How reversible is methylotrophic methanogenesis? Distinguishing gross from net uptake using clumped isotopes in methanol

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Isotopic fractionations among carbon compounds are often used to distinguish the metabolic pathways of methanogens in pure cultures and natural environments. However, such techniques are typically only sensitive to *net* changes in the abundance and isotopic composition of inputs and outputs. *Gross* substrate uptake rates may be larger, but cryptic, if the first metabolic steps are highly reversible and only the end-product methane is observed. The clumped ^{13}C–^{2}H composition of biogenic methane depends on the reversibility of the metabolism. When H_2 is abundant, hydrogenotrophic methanogenesis is unidirectional and product methane is far from clumped isotope equilibrium; when H_2-limited, the pathway is more reversible and methane C–H bonds equilibrate with ambient temperature. Here we leverage a new method for measuring the clumped ^{13}C–^{2}H composition of methyl groups in methanol to detect re-equilibration of C–H bonds in the substrate and inheritance of clumped compositions in the product of methylotrophic methanogenesis.

We grew *Methanosarcia acetivorans* on methanol in batch cultures. Parallel cultures were stopped in sequence to capture both the logarithmic growth and stationary phases. Abundances and clumped isotopic compositions of methanol, methane, and carbon dioxide were characterized by ultra high-resolution IRMS. Methoxy groups were also extracted from the cell biomass, producing a site-specific record of the isotopic evolution of the C1 metabolic intermediates for the first time. By monitoring the intramolecular isotopic compositons of inputs, outputs, and intermediates, we address key outstanding questions regarding the metabolism of methanogens, such as: what fraction of methane C–H bonds are inherited from substrate and do these contribute to non-equilibrium clumped isotope values? And, what is the true ratio of methanol input to methane output?