Supplementary Material

Multiple modes of methanogenesis in deep hydrothermally-influenced subsurface sediments

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Supplementary methods

Methanogenic activity

Radiotracer assays were conducted using sediment from four sites spanning different geochemical and thermal gradients. Rates of methanogenesis were determined by tracking generation of ^{14}C-methane from five ^{14}C-labeled substrates, acetate, bicarbonate, formate, methanol, and methylamine, as described previously (Bowles et al. 2011; Zhuang et al. 2018). Briefly, 100 μL of ^{14}C-bicarbonate (600 kBq), ^{14}C-formate (~200 kBq), 2-^{14}C-acetate (210 kBq), ^{14}C-methanol (298 kBq) and ^{14}C-methylamine (MA) (~143 kBq) in anoxic, sterile circumneutral saline solution were injected into sediment samples in cut-end Hungate tubes. Each treatment included one killed control and three live replicates. Killed controls were generated by adding 3 mL of 2 M NaOH to samples; samples were then homogenized before injecting radiotracers.

We minimized the amounts of added tracer while ensuring we could detect production of ^{14}C-methane production. Injection of the ^{14}C-labeled substrates resulted in the addition of 91 μM bicarbonate, 30 μM formate, 32 μM acetate, 45 μM methanol and 22 μM MA to the samples. The *in situ* concentrations of methanogenic substrates in sediments ranged from 1 mM to 73 mM for DIC, 16 μM to 608 μM for acetate, 0.1 μM to 4 μM for methanol, 2 nM to 820 nM for porewater trimethylamine; we do not have concentration data for methylamine and formate. Due to the tracer-associated substrate addition, the measured rates reflect potential rather than *in situ* rates. Nevertheless, comparisons across sites and depths are valid and environmentally relevant, as the concentrations of most tracers were in similar magnitude (e.g., acetate, bicarbonate, and formate) to *in situ* concentrations. All samples were incubated at *in situ* temperature on board the drill ship for 20 days. Incubations were terminated by

addition of 3 mL of 2 M NaOH into the sample, halting microbial activity. Samples were vortexed vigorously to mix and a headspace was created by gently pulling the plunger to the bottom of the Hungate tube while introducing CO_2-free air into the sample.

The methane production rate was determined by quantitatively converting $^{14}CH_4$ in the headspace to $^{14}CO_2$ using a combustion furnace and then trapping the evolved $^{14}CO_2$. Samples were purged with CO_2-free air to move the sample headspace through a series of traps to capture parent tracer and then through a combustion furnace to convert $^{14}CH_4$ to $^{14}CO_2$. The initial ^{14}C-labeled substrates, that is $^{14}CO_2$, ^{14}C-formate, ^{14}C-acetate, ^{14}C-methanol and ^{14}C-methylamine, were removed from the gas stream via a series of in-line traps. For bicarbonate, formate, acetate, and methanol, trap 1 contained 8 mL of 1 M NaOH and trap 2 contained 5 mL of NaOH pellets. For methylamine, trap 1 contained 8 mL of 1 M H_2SO_4 and trap 2 contained 5 mL of NaOH pellets. After these traps, the gas stream was directed through a titanium-nickel alloy column heated to 850 °C and filled with copper oxide to facilitate the oxidation of $^{14}CH_4$ to $^{14}CO_2$. The $^{14}CO_2$ was trapped in 4.5 mL of ScintiSafe Gel cocktail mixed with 1.5 mL of Carbosorb E and radioactivity was quantified on a Tri-Carb 3110TR liquid scintillation counter (PerkinElmer, USA) after resting the sample for 24 hours to minimize chemiluminescence.

The detection limit of methanogenesis rates measurement was calculated as the mean counts from killed controls plus three times the standard deviation of the control counts. Sample counts below this value were considered below the detection limit. The recovery of the combustion system was >90% as determined by the addition of known activities of $^{14}CH_4$. Methanogenesis rates were calculated using total substrate concentrations (porewater + radiotracer samples), and activities recovered from the

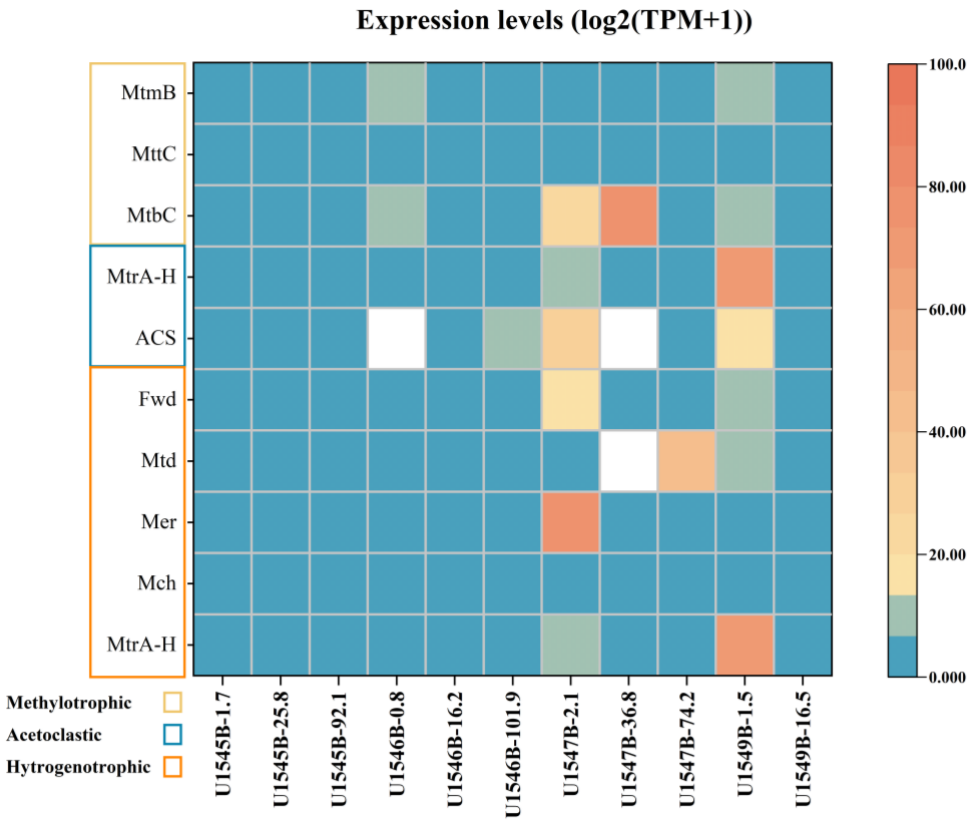
$^{14}CH_4$ pool (Eq. 1):

$$\text{MOG rate} = C_{substrate} \times \alpha/t(\text{DPM-}^{14}CH_{4product}/\text{DPM-}^{14}C_{substrate})/\rho \quad (1)$$

where MOG is the rate of methanogenesis from bicarbonate, acetate, methanol and MA (nmol substrate reduced cc^{-1} d^{-1}), $C_{substrate}$ is the total concentration of methanogenic substrates, ρ is the porosity (vol %), *in situ* levels plus added tracer (nmol cc^{-1}), α is the isotopic fractionation factor (assumed to be 1.04 for bicarbonate, 1.02 for acetate and formate, 1.07 for methanol, and 1.06 for MA [Krzycki et al. 1987; Summons et al. 1998; Whiticar 1999]), *t* is the incubation period (days), DPM-$^{14}CH_4$ is the activity recovered in the product pool, DPM-$^{14}C_{substrate}$ is the activity of the parent tracer injected into the sample (DPM: disintegrations per minute). Relative turnover times for ^{14}C-labeled substrates were compared and calculated based on the time (in days) required to convert the total amount of ^{14}C-substrate added completely to methane (Eq. 2):

$$\text{Turnover time} = (\text{DPM-}^{14}C_{substrate} / \text{DPM-}^{14}CH_{4product}) / t \quad (2)$$

All rate and turnover time calculations were corrected for the killed control by subtracting kill counts from live sample counts; killed controls were comparable to the instrument blanks.



Supplementary Figure S1. Heatmap showing the normalized expression levels [log2(TPM + 1)] of metatranscriptomic reads mapped to key methanogenesis genes across different holes in the Guaymas Basin (x-axis: site-depth in meters below seafloor, mbsf). White squares indicate log2(TPM + 1) values greater than 150.