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Potential contribution of acetogenesis to anaerobic degradation in methanogenic rice field soils

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ABSTRACT

Acetate is an important intermediate in the anaerobic degradation of organic matter. It is not only produced by fermentation but also by the reduction of CO_2 via the acetyl-CoA pathway (acetogenesis). However, the interplay of this process in methanogenic rice field soils is not fully understood. Chemolithotrophic acetogenesis results in a rather strong depletion of the ^{13}C of acetate. Therefore, we measured the $\delta^{13}C$ of acetate, CO_2 and CH_4 that were produced during methanogenic degradation of organic matter in rice paddy soils from two geographical origins (Philippines and Italy) and used three different strategies to estimate the contribution of acetogenesis to acetate formation: (1) Incubation of soil slurries under elevated concentrations of H_2/CO_2 to specifically activate the H_2-dependent communities; (2) incubation at three different temperatures (15, 30, 50 °C) to shift the conditions for H_2 consumption; (3) incubation in the presence of inhibitors presumed to inhibit acetogenesis (KCN) or methanogenesis (BES). Only incubations under elevated H_2/CO_2 resulted in ^{13}C-depleted acetate ($\delta^{13}C$ of −68 to −65‰) compared to the control ($\delta^{13}C$ of −25‰). Temperature and presence of inhibitors also affected the $\delta^{13}C$ of acetate, CO_2 and/or CH_4, but $\delta^{13}C$ of acetate was never as low as after addition H_2/CO_2. A significant ^{13}C enrichment of acetate at 15 °C in presence of BES and KCN indicated that H_2-dependent acetogenesis is a favoured process at low temperature. Copy numbers in the Philippine soil of the *fhs* gene coding for the formyltetrahydrofolate synthetase of the acetyl-CoA pathway were on a similar order of magnitude (10^6 per gram dry soil) irrespectively of the different incubation conditions. Our results indicate that chemolithotrophic acetogenesis was operative in methanogenic rice soil at 15 °C but was more important at elevated H_2/CO_2 concentrations.

1. Introduction

The anaerobic degradation of organic matter accounts for a significant part of the global carbon cycle and involves the production of the greenhouse gas CH_4 (Reeburgh, 2003). While the terminal processes liberating CH_4 have been thoroughly investigated using isotopic techniques (Conrad, 2005), the contribution of other pathways during the anaerobic degradation is not well investigated. In this study we aimed to specify the contribution of acetogenesis to the acetate pool in rice paddy soils from two geographical origins (Philippines and Italy).

The unifying trait of acetogenic bacteria is that acetate is their major product, which is mainly formed by the acetyl-CoA pathway reducing CO_2 to acetate. However, the usage of the acetyl-CoA pathway in acetogens is versatile (Drake et al., 2002; Schuchmann and Muller, 2016): acetogens can compete with primary fermenters for monomeric compounds, e.g., glucose, fructose or xylose; they can act as secondary fermenters using lactate, ethanol or even methoxylated aromatics; many acetogens can grow chemolithotrophically converting H_2 and CO_2 to acetate; and some acetogens can even grow in syntrophic associations at the expense of acetate running the acetyl-CoA pathways in the reverse direction (Hattori et al., 2000; Hattori, 2008).

Even though acetate is the major end product of acetogens, their environmental role is hard to address since many anaerobic bacteria also produce acetate by other biochemical pathways than the acetyl-CoA pathway. To investigate the function of chemolithotrophic acetogens in rice field soils we studied their contribution to the acetate pool using isotope techniques. This is a reasonable approach since studies of microbial pure cultures have shown that the stable carbon isotope fractionation of most fermentative processes is almost negligible (< 5‰) (Blair et al., 1985; Penning and Conrad, 2006; Botsch and Conrad, 2011), while in contrast the fractionation by chemolithotrophic acetogenesis ($4\ H_2 + 2\ CO_2 \rightarrow CH_3COOH + 2\ H_2O$) is strong (−38‰ to −68‰) (Gelwicks et al., 1989; Preuss et al., 1989; Blaser et al., 2013). One has to keep in mind that the acetate formed by heterotrophic

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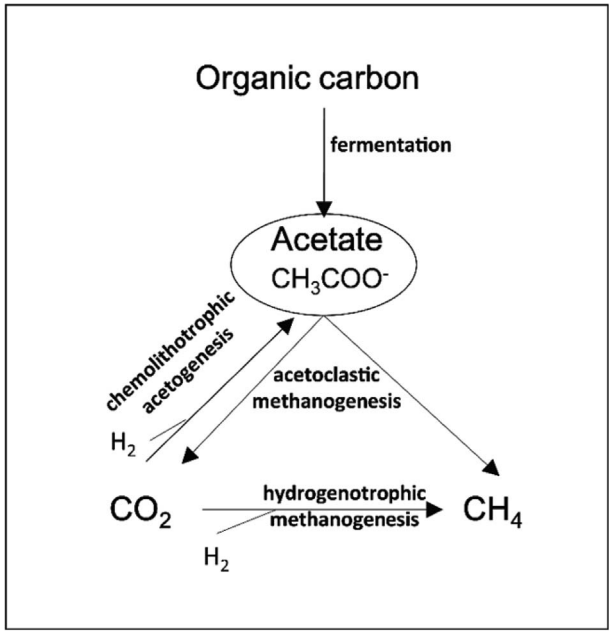

Fig. 1. Scheme illustrating acetate as important intermediate in the anaerobic degradation of organic matter: fermentation or heterotrophic acetogenesis, chemolithotrophic acetogenesis, aceticlastic methanogenesis, hydrogenotrophic methanogenesis.

acetogenesis originates partially from fermentation of organic matter and partially from the reduction of CO_2 (Freude and Blaser, 2016).

However, while the differentiation of aceticlastically and hydrogenotrophically formed CH_4 is rather straight forward (Conrad, 2005; Blaser and Conrad, 2016), the application of such techniques for the investigation of chemolithotrophic acetogenic bacteria is much more complex. In contrast to CH_4, which is an end product in most anaerobic sediments, acetate is produced and consumed by different metabolic pathways, which all imprint a signature on the stable carbon isotope composition ($^{13}C/^{12}C$) of acetate (Fig. 1) (Conrad et al., 2014).

Hence, the stable carbon isotopic composition of acetate is basically governed by three factors: (1) the isotopic composition of its precursors, (2) the isotopic fractionations (enrichment factors) associated with its formation and consumption, and (3) the relative rates of all acetate producing and consuming processes which influence its pool size. The knowledge of all three factors is of great importance for the evaluation of ^{13}C values of acetate determined in environmental samples. The isotopic composition of the precursors can be measured, since CO_2 is the exclusive C substrate for chemolithotrophic acetate formation, while soil organic carbon is the substrate for fermentatively produced acetate. We used three strategies to estimate the contribution of chemolithotrophic acetogenesis to acetate formation: (1) Incubation of soil slurries under elevated concentrations of H_2/CO_2 to specifically activate the H_2-dependent communities; (2) incubation at three different temperatures to shift the conditions for H_2 consumption; (3) incubation in the presence of inhibitors to control the competitive role of chemolithotrophic acetogens and methanogens under natural H_2 concentrations.

In many anoxic systems the supply of H_2 is low, and hence microbial populations will consume H_2 to the threshold of bioenergetic limitation (Cordruwisch et al., 1988; Hoehler et al., 2002). In such systems, the competition of acetogens and methanogens for H_2 is of great ecological importance (Kotsyurbenko et al., 2001). Even though methanogens are expected to outcompete acetogens for H_2 because of thermodynamic reasons (Hoehler et al., 2002), there are additional constraints. Thus, at low temperatures (5–15 °C) acetogens have often been found to be better adapted than methanogens and outcompete them for H_2 (Schulz and Conrad, 1996; Schulz et al., 1997; Kotsyurbenko et al., 2001; Glissmann et al., 2004; Nozhevnikova et al., 2007; Liu and Conrad, 2011). Then, CH_4 has been generated by a two-step process: First acetogens reduce CO_2 to acetate which in turn is cleaved by aceticlastic methanogens. In contrast at elevated temperatures (> 55 °C), CH_4 production has frequently been found to be driven by syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis (Hattori et al., 2000; Hattori, 2008; Conrad and Klose, 2011). Syntrophic acetate oxidation is the reversal of chemolithotrophic acetogenesis and presumably exhibits a low carbon isotope fractionation (Hattori et al., 2000; Hattori, 2008; Conrad and Klose, 2011). Thermophilic syntrophic acetate oxidizers were found to be able also operatíng in the reverse mode (i.e., chemolithotrophic acetogenesis) if supplied with H_2 plus CO_2 (Liu and Conrad, 2011). To elucidate the environmental relevance of acetogenically formed acetate we therefore used different temperature regimes to shift conditions and estimate the contribution of acetogenic and methanogenic pathways.

Finally, different inhibitors may constrain the influence of methanogens and acetogens. While 2-bromoethanesulfonate (BES) has been shown to be an effective inhibitor for methanogens (Sparling and Daniels, 1987), the inhibition of acetogenic bacteria is much more difficult. We used cyanide (KCN) as possible inhibitor, as it interferes with the electron transport via cytochromes (Cooper and Brown, 2008). Cytochromes are used by several acetogens, which rely on a proton gradient for energy translocation (e.g. *Moorella thermoacetica* (Pierce et al., 2008) or *Clostridium aceticum* (Poehlein et al., 2015)). Other acetogens however rely on ferredoxins instead of cytochromes for electron transportation (e.g. *Acetobacterium woodii* (Poehlein et al., 2012), *Clostridium ljungdahlii* (Kopke et al., 2010), *Thermoacetogenium phaeum* (Oehler et al., 2012) and *Thermoanaerobacter kivui* (Hess et al., 2014)). Likewise KCN directly inhibits carbon monoxide dehydrogenase (CODH), which is one of the key enzymes of the reductive acetyl CoA pathway (Ensign et al., 1989). Therefore, pathways involving CODH should be more susceptible to cyanide inhibition than fermentation processes.

Distinct isotopic fractionations have been observed in various pure microbial cultures of acetate-producing and consuming cultures (Meinschein et al., 1974; Rinaldi et al., 1974; Blair et al., 1985; Krzycki et al., 1987; Gelwicks et al., 1989, 1994; Preuss et al., 1989; Penning et al., 2005; Penning et al., 2006; Penning and Conrad, 2006; Londry et al., 2008; Goevert and Conrad, 2009; Blaser et al., 2013; Freude and Blaser, 2016). Most of them focused on carbon isotope fractionation during the consumption of acetate, in particular by aceticlastic methanogenic archaea which show isotopic enrichment factors of −21‰ to −35‰ in *Methanosarcinaceae* (Krzycki et al., 1987; Gelwicks et al., 1994; Londry et al., 2008; Goevert and Conrad, 2009) and −7‰ to −10‰ in *Methanosaetaceae*, respectively (Valentine et al., 2004; Penning et al., 2006). So far only few studies have analyzed the stable carbon isotopic composition of acetate in natural sediments under in situ conditions. The isotopic composition of acetate has been shown to be highly variable in natural sediments, ranging from −85‰ up to −3‰ (Blair et al., 1987; Blair and Carter, 1992; Heuer et al., 2006, 2009, 2010; Conrad et al., 2014). Detailed analyses to study the contribution of acetogenesis have been performed using freshwater sediments under elevated H_2 concentrations using BES to inhibit methanogenesis and at low temperature (8 °C) to follow acetate concentrations and their $\delta^{13}C$ values (Heuer et al., 2010). A similar approach using peat soil under elevated H_2 concentrations was used to monitor the acetogenic community (Haedrich et al., 2012). However, the contribution of acetogenic bacteria in rice paddy soils using inhibitors or natural abundance of stable carbon isotopes has barely been investigated.

Molecular studies have shown that acetogens can be found in almost every anoxic and also some oxic environments (Leaphart and Lovell, 2001; Leaphart et al., 2003; Conrad, 2005; Henderson et al., 2010; Hunger et al., 2011). They reach 10^6 to 10^8 gene copies per gram dry weight (Xu et al., 2009). However, acetogens cannot be targeted using 16 S rRNA-based approaches, because they do not form a