Accumulation of ammonium owing to the metabolic imbalance of carbon and nitrogen might inhibit the central metabolism in *Methylomonas* sp. ZR1

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Abstract:

The metabolic intermediates of nitrogen source have been proved to have multiple functions on the metabolism of mehthanotrophs. In this study, accumulation and assimilation mechanism of the nitrate metabolic intermediate ammonium in the fast growing *Methylomonas* sp. ZR1 was analyzed. Although, nitrate salt was the best nitrogen source supporting the growth of ZR1, its metabolic intermediate ammonium would accumulate and inhibit ZR1. Kinetic studies indicated that accumulation of $NH4^+$ was deduced from the imbalance of nitrogen and carbon metabolism. Compensation of carbon skeleton α-keto-glutaramate could effectively relieve the inhibition of $NH4^+$ to ZR1, which further approved

the assumption. qPCR analysis indicated a third ammonium assimilation pathway Glycine synthesis system may function in ZR1 under high ammonium tension. In the presence of ammonium, ZR1 might employ two strategies to relieve the ammonium stress, one was assimilating the excess ammonium, and another one was cutting off the nitrogen reduction reactions. Investigation of the nitrogen metabolism and its influence to the carbon metabolism is meaningful to systematically understand and control the C1 feedstock bioconversion process in methanotrophs.

Importance:

The nitrogen metabolism in methanotrophs has long been concerned. However, there are lots of research problems yet to be solved. In this study, the accumulation and assimilation mechanism of the nitrogen metabolic intermediate ammonium in the fast growing *Methylomonas* sp. ZR1 was analyzed. Owing to the imbalance metabolism of carbon and nitrogen source, ammonium would accumulate to high concentrations to inhibit cell growth. Compensation of carbon skeleton was an effective strategy to relieve the inhibition of NH_4^+. A third ammonium assimilation pathway related genes were proved actively expressing in ZR1 when it confronted with high ammonium tension. When confronted with ammonium tension, ZR1 might employ different strategies to relieve the ammonium stress according to the edible carbon source. Revealing the endogenous ammonium accumulation mechanism and its

metabolic adjustment effect on the central metabolism of methanotrophs, was meaningful to reveal the complex coordination metabolic mechanism of nitrogen and carbon in methanotrophs.

Keywords: nitrogen metabolism, ammonium, *Methylomonas*

Introduction：

Methanotrophs are excellent C1 compounds assimilating microorganisms, and its industrial application has a long history since 1960s. At the beginning methanotrophs were used to produce single cell protein from methane or methanol. Nowadays, with the development of biotechnology, methanotrophs were genetic modified to produce lactic acid (Henard et al., 2016; Garg et al., 2018), astaxanthin (Ye and Kelly, 2012) and α-hemelune (Sonntag et al., 2015) from methane. However, the slow growth nature of methanotrophs has constrained its application in industrial biotechnology.

Efforts to optimize fermentation technologies to improve the growth rates of methanotrophs on methane have been reported over the past years (Yu et al., 2003; Park et al., 1991b; Shah et al., 1996; Xing et al., 2006; Han et al., 2009) (Myung et al., 2016). Other factors such as the addition of citrate acid (Xing et al., 2006) and adjusting the ion concentration (Leak and Dalton, 1986) were also reported to have effect in improving the growth rate of methanotrophs from methane. These studies indicated that the growth rate of methanotrophs might be result from multi-factor

combined effects. Many studies have also proved that nitrogen sources have strong effect on the growth of methanotrophs. Park et al. studied several factors including the nitrogen source affected the growth of OB3b, and found that nitrate depletion was responsible for the diauxic growth pattern in the batch cultivation of OB3b in the bioreactor. However, its growth declined much with 40 mM nitrate (Park et al., 1991). Hoefman et al. found niche partitioning among methanotrophic species, with methane oxidation activity responses to changes in nitrogen content being dependent on the in situ methanotrophic community structure (Hoefman et al., 2014). Strains have developed a complex mechanism to balance the carbon and nitrogen metabolism (Commichau et al., 2006). Recent studies have shown that many rapidly proliferating cells are dependent on the nitrogen metabolic intermediate such as serine and glutamine (Yang and Vousden, 2016). These studies indicated that nitrogen and its metabolic intermediate might have multiple functions on the central metabolism of methanotrophs, and subsequently affected its growth.

In methanotrophs, the nitrogen metabolism has long been concerned. 1983, Dalton et al. studied ammonia assimilation in the type X methanotroph *Methylococcus capsulatus* Bath, type I methanotroph, *Methylomonas methanica* S1, and the type Ⅱ methanotroph, *Methylosinus trichosporium* OB3b, and found that, Bath and S1 possess both the glutamine synthase/ glutamine 2-oxo-glutarate amino transferase

(GS-GAGOT) and alanine dehydrogenase (ALAD) pathways for the assimilation of ammonia, but operated according to the nitrogen source (Murrellt and Dalton, 1983). Loginova NV et al. (Loginova et al., 1982) studied enzymes involved in ammonium assimilation by 15 bacterial strains of different taxonomy, and found that bacteria were found to differ in the enzymes for ammonium assimilation according to the pathways of primary C1-metabolism. Nyerges (Nyerges, 2008) assessed the differerences in ammonia co-metabolism among four methanotrophs isolates, found the investigated strains exhibited different levels of ammonia and hydroxylamine oxidation, and inhibition of methane-oxidizing activity by ammonia and nitrite. Dam et al. found that ammonium induces differential expression pattern of methane and nitrogen metabolism-related gene in *Methylocystis* sp. strain SC2 (Dam et al., 2014). Kits et al. found that in *Methylomonas denitrifican*, sp. nov. type strain FJG1, methane oxidation could couple to nitrate reduction under hypoxia (Kits et al., 2015). These results revealed that nitrogen metabolism might play an important role in the global metabolism of methanotrophs, and nitrogen metabolism mechanism might be distinct among methanotrophs species.

In this study, the nitrogen metabolism during the cell growth of *Methylomonas* sp. ZR1 were studied at kinetic and gene expression level. It was found that, ZR1 might employ different nitrogen metabolic pattern

111 according to the ediable carbon source, the nitrogen metabolic
112 intermediate ammonium which was also identified as a growth inhibiter
113 was found to accumulate when nitrogen sources were relative surplus in
114 comparison with carbon sources. Carbon skeleton supplementation was
115 found to be an efficient strategy to relieve the inhibition effect of
116 ammonium on the growth of ZR1. qPCR analysis of the carbon and
117 nitrogen metabolic key gene indicated the gene expression diversity when
118 ZR1 was under the ammonium tension condition, and a third ammonia
119 assimilation pathway were found highly expressed with methane as
120 carbon source. All these results further indicated that ammonium might
121 have multidimensional effect on the central metabolism of ZR1.

122 **Material and Methods**

123 **Strains and culture method**

124 *Methylomonas* sp. ZR1 was isolated by our group and deposited in China
125 General Microbiological Culture Collection Center with the accession
126 number CGMCC No. 9873. It can be cultivated using liquid or solid
127 mineral medium with methane or methanol as the growth substrate. The
128 medium used for ZR1 cultivation was nitrate mineral salts (NMS)
129 medium (Whittenbury et al., 1970). For nitrogen source screening, NMS
130 medium without nitrate salt was added with 1 g/l KNO_3, 1 g/l $NaNO_3$, 0.5
131 g/l NH_4Cl, 0.5 g/l $(NH_4)SO_4$, 0.5 g/l urea, 1 g/l trypton or 1 g/l yeast
132 extract separately. Samples having no nitrogen substate were performed

as control. To test the ammonium inhibition effect, ammonium chloride at different concentration were added into the NMS medium with or without 1 g/l KNO_3. And for carbon skeleton compensation test, 0.1 g/l ammonium chloride with 0.3 g/l α-ketoglutaramate （α-KG）, 0.4 g/l glutamate, 0.3g/l malic acid, or 0.3g/l pyruvate were simultaneously added into the NMS medium. ZR1 was cultured in flask or bubble column reactor according to the method described by Guo et al. (Guo et al., 2017). When using methanol as carbon source, the initial methanol concentration was 6 g/l without specific instruction. When using methane as carbon source, ZR1 was cultured in bubble column reactor for the ammonium accumulation study; and cultured in flask with gas refreshing every 12 hours for the nitrogen source screening, ammonium inhibition test and carbon skeleton replenishing study.

Total nitrogen and ammonium concentration analysis method

Total nitrogen concentration in the fermentation broth was analyzed using the TOC/TN analyzer Multi N/C 2100s (Analytik Jena AG, German). Fermentation broth was first centrifuged, and the supernatant were diluted using ddH_2O into suitable concentration for the analysis.

Ammonium was analyzed using the indophenol blue reaction according to the method described by Xie et al. (Xie et al., 2005). The fermentation broth was first centrifuged, and 0.1 ml of the supernatant were added with 0.5ml reaction solution 1 (3.5 g phenol and 0.04 g sodium nitroprusside

in 100 ml ddH_2O), and 0.5 ml reaction solution 2 (1.8 g sodium sodium hydroxide and 4.0 mmol sodium hypochlorite in 100 ml ddH_2O). The mixture was maintained at 37℃ for 1 hour, and the absorbance of the solutions was read on a spectrophotometer at 625 nm. The concentration of the ammonium in the samples was calculated according to the calibration curve established using ammonium chloride.

Methanol concentration

Methanol concentration in the fermentation broth were measured by a GC (GC9790, Fuli Instrument, China) equipped with a flame ionization detector (FID) and a capillary column (0.25μm, 60m×0.25mm, 7KG-G013-11 Zebron^{TM}, Phenomenex).

Kinetic analysis of the carbon and nitrogen metabolism to cell growth

The growth, carbon and nitrogen assimilation curve were first fitted using the logistic model in Origin 9.0. Then the simulated curve were took derivative with respect to OD_{600} of ZR1 using the mathematic module of origin 9.0.

qPCR analysis of expression level of the genes concerns carbon and ammonium metabolism

Strain ZR1 was initially grown up to log phase (OD_{600} achieved 0.8) in 50 ml of NMS medium in a 250 ml bottle fitted with butyl rubber septum and with 20% methane (v/v) in the headspace. For methanol as carbon sources, strains were cultured with 6 g/l methanol in 50 ml NMS medium

177 in 250 ml flask. For preparing ammonium inhibition samples, 0.1 g/l of
178 NH_4Cl were then added into the medium. For carbon compensation
179 samples, 0.1 g/l of NH_4Cl and 0.3 g/l of α-keto-glutaramate （α-KG）
180 were added, and samples without any operation were performed as
181 control. All samples were incubated for 2 hours, then, cells were collected
182 by centrifugation and washed with TE buffer. RNA was extracted
183 immediately using the RNAprep Pure Cell/Bacteria Kit (Tiangen Biotech
184 (Beijing) CO., Ltd), according to the product instructions. The extracted
185 RNA was used as template to construct cDNA using the Takara Prime
186 Script RTreagent Kit with gDNA Eraser (Perfect Real Time). Then genes
187 were amplified using the SYBR Premix EX Taq kit, using Applied
188 Biosystems 7500 fast Real-Time PCR system. Standard housekeeping
189 gene 16S rDNA was selected as internal control gene. qPCR data
190 obtained were analyzed using the method described by Pfaffl (Pfaffl,
191 2001).
192 Primers used are listed in table 1.
193 Table 1. Primers designed for qPCR

Gene	Forward primer	Reverse primer	Product length bp
16s rDNA	ATGCAAGTCGAACGGTAGCA	AGGGCGTATGCGGTATTAGC	125
*gln*A	TGGCGTTTTCTGCCATGTTG	ACGAGTCAAGAATTCGCGGT	185
*glt*B	CGCTCTGGTTAAATCGCACG	CAACGGGATGTTGCGTTTGT	143

*ala*D	CAACAAGCATTGCGGGACAA	TCAGCTCAGAAACTGCTCCG	129
*gcv*T	GCAGTTTCTCGCCGAGTCTA	GTAACGGTGCGGAACGAAAG	149
nir	ACCTGGATAGATGTGTGCGG	CCGCCTATGTCGCCTATCAT	185
nif	TTGCGACCCTAAAGCCGATT	TTTAACGTCGCGGTAACCCA	142
fae	GCACTATCCCTGCTGACGAA	GCGATAGCTTCTTTGGTGGC	124
hps	CACCGGTTTGGATGCACAAG	CGCCATAGATAGCAGCACCA	176
pdh	AACAGCAAATGCGTTGTCCC	CGGCAAATCACCGCCTTTAG	146
cs	ATGACCAGGACCGCGTTATC	GGGTAGCCCCGATACAACAG	193

All culture conditions were performed in triplicate biological replicates, and for qPCR analysis, each biological sample was carried out in triplicate. Raw data of the qPCR result were shown in supplementary Table S1.

Result and discussion

1. Nitrogen source effect for the growth of ZR1 from methane and methanol.

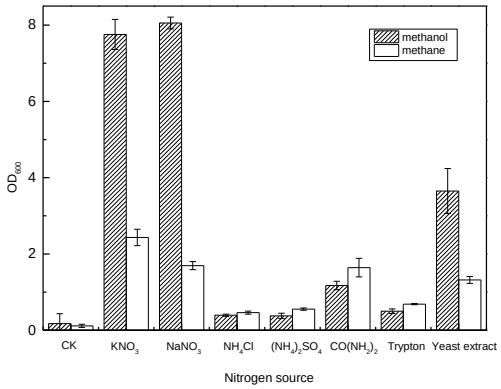

Figure. 1 Growth of ZR1 in the presence of different nitrogen substrate

from methane and methanol

Seven categories of nitrogen substrate were tested for the growth of ZR1 from both methane and methanol (Figure 1). According to Figure 1, nitrate salts were the best nitrogen sources supporting the growth of ZR1 from both methanol and methane among the tested compounds. And the growth of ZR1 from both methanol and methane with ammonium salts was much weaker than from nitrate salts. Meanwhile, the effects of the nitrogen sources on the growth of ZR1 from methane and methanol were somewhat different. With methane as carbon sources the cell density ($OD6_{00}$) of ZR1 achieved 2.5 with potassium nitrate, 1.8 with sodium nitrate, 1.8 with urea, 1.5 with yeast extract, 0.5 with tryptone, and 0.34 with ammonia chloride, 0.35 from ammonia sulfate. With methanol as carbon sources the cell density of ZR1 achieved 8.2 from sodium nitrate, 7.9 from potassium nitrate, 3.9 from yeast extract, 1.8 from urea, 0.4 from tryptone, 0.35 from ammonia chloride, and 0.33 from ammonia sulfate. It can be seen that nitrate salt was the most suitable nitrogen source and ammonia salt could not effectively support the growth of ZR1 from methane and methanol. It was generally regarded that ammonium radicals were the competitive inhibitor of particular methane monooxygenase (pMMO) (He et al., 2017; Hu and Lu, 2015; Dam et al., 2014; Nyerges et al., 2010; Nyerges and Stein, 2009; Nyerges, 2008; Dunfield and Knowles, 1995; Schnell and King, 1994; Carlsen et al., 1991; Murrellt

and Dalton, 1983), which will resulted in the lower growth of strains from methane. However, this study also proved that ammonium would hinder the growth of ZR1 from methanol, although its supposed competent object pMMO is not the key enzyme for the metabolism of methanol. It means that ammonium might have other inhibition effects for the growth of ZR1, besides its competitive inhibition effect to pMMO.

2. Effect of nitrate concentration on the growth of ZR1 from methane and methanol

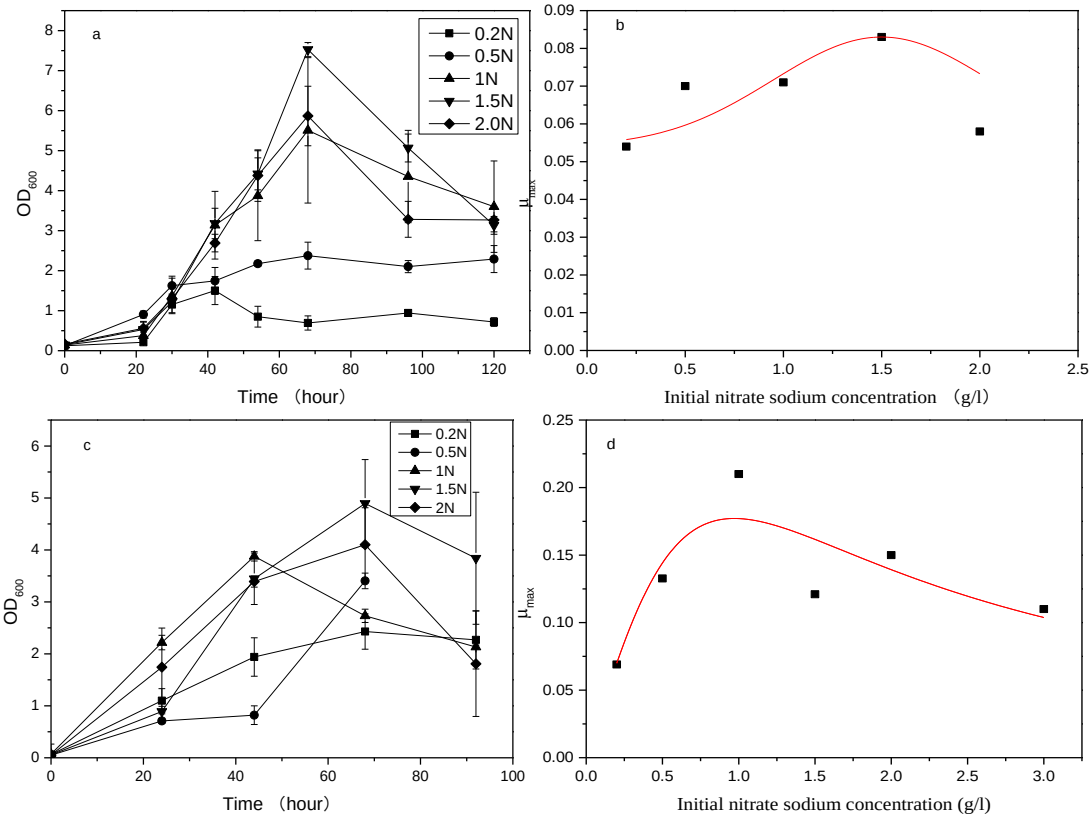

Figure 2. Effect of nitrate concentration on the growth of ZR1 from methane and methanol a, growth of ZR1 with different initial nitrate sodium concentration from methanol; b, specific growth rate of ZR1 with different initial nitrate concentration from methanol; c, growth of ZR1

with different initial nitrate sodium concentration from methane; d, specific growth rate of ZR1 with different initial nitrate concentration from methanol;

According to Figure 1, nitrate salts were supposed to be the most suitable nitrate source that supporting the growth of ZR1 from both methane and methanol, and effects of potassium nitrate concentration on the growth of ZR1 were investigated subsequently. According to Fig. 2, the obvious substrate inhibition effects of nitrate salts on the growth of ZR1 were investigated. The highest cell density of ZR1 achieved 7.8 with 1.5 g/l potassium nitrate （Figure 2a）. Meanwhile, Figure 2b indicated that the growth rate of ZR1 is first ascend with the increase in the substrate level and approaches a maximum value at 1.5 g/l of potassium nitrate. Then, a subsequent increase in the nitrogen concentration leaded to a decrease in the specific growth rate of ZR1. The situation with methane as carbon sources is similar to that of methanol. The highest cell density of ZR1 achieved 4.8 with 1.5 g/l potassium nitrate （Figure 2c）, however the specific growth rate of ZR1 achieved 0.23 with 1 g/l of potassium nitrate (Figure 2d). It has been reported that growth of type Ⅱ methanotrophs OB3b were also inhibited by 40 mM nitrate (4 g/l)(Park et al., 1991a). These phenomenon indicated that nitrate as nitrogen source when its concentration achieved a certain value might inhibit the growth of methanotrophs. According to the substrate inhibition theory (Muchandani

and Luong, 1989), an increase in the substrate concentration could cause an alteration in the cell metabolism such as an overproduction of a molecule by one pathway which results in the feedback inhibition of a second related pathway. Thus the nitrate metabolism pathway of ZR1 was further explored.

3. The formulation of ammonium, and its inhibition effect to ZR1

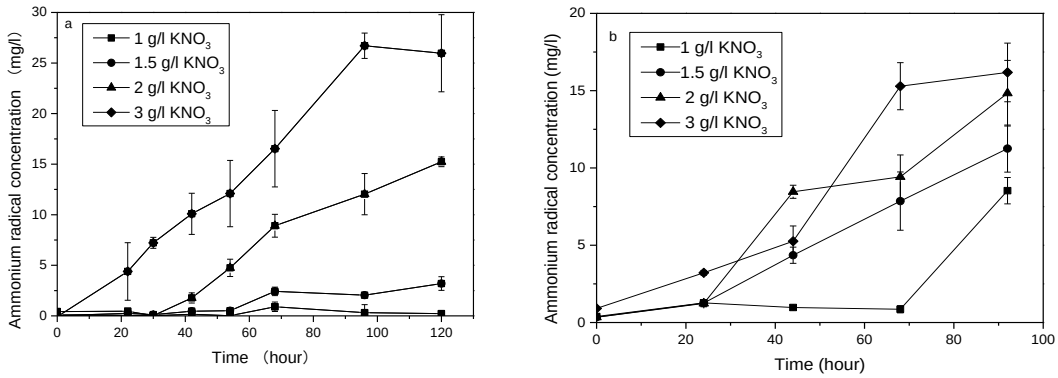

Figure.3 Ammonium accumulation of ZR1 in the fermentation broth with different initiate nitrate concentration from methanol and methane

Considering the nitrate metabolism pathway of methanotrophs, nitrogen metabolism intermediate NH_4^+ was supposed to be accumulated during the process. Thus ammonium concentration in the fermentation broth was analyzed during the fermentation process of ZR1 from methane and methanol. According to Figure 3, the concentration of NH_4^+ in the fermentation broth grew higher and higher during the fermentation process, which indicated that NH_4^+ might accumulated accompanied with the growth of ZR1 when excess nitrate were supplied. With the increasing concentration of nitrogen source, the starting time of NH_4^+ accumulation

has been moved up. Meanwhile the final concentration of NH_4^+ increased with the increase of the initial concentration of KNO_3. With 3 g/l of KNO_3, the final accumulated NH_4^+ achieved 25 mg/l with methanol as carbon sources and 16 mg/l with methane as carbon source. According to Fig. 1, NH_4^+ was supposed to be an inhibitor of the growth of ZR1. The accumulated NH_4^+ during the growth process of ZR1 might inhibit the growth of ZR1. Thus the effect of NH_4^+ on the growth of ZR1 was further analyzed.

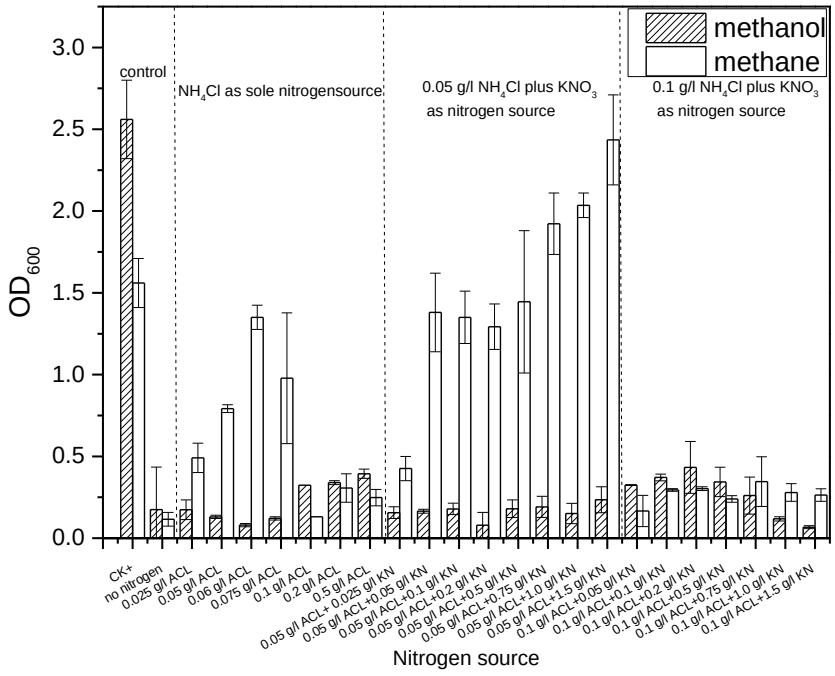

Figure. 4 Effect of Ammonium on the growth of ZR1 from methane and methanol.

ACL, NH_4Cl; KN, KNO_3;

Growths of ZR1 with NH_4Cl as sole nitrogen source or with both NH_4Cl and KNO_3 at different concentrations were investigated. It was found that,

when NH_4Cl were used as the mono-nitrogen sources, cell growth of ZR1 first rise up with the going up of the NH_4Cl concentration, the highest cell growth achieved 1.5 when NH_4Cl achieved 0.05, and then growth of ZR1 from methane decreased when the initial NH_4Cl concentration growing higher, and OD_{600} of ZR1 with NH_4Cl at 0.1-0.5 g/l only achieved 0.42, much lower than the control (with 1 g/l KNO_3 as nitrogen source). When 1 g/l of KNO_3 with addition of 0.05 g/l of NH_4Cl was used as nitrogen sources, the highest cell density of ZR1 from methane achieved 2.5, much higher than control. While the highest cell density of ZR1 from methanol only achieved 0.42 under the same condition. When 1 g/l of KNO_3 with addition of 0.1 g/l of NH_4Cl (NH_4^+ 34 mg/l, 1.86 mmol/l) was used as nitrogen sources, the highest cell density of ZR1 only achieved 0.7 with methane and 0.8 with methanol. These results showed that, with the existence of KNO_3, growth of ZR1 from methane and methanol was inhibited by NH_4Cl at concentration higher than 0.1 g/l. Growth of ZR1 from methane could resist with 0.05 g/l of NH_4Cl (0. 93 mmol/l), and were strictly inhibited with 0.1 g/l of NH_4Cl (1.86 mmol/l), while growth of ZR1 from methanol were totally inhibited by 0.05 g/l of NH_4Cl, which indicated that growth of ZR1 from methanol is more sensitive to NH_4^+ than that from methane. And according to Figure 3 the final accumulated NH_4^+ with initial higher concentration of nitrate salts could achieve 25 mg/l (0.89 mmol/l), which was high enough to inhibit the growth of ZR1

331 from methanol.

332 It was generally regarded that ammonium radicals were the competitive
333 inhibitor of pMMO (He et al., 2017). In this study, ammonium was also
334 identified to inhibit the growth of ZR1 from methanol and accumulate
335 during the growing process. Recently, ammonium was reported to have
336 effects on global gene expression of methane and nitrogen
337 metabolism-related gene in methanotrophs (Dam et al., 2014). Being the
338 nitrogen metabolic intermediate, accumulation of ammonium may have
339 multiple effects on the growth of methanotrophs. Revealing its
340 accumulation and assimilation mechanism is meaningful to understand
341 the metabolism mechanism of methanotrophs.

342 4. Carbon and nitrogen metabolic kinetic analysis of ZR1 from methanol

343 According to Figure 4, concentration of the accumulated NH_4^+ was
344 proportional to the initial nitrogen concentration in the fermentation broth.
345 And the accumulation of NH_4^+ might be result from the high initial
346 nitrogen source concentration (in another words the lacking of the carbon
347 skeleton). Thus, co-metabolism of the carbon and nitrogen and
348 accumulation of ammonium in ZR1 with methanol as carbon source was
349 further analyzed.

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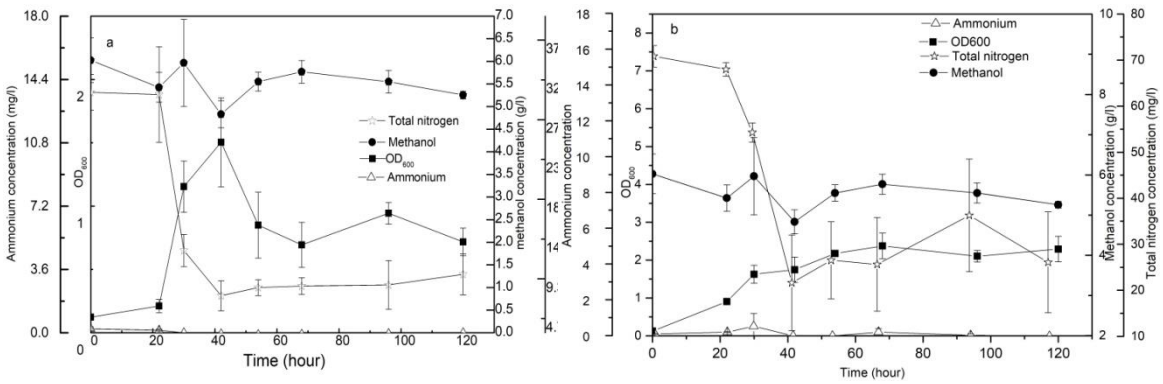


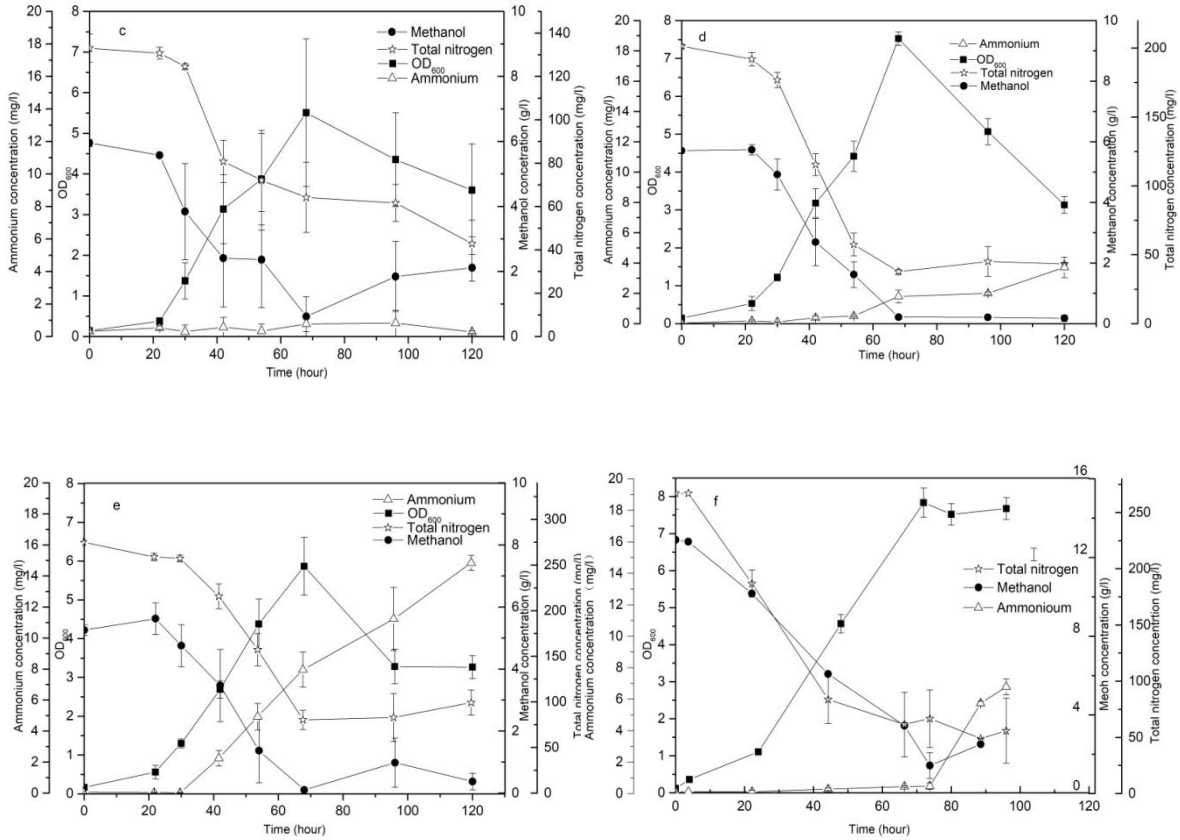


Figure 5. Kinetic analysis of the carbon and nitrogen metabolism of ZR1 from methanol. a, 6 g/l methanol and 0.2 g/l nitrate sodium; b.6g/l methanol and 0.5g/l nitrate sodium; c. 6 g/l methanol and 1 g/l nitrate sodium; d. 6 g/l methanol and 1.5 g/l nitrate sodium; e. 6 g/l methanol and 2 g/l nitrate sodium; f. 12g/l methanol and 2g/l nitrate sodium

According to Figure 5, it can be found that with methanol under the same concentration of 6 g/l, ammonium accumulation increased with the increscent of nitrogen concentration. Nonetheless, increasing the carbon sources to 12 g/l (Figure 5e), the accumulated ammonium will subsequently decrease. These results indicated that ammonium accumulation was deduced from the imbalance of the carbon and nitrogen

metabolism. And according to the nitrogen test result (Figure 1, Figure 3), the accumulated ammonium could achieve 25-30 g/l which was high enough to inhibit the growth of ZR1 from methane and methanol as carbon sources.

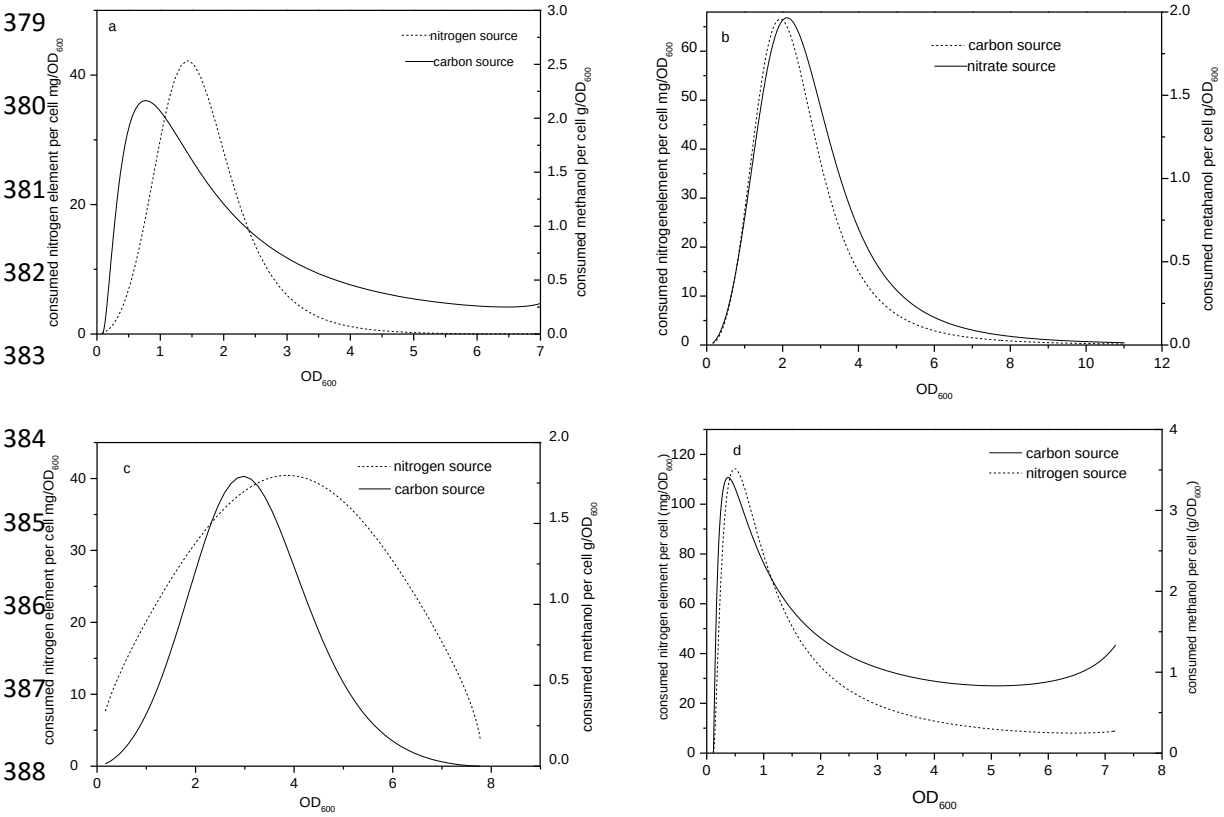


Figure 6. Kinetic analysis of the carbon and nitrogen metabolism to cell growth a. 6 g/l methanol and 1g/l nitrate sodium; b.6g/l methanol and 1.5g/l nitrate sodium; c. 6 g/l methanol and 2 g/l nitrate sodium; d. 12g/l methanol and 2g/l nitrate sodium

To further compare the carbon and nitrogen metabolism pattern, specific consuming rate of the carbon and nitrogen during the growth process of ZR1 were further analyzed. According to Figure 6, it can be seen that the specific uptake rates of nitrogen and carbon dynamically changed with

the nitrogen-carbon ratio (NCR). High NCR will result in delayed carbon consuming, and the carbon and nitrogen uptake will keep in step when NCR fall into a suitable value.

5. Relieving of ammonium inhibition effect by the carbon metabolites

According to the nitrogen sources metabolic pathway (Murrellt and Dalton, 1983; He et al., 2017; Nyerges, 2008a), methanotrophs mainly assimilate ammonium through the glutamine synthase/ glutamine 2-oxo-glutarate amino transferase (GS-GAGOT) and alanine dehydrogenase (ALAD) pathways. To further confirm that accumulation of NH_4^+ was derived from the shortness of the carbon skeleton, several carbon metabolic mediates pyruvate, malic acid, citrate acid, α-KG which are related with the nitrogen metabolism were added to the ammonium accumulation samples (Figure. 7). According to Figure 7, α-KG and glutamate were the most effective carbon metabolic mediates in relieving of the NH_4^+ inhibition effect with methane as carbon sources. However，with methanol as carbon source only α-KG could effectively relieve the inhibition effect of NH_4^+ on the growth of ZR1. Carbon metabolic mediates upstream or downstream of α-KG, has small relieving effect on NH_4^+ inhibition. It was also found that, direct supply of carbon skeleton such as pyruvate, citric acid, α-KG, glutamate or malic acid have somewhat inhibit effect on the growth of ZR1 from methanol. Meanwhile, α-KG and glutamate were found to have stimulate effect on

the growth of ZR1 from methane.

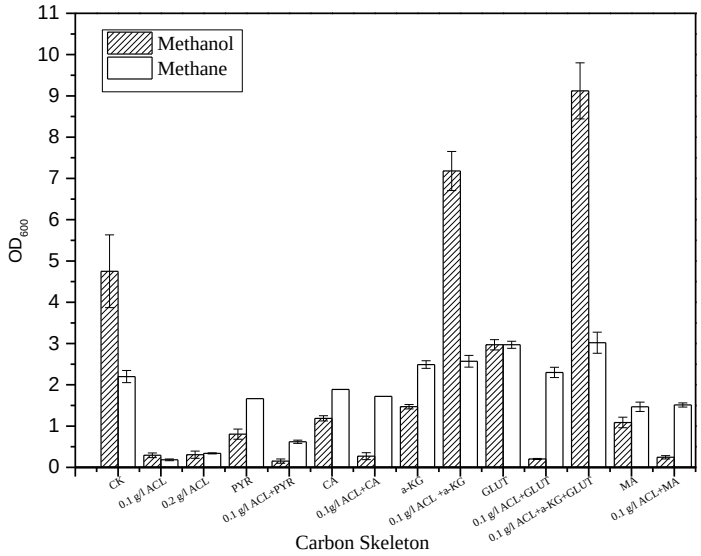


Figure 7. Relieve Effect of the carbon skeleton to ammonium inhibition

CK: control; ACL, NH_4Cl; PYR, pyruvate; CA, citrate acid; α-KG, α-ketoglutaramate ; GLUT, glutamate; MA, malic acid;

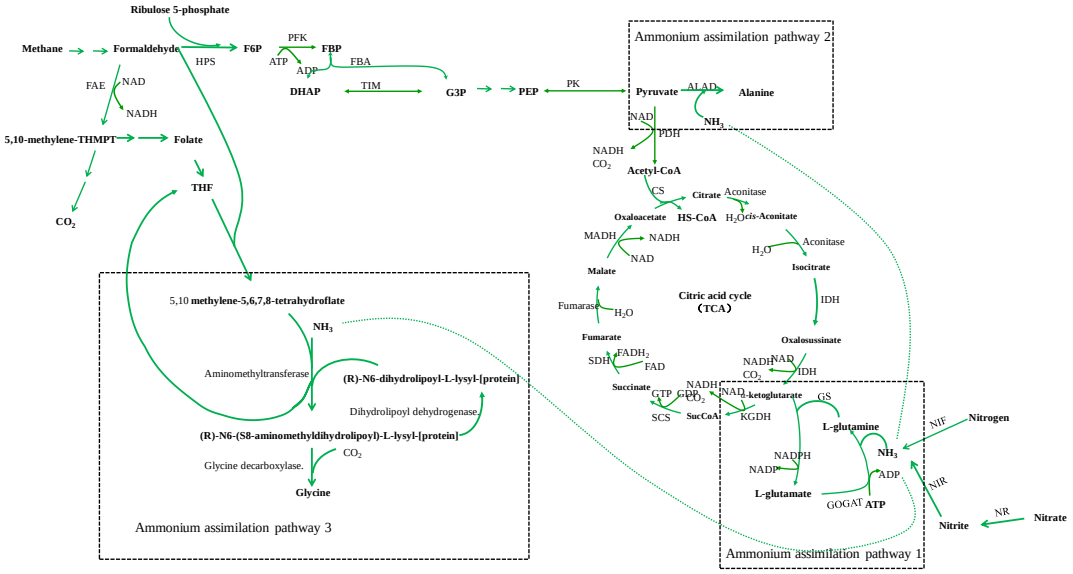
It means that, ZR1 may utilize different carbon and nitrogen metabolic mechanism to balance the carbon and nitrogen metabolism. Nonetheless, α-KG play more important role in assimilation of the nitrogen metabolic intermediate ammonium.

6. Transcript level analysis the carbon and nitrogen metabolism in ZR1 with the accumulation of ammonium

According to the nitrogen metabolism pathway of microorganisms, many studies indicated that, ammonia is a competitive inhibitor of pMMO, which deduced the inhibition effect of methanotrophs from methane. However, this study also identified the inhibition effect of NH^+_4 on ZR1 with methanol as carbon source. So, besides the competitive inhibition

441 effect of MMO, NH_4^+ as a nitrogen metabolism intermediate may have
442 multiple complex effects on the metabolic pathway of methanotrophs.
443 Thus transcript level analysis of the genes relevant with carbon and
444 nitrogen metabolism in ZR1 was carried out by qPCR.

445 Ammonium mainly transferred to the C skeletons of pyruvate or
446 intermediates of citric acid cycle (glycogenic) and others to form amino
447 acid. Most amino acid participate in transamination reactions with TCA
448 cycle mediate such as oxaloacetate, or α-ketoglutarate to form aspartate,
449 or glutamate, respectively, and the α-KG corresponding to the original
450 ammonium assimilation. Based on the genomic analysis result of ZR1,
451 besides the ALAD and GS/GOGAT pathway, another special ammonium
452 assimilation mechanism the Glycine synthase system may exist and
453 function in methanotrphs(Figure 8). In this study, the GS-GAGOT,
454 ALAD, and Glycine synthesis system on behalf of three main pathways
455 of the ammonium assimilation were studied by qPCRT, the data quality of
456 the qPCR result was presented in supplementary Table 1.
457 E-supplementary data of this work can be found in online version of the
458 paper. The relative expression fold of the target genes was listed in Table
459 2.



460

461 Figure 8. Nitrogen metabolism pathway of ZR1

462 HPS:3-hexulose-6-phosphate synthase, DHAP, Dihydroxyacetone phosphate; F6P,
463 Fructose-6-phosphate; FBP, Fructose- 1, 6 –bisphosphate; G3P, Glyceraldehyde
464 3-phosphate; PEP, Phosphoenolopyruvate; SucCoA, Succinyl-CoA; TIM,
465 Triosephosphate isomerase; GAPD, Glyceraldehyde 3-phosphate dehydrogenase; PK,
466 pyruvate kinase; PFK , Phosphofructose kinase; CS, Citrate synthase; IDH, Isocitrate
467 dehydrogenase; KGDH, Ketoglutarate dehydrogenase; SCS, Succinyl-CoA synthetase;
468 SDH, Succinate dehydrogenase; MDH, Malate dehydrogenase; GS, Glutamine
469 synthetase; GOGAT, Glutamate synthase; cd1 NIRs, Cytochrome cd1 nitrite reductase;
470 ALAD, Alanine dehydrogenase; NIF, Nitrogenase; FAE, Formaldehyde activating enzyme;

471 Nitrogen assimilation pathway 1:

472 $$(1)\mathrm{NH_4^+ + ATP + L-glutamate \xrightarrow{GS} L-glutamine + ADP + Pi + H^+}$$

473 $$(2)\mathrm{NADH + \alpha KG + L-glutamine + H^+ \xrightarrow{GOGAT} 2 \times L-glutamate + NAD}$$

474 $$(1)+(2)\quad \mathrm{NH_4^+ + NADH + \alpha KG + ATP \xrightarrow{GS-GOGAT} L-glutamate + ADP + Pi + NAD^+}$$

475 Nitrogen assimilation pathway 2:

476 $$NH_4^+ + \text{pruvate} + NADH \xrightarrow{ALD} L-\text{alanine} + H_2O + NAD^+$$

477 Nitrogen assimilation pathway 3:

$$(1)\ (6R)-5,10-\text{methylene}-5,6,7,8-\text{tetrahydrofolate}+(R)-N^6-\text{dihydrolipoyl}-L-\text{lysyl}-[\text{protein}]+NH_4^+ \longleftrightarrow (6s)-5,6,7,8-\text{tetrahydrofolate}+(R)-N^6-(S^8-\text{aminomethyldihydrolipoly})-L-\text{lysyl}-[\text{protein}]$$

$$(2)\ (R)-N^6-(S^8-\text{aminomethyldihydrolipoyl})-L-\text{lysyl}-[\text{glycine}-\text{cleavage complex H protein}]+CO_2 \longleftrightarrow \text{glycine}+H^+ + N^6-\text{lipolyl}-L-\text{lysyl}-[\text{glycine}-\text{cleavage complex H protein}]$$

$$(3)\ (R)-N^6-\text{lipoyl}-L-\text{lysyl}-[\text{protein}]+H^+ + NADH \longleftrightarrow (R)-N^6-\text{dihydrolipoyl}-L-\text{lysyl}-[\text{potein}]+NAD^+$$

$$(1)+(2)+(3)\ (6R)-5,10-\text{methylene}-5,6,7,8-\text{tetrahydrofolate}+NADH+NH_4^+ + CO_2 \longleftrightarrow \text{glycine}+NAD^+ + (6s)-5,6,7,8-\text{tetrahydrofolate}$$

478

479 Direction 1 is catalyzed by two enzymes, glutamine synthase and
480 glutamine 2-oxo-glutarate amino transferase. Direction 2 is catalyzed by
481 the alanine dehydrogenase. Direction 3 is catalyzed by the Glycine
482 synthesis system.

483 Table 2. Relative expression level of the genes concerning nitrogen and
484 carbon metabolism

		Mean fold change in gene expression of samples						
genes	**protein**	$N_{control}$	N_{ACL}	N_{NCL-KG}	$M_{control}$-$N_{control}$	$M_{control}$	M_{ACL}	M_{ACL-KG}
*gln*A	Glutamine synthase	1	1.92	15.73	0.69	1.00	0.89	1.34
*glt*B	Glutamate synthase large subunit	1	7.48	12.64	42.38	1.00	0.45	0.12
*ala*D	Alanine dehydrogenase	1	30.84	17.84	25.07	1.00	0.48	0.08
*gcv*T	Aminomethyltransferase	1	19.08	2.96	1.56	1.00	0.95	2.17
nir	Nitrite reductase	1	8.74	1.93	2.01	1.00	0.48	0.92

nif	Nitrogenase	1	4.59	4.14	2.15	1.00	0.50	0.92
fae	Formaldehyde activating enzyme	1	4.32	2.94	2.99	1.00	1.07	1.04
hps	3-hexulose-6-phosphate synthase	1	13.30	2.53	5.70	1.00	1.42	0.75
pdh	Pyruvate Dehydrogenase	1	1.66	1.35	0.05	1.00	1.88	2.06
cs	Citrate synthase	1	1.15	1.52	8.32	1.00	1.24	1.21

485 $N_{control}$, Samples cultured with methane; N_{ACL}, Samples cultured with
486 methane under 0.1 g/l NH_4Cl condition; $N_{ACL\text{-}KG}$, Samples cultured with
487 methane under 0.1 g/l NH_4Cl and 0.3 g/l α-KG; $M_{control}$, Samples
488 cultured with methanol; M_{ACL}, Samples cultured with methanol under 0.1
489 g/l NH_4Cl condition; $M_{ACL\text{-}KG}$, Samples cultured with methanol under 0.1 g/l
490 NH_4Cl and 0.3 g/l α-KG;

491 According to Table 2, with methane as carbon source, when 0.1 g/l
492 ammonium accumulated, the *ala*D and *gcv*T gene expression levels were
493 30.84 and 19.08 times higher than the control, while the genes *gln*A and
494 *glt*B which responsible for the assimilation of NH_4^+ with the C_5 substrate
495 expressed 1.92 and 7.48 times higher than that of the control. This
496 indicated that ZR1 may rely more on the C_3 carbon skeleton to assimilate
497 the extra NH_4^+. Another interesting phenomenon is the high expression
498 level of *gcv*T gene (19.08) when ZR1 confronted with 0.1g/l of NH_4Cl
499 with methane as carbon source and its expression level returned to 2.96

when the C5 carbon skeleton was replenished. According the third direction of nitrogen assimilation, this reaction consumes much 5,10-methylene-5,6,7,8-tetrahydrofolate which is a derivative of C1 substrate. And the exhaustion of the C1 and C3 substrate may reduce the flux distribution of the downstream carbon metabolic pathway and cause the worse growth of ZR1 when high concentration ammonium appeared. Furthermore, NH_4^+ accumulation may also induce the expression of the *hps* gene which is responsible for the assimilation of C1 substrate. Surprisingly, expression level of genes concerns the nitrogen assimilation and formaldehyde oxidization was also up-regulated 4-8 times. On the other hand, with methanol as carbon sources, ZR1 possess a relative high expression background with the *glt*B, *ala*D, *hps* and *cs* genes. And genes changed slightly with NH_4^+ accumulation in comparison with control. However, if considering the relative gene expression level of the samples with methanol as carbon sources in comparison with methane, the C5 and C3 carbon skeleton assimilation gene *glt*B and *ala*D of ZR1 still kept 19.07 and 12.03 times higher than the methane control. Thus, when confronted with high ammonium concentration, ZR1 may mainly assimilate NH_4^+ through pathway 1 and 2. Meanwhile, decreasing the *nir* and *nif* gene expression level to decrease the formulation of ammonium might be the main strategy employed by ZR1. With the replenishment of the C5 skeleton, the expression level of ammonium assimilation gene

522 *glt*B and *ala*D using C5 and C3 carbon skeleton decreased to 0.12 and
523 0.08, and the nitrate and nitrogen reduction gene return to normal level.
524 However, the *gcv*T gene in the third ammonium assimilation direction
525 expressed 2 times higher.

526 Nitrate and its metabolic intermediate were found to have multiple
527 functions for the primary metabolism in biology (Stitt, 1999; Commichau
528 et al., 2006; Cueto et al., 2016). Ammonium was found to be an important
529 signal molecular to affect the methane and nitrogen metabolism in
530 methanotrophs (Dam et al., 2014; Bodelier and Laanbroek, 2004). In this
531 study，it was also found that, owing to the imbalance metabolism of
532 carbon and nitrogen source, ammonium would accumulated to
533 congcentrations high enough to inhibited cell growth. High concentration
534 ammonium will result in high level expression of several genes concerned
535 the carbon and nitrogen metabolism.

536 **Conclusions:**

537 Nitrate and its metabolic intermediate were found to have multiple
538 functions for the primary metabolism in biology (Stitt, 1999; Commichau
539 et al., 2006; Cueto-Rojas et al., 2016). Ammonium was found to be an
540 important signal molecular to affect the methane and nitrogen metabolism
541 in methanotrophs (Dam et al., 2014; Bodelier and Laanbroek, 2004). In
542 this study，it was also found that, owing to the imbalance metabolism of
543 carbon and nitrogen source, ammonium would accumulated to

544 concentrations high enough to inhibited cell growth. High concentration
545 ammonium will result in high level expression of several genes concerned
546 the central carbon and nitrogen metabolism, and thus change the
547 metabolic mode of cells.

548 First, effect of nitrogen substrate to the growth of ZR1 from methane and
549 methanol were analyzed. Nitrate salts were proved to be the best nitrogen
550 substrate to support the growth of ZR1 from methanol and methane as
551 carbon source. The nitrate intermediate metabolite ammonium was found
552 to inhibit the growth of ZR1 from methanol and methane. High nitrate
553 concentration inhibition phenomenon has long been investigated in 1991
554 by Park et al.(Park et al., 1991), however its inhibition mechanism in
555 methanotrophs still lack studying. According to the substrate inhibition
556 theory (Muchandani and Luong, 1989), the nitrate metabolic intermediate
557 ammonium were found to accumulate, and might inhibit the growth of
558 ZR. Kinetic study revealed that the concentration of accumulated
559 ammonium was proportional to the original nitrate concentration in the
560 medium. With high initiate nitrate salt, the metabolic mediates NH_4^+
561 would accumulate to more than 10 mM which was high enough to inhibit
562 the growth of ZR1. These results indicated that worse growth of ZR1
563 might be owing to the disequilibrium of carbon and nitrogen metabolism.
564 Supplying carbon skeleton to assimilate extra ammonium was proven to
565 be a suitable strategy to relieve the inhibition effect of NH_4^+. The carbon

skeleton replenish effect of several carbon substrate to ammonium inhibition was found to be different with methanol and methane as carbon source. However α-KG was found to be the best carbon skeleton to relieve the ammonium inhibition effect. qPCR analysis indicated that, *gcvT*, *gln*A, and *ala*D genes were expressed relatively higher when ZR1 confronted with the high ammonium concentration with methane as carbon source. With addition of the C5 carbon skeleton α-KG, *gln*A gene expressed higher, and the *gcv*T gene decreased 8 times lower to ammonium accumulation condition, which indicated that, ZR1 may rely more on the third direction to assimilate extra ammonium. With methanol as carbon source, the *glt*B and *ala*D gene expressed at a relative high level at normal condition in comparison with methane as carbon source. When confronted the ammonium inhibition condition, ZR1 may decrease its *nir* and *nif* gene expression level and up-regulate the *hps* gene to further prevent the accumulation of ammonium.

In this study it was found that the nitrogen metabolic intermediate ammonium might be a signature of the nitrogen metabolism in *Methylomonas*, and induce related genes to balance the carbon and nitrogen metabolism. Besides the normal ALAD and GS/GAGOT ammonium assimilation pathway, the third Glycine synthase using the C1 carbon skeleton may also actively expressed in ZR1. Methanotrophs may utilize a complex strategy to balance the carbon and nitrogen metabolism

588 according to the available carbon source. These findings are meaningful
589 to reveal the complex coordination metabolic mechanism of nitrogen and
590 carbon in methanotrophs. However, the ammonium signal transduction
591 pathway of methanotrophs needs further study to reveal its induction
592 mechanism.

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598 **Conflicts of interest**

599 The authors have declared that no conflicts of interest exist.

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