaerobic microbial community in biological treatment process has become increasingly prevalent. This review examines the ways in which microbial sequencing methods can be applied to achieve an extensive understanding of the phylogenetic and functional characteristics of microbial assemblages in anaerobic reactor if the substrate is contaminated by antibiotics which is one of the most important toxic compounds. It will discuss some of the advantages and disadvantages associated with microbial sequencing techniques that are more commonly employed and will assess how a combination of the existing methods may be applied to develop a more comprehensive understanding of microbial communities and improve the validity and depth of the results for the enhancement of the stability of anaerobic reactors.

Keywords Anaerobic treatment process · Anaerobic microbial community · Microbial sequencing techniques · Antibiotics

Introduction

The occurrence of antibiotics, one kind of important contaminants due to their potential for long-term adverse effects on the microorganism, has become a growing concern in the aquatic environment. Because the active compounds of antibiotics cannot be completely metabolized by humans and removed in conventional wastewater treatment plants (WWTPs), antibiotics can go into all parts of the aquatic and terrestrial environment via wastewater discharges and agricultural runoff. Even if the concentration of antibiotics is comparatively low in wastewater, the effluents originated from hospital and pharmaceutical industry have considerably high concentration of antibiotics ranging from 100 to 500 mg/L (Amin et al. 2006; Aydin et al. 2015a, b, c). Accumulation of antibiotics in the environment causes the dissemination and improvement of antibiotic-resistant bacteria (Aydin et al. 2015b). Thus, the native microbial community in the ecosystem is changed, and it threatens public health. Antibiotics also have an impact on the efficiency and performance of WWTPs, if they are in high levels in the wastewaters.

Anaerobic treatment is remarkable due to the production of biogas and biodegradation efficiency of anaerobic microorganisms to adjust to unstable environmental conditions. Since pharmaceutical industry wastewaters contain antibiotics and a high concentration of COD level, anaerobic biotechnology is obviously a sufficient alternative for biological treatment of water contaminated by antibiotics (Amin et al. 2006; Oktem et al. 2008; Cetecioglu et al. 2013; Resende et al. 2014; Aydin et al. 2015a, b, c, 2016b). Antibiotics have different effect compared to other pollutants present in the biological treatment process, which have a direct biological action on microbial community. Previous studies of anaerobic treatment process have also revealed that antibiotics under balance of diverse microbial population in WWTPs could be efficiently biodegraded, and failure to maintain the stability of these microorganisms resulted in a decrease in the performance and stability of the anaerobic reactor (Aydin et al. 2015c). Consequently, the quantitative detection of the microbial

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community in bioreactors is considerably significant to keep efficiency of reactors operation.

Anaerobic treatment involves four steps consisting of hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Anaerobic process and targeting of important genes are given in Fig. 1. Hydrolysis is the first rate-limiting step in anaerobic degradation of organic matter. The hydrolysis of macromolecules (lipids, proteins, and carbohydrates) under anaerobic conditions is converted by specific extracellular enzymes, the reaction rates of which are influenced by pH, cell residence time, and the waste constituents in the digester produced by hydrolytic bacteria, which is quite heterogenic (*Clostridium thermocellum*, *Clostridium bifermentas*, *Peptococcus* sp., *Clostridium butyricum*, *Bacillus subtilis*). Following this stage, soluble oligomers and monomers are taken up by facultative and obligatory anaerobic bacteria and are converted to VFAs (acetate, probionate, butyrate, lactate) and alcohols in acidogenesis, which is mainly formed by *Propionibacterium* sp. and *Clostridium* sp. Acidogenesis stage is followed by acetogenesis. Two different types of acetogenic mechanisms can be pronounced. First of them is acetogenic hydrogenation which involve the production of acetate as an end product, either from the fermentation of hexoses or from CO_2 and H_2. Second of them is the acetogenic dehydrogenation which refers to the anaerobic oxidation of long and short chain volatile fatty acids (Stams et al. 2012). Methanogenesis is the final and may be the most important step of the anaerobic digestion process, which is a special group of Archaea called Methanogens (Aydin et al. 2015b). This population is strictly anaerobic and considered the rate-limiting step in the whole anaerobic digestion process due to the slow growth rate of the methanogens comparing to acidogens and accordingly, the performance of anaerobic digesters and the quality of the reactors depend on the activity of methanogens (Ma et al. 2013; Aydin et al. 2015b). Methanogens are categorized in two main groups according to their substrate conversion capabilities; acetate utilizers (Acetotrophic methanogens) and hydrogen utilizers (Hydrogenotrophic methanogens). The percentage of these two groups in anaerobic reactors can change due to operational parameters (temperature, pH, type of the substrate, alkalinity, HRT, SRT, reactor configuration) and instabilities or differences in these conditions such as the occurrence of antibiotics (Ma et al. 2013). Nevertheless, the various microbial species in a complex microbial community and their distribution in the anaerobic process are not understood enough in terms of the treatment of wastewater that includes antibiotics. Therefore, comprehensive research into this subject is required based on DNA, RNA, and functional genes to understand the microbial community dynamics and enhanced the biodegradation capacity of antibiotics (Abbassi-Guendouz et al. 2013; Aydin et al. 2015a, b, c).

Culture-based methods are commonly employed as a means of investigating the microbial ecology of both natural, untouched environments and those that have been anthropogenically altered by human activity. However, gaining in-depth insights into microorganisms can be extremely challenging because many of the groups of interest to scientists cannot be cultured in a laboratory setting and have only been detected through the use of culture-independent approaches. Previous attempts to cultivate environmental communities have only successfully developed less than 1 % of the total prokaryotic species present in the sample (Wittwer et al. 1997;

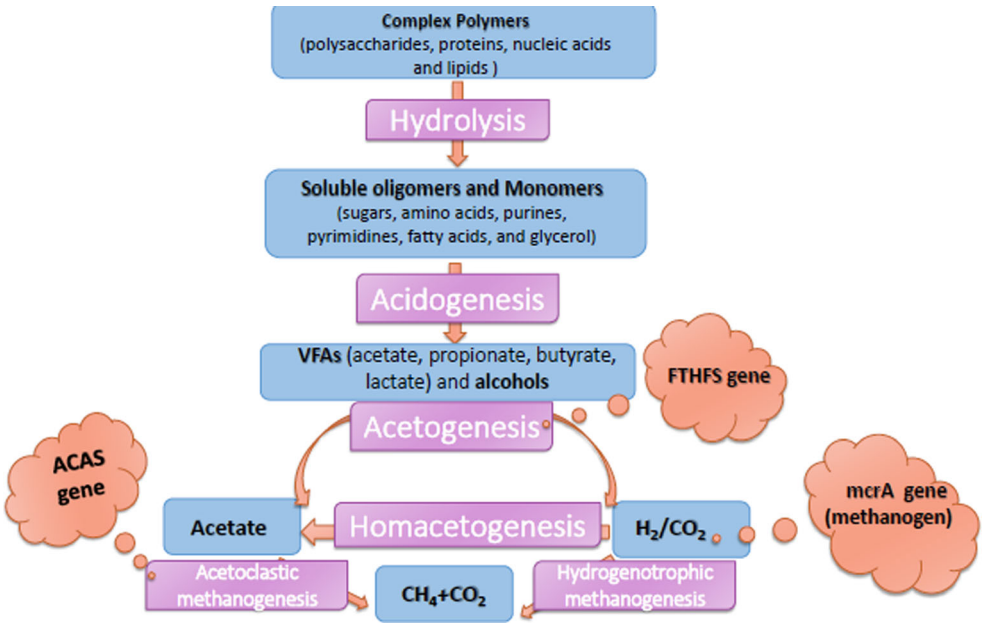

Fig. 1 Anaerobic process and targeting metabolic genes encoding formyltetrahydrofolate synthetase (FTHFS), methylcoenzyme M reductase (mcrA), and acetyl-coA synthetase (ACAS) (Aydin et al. 2015c)

Yu et al. 2006; Nagarajan and Loh 2014). Microorganisms living in anaerobic environment are also hard to grow because of low growth rates, syntrophic interactions. Molecular methods that involve the isolation and assessment of DNA, RNA, proteins, metabolites, and stable/radioactive isotopes from environmental samples have been successfully deployed, and these can provide valuable insights into the structure and functional behavior of microbial communities as seen in Fig. 2 (Abbasi-Guendouz et al. 2013; Nagarajan and Loh 2014; Vanwonterghem et al. 2014).

Environmental microbiology as a field of study has undergone significant developments over the past 10 years as a result of applications of microbial sequencing technologies. It is now much easier to observe the structural and functional microbial community dynamics in anaerobic bioreactor. The main objective of this review is to examine the applications of next-generation sequencing and metagenomics techniques that are available in order to identify the advantages and disadvantages associated with its use and how anaerobic bioreactors can be affected by substrate which is contaminated by toxic compounds like antibiotics.

Current molecular approaches to assess anaerobic microbial community

Next-generation sequencing

Microbial sequencing, a new tool in the field of molecular biology, has great potential for the development of environmental analysis. The first automated sequencing process, developed from Sanger sequencing, produces 550–900 bp read lengths but its sequencing capacity is just 96 reads per run; the process can also be costly and fraught with errors (Cardenas and Tiedje 2008). However, sequencing has become more financially manageable with the development of next-generation technologies, which means that many smaller organizations and research groups have more access to these extremely powerful sequencing tools (Cardenas and Tiedje 2008; Shendure and Ji 2008). The first marketed deep sequencing tool made available to researchers was introduced by 454 Life Sciences (Branford, CT). Other systems also became available including the Solexa system (Illumina, San Diego, CA), the SOLID system (Applied Biosystems,

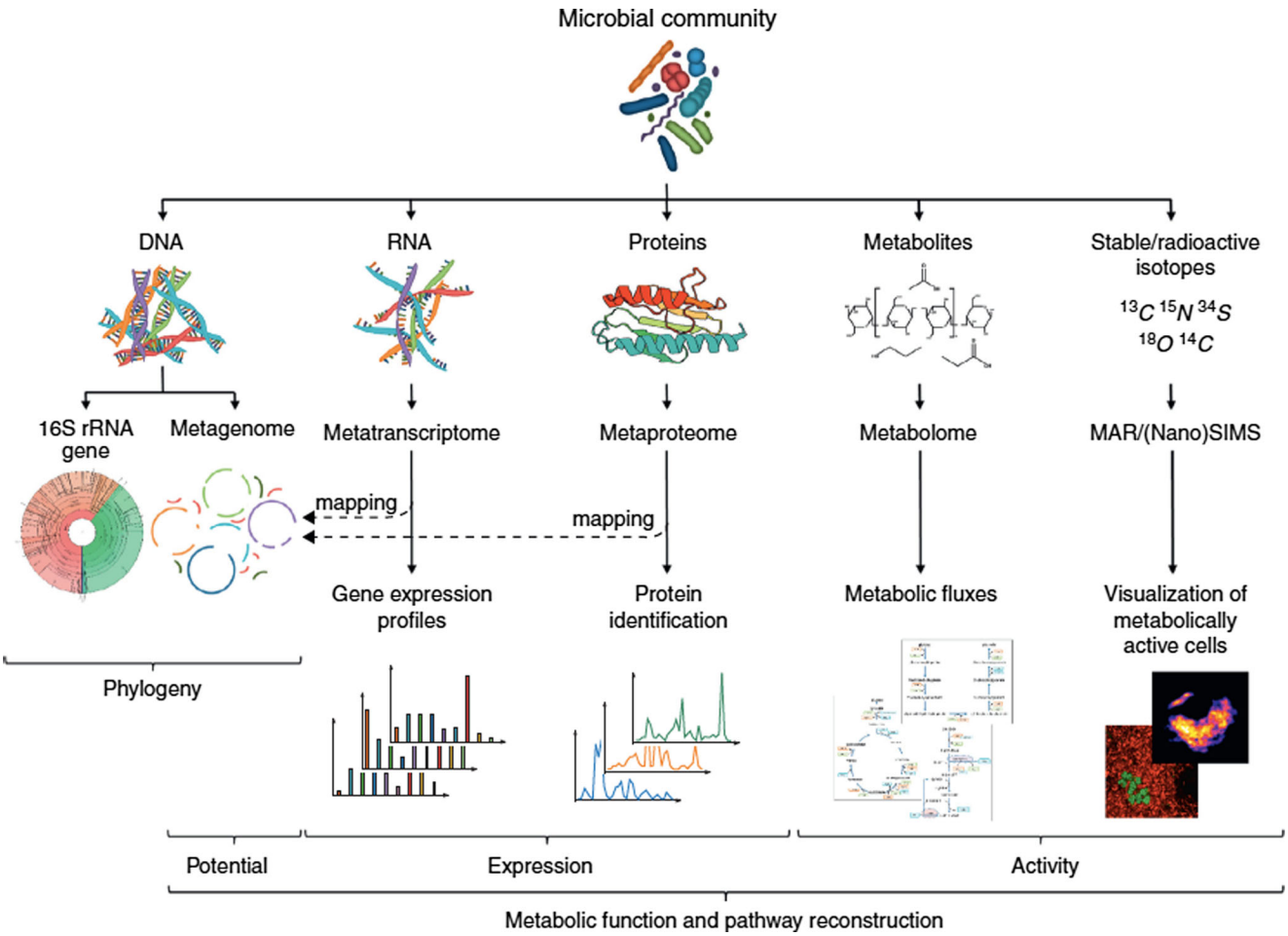

Fig. 2 A combination of molecular methods from environmental samples (Vanwonterghem et al. 2014)

Carlsbad, CA), the HeliScope system (Helicos Biosciences, Cambridge, MA), and the Polonator system (Dover Systems, Salem, NH). All of these systems have high data output and various length capacities (Rothberg and Leamon 2008; Shendure and Ji 2008). Next-generation sequencing technologies have made it possible to characterize molecular diversity among microbial communities, as well as achieve entire functional analyses of bacterial and archaeal communities and perform various genomic analyses (Cardenas and Tiedje 2008; Metzker 2010; Prest et al. 2014).

Pyrosequencing is an innovative next-generation sequencing system with a promising position in environmental samples with huge genetic diversity. The method makes use of specific enzymes associated with light reactions to record each nucleotide inserted into a complementary DNA strand; this is based on a "sequencing-by-synthesis" process that enables the parallel sequencing of millions of DNA strands (Rothberg and Leamon 2008). However, more research is needed to improve certain problems with the pyrosequencing process. For example, strings of following identical bases, or homopolymers, may have high error rates due to reliance on light signals (Shendure and Ji 2008). The average cost-per-sequence is lower than other next-generation methods, but the maintenance and running cost of the sequencer platform can be high (Hamady and Knight 2009). Additionally, higher maintenance and running costs can be inhibiting for single labs and smaller organizations (Sanapareddy et al. 2009; Lautenschlager et al. 2014). An anaerobic membrane bioreactor bioaugmentated with costal sediment (B-AnMBR) was worked out by Ng et al. (2015) in order to treat pharmaceutical wastewater containing penicillin. They also worked out a control anaerobic membrane bioreactor (C-AnMBR) in addition to bioaugmented anaerobic membrane bioreactor (B-AnMBR). In this study, microbial communities in the anaerobic membrane bioreactors were identified using a 454 high-throughput pyrosequencing method (based on a partial 16S rRNA gene fragment) at steady-state conditions. Furthermore, 465 bacterial 16S rRNA gene sequences were characterized from the C-AnMBR and 398 bacterial 16S rRNA gene sequences were characterized from the B-AnMBR. Methanogenic archaea which are resistant to penicillin were found in B-AnMBR as results of the study. The importance of antibiotics in shaping the bacterial community was also researched by Meng et al. (2015). An anoxic-aerobic membrane bioreactor was operated to comprehend the effect of fluoroquinolones, which is one class of representative antibiotics in wastewater. The microbial community in the reactors was analyzed by 16S rRNA-targeted high-throughput pyrosequencing. It was showed that an obvious degradation in the microbial species abundance when fluoroquinolones were added and important fluctuations of bacterial community dynamics at phylum and genus levels as a consequence of 16S rRNA gene-targeted 454-pyrosequencing. Additionally, Ng et al. (2016) studied with anaerobic membrane bioreactor (AnMBR) and an anaerobic bio-entrapped membrane reactor (AnBEMR) in order to treat pharmaceutical wastewater including high total chemical oxygen demand (TCOD) and salinity. Then, 454 high-throughput pyrosequencing methods were used to analyze the microbial populations and communities. Throughout DNA pyrosequencing analysis, both the anaerobic membrane bioreactors presented similar main species of bacteria and archaea. Nevertheless, *Elusimicrobia* was only found in the anaerobic bio-entrapped membrane reactor; the higher abundance of *Methanimicrococcus* detected in the AnBEMR could play a critical role in biodegradation of the major organic pollutant (i.e., trimethylamine) existing in the pharmaceutical wastewater.

While many different approaches can be used to generate next-generation sequencing, in recent times, the Illumina sequencing technology has arisen as the most successful and widely adopted next-generation sequencing technology worldwide (Metzker 2010; Hayes et al. 2013). The Illumina Genome Analyzer utilizes a proven sequencing by synthesis (SBS) approach that, as opposed to using beads, involves randomly attaching fragments to the flow cell and constructing clusters via a process of build-by-bridge amplification (Bentley et al. 2008). Using this approach, during each respective cycle of the sequencing process, each of the four nucleotides is labeled with an allocated dye and is then simultaneously bound to the flow cell as seen in Fig. 3. Each nucleotide incorporates a chemically blocked 3′-OH group, meaning that only one nucleotide is incorporated per sequencing cycle; the unbound nucleotides are washed away so that the incorporated nucleotides can be identified in an imaging step and the next round of sequencing can commence (Fig. 3a). Figure 3b provides an overview of two sequencing cycles, while Fig. 2c demonstrates the use of a sequence of images to verify the nucleotide sequence of each cluster (Metzker 2010). When a paired-end approach is applied to sequencing activities, both ends of a fragment are sequenced. This process commences by sequencing the first read. The template strand is then employed to develop a bridge that can be used to re-synthesize the second strand. The output of the synthesis of the second strand is, in turn, used as a template strand for the second read of the read pair.

Illumina have been used for identifying the entire microbial communities in the environment research. For example, Yang et al. (2014) worked out with Illumina sequencing technology in order to research the wide spectrum profiles and fate of antibiotic resistance genes (ARGs) in a full-scale sewage treatment plant. Sequencing by synthesis technology was carried out using Illumian Hiseq 2000 instrument. As a result of the study, 271 ARGs subtypes having 18 ARGs types were found in influent, effluent, anaerobic digestion sludge, and activated sludge by Illumina analysis. It was also investigated that there is an important relation between 6 genera and their antibiotic

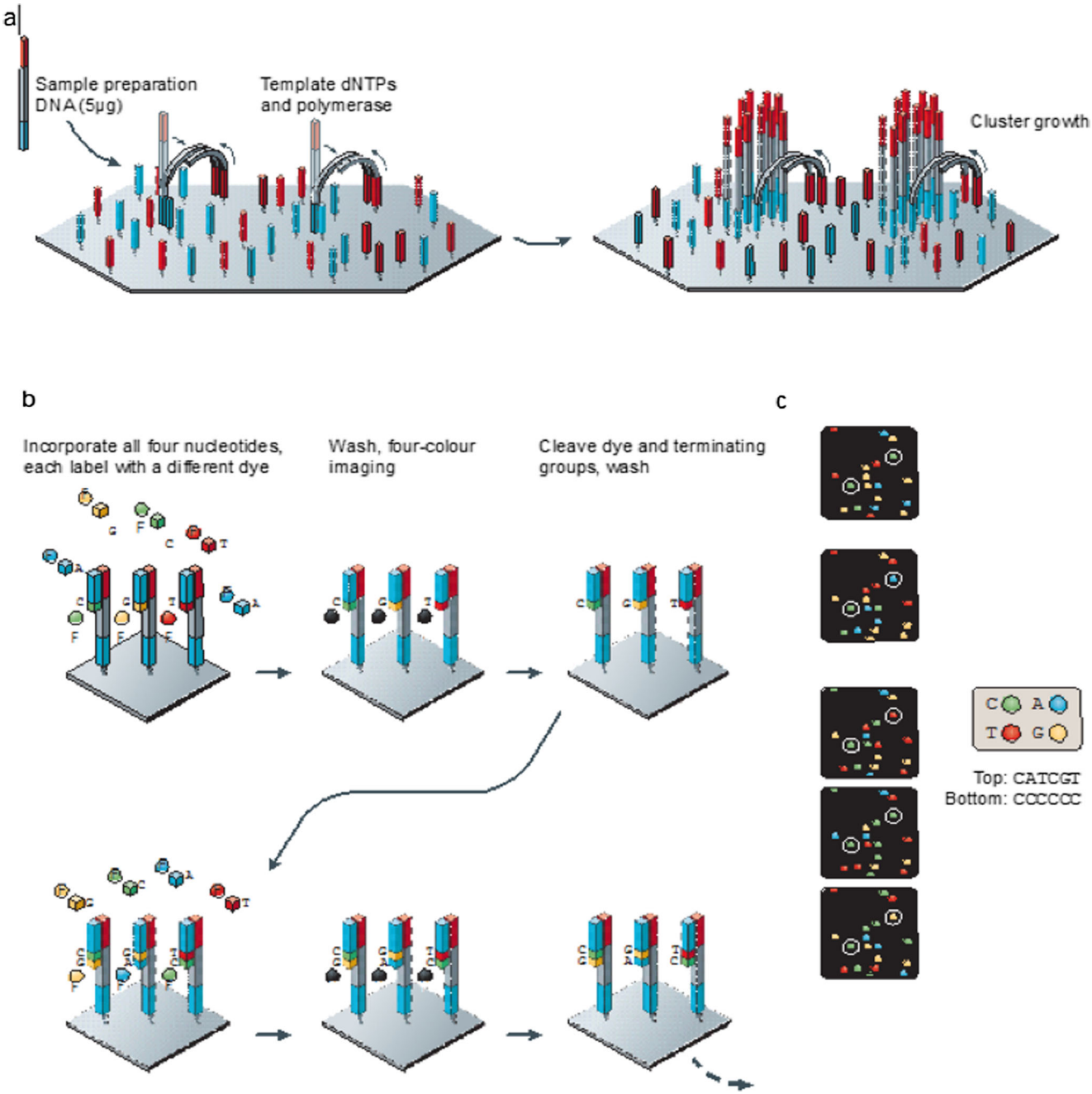

Fig. 3 Illumina sequencing. **a** Bridge amplification; **b** determine first base; **c** sequence read over multiple chemistry cycles (Metzker 2010)

resistance genes. Moreover, removal efficiency of antibiotic resistance genes in both thermophilic and mesophilic anaerobic digestion was studied using bench-scale reactors by Zhang et al. (2015). Illumina sequencing instruments and reagents were applied to determine removal potential through anaerobic treatment process. Illumina Hiseq 2000 sequencing system was used to conduct high-throughput sequencing method. Illumina helped to find out the efficiency for anaerobic digestion to decrease a wide array of ARGs in the wastewater contaminated with antibiotics. According to Illumina sequencing results, it was found that 12 ARG types are detected, and there is no conspicuous decrease in total ARG abundance in thermophilic anaerobic digestion or mesophilic anaerobic digestion. These studies consistently indicate that HiSeq 2000 makes it possible to take on the largest and most complex sequencing studies at the lowest cost.

The Ion Torrent is an innovative sequencing system that operates according to a similar concept as pyrosequencing; however, it uses proprietary ion sensor technology to conduct sequencing activities via the establishment of direct connections between chemical and technical information as opposed to utilizing an approach that involves the indirect detection of

the pyrophosphate released by light and thus the positively charged hydrogen ion that is released along with it (Parson et al. 2013). The Ion Torrent has undergone several different release cycles and has been upgraded a number of times. One of the more notable developments with this technology involved the release of disposable sequencing "chips." The current version of the Ion Torrent offers the functionality to achieve a read length of 200 bp at a fraction of the cost per base associated with a pyrosequencing approach; however, it is widely acknowledged that the results are less accurate with the former method (Camarinha-Silva et al. 2014). Although there is not enough study about application of Ion Torrent techniques on anaerobic antibiotic treatment of antibiotics, Aydin et al. (2016a) used Ion Torrent sequencing in their research in order to investigate microbial community dynamics relating to additional antibiotics. Two anaerobic sequencing batch reactors (SBRs) feeding with wastewater containing pharmaceutical antibiotics were performed in this study so as to evaluate the relation between bacterial diversity and different antibiotic combinations and concentrations. In addition to Ion Torrent, qPCR and UV-HPLC were used to analyze microbial community changes. As a result, it was showed that the performance of biodegradation, treatment of antibiotic combinations, and existence of antibiotic resistance genes are changed due to alteration of the microbial community structure.

Metagenomics

Metagenomics excludes any approaches that reply solely on the interrogation of PCR-amplified selected genes, as these methods are limited to developing an understanding of the genetic diversity of the genes under investigation (Schloss and Handelsman 2003). The basic underlying principle of metagenomics is that it allows for the sequencing and analysis of the complete genetic composition of environmental microbial communities. The use of small-insert DNA libraries allows researchers to investigate the phenotypes encoded by single genes. It also makes it simpler to reconstruct metagenomes for the purposes of genotypic analysis. However, large-fragment metagenomic libraries (100–200 kb) are of more use in studies that involve the investigation of multigene biochemical pathways. There are two methods of screening metagenomic libraries: sequence-driven metagenomic analysis, which contains massive high-throughput sequencing, or functional screening of expressed phenotypes. There are several advantages associated with sequence-driven analysis. Massive whole-genome metagenomic sequencing gains detailed insights into a range of different genomic aspects, including the presence of redundant functions in a community, genomic organizations, and the traits distinctly related to taxa acquired via horizontal gene transfers (Taupp et al. 2011).

16S ribosomal RNA (rRNA) genes encode small subunit ribosomal RNAs are highly conserved in bacterial organisms, but it has many various genetic regions. This variety of regions, which are the fingerprints, can be used to identify an organism and help to establish its evolutionary relationship to other microbes when compared to a 16S rRNA database. Because of this, 16S rRNA sequencing is a common amplicon sequencing approach used to identify and compare microorganism present in samples. It is also a well-established technique for examining phylogenetic and taxonomic classification from complex microbial communities that are difficult or impossible to study. The 16S method only sequences amplified ribosomal DNA, but metagenomic sequencing or "whole-genome shotgun sequencing" sequences all DNA extracted from a community. The sequencing output is a huge collection of random DNA fragments from the community. This method can analyze multiple arrays of genes, instead of only one, and collect information about functional identifiers, such as genes and metabolic pathways, as well as provide a more comprehensive view of the microbial ecosystem (Tringe and Hugenholtz 2008; Simon et al. 2009). If enough coverage is achieved, the assembly of whole genomes from metagenomic sequence data is possible. But some achievements have only been demonstrated in low-diversity environments; computing and sequencing demands are currently too great for extremely complex environments. The increase of read length capabilities of pyrosequencing will continue to improve the accuracy of sequence classification for metagenomic studies (Simon et al. 2009; Cardenas and Tiedje 2008).

Christgen et al. (2015) studied with metagenomic approaches to investigate the fate of antibiotic-resistant genes in anaerobic, aerobic, and anaerobic–aerobic sequence bioreactors treating domestic wastewater. Shotgun metagenomic analyses were used to detect the variety and relative abundance of antibiotic-resistant genes in the bioreactors. Metagenomic data revealed that anaerobic–aerobic sequence treatment bioreactors are generally more effective than the others, and they can ensure effective treatment performance and reduction of ARG. Overall, this research highlighted the beneficial effects of AAS reactors, which can reduce more ARGs for 32 % less energy.

Advantages and drawbacks of recent molecular analyses for evaluating the effect of antibiotic on anaerobic microbial community

Polymerase chain reaction (PCR) has allowed researchers to better comprehend the phylogenetic and functional characteristics of microorganisms. Techniques that operate by extracting nucleic acids rely on the enzymatic amplification of certain genes from the complex genomic DNA or cDNA of samples (Aydin et al. 2015b, c). Although PCR is so effective method in molecular biology, there are some limitations due to DNA polymerase, which cannot be totally reliable for

transcribing DNA. Roughly 0.02–0.3 % incorrect nucleotides add throughout amplification. Because the contamination of humic acids, phenolic compounds, or chelating agents decreases efficiency and fidelity of Taq polymerase, the DNA purification methods were developed. The error rate is raised by decrease of nucleotides owing to proceeded features of Taq polymerase. Another drawback that arises when objective primers are shared in other DNAs is the production of recombinant products. The power of PCR to amplify DNA fragments also leads to limitation. Thus, advanced sterilization and care are required to perform PCR. In addition, contamination originated from foreign DNAs can be prohibited by a negative control without a DNA template or DNAse treatment of reagents (Kim et al. 2013; Aydin et al. 2015c).

The understanding of molecular approaches and comprehension of the way in which they interact with the bioreactor has also been aided by techniques such as genetic fingerprinting. For instances, comparing gene sequences can give scientists valuable insights into the nature of existing microorganisms. Prime examples of this are fingerprinting tools like denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment polymorphism analysis (T-RFLP). Both of these techniques allow scientists to observe both the general composition of the microbial communities and the way in which they change over time (Rastogi and Sani 2011). Some studies required the long-term storage of samples. Although band excising is an effective feature of DGGE, there are some drawbacks because of band consisting of 150–200 bp DNA that is shorter for a phylogenetic analysis. As another problem, poor identification generating from co-migration of bands can be solved by clone library screening (Aydin et al. 2015a). It can be said that clone library includes both techniques' strong aspects. Extent of two bands is the other problem for excision. If distance of bands is too close, suitable excision cannot be occurred. Additionally, DNA can be affected by UV in excision step, and ambiguous sequences may be formed in re-amplification. Because DGGE and T-RFLP are a PCR-dependent method, it has also biases like binding error of polymerase or chimeric products. Because of these reasons, DGGE has been considered as a semiquantitative method. The selection of the primers and determination of the gel conditions of running are the fundamental limitations and difficulties for comparison patterns in the gel if samples have countless bands.

It is also possible to quantify different nucleic acid sequences by employing a real-time quantitative polymerase chain reaction (qPCR) analysis method, which works by investigating the concentration of specific nucleic acid sequences as they are enzymatically amplified in vitro (Aydin et al. 2015b, c). However, qPCR-based approaches have a common limitation of the requirement for sequence data of the specific target gene of interest (Smith and Osborn 2009). Fluorescence in situ hybridization (FISH) is a benefical approach that can be used to count and detect live cells as seen in Fig. 4. On the other hand, the bias of FISH analysis is the essential for genes or RNA with a high-copy number and is not appropriate for detection of whole microbial diversity. These analyses have also a common limitation of being low-throughput approaches, largely because of difficulties in optimizing the reaction conditions, mainly for the hybridization and washing phases (Kim et al. 2013).

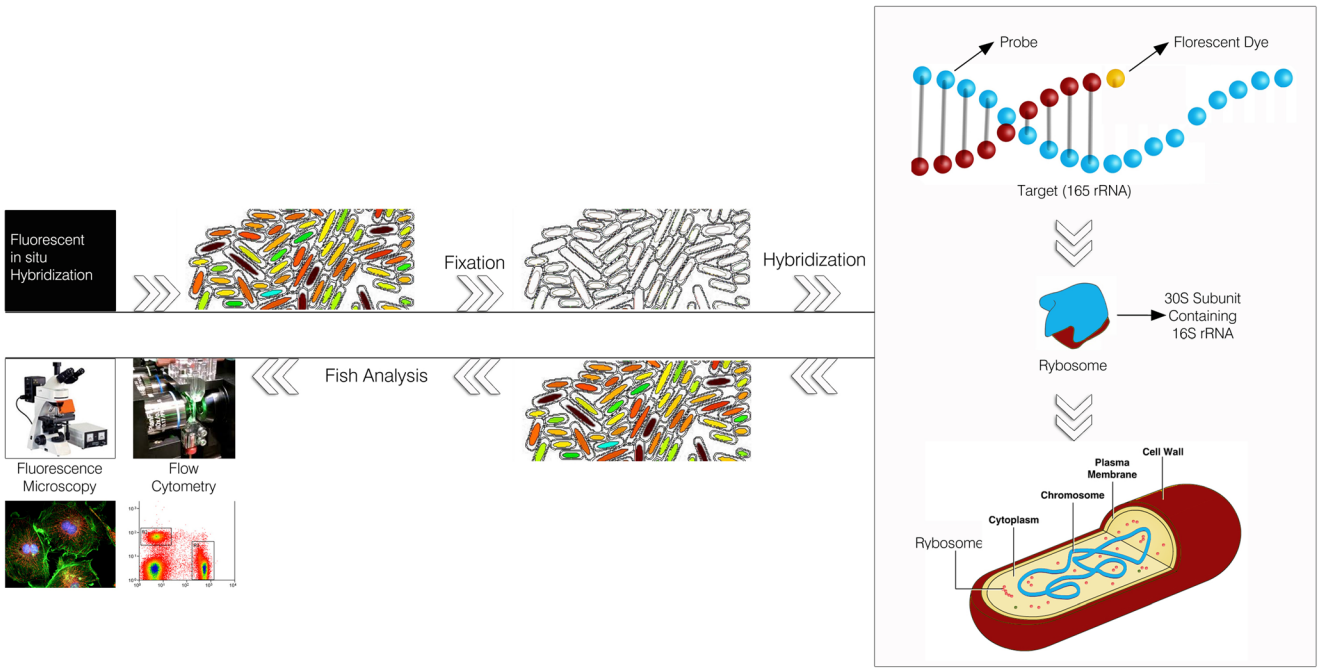

Fig. 4 The basic steps of the FISH procedure

At present, the various microbial sequencing techniques that are available for the purposes of community structure and function testing have their own inherent advantages and disadvantages and none of them are currently able to offer a comprehensive understanding of the genetic and functional diversity of complex microbial communities through anaerobic digestion process. While significant advancements have been made with regards to the characterization of microbial communities in anaerobic reactor, there is still a general lack of understanding of the functional behavior of uncultured organisms, and improving knowledge in this area remains an intimidating task because the majority of genes that have been identified thus far have no homologous representatives in the existing databases. The applications of new microbial sequencing techniques, such as Illumina sequencing, have improved understanding; however, a number of significant technical challenges remain. In order to improve the effectiveness and depth of study results, scientists should apply a combination of several techniques in their quest to understand the diversity, function, and ecology of microbial assemblages. There is a specific need for further development of the bioinformatics methods that are available so that the significant amount of data that is generated through the whole-genome analysis and metagenomic and metatranscriptomics tools can be better understood and applied. Producing an adequate quantitative understanding of microbial communities represents one of the most challenging hurdles because there are significant biases related to the nucleic acid isolation, PCR, and the more sophisticated DNA/RNA extraction methods. However, the approaches that are available are very much in a process of development, and further technical advancements are expected in the near future.

Conclusions

These studies consistently indicate that there is an association between microbial community dynamics and stability of anaerobic systems. Molecular monitoring of anaerobic process is essential to optimize reactors performance and prevent possible system failures. Through the use of microbial sequencing methods to achieve high sequencing depth, it is possible to control the changes in the structure of a microbial community with a more in-depth understanding that successfully facilitates biogas production. Changes in the structure of a microbial community lead to changes in degradation efficiency of antibiotics and occurrence of antibiotic resistance genes. The increased biodegradation efficiency seemed to generate changes in the structure of the microbial population. However, next-generation analyses still remain at a high cost compare to fingerprinting approach. Applications of microbial sequencing technologies have become much more affordable in the last few years and will likely become more so in the future.

Acknowledgments The authors thank Republic of Turkey Ministry of Science, Industry and Technology (Project No: 0328.TGSD.2015) for their support of this research.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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