Thermodynamic restrictions determine ammonia tolerance of methanogenic pathways in *Methanosarcina barkeri*

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Abstract

Ammonia is a ubiquitous potential inhibitor of anaerobic digestion processes, mainly exhibiting inhibition towards methanogenic activity. However, knowledge as to how ammonia affects the methanogens is still limited. In this study, we cultured a multitrophic methanogen, *Methanosarcina barkeri* DSM 800, with acetate, H_2/CO_2, and methanol to evaluate the influence of ammonia on different methanogenic pathways. Aceticlastic methanogenesis was more sensitive to increased ammonia concentrations than hydrogenotrophic and methylotrophic methanogenesis. Theoretical maximum NH_3 tolerances of *M. barkeri* fed with acetate, H_2/CO_2, and methanol were calculated to be 39.1 ± 9.0, 104.3 ± 7.4, and 85.7 ± 1.0 mg/L, respectively. The order of the ΔG range of *M. barkeri* under three methanogenic pathways reflected the order of ammonia tolerance of *M. barkeri*. Our results provide insights into the role of the thermodynamic potential of methanogenesis on the tolerance of ammonia stress; and shed light on the mechanism of ammonia inhibition on anaerobic digestion.

Keywords: Ammonia tolerance, *Methanosarcina barkeri*, Gibbs free energy, Thermodynamic restriction

1 Introduction

Methanogenic degradation of organic waste to methane and carbon dioxide is a common biochemical process performed by the cooperation of bacteria and methanogens in oxygen-limited environments, and increasingly applied as an effective and environmentally friendly technology for energy recovery from the recycling of organic waste (Demirel et al. 2008). In anaerobic digestion (AD), the final methanogenic pathways are normally divided into three categories according to their substrate utilization spectrum: CO_2 reducing (hydrogenotrophic), acetate splitting (aceticlastic) and methyl reducing (methylotrophic) pathway(Angel et al. 2011). In addition, two novel pathways capable of methanogenic degradation of methoxylated aromatic compounds (Mayumi et al. 2016) and long-chain alkanes (Zhou et al. 2022) have recently been reported, however, these pathways are always restricted to natural and subsurface environment.

Ammonia is a common inhibitor in AD, especially in reactors treating protein-rich substrates (Rajagopal et al. 2013), and mainly inhibits the methanogenic activity (Calli et al. 2005, Duan et al. 2022). Excessive ammonia results in an increase in lag-phase and a decrease of methanogen growth rate and methane production (Hansen et al. 1998, Yu et al. 2020). In most reported cases of AD, aceticlastic methanogens were observed to be much more sensitive than hydrogenotrophic methanogens under ammonia stress (Lue et al. 2013, Ruiz-Sánchez et al. 2017, Lv et al. 2019). The un-ionized ammonia (NH_3), rather than ionized ammonium is considered the more toxic species (Tian et al.

2018a, Astals et al. 2018). Mechanisms proposed to explain ammonia inhibition of methanogens include efflux of intracellular K^+ (Sprott et al. 1984, 1985, 1986) and inhibition of genes related to methanogenic metabolism (Duan et al. 2022, Kato et al. 2014, Kato et al. 2008, Zhang et al. 2018, Zhang et al. 2014, Yan et al. 2020) . However, these generic mechanisms cannot explain the observed variation of ammonia tolerance of diverse methanogenic pathways in the methanogenic consortia (Lue et al. 2013, Ruiz-Sánchez et al. 2017, Lv et al. 2019). In order to unlock the black box of ammonia inhibition in AD, it is necessary to further research the flexible response of different methanogenic pathways to ammonia.

In this study, we cultivated a multitrophic methanogen, *M. barkeri* strain DSM 800, with acetate, H_2/CO_2 or methanol as sole carbon and energy source, respectively. Based on physiological profiles, transcriptomic analyses and thermodynamic calculations, the ammonia tolerance of three methanogenic pathways of *M. barkeri* was compared. This comparison gave an in depth understanding of the mechanism of ammonia inhibition in methanogenesis.

2 Materials and methods

2.1 Cultures and growth conditions

Methanosarcina barkeri DSM 800 was obtained from the China Collection of Anaerobic Microorganisms (CCAM). *M. barkeri* was grown using a medium with the following composition (in g/L): K_2HPO_4, 0.35; KH_2PO_4, 0.23; NH_4Cl, 1.0; $MgSO_4$, 0.25; NaCl, 2.25; yeast extract, 2.0; cysteine-HCl·H_2O, 0.5; $Na_2S·9H_2O$, 0.25; trace

60 element solution SL-10, 1 mL (Chin et al. 1998); vitamin solution, 1 mL (Wolin et al.
61 1963); 0.1% (w/v) resazurin solution, 1 mL. *M. barkeri* was incubated in 650-mL sealed
62 nitrogen-filled serum bottles with a 270-mL medium volume. These serum bottles were
63 divided into several treatments according to carbon source and the concentration of
64 ammonia (Table S1). The culture bottles were inoculated with 30 mL of inoculum
65 cultivated with the corresponding carbon source and headspaces were filled with N_2.
66 The initial pH values of all treatments were controlled at 7.0 ± 0.1 and MOPS buffer
67 (final concentration of 50 mM) was added as a pH buffer. Triplicate and quintuplicate
68 incubations were conducted for blank treatment and treatment with added substrate,
69 respectively, and incubated at 37 °C with shaking (100 rpm).

70 **2.2 Chemical analysis**

71 CO_2 and CH_4 in gas samples were determined by gas chromatography (Agilent
72 7820A, USA) (Zhou et al. 2022). H_2 was analyzed by gas chromatography (Pu Chuang
73 GC 6800T, China) (Jiao et al. 2021). Acetate and methanol were measured by high-
74 performance liquid chromatography (Agilent HPLC 1200, USA) (Cheng et al. 2013).
75 Gas pressure and pH were measured by pressure gauge (Ashcroft 2084, USA) and
76 pH meter (Horiba B-712, Japan), respectively (Zhou et al. 2022). Total ammonia
77 concentration was quantified by Nessler's reagent colorimetric method following the
78 standard methods (Greenberg et al. 1992).

79 **2.3 RNA extraction and transcriptomic analysis**

80 Samples from the control treatments (0.5 g/L NH_4Cl) and treatments with high
81 NH_4Cl concentration (10 g/L NH_4Cl for acetate, 20 g/L NH_4Cl for H_2/CO_2 and
82 methanol) at multiple time points over the logarithmic period were selected for
83 transcriptomic analysis. Triplicates in each treatment were used for RNA transcripts.
84 RNA was extracted using RNAprep pure Cell/Bacteria Kit (Cat. No. DP430;
85 TIANGEN, Beijing, China). The genome of *M. barkeri* DSM 800 (RefSeq:
86 GCF_000970025.1) was downloaded from National Center for Biotechnology
87 Information (NCBI, https://www.ncbi.nlm.nih.gov/). Quality trimming of the
88 transcriptomic sequences was performed by Trimmomatic v0.36 and mapped to CDS
89 (coding sequence) using the BWA (http://bio-bwa.sourceforge.net). RNA expression
90 was calculated by transcripts per kilobase of exon model per million mapped reads
91 (TPM) (Wagner et al. 2012).

92 **2.4 Statistical analyses**

93 The ammonia effect on the general changes of gene expression was assessed by
94 principal component analysis (PCA) in R (http://www.r-project.org/), using the vegan
95 package (Mendes et al. 2008). The t-test was used to determine whether the individual
96 gene expression varies in the different treatments (Only differences between treatments
97 with the same carbon source but different ammonia concentrations for the same period
98 were shown). The absolute value of log_2foldchange >1 and p value <0.05 were used to
99 identify differentially expressed genes (DEGs). To reflect the similarities and

differences in DEGs in the different carbon source treatments, the Venn package (Mamakani et al. 2012) in R was used. Pearson correlation test was performed using R software (packages: psych (Revelle 2008)).

2.5 Calculations

The moles of H_2, CH_4 and CO_2 were calculated from the equilibrium of Eq. (1).

$$n_i = P_i * V / R * T \quad (1)$$

Where n_i is mole of i, P_i is partial pressure of i V is the volume over the bottle, R is the universal gas constant (8.314 J/K·mol) and T is the temperature in Kelvin.

Free ammonia concentrations were calculated from the equilibrium of Eq. (2) as described previously (Calli. et al. 2005).

$$[NH_3] = \frac{[TNH_3]}{1+10^{\left(0.09018+\frac{2729.92}{T(K)}-pH\right)}} \quad (2)$$

where $[NH_3]$ is the free ammonia concentration, $[TNH_3]$ is the total ammonia concentration and T(K) is the kelvin temperature.

The maximum specific methane production rate (μ_{max}) of *M. barkeri* DSM 800 was calculated from methane accumulation curve in the logarithmic period as described in detail by Zhou ((Zhou et al. 2022).

For the thermodynamic calculations, the reactions evaluated were as follows: $CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$, $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$, $4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$,

For a reaction aA + bB → cC + dD, ΔG was calculated from the equilibrium of Eq. (3) as described previously (Dolfing 2015).

$$\Delta G = \Delta G^{\theta} + RT\ln\{[(C)c(D)d]/[(A)a(B)b] \quad (3)$$

Where ΔG^{θ} values were taken from Amend and Shock (Amend 2001), R is the universal gas constant (8.314 J/K·mol) and T is the temperature in Kelvin.

3 Results

3.1 Effects of ammonia on the growth of *M. barkeri*

Incubation of *M. barkeri* with acetate, methanol, and H_2/CO_2 respectively, was performed under different NH_4Cl conditions. The physiological response of *M. barkeri* to NH_4Cl stress was dramatically different under three methanogenic carbon source conditions (Fig. 1, Table 1). No methane was detected in 15, 40 and 30 g/L NH_4Cl groups for the acetate, methanol, and H_2/CO_2 treatment, respectively (Fig. 1, Table 1). The lag period increased from 2 to 26 days for acetate treatment when the concentration of NH_4Cl was increased from 0.5 to 10 g/L (Fig. 1a, Table 1). However, the delay was only 3 days and 1 day for methanol and H_2/CO_2 treatments when NH_4Cl increased to 20 and 30 g/L, respectively (Fig. 1a, Table 1).

pH in three substrate treatments fluctuated, within an average range of 0.3 units in the methane production period (Fig. S1). For the total ammonia concentration remained essentially constant (Fig. S2), the NH_3 of different substrates calculated based on Eq. (1) (Calli. et al. 2005) varies slightly(Fig. S2). The μ_{max} decreased with increasing NH_3 concentration under all three substrate treatments (Fig. 2, Table 1), and exhibited a negative linear correlation with NH_3 concentration ($R^2 > 0.96$) (Fig. 2a, Table 2). Based on these regression equations, the theoretical maximum NH_3 tolerance for acetate, methanol, and H_2/CO_2 utilization were calculated to 39.1 ± 9.0, 104.3 ± 7.4, and 85.7

142 ± 1.0 mg/L (i.e., the NH_3 concentration where μ_{max} = 0), respectively (Fig. 2a). The
143 maximum ammonia tolerance of *M. barkeri* from methanogenic precursors was
144 H_2/CO_2 > methanol > acetate, which indicated the aceticlastic methanogenic pathway
145 was much more sensitive than hydrogenotrophic and methylotrophic methanogenic
146 pathways.

147 The ΔG values calculated during the methane accumulation stage gradually
148 increased from −65 to −30 kJ/reaction for aceticlastic methanogenesis, from −108 to
149 −73 kJ/reaction for methylotrophic methanogenesis, and from −136 to −90 kJ/reaction
150 for hydrogenotrophic methanogenesis respectively (Fig. S3a). Standard Gibbs free
151 energies are -80, -107, and -126 kJ/mol for acetate, methanol and H_2/CO_2, respectively
152 (Table S2). The order of the ΔG range of *M. barkeri* under three methanogenic
153 pathways was in agreement with the ammonia tolerance of *M. barkeri,* i.e., H_2/CO_2 >
154 methanol > acetate. Noteworthy, a positive linear relationship between the theoretical
155 maximum NH_3 tolerance for different substrates and their ΔG was observed. These
156 trends remained stable under different incubation time ranged from the initial time point
157 (t_0), to the half concentration of substrate consumed (t_{50}), or standard conditions (R^2 >
158 0.99) (Fig. 2b).

159 **3.2 Transcriptomic analysis**

160 PCA analysis results showed gene expression of different samples with the same
161 substrate and ammonia concentration at different sampling periods was clustered (Fig.
162 S4), indicating that the substrate and ammonia had significant impact on the gene

163 expression of *M. barkeri*. For treatments of acetate, methanol and H_2/CO_2, there were
164 516-546, 535-445, and 311-348 down-regulated genes and 471-398, 446-522, and 290-
165 372 upregulated genes in response to high ammonia stress at the two sampling time
166 points, respectively (Fig. S5 and S6). *M. barkeri* were more significantly affected by
167 ammonia when using acetate and methanol than when using H_2/CO_2.

168 The genes involved in individual methanogenic pathways were not specifically
169 down-regulated. Down-regulation of genes was essentially consistent between acetate
170 and methanol treatments, including all subunits of tetrahydromethanopterin S-
171 methyltransferase (*mtr*), heterodisulfide reductase subunit E (*hdr*E), and most of the
172 subunits of V-type ATP synthase (*atp*) (Fig. 3). Sodium/proton antiporter (*nha*) was
173 observed to be significantly down-regulated in both H_2/CO_2 and methanol groups (Fig.
174 3). In H_2/CO_2 treatment *atp* and *mtr* were no statistically but differentially down-
175 regulated (Fig. 3). In general, inhibition of methanogenic pathways by ammonia was
176 general rather than selective.

177 Previous studies have reported that ammonia stress causes efflux of intracellular
178 K^+ (Sprott et al. 1985, 1984, 1986). Therefore, we further investigated the expression
179 of genes involved in the K^+ transport systems. Trk system K^+ uptake protein (*trk*) and
180 glutathione-regulated K^+-efflux system ancillary protein (*kefG*) were up-regulated in
181 the presence of high ammonia in acetate treatment (Fig. 4). In methanol treatment, the
182 up-regulated genes were K^+-efflux system ancillary protein (*kefG*), K^+ channel protein
183 (*kch*), and large conductance mechanosensitive channel (*mscL*) (Fig. 4). No
184 significantly regulated gene responsible for K^+ transport was observed in H_2/CO_2

treatment (Fig. 4). According to transcriptomic results, the degree of K^+ efflux caused by ammonia was different in different treatments.

4 Discussion

We observed that the impact of ammonia stress on methanogenic activity was directly related to the active methanogenic pathway, which could not be explained by the hypotheses previously put forward in the literature, such as K^+ flux and selective inhibition of the expression of key genes involved in methanogenesis (Yu et al. 2020, Sprott et al. 1986, Kato et al. 2014, Wang et al. 2015). Sprott et al. (1986) proposed ammonia induced efflux of the cytoplasmic K^+ through an ammonia/K^+ exchange reaction. The higher the concentration of ammonia, the larger the K^+ loss, and the more severely the cells are inhibited. However, the effects of the same methanogen grown under different methanogenic pathways were significantly different, the differential response could not be fully explained by the hypothesis of K^+ loss, which has also been argued by Hendriksen et al. (1991).

Functional genes involved in different methanogenic pathways were possibly specifically inhibited (Yu et al. 2020, Kato et al. 2014). However, our transcriptomic analysis showed that ammonia repressed the expression of genes involved in different methanogenic pathways in a general rather than a specific way. The relevant genes involved in only a single methanogenic pathway were not significantly down-regulation under ammonia stress. Instead, genes *mtr*, *nha* and *atp* involved in energy metabolism of all three pathways simultaneously were significantly regulated by ammonia (Fig. 3),

206 others studies have found those genes down-regulated in response to high ammonia
207 stress by transcriptomic analysis (Kato et al. 2010, Yan et al. 2020, Xing et al. 2021,
208 Kato et al. 2014). A decrease in ATP content was also observed as ammonia
209 concentration increased (Wang et al. 2022).

210 Taking the above findings into account, the previous hypotheses to explain the
211 difference in ammonia tolerance of different methanogenic pathways had some
212 drawbacks. It was reported that variable capabilities of ammonia tolerance might
213 depend on the difference in ΔG of reactions with different substrates (Yan et al. 2020),
214 and higher energy metabolism level might be conducive to counteract ammonia stress
215 (Wang et al. 2022). However, these findings could not exclude influence of other
216 microorganisms because these results were obtained from complex microflora. We
217 cultivated a multitrophic methanogen with three methanogenic pathways to circumvent
218 this effect and demonstrated more clearly, that the toxicity of NH_3 was related to ΔG of
219 methanogenesis with corresponding precursors. ΔG is a thermodynamic potential and
220 the maximum amount of energy available from one chemical reaction (Zhang and
221 Reece 2019), which is conserved in the form of reducing power and ATP in
222 methanogens. The reducing power decreased because the intracellular NH_3 competed
223 for protons with endoenzymes that catalyzed the production of reducing power (Yu et
224 al. 2021). The latter was consumed when the cells counteracted the presence/damage
225 of NH_3 (Yan et al. 2020). Consequently, less energy was produced and more was
226 consumed, causing an energy drain. Methanogenic processes that produce more energy
227 will consequently be more resistant to ammonia stress. On the other hand, the same

228 energy was required for cell maintenance in same cell, however, the energy produced
229 by cells cultured with different substrates was different. Which meant that the ratio of
230 energy flux between maintenance and production was different for each substrate. The
231 cells with a higher relative maintenance energy contribution were more sensitive to
232 ammonia and stopped growing at a lower ammonia concentration.

233 Our findings demonstrate that, due to the influence of thermodynamic limitation,
234 the ranking of ammonia tolerance of the typical three methanogenic pathways of *M.*
235 *barkeri* is hydrogenotrophic > methylotrophic > aceticlastic. Similar results were found
236 at the community level despite the variability of species and methanogenic pathways.
237 A number of studies found that the ammonia tolerance of hydrogenotrophic and
238 methylotrophic methanogens was higher than that of aceticlastic methanogens in
239 complex microbial community systems (Lue et al. 2013, Ruiz-Sánchez et al. 2017, Lv
240 et al. 2019, Yan et al. 2020, Fujishima et al. 2000, Chen et al. 2016, Tian et al. 2018b,
241 Sun et al. 2021). Kato et al. (2014) evaluated the inhibitory effects of ammonia on two
242 different degradation pathways using a pure culture (aceticlastic methanogen) and
243 defined co-culture (hydrogenotrophic methanogen and syntrophic acetate-oxidizing
244 bacteria), the results demonstrated that aceticlastic methanogen was sensitive to
245 ammonia stress than hydrogenotrophic methanogen. Although the resistance of acetate-
246 consuming methanogens to high ammonia stress has also been reported, however these
247 aceticlastic methanogens reported were *Methanosarcina* (Fotidis et al. 2013, Hao et al.
248 2015), which are a multitrophic methanogens. Indeed, there are indications that
249 *Methanosarcina* may act as hydrogen-utilizing methanogen in microbial communities

under ammonia stress (Westerholm et al. 2012), which suggests that under duress *Methanosarcina* may shift to the thermodynamically best option. The multiple methanogenic metabolisms of *Methanosarcina* confers adaptability and contributes to its adaptation to environmental stress.

5 Conclusions

This study revealed that the ranking (highest to lowest) of ammonia tolerance of methanogenic pathways is hydrogenotrophic > methylotrophic > aceticlastic. And our results clearly showed the relationship between thermodynamic potential and ammonia tolerance, revealing the ammonia tolerance of *M. barkeri* was determined by thermodynamic restriction. The study provides a theoretical basis for a relative ammonia inhibition tolerance for H_2 vs acetate. These findings advance understanding of how ammonia inhibits methanogens, extending and refining the model of ammonia inhibition in methanogens. Further physiological and molecular biological experiments are needed to comprehensively understand ammonia inhibition in methanogenic ecosystems and beyond.

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Data Availability Statement

The raw reads of the transcriptomics sequencing are available at the NODE database (https://www.biosino.org/node/) by accession number OEP003314.

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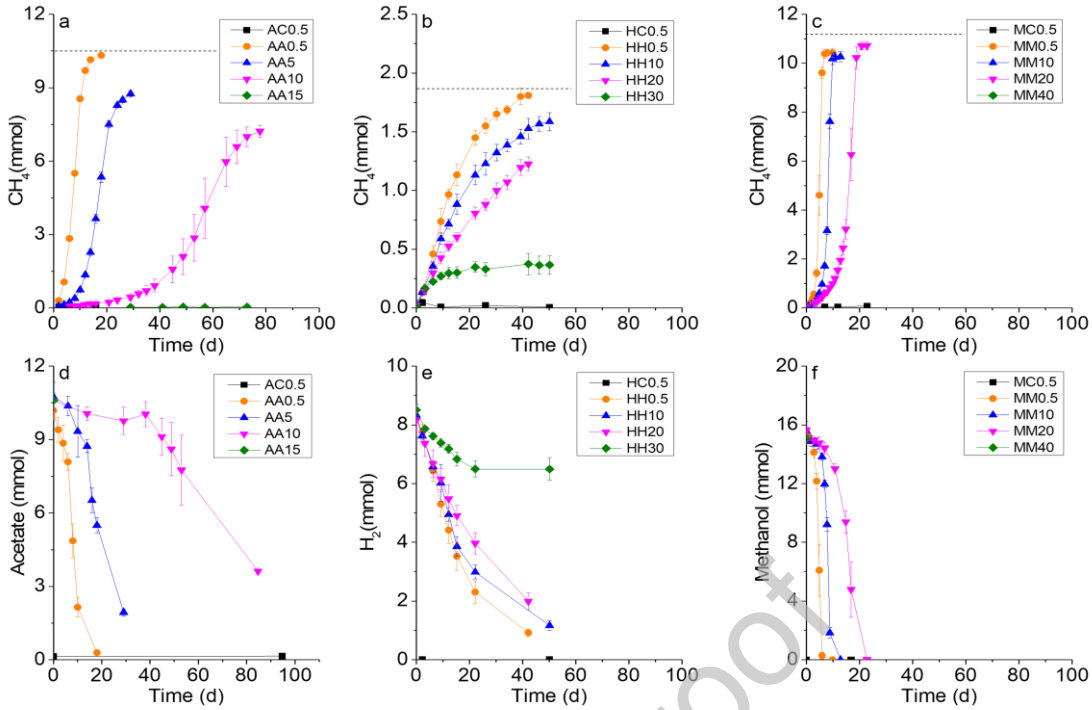


Fig. 1 Methane production and substrate consumption in treatments supplemented with different substrates and different ammonia concentration. (a and d) Acetate treatment; (b and e) H_2/CO_2 treatment; (c and f) Methanol treatment. The dashed line is the theoretical methane production when the substrate is completely consumed.

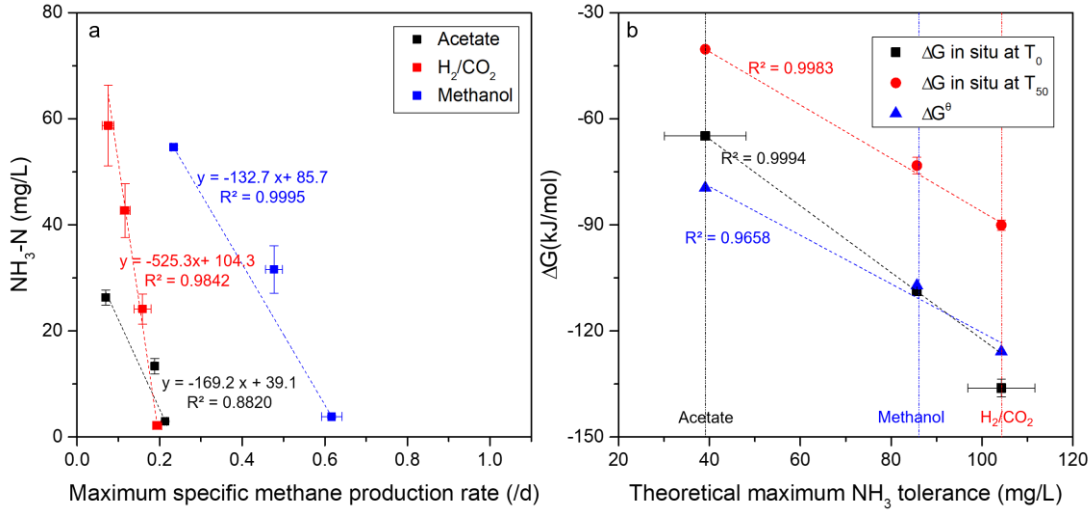


Fig. 2 Relationship between NH_3-N concentration and maximum specific methane production rate (a) and relationship between standard Gibbs free energy and theoretical maximum NH_3 tolerance (b). t_0: Initial time point, t_{50}: Time where 50% of the substrate has been consumed. The CH_4 and CO_2 concentrations at t_0 were assumed to be 0.1%.

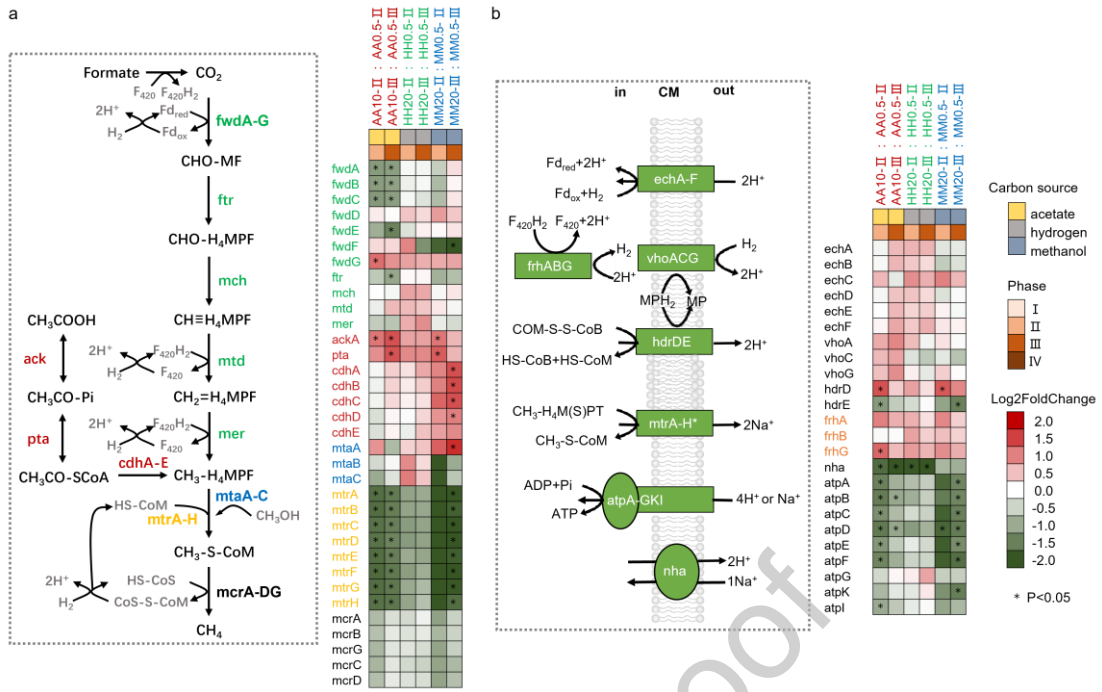


Fig. 3 Metabolic pathway and heatmap of log_2foldchange for key genes involved in methanogenesis of *M. barkeri* under ammonia stress. * : To avoid duplication, this gene was only shown in the first heatmap. Genes involved in different pathways was colour coded as follows: green (hydrogenotrophic), red (aceticlastic), blue (methylotrophic), yellow (hydrogenotrophic and aceticlastic), orange (hydrogenotrophic and methylotrophic), and blank (all pathway). a: methanogenic pathway, b: energy conservation.

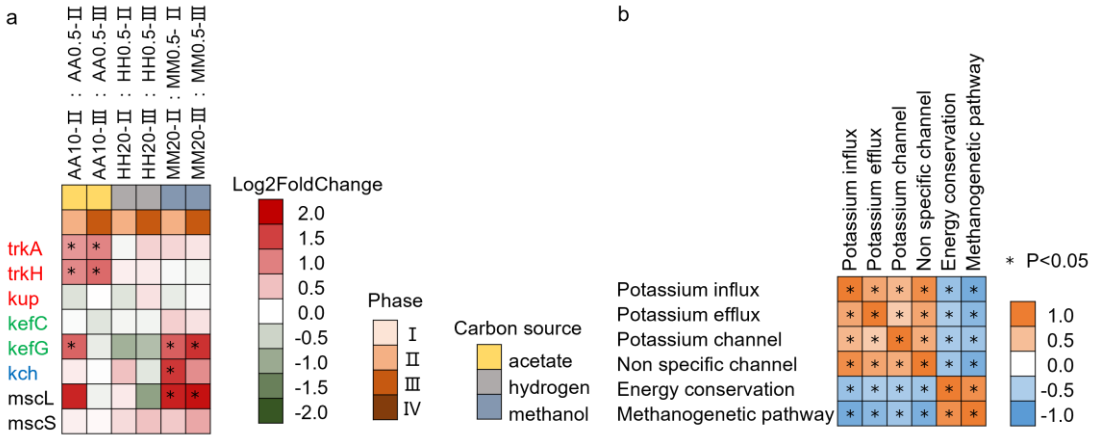


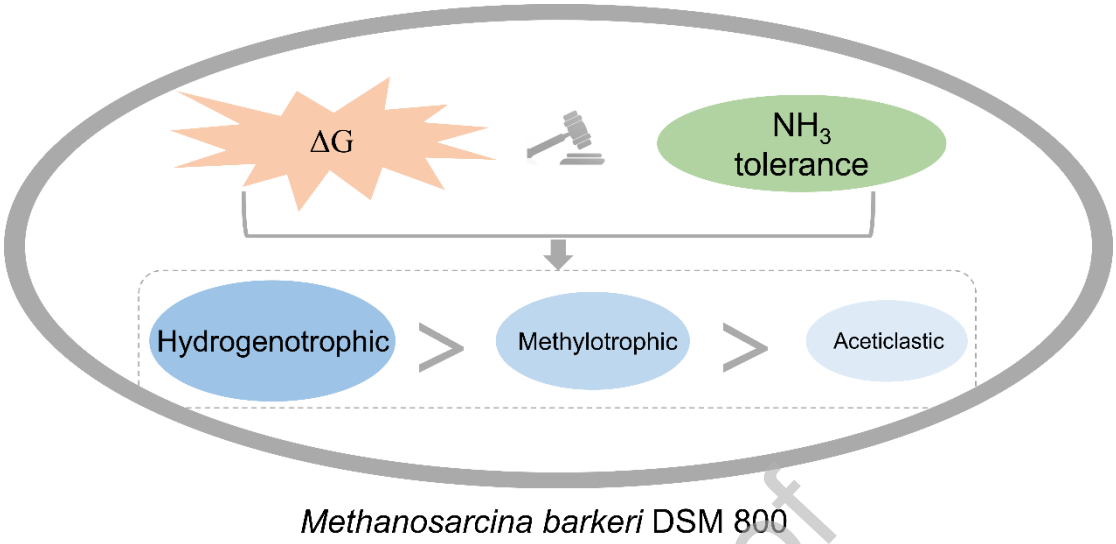
Fig. 4 Heatmap of log_2foldchange for gene involved in K^+ transport system of *M. barkeri* under ammonia stress.

Table 1 Ammonia concentration and growth properties of *M. barkeri* DSM 800

Carbon source	Treatment	NH_4Cl added (g/L)	Detected NH_4^+-N (mg/L)*	Calculated NH_3-N (mg/L)*	Lag period (d)	Maximum specific methane production rate (/d)
Acetate	AC0.5	0.5	NT	NT	NT	NT
Acetate	AA0.5	0.5	262.6 ± 8.1	3.0 ± 0.2	<2	0.21 ± 0.01
Acetate	AA5	5	1126.5 ± 65.7	13.4 ± 1.5	<8	0.19 ± 0.01
Acetate	AA10	10	2391.5 ± 61.9	26.3 ± 1.4	<26	0.07 ± 0.01
Acetate	AA15	15	NT	NT	NT	NT
H_2/CO_2	HC0.5	0.5	NT	NT	NT	NT
H_2/CO_2	HH0.5	0.5	271.1 ± 2.9	2.2 ± 0.3	<2	0.19 ± 0.01
H_2/CO_2	HH10	10	2776.8 ± 37.4	24.1 ± 2.8	<2	0.16 ± 0.02
H_2/CO_2	HH20	20	5320.1 ± 68	42.7 ± 5.1	<3	0.12 ± 0.01
H_2/CO_2	HH30	30	7204.5 ± 162.1	58.7 ± 7.6	<3	0.08 ± 0.01
H_2/CO_2	HH40	40	NT	NT	NT	NT
Methanol	MC0.5	0.5	NT	NT	NT	NT
Methanol	MM0.5	0.5	278.5 ± 6.9	3.8 ± 0.2	<3	0.62 ± 0.03
Methanol	MM10	10	2559.2 ± 42	31.6 ± 4.5	<5	0.48 ± 0.02
Methanol	MM20	20	4935.5 ± 48.4	54.6 ± 0.3	<6	0.23 ± 0.01
Methanol	MM40	40	NT	NT	NT	NT

* : Initial concentration of NH_3-N, NT: Not tested

Graphical abstract



Highlights

1. Differential ammonia tolerance of *M. barkeri* was observed under three different methanogenic pathways.
2. The Maximum NH_3 tolerances of *M. barkeri* fed with acetate, H_2/CO_2, and methanol were 39.1 ± 9.0, 104.3 ± 7.4, and 85.7 ± 1.0 mg/L, respectively.
3. Thermodynamic restrictions were the key factor determining the ammonia tolerance of the three methanogenic pathways.