Microbial trophic interactions and mcrA gene expression in monitoring of anaerobic digesters

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INTRODUCTION

Anaerobic digestion (AD) is a series of unique processes that involve the reduction and oxidation of organic molecules by the complex metabolic interactions between several microbial groups. AD is widely used worldwide for the treatment of organic wastes, such as animal manures, municipal, and industrial wastewaters, and solid organic wastes such as sludge, crop, and food wastes. Carbon present in the biomass is recovered in the form of methane; a renewable source of energy and the effluent can be used as organic fertilizer as it is rich in nutrients (Cho et al., 2013).

For years, several studies have focused on optimization of the design of biodigesters (Ince, 1998; Raynal et al., 1998; Bouallagui et al., 2005; Martí-Herrero, 2011), or treatment conditions (Kim et al., 2002, 2003) and the characterization and preparation of adequate waste mixtures to obtain proper C:N ratio (Hills, 1979; Yen and Brune, 2007). However, the performance and efficiency of AD depends greatly on the interactions between different active microbial groups (Talbot et al., 2008; Ali Shah et al., 2014). Therefore, characterization of the microbial community structure and the comprehension of the metabolic networks are critical to improve digestion efficiency (Shin et al., 2010). Several molecular biological analytical tools, including polymerase chain reaction (PCR) and its many variants, denaturing gradient gel electrophoresis (DGGE), fluorescence in situ hybridization (FISH), restriction fragment length polymorphism (RFLP) among others, have been applied in the study of the microbial communities in AD (Montero et al., 2009; Shin et al., 2010; Nelson et al., 2011; Supaphol et al., 2011).

Usually AD is conceptually divided into three or four stages, hydrolysis and/or fermentation, acetogenesis and methanogenesis. During the first stage insoluble particles of cellulose or hemicellulose contained in the substrates are hydrolyzed and converted into simple and soluble products, which are catabolized by fermentative bacteria into alcohol and fatty acids. Subsequent steps involve the oxidation of such alcohols and fatty acids with carbon chain longer than C2 to acetate by syntrophic bacteria and their activity depends on the removal of hydrogen either by CO2 or sulfate reduction. Finally, during methanogenesis, acetate and other methyl-containing Cl compounds are reduced to methane by acetoclastic and methylotrophic methanogens and CO2 is reduced by H2-oxidizing methanogens. Methanogens belong to the domain Archaea and are characterized by their substrate specificity, slow growth rate, and susceptibility to environmental changes, but their growth and activity is vital for the efficient functioning of AD process (Balagurusamy and Ramasamy, 1999).

Methanogenesis requires reduction of the methyl group of methyl coenzyme M to CH4 by the enzyme methyl coenzyme M reductase (MCR), involving a nickel-containing factor F430 (Pramanik and Kim, 2013). All known genomes of the methanogenic archaea encode at least one copy of the mcrBDCGA operon, which is composed of two alpha (mcrA), beta (mcrB), and gamma (mcrG) subunits (Luo et al., 2002). Moreover, all known methanogens...
express MCR, which catalyzes the last step in the methanogenesis (Ferry, 1999). Therefore, the presence of this enzyme is a reliable diagnostic indicator of methanogenesis in diverse environments (Reeve et al., 1997; Luton et al., 2002; Steinberg and Regan, 2009; Palacio-Molina et al., 2013). Currently various groups are involved in developing strategies to combine the analysis of differential gene expression of mcr alpha subunit and the traditional approaches to monitor the performance of biodigesters on real time basis. The present paper discusses the currently available knowledge on this new strategy for management of AD process.

**ANAEROBIC DIGESTION: THE PLAYERS**

Although many of the microorganisms involved in the process are still to be identified or cultured, at least 11 groups have been reported to interact with each other in a series of specific reactions in anaerobic ecosystems (Figure 1).

Hydrolytic bacteria are extremely diverse in anaerobic biodigesters, reflecting their enormous metabolic flexibility. One of the most important polysaccharides in biodigesters is cellulose, the main substrate of anaerobic cellulolytic bacteria. Cellulolytic anaerobes possess cellulosome, a multienzymatic complex, which degrades cellulose by binding to the substrate. In general, hydrolysis of polysaccharides is a slow process under anaerobic conditions. The rate and efficiency of cellulose hydrolysis in a biodigester is intrinsically related with the abundance of particulate-bound hydrolytic bacteria. In fact, the performance of a bioreactor may vary depending on the hydrolytic species that form the microbial community (Ren et al., 2007).

In one study, the microbial community was examined by DGGE and dot-blot hybridization during the start-up of two acidicphilic systems at mesophilic (35°C) and thermophilic (55°C) conditions (Liu et al., 2002b). The reactors were fed dairy wastewater and inoculated with granular sludge. In both systems, mesophilic and thermophilic Bacteria predominated during hydrolysis, specifically the phylum Firmicutes. Actually, it is well known that in cellulosolytic environments, Clostridium predominates (Balagurusamy, 2007). In addition, Actisetivibrio, Bacteroides, Selenomonas, and Ruminococcus are some of the most common hydrolytic bacteria in the anaerobic bioreactors (Balagurusamy and Ramasamy, 1999).

In the rumen, the most similar natural environment to biodigesters, *Ruminococcus albus* and *R. flavefaciens* are the predominant gram-positive, fiber-degrading bacteria, while *Fibrobacter succinogenes* is the most abundant Gram-negative (Wanapat and Cherdhong, 2009). Commonly, hydrolytic bacteria adhere to the substrate particles along with some anaerobic fungi, which are also present in biodigesters. However, the growth of anaerobic hydrolytic fungi is slower than that of bacteria, which explains their limited presence in the community structure studies. *Neocallimastix*, is one of the most studied anaerobic fungi in rumen, which also include *Orpinomyces*, *Anaeromyces*, *Piromyces*, and *Caeecomyces* (Lynd et al., 2002; Gallet and Winter, 2005). As hydrolysis of complex compounds is catalyzed by a defined group of specific enzymes such as cellulases, proteases and lipases, this step is known as one of the most catalytically active and one that could greatly benefit from new monitoring strategies involving the analysis of gene expression profiles of key enzymes (Amani et al., 2010).

In general, hydrolysis of recalcitrant materials, such as lignin, cellulose, or hemiceluloses is a relatively slow process, and hence is often a limiting-step, normally overcome in thermophilic treatments, especially when solids contents are kept below 7%. In comparison, hydrolysis of proteins and lipids is faster (Ortega-Charleston, 2008). Proteins are generally hydrolyzed to amino acids by proteases. Microorganisms that are responsible of this reaction include species of the genera *Bacteroides*, *Butyrivibrio*, *Clostridium*, *Fusobacterium*, *Selenomonas*, and *Streptococcus* (Amani et al., 2010).

Monomeric compounds generated after hydrolysis are taken by fermentative bacteria and transformed to alcohols, volatile fatty acids (VFAs), CO₂, or H₂. In biodigesters, alcohols and VFAs are further transformed into the substrates for methanogenesis, namely, acetate, formate, H₂, and CO₂. In the presence of electron acceptors such as sulfate and nitrate, the intermediates for methanogenesis are diverted to anaerobic respiration.

Representatives of domain Bacteria are largely responsible for fermentation reactions. Among the fermentative microorganisms in the rumen are several species of *Clostridium* and *R. albus*. Meanwhile in the biodigesters fed with cow manure, members of *Clostridium*, *Eubacterium*, and *Bacteroides* are the abundant ones (Sivakumaran et al., 1991; Delbes et al., 2000). *Streptococcus* sp., *Lactobacillus* sp. and *Propionibacterium* are also fermentative microorganisms commonly found in the biodigesters, producing lactate or lactate and ethanol plus CO₂ and H₂ (Insam et al., 2010). From the phylogenetic point of view, acidogenic bacteria are widely diverse. Most of the microorganisms of this group found in biodigesters include members of the genera *Clostridium*, *Eubacterium*, and *Ruminococcus* (Drake et al., 2013).

The products generated by fermentation are typically VFAs, which decrease the pH and are the most common cause of failure in anaerobic systems (Chen et al., 2008; Wang et al., 2009). This acidification is a consequence of the imbalance between fermentative and syntrophic bacteria, especially obligatory hydrogen aceticogenic bacteria. Although the simple monitoring of the profile of VFAs can help to prevent failures, differences between microbial populations are already very large when a decrease in the pH has been perceived (Amani et al., 2010). Hence, knowledge on the activities of microbial communities might help to anticipate this common failure even before it arises. This is probably the most important reason that microbial diversity alone is not helpful in the monitoring of anaerobic biodigesters.

Further, inhibition thresholds of VFAs vary greatly, as they depend on multiple factors, such as temperature, characteristics of feeding, source of inoculum, type of system, organic load, their state of ionization, among many others (Chen et al., 2008). But in general, acetic acid requires higher concentrations; about 2.4 g L⁻¹, than the other acids to be inhibitory, and it is also a major substrate for methane production. In contrast, concentrations of propionic acid below of 900 mg L⁻¹ are a sign of good performance (Wang et al., 2009).

During AD syntrophic aceticogenic bacteria oxidize VFAs greater than C3 into hydrogen, acetate, and CO₂ in association with methanogens or sulfate reducing bacteria. The oxidation of VFAs...
by syntrophic acetogenic bacteria is not a thermodynamically favorable process \( (\Delta G \geq 0) \). These reactions are favorable only under low partial pressure of hydrogen, which is achieved either by methanogenesis or sulfate reduction (Schink, 1997). Therefore, hydrogen metabolism is crucial in AD since at high partial pressures of hydrogen \( (> 10 \text{ Pa} \times 10^{-4} \text{ atm}) \) will result in accumulation of VFAs and will result in acidification of biodigesters. It has been reported that hydrogen partial pressure in biodigesters should not exceed \( 10^{-6} \text{ atm} \) for the efficient oxidation of VFAs (Schink, 1997; Sieber et al., 2012).

Methane formation is the final step in AD, and it is also the most sensitive to imbalance. As a matter of fact, the amount of viable methanogens is probably the most effective indicator of a stable and effective system. Methanogenic communities are not as diverse as the others within the digesters, and they possess a specialized metabolism, characteristics that make them more likely to be inhibited. Among the seven orders of known methanogens, three are found with more frequency in the biodigesters: Methanobacterales, Methanomicrobiales, and Methanosarcinales (Demirel and Scherer, 2008). Members of the order Methanococcales are rarely found in biodigesters; however, there is a report about the finding of these microorganisms in granular sludge treating brewery wastewater (Liu et al., 2002a). The fifth order, Methanopyrales, includes only one hyperthermophilic species, which is unlikely to be found into anaerobic biodigesters (Bapteste et al., 2005). The recently recognized sixth order of Methanocellales contains only one genus, Methanocella, a hydrogenotrophic methanogen that was first isolated from a propionate-degrading culture obtained from rice paddy soils (Sakai et al., 2008). Meanwhile, the newest proposed order, Methanoplasmatales, was derived from

![Figure 1](https://www.frontiersin.org)
samples of hindguts of termites and wood-feeding cockroaches. Methanoplasmatales includes members, which were previously believed to be distantly related to a different lineage in the phylum Euryarchaeota (Paul et al., 2012).

Members of Methanobacteriales, Methanococcales, and Methanomicrobiales utilize CO₂ as electron acceptor. Hydrogen is commonly used as electron donor in this case, but some species also use formate and/or alcohols such as ethanol or isopropanol. With the exception of Methanospirillum (from Methanobacteriales), members of these orders cannot use acetate or one-single carbon compounds (Bonin and Boone, 2006).

Methanosarcinales are the most diverse in terms of metabolism. Acetate, hydrogen, formate, ethanol, isopropanol, and methylated compounds can be metabolized by members from this order (Kendall and Boone, 2006). *Methanoaetaceae* is the only family within Methanosarcinales that includes strictly aceticlastic anaerobes (Smith and Ingram-Smith, 2007).

In natural environments, such as swamps or rumen, populations of hydrogenotrophic methanogens are predominant, while in biodigesters; usually there are more aceticlastic methanogens (Ferry, 2010). This difference appears to be related to the amount of substrates and the presence of relatively high levels of various inhibitory compounds in the biodigesters, such as ammonia, H₂S, and VFAs.

In general, the methanogenic pathway itself has captured the curiosity of many for decades. The pioneer work of several scientists has allowed us to know the biochemically distinct features of methanogens. However, our understanding of how methanogenesis is coupled to energy conservation has been slower to develop (Leigh et al., 2011).

**METHANOGENESIS AND METHANOGENS**

Methanogenesis usually occurs in a variety of natural anaerobic environments such as marine and freshwater sediments, rice paddies, landfill, animal digestive tracts, and hydrothermal vents. However, it has been demonstrated that these microorganisms are also able to grow in aerated places like deserts soils (Aschenbach et al., 2013). Annually, approximately 600 million metric tons of methane is produced. Due to its potential greenhouse effect, which is 21 times higher that of CO₂, methane emission into the atmosphere is an important concern (EPA, 2014). In this sense, AD represents an economical and effective alternative for reducing the emission of methane from organic wastes since it recovers methane as an energy source.

Methanogens can obtain energy for growth by converting a limited number of substrates to methane under anaerobic conditions. In thermodynamic terms, methanogenesis will only occur when other electron acceptors such as oxygen, nitrate, and sulfate are absent, as methanogens require a low redox potential, around −300 mv for growth and activity. Given that CO₂ is the only electron acceptor that does not owe its abundance to photosynthesis, methanogenesis was a favored metabolism early on earth (Kasting and Siepert, 2002).

For methanogens, methane is actually a waste product. The heterodisulfide CoM-S-S-CoB formed as an intermediate in the pathway is of vital importance for the cell since its reduction is coupled to energy conservation, making the heterodisulfide the terminal electron acceptor in the respiratory chain of methanogens (Hedderich and Whitman, 2013). Methanogens use 2-mercaptoethanesulfonate (coenzyme M or CoM) as the terminal methyl carrier in methanogenesis and have four enzymes for CoM biosynthesis. Coenzyme B-Coenzyme M heterodisulfide reductase (Hdr), required for the final reaction of methanogenesis, is divided into two types, cytoplasmic HdrABC in most methanogens and membrane-bound HdrED in *Methanosarcina* species (Kaster et al., 2011). Currently, only two types of methanogenic pathways are known, (1) methanogenesis from H₂/CO₂ or formate, (2) from acetate and methyl group containing C₁ compounds. The conversion of methyl group to methane is common in both pathways as shown in Figure 2 (Ferry, 2011).

In order to establish an accurate classification of methanogens, phylogenetic analysis have been made with the purpose of organize these microorganisms according to their evolutionary history (Fox et al., 1977; Bapteste et al., 2005). Initially, Bapteste et al. (2005) divided the five orders of methanogens known until that date into two major groups which they named Classes. Class I included the orders Methanobacteriales, Methanococcales and Methanopyrales and Class II comprised Methanomicrobiales and Methanosarcinales. However, it was acknowledged that Methanomicrobiales shared more traits with Class I members than with Methanosarcinales. Therefore, in 2009, an updated view for methanogens differentiation was presented. This new classification divided methanogens into three classes according to seven core methanogenesis enzymes and cofactor biosynthesis (Anderson et al., 2009). In this arrangement, Methanomicrobiales and Methanosarcinales, orders that used to be grouped in the same class, were separated into Class II and Class III, respectively, due to several unique protein signatures observed. Nevertheless, with the discovery of two novel orders, Methanocellales and Methanoplasmatales, the classification of methanogens must be updated.

On the other hand, methanogens also can be divided into two categories based on the presence or lack of cytochromes (Thauer et al., 2008). All members of Methanosarcinales possess cytochromes and methanophenazine while members of the remaining orders lack of both of them. Additionally, Methanosarcinales have the ability to grow on acetate, methanol, and H₂/CO₂ with a higher growth yield.

It is well documented that methanogenic communities in biodigesters are susceptible to environmental changes, especially low pH and temperature (Demirel and Scherer, 2008). However, methanogenesis in natural ecosystems is known to proceed in cold and acidic conditions that are inhospitable for biodigesters (Steinberg and Regan, 2008; Aschenbach et al., 2013). This difference between biodigesters and natural ecosystems could be attributed to differences in the composition of the methanogenic communities (Liu and Whitman, 2008; Steinberg and Regan, 2008; Liu, 2010). Therefore, a better knowledge of these differences might lend insights into community-based strategies to increase digester stability with reduced chemical and energy inputs necessary to maintain narrow operating conditions (Steinberg and Regan, 2008).

Community studies of methanogenic population most frequently involve culture-independent techniques and molecular analysis has taken a major role in recent years. Recently
biochemical markers using archaeol (2,3-diphytanyl-O-sn-glycerol) also have been developed (McCartney et al., 2013a).

In the case of molecular analysis, various methanogen specific primers targeting 16S rRNA gene have been developed (Castro et al., 2004; Yu et al., 2005; Zhou et al., 2011). To eliminate potential problems with non-specific amplification, some researchers have developed primers for the gene sequence of the alpha subunit of the MCR, \textit{mcr}A (Springer et al., 1995; Hales et al., 1996; Luton et al., 2002; Denman et al., 2007; Steinberg and Regan, 2008). Phylogenetic inference with \textit{mcr}A sequence is similar to that obtained with 16S rRNA, suggesting non-lateral gene transfer. Due to the fact that methanogens may be examined exclusively from other bacteria present in the biodigesters, \textit{mcr}A has been increasingly used for phylogenetic analysis coupled with or independently of 16S rRNA studies. Primers and methods targeting both genes for monitoring of methanogens have also been reviewed (Narihiro and Sekiguchi, 2011).

**COMPARISONS BETWEEN \textit{mcr}A AND 16S rRNA**

Methyl coenzyme M reductase is the unique enzyme that catalyzes the reduction of CH$_3$-CoM to CH$_4$ and is highly conserved in all methanogens. Two iZoenzymes of MCR designated MCR I and MCR II are known and their respective operons are shown in **Figure 3**. The operon encoding MCR I, \textit{mcr}BDCGA, prevails in all known methanogens while MCR II operon, \textit{mrt}BDGA, is only found in some members from the orders Methanobacteriales and the Methanococcales (Garcia et al., 2000; Luton et al., 2002). Kinetic parameters are different for both isoenzymes and expression of either MCR I or MCR II seems to be dependent on hydrogen concentrations (Reeve et al., 1997). MCR II in \textit{Methanothermobacter thermoautotrophicus} (formely known as \textit{Methanobacterium thermoautotrophicum}) was shown to be expressed only in the earlier stages of batch cultures, then it was replaced with MCR I in late growth and stationary phases where hydrogen concentrations were lower (Pihl et al., 1994; Nölling et al., 1995).

Earlier, Rouvière and Wolfe (1987) showed that phylogenetic relationships obtained with different subunits of MCR corresponded at genus level of methanogens with those derived with 16S rRNA gene. After the genes encoding for MCR subunits were available, the \textit{mcr}A gene, which encodes the \alpha-subunit of MCR I was selected as a phylogenetic tool for the analysis of members of the family \textit{Methanosarcinaceae} (Springer et al., 1995). The relationships calculated with the sequences of \textit{mcr}A and 16S rRNA showed high similarity. Subsequent studies have also confirmed that similar
phylogenetic relationships can be obtained by use of 16S rRNA and mcrA in methanogens (Luton et al., 2002; Baptiste et al., 2005). Both strategies showed higher concurrence within the same environmental sample. However, it was also reported that 16S rRNA library of the biodigester showed less diversity than the library of mcrA gene (Springer et al., 1995; Steinberg and Regan, 2008).

Since then, mcrA gene has been established as a molecular marker for methanogenic archaea (Lueders et al., 2001) and several studies have identified the presence of mcrA with methanogenic activity. In 2003, environmental mcrA sequences were reported for the first time from a eutrophic lake (Earl et al., 2003) as well as in salt marsh sediments (Castro et al., 2004). Subsequent studies of vertebrate guts also revealed the presence of mcrA genes in the cow rumen (Denman et al., 2007); feces of pigs, chickens and horses (Uhnar et al., 2007a); the guts of humans (Scanlan et al., 2008; Mihajlovski et al., 2010), and the foregut of wallabies (Evans et al., 2009). A comparison between 16S rRNA and mcrA clone frequencies in samples of insect guts showed their strong accordance (Paul et al., 2012). As a result of this comparison arose the differentiation of a separate lineage into a new order of methanogens, the Methanoplasmatales. It can be observed that use of mcrA gene is a potential tool in the analysis of methanogen diversity in samples from different and varied sources (Ellis et al., 2012; Iwin and Matsui, 2014).

One of the advantages of mcrA gene is that only one or two copies of mcrA have been found in sequenced methanogens genomes, making it a more precise tool for estimating the number of these archaea in the biodigesters than the 16S rRNA gene, which can have up to four copies per genome (Ellis et al., 2009; Ma et al., 2012). A strong correlation between mcrA copy number and methane production has been reported in H2/CO2- enriched cultures (Morris et al., 2014). Moreover, transcription of mcrA has been used to demonstrate that methanogens are metabolically active (Juutinen et al., 2008), as it is well known that these microorganisms are capable of dormancy when conditions are not optimal (Speece, 1983). Thus, identifying active members of the methanogenic population can provide a real insight into the digester performance. Likewise, because transcription is more closely related to activity, determination of mcrA transcript number promises to be a better indicator of good performance rather than the only mcrA copy number (Morris, 2011). This was proven by studies in paddy field soils, where it was discovered that under different environmental conditions, abundance of mcrA transcripts changed while mcrA gene copy number remained almost the same, suggesting that only certain members of the methanogenic community were metabolically active and responsible for methane emissions (Watanabe et al., 2009; Ma et al., 2012).

However, it was reported that quantification of gene transcript abundance in peat soils was not a reliable method since the presence of inactive and dormant cells overestimated the final values (Freitag and Prosser, 2009). Besides, MCR activity is strongly temperature dependent (Goenrich et al., 2005), and it is still unknown if post-translational modifications affect the expression of the enzyme (Kahnt et al., 2007). Therefore, the analysis of mcrA transcripts solely may not be efficient as a tool for monitoring biodigesters. Moreover, it should be taken into consideration that sequences of isoenzyme mcrA can also be part of the targets of the mcrA primers. Hence, overestimation of transcripts is also possible (Nettmann et al., 2008), if members of Methanobacteriales and Methanococcales are present in the samples, since mrtA has been observed only in both of these orders (Luton et al., 2002).

**LINKING THE mcrA GENE TRANSCRIPTS TO THE DYNAMICS OF THE METHANOCOCCAL COMMUNITY IN ANAEROBIC BIODIGESTERS**

Some of the problems in using and comparing methods of mcrA expression studies are the choice of primers (McCartney et al., 2013b) and the differences in the PCR conditions (Steinberg and Regan, 2008). A comparison between the methods and the outcomes in each experiment requires a much more detailed analysis and for those interested on other molecular methods for environmental monitoring of methanogens, Narihiro and Sekiguchi’s (2011) review is suggested. This review discusses only the relationship between the mcrA gene expression and methanogenic activity, thrusting forward our knowledge on the importance of mcrA as a tool to monitor the functioning of the biodigester. The use of mcrA
Table 1 | Application of mcrA gene in studies related to biodigester and other natural anaerobic environments.

| Type of study                  | Primers/probes | Environment                        | Reference                  |
|--------------------------------|----------------|------------------------------------|----------------------------|
| Phylogenetic relations         | MCR/MCRr*      | Pure cultures                      | Springer et al. (1995)     |
| Community composition          | ME1/ME2*       | Blanket peat bog                   | Hales et al. (1996)       |
| Community composition          | MCRf/MCRr*     | Rice field soil                    | Lueders et al. (2001)     |
| Community composition          | ME1/ME2b       | Oligotrophic fen                   | Galand et al. (2002)      |
| Community composition          | mcrAF/mcrAR*   | Landfill material                  | Luton et al. (2002)       |
| Community composition          | ME1/ME2b       | Lake sediments                     | Earl et al. (2003)        |
| Community composition          | mcrAF/mcrARc   | Freshwaters marshes                | Castro et al. (2004)      |
| Community composition          | MCRf/MCRr*     | Hydrothermal sediments             | Dhillon et al. (2005)     |
| Community composition          | MCR/MCRr*      | Peatland soil                      | Juottonen et al. (2006)   |
| Community composition          | mcrAF/mcrARc   | Animal feces                       | Ufnar et al. (2007b)      |
| Community composition          | MeA 1048f/MeA 1435r* | Biodigester                  | Bauer et al. (2008)      |
| Community composition          | mcrAF/mcrARc   | Biodigester                        | Nettmann et al. (2008)    |
| Community composition          | ME1/ME2b       | Human feces                        | Scanlan et al. (2008)     |
| Community composition          | ME1b ,mcrAFc , mlas*/mcrA-rev* | Acidic peat bog, Biodigester    | Steinberg and Regan (2008) |
| Community composition          | MM-01_pSTC/MM-02* | Human feces                      | Mihajlovski et al. (2010, 2008) |
| Community composition          | mcrAF/mcrARc   | Marine sediments                   | Merkel et al. (2010)      |
| Community composition          | ME3F/M2b*      | Biodigester                        | Tale et al. (2011)        |
| Community composition          | mcrAF/mcrARc   | Biodigester                        | Zhu et al. (2011)         |
| Community composition          | MCRf/MCRr*     | Biodigester                        | Ellis et al. (2012)       |
| Community abundance            | mlas/mcrA-rev* | Biodigester                        | Traversi et al. (2012)    |
| Community composition          | MCRf/6-FAM-MCRr* | Biodigester                      | Ma et al. (2013)          |
| Community composition          | mcrAF/mcrARc   | Rumen fluid                        | Sirohi et al. (2013)      |
| Detection of methanogenic activity | mcrAF/mcrARc  | Boreal mire                        | Juottonen et al. (2008)   |
| Changes in community composition | mcrAF/mcrARc  | Biodigester                        | Rastogi et al. (2008)     |
| Changes in community composition | mcrAF/mcrARc  | Biodigester                        | Cardinili-Rezende et al. (2009) |
| Changes in community composition | TET-mcrAF/mcrARc | Biodigester                      | Ács et al. (2013)         |
| Quantification and community composition | ME1b /M2b*  | Biodigester                        | Shigematsu et al. (2004)  |

(Continued)
Table 1 | Continued

| Type of study                                    | Primers/probes                                                                 | Environment                        | Reference                   |
|-------------------------------------------------|-------------------------------------------------------------------------------|------------------------------------|-----------------------------|
| Quantification and composition of communities   | mcrAF/mcrAR<sup>2</sup>                                                        | Rumen fluid                        | Denman et al. (2007)        |
|                                                 | qmcrA-F/qmcrA-R*                                                               |                                    |                             |
| Community composition and transcript quantification | mcrAF/mcrAR<sup>2</sup>                                                        | Rumen fluid                        | Guo et al. (2008)           |
|                                                 | qmcrA-F/qmcrA-R<sup>I</sup>                                                    |                                    |                             |
| Quantification and community composition        | ME3MF*/ME2B                                                                    | Biodigester, Marine sediments      | Nunoura et al. (2008)       |
| Quantification and composition of communities   | mcrAF/mcrAR<sup>2</sup>                                                        | Foregut of the Tammar Wallaby      | Evans et al. (2009)         |
|                                                 | qmcrA-F/qmcrA-R<sup>I</sup>                                                    | (Macropus eugenii)                 |                             |
| Quantification and community composition        | mlas/mcrA-rev                                                                 | Biodigester                        | Steinberg and Regan (2009)  |
|                                                 | mbac-mcrA*                                                                     |                                    |                             |
|                                                 | mrtA*                                                                         |                                    |                             |
|                                                 | mcp*                                                                          |                                    |                             |
|                                                 | msp*                                                                          |                                    |                             |
|                                                 | MCR-7*                                                                         |                                    |                             |
|                                                 | MCR-2a*                                                                        |                                    |                             |
|                                                 | MCR-2b*                                                                        |                                    |                             |
|                                                 | fen*                                                                          |                                    |                             |
|                                                 | msar*                                                                         |                                    |                             |
|                                                 | msa*                                                                          |                                    |                             |
| Quantification and changes in community composition | mlas/mcrA-rev<sup>2</sup>                                                        | Biodigester                        | Steinberg and Regan (2011)  |
|                                                 | mrtA<sup>2</sup>                                                               |                                    | Traversi et al. (2011)      |
|                                                 | mcp<sup>2</sup>                                                                |                                    |                             |
|                                                 | msp<sup>2</sup>                                                                |                                    |                             |
|                                                 | MCR-7<sup>2</sup>                                                              |                                    |                             |
|                                                 | MCR-2a<sup>2</sup>                                                             |                                    |                             |
|                                                 | MCR-2b<sup>2</sup>                                                             |                                    |                             |
|                                                 | fen<sup>2</sup>                                                                |                                    |                             |
|                                                 | msar<sup>2</sup>                                                               |                                    |                             |
|                                                 | msa<sup>2</sup>                                                                |                                    |                             |
| Quantification and community composition        | mcrAF/mcrAR<sup>2</sup>                                                        | Biodigester                        | Kampmann et al. (2012)      |
| Variations in transcripts and community composition | MCR<sup>T</sup>/MCR<sup>R</sup>                                             | Rice field soil                    | Ma et al. (2012)            |
|                                                 | mlas/mcrA-rev<sup>2</sup>                                                        |                                    |                             |
| Quantification and Community composition        | mcrAF/mcrAR<sup>2</sup>                                                        | Feces of horse and pony            | Lwin and Matsui (2014)      |
| Transcript and gene copy number quantification  | qmcrA-F/qmcrA-R<sup>I</sup>                                                    | Peat soil                          | Freitag and Prosser (2009), |
|                                                 | mcrAF/mcrAR<sup>2</sup>                                                        |                                    | Freitag et al. (2010)       |
| Gene abundance                                  | mcrAF/mcrAR<sup>2</sup>                                                        | Rumen fluid                        | Li et al. (2012)            |
| Transcript and gene copy number quantification  | MeA 1048f/MeA 1435r<sup>h</sup>                                               | Biodigester                        | Munk et al. (2012)          |
| Gene copy number quantification                 | mlas/mcrA-rev<sup>2</sup>                                                        | Cold desert soil                   | Aschenbach et al. (2013)    |

(Continued)
in different studies related to anaerobic biodigesters and natural anaerobic environments is summarized in Table 1.

CORRELATION BETWEEN OPERATIONAL AND ENVIRONMENTAL CONDITIONS OF BIODIGESTERS AND mcRA

Usually, in balanced anaerobic reactors it is reported that the majority of the methanogens are aceticlastic. The effect of dilution rate and their relation to methanogenic pathways using 13C-labeled acetate and phylogenetic analysis of mcRA gene transcripts showed that transcripts of *Methanosarcina* species were the most abundant at high dilutions and that aceticlastic pathway was the major pathway for cleavage of acetate and methane production at those dilutions (Shigematsu et al., 2004). However, at low dilution rates, transcripts of *Methanoculleus* were the most abundant ones and the pathway shifted towards syntrophic acetate oxidation where hydrogenotrophic pathway was the major source for methane production. Traversi et al. (2011) reported a positive correlation between the biogas production and the presence of *Methanosarcina* and *Methanaosacta* were found in biodigesters even when most of the mcRA genes corresponded to members of Methanomicrobiales. It was proposed that the abundance of *Methanosarcina* was a better indicator to understand the efficiency AD process. However, other investigators have had a different experience, and the use of *Methanosarcina* species alone is not sufficient to monitor the efficiency of the biodigesters. For example, in bioreactors recovered from organic overload by addition of propionate-degrading microorganisms, mcRA gene copies obtained from samples of these bioreactors were associated with *Methanospirillum hungatei* and *Methanobacterium beijingense*, both hydrogenotrophic methanogens (Tale et al., 2011). The study does not report the presence of *Methanosarcina* species, and methanogenic activity is attributed to *M. hungatei* and *M. beijingense*.

Recently, analysis of mcRA-based libraries showed that methanogenic populations shifted substantially with modifications in substrate composition (Acs et al., 2013). Microbial community analysis of a large scale mesophilic biodigester with swine manure as substrate showed that 123 clones of mcRA library were assigned to 28 OTUs, of which *Methanobrevibacter* spp. (an hydrogenotrophic methanogen) was the most abundant (Zhu et al., 2011). Similarly, the predominance of hydrogenotrophic phyla (60–90%) over aceticlastic ones in six large-scale biodigesters fed with different industrial wastes has been reported (Regueiro et al., 2012). A higher predominance of hydrogenotrophic methanogens was found in a continuous anaerobic biodigester treated a mixture of fruit and meal leftovers (Cardinali-Rezende et al., 2009). Similarly, a higher proportion of OTUs clustered within the order of Methanomicrobiales for both mcRA and 16S rRNA libraries (79–88%) in an agricultural biogas plant fed with catle manure and maize silage under mesophilic conditions (39°C) was reported (Nettmann et al., 2008). Likewise, it was stated that H2/CO2 was the main substrate for methanogenesis in acidic peat (Castro et al., 2004). It was also observed that casein addition modified the population of fermenting bacteria, as well as the available hydrogen and the methanogenic community. After...
5 weeks, Methanoculleus marisnigri increased almost twice when casein was added, and with addition of pig blood, Methanomicrobiaeae increased its abundance by 10 times. Clones related to Methanocorpusculum parvum, Methanomassiliicoccus luminyensis, and Methanoculleus bourgensis were more abundant after casein addition and decreased with pig blood. M. luminyensis is a methanogen that produces methane from H₂ and methanol (Dridi et al., 2012), whereas M. marisnigri, M. parvum, and M. bourgensis as members of the Methanomicrobiaeae are strictly hydrogenotrophic methanogens. Similarly in another study with casein, starch and cream as substrates showed that copy numbers of mcrA were higher in casein fed biodigesters than the other two substrates (Kampmann et al., 2012). In the starch-fed reactor, the predominant methanogenic populations were Methanoculleus bourgensis and Methanobrevibacter millerae. These methanogens utilize H₂ and CO₂ for their metabolism. Similarly, the dominance of Methanobrevibacter and Methanospiillum together with uncharacterized methanogens was reported in biodigesters fed with swine manure (Zhu et al., 2011). Hydrogenotrophic methanogens, specifically of the genus Methanoculleus and of the order Methanomicrobiaeae were reported to be predominant in pulp mill wastewater treating biodigesters (Yang et al., 2013).

All these studies contradict the previously established ratio of acetate and H₂/CO₂ on methane production in biodigesters, 70 and 30%, respectively (Ahring, 2003). Although it is reported that stirred tank reactor conditions affected the conglomeration and of the family Methanobacteriaceae increased its abundance by 10 times. Clones related to Methanosarcina species or members of the Methanomicrobiaeae as low as 1–5°C, and were highly abundant in both summer (36°C) and winter (25°C; Rastogi et al., 2008). In contrast, clones related to Methanosaeta concilii were present only during summer.

Zhang et al. (2014) studied the response of methanogens to different concentrations of ammonia using mcrA transcripts. While T-RFLP analysis showed that members of Methanosetaeaceae were the dominant ones in all samples, the abundance of transcripts displayed variations according to the ammonia concentrations. In the case of transcripts, Methanobacteriaeae recorded higher number at high concentrations of ammonia. Transcripts of Methanosarcinaeae increased during the last stages of the experiments and this coincided with the decrease in concentration of free ammonia. In another study, addition of tea saponins recorded only 8% decrease in methane production by rumen microorganism, but decreased mcrA gene transcription by 76% (Guo et al., 2008). This reduction was attributed to a 79% decrease on protozoa population. It is well known that methanogens are associated with ciliates protozoa of the genera Entodinium, Polyplastron, Epidinium, and Ophryoscolex (Hook et al., 2010).

Even though mcrA gene is mainly employed to determine the presence and community composition of methanogens, transcriptional analysis of this gene can give us a major insight to the dynamics and performance of anaerobic digesters. Inspite of observed variations, mcrA gene could become an important tool for the monitoring of presence and activity in methanogens in different environments in combination with other unique biochemical properties of methanogens.

CONCLUSION
It is a common perception and widely accepted that acetoclastic methanogens contribute nearly 70% of methane produced in animal wastes fed biodigesters. Most of the time, data on methanogenic community analysis support this idea. However, analysis of mcrA gene expression has broadened our knowledge on the composition and activity of methanogenic communities in biodigesters and in other anaerobic environments. It is clear that hydrogenotrophic methanogens are widely distributed, active and under some operational conditions even dominate over acetoclastic methanogens. Hence, making assumptions based only on the presence and abundance of certain methanogens groups is not a valid parameter to monitor the state of biodigesters. It can be concluded that gene expression of mcrA can be a potential tool in determining the active members of the methanogenic community since it gives a better insight on the metabolic dynamics within biodigesters,
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