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Monomethylamine as a nitrogen source for non-methylotrophs

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Abstract

Monomethylamine can be used by non-methylotrophs as a sole nitrogen source but not as a carbon source, however, little is known about the **genes and enzymes** involved. The γ-glutamylmethylamide/ N-methylglutamate pathway for monomethylamine utilization by methylotrophs has recently been resolved. We have identified genes encoding key enzymes of this pathway in non-methylotrophs (e.g. *Agrobacterium tumefaciens*) and demonstrated that this pathway is also involved in the utilization of monomethylamine as a nitrogen source by non-methylotrophs.

Monomethylamine (MMA, CH_3NH_2) is ubiquitous in the environment and is released during the degradation of many nitrogen containing compounds (1, 2, 6). Bacteria can use MMA as either a sole carbon (C) and/ or sole nitrogen (N) source (1). For methylotrophic bacteria that use MMA as both C and N source, different pathways have been elucidated, including the MMA dehydrogenase pathway, the MMA oxidase pathway and pathways involving methylated glutamate, i.e. γ-glutamylmethylamide (GMA) and N-methylglutamate (NMG) (1). Recently the genes involved in the GMA/ NMG-mediated MMA utilization pathway, which includes GMA synthetase (*gmas*), 'NMG synthase' (*mgsABC*) and NMG dehydrogenase (*mgdABCD*), have been identified in the methylotroph *Methyloversatilis universalis* (**Figure 1a**) (11). Although MMA can serve as a sole N but not as a C source for many non-methylotrophs (3, 5), the mechanisms for this are unclear. A search for GMA/ NMG gene clusters in microbial genome sequence databases revealed that similar clusters are present in many non-methylotrophs, including *Agrobacterium tumefaciens* C58, *Rhizobium leguminosarum* bv. viciae 3841, *Mesorhizobium loti* MAFF303099 and *Ruegeria pomeroyi* DSS-3. We tested the hypothesis that the GMA/ NMG-mediated MMA utilization pathway is also involved in the metabolism of MMA as sole N source by non-methylotrophs.

Identification of the gene clusters involved in metabolism of MMA via the GMA/ NMG pathway in non-methylotrophs

An eight-gene cluster which encodes three key enzymes involved in the GMA/ NMG pathway for MMA oxidation in methylotrophs was identified (11, Chen unpublished

data). The overall reaction for MMA oxidation in methylotrophs containing this pathway is the conversion of MMA to formaldehyde and ammonium ($CH_3NH_2 \rightarrow HCHO + NH_4^+$), which are used as a C and energy source and as a N source, respectively (**Figure 1b**). Searches for similar gene clusters encoding the GMA/ NMG pathway in other microorganisms were carried out using the integrated microbial genomes (IMG) tool (http://img.jgi.doe.gov). Similar gene clusters are present in many methylotrophs, but also in non-methylotrophs such as *Agrobacterium tumefaciens* (**Figure 1a**). Therefore, the same enzymes encoded by this gene cluster might be used by non-methylotrophs to metabolize MMA as a sole N source. If so, the ammonium released from MMA oxidation would serve as the N source. The formaldehyde produced is toxic and would need to be detoxified. Indeed a formyltetrahydrofolate deformylase (*purU*) gene is found immediately downstream of this gene cluster (**Figure 1a**) in *A. tumefaciens* and a *folD* gene (encoding methylenetetrahydrofolate dehydrogenase/ cyclohydrolase) is also present in the genome. Therefore it is likely that formaldehyde released from this pathway in non-methylotrophs is converted to formate via 5,10-methylenetetrahydrofolate, 5,10-methenyltetrahydrofolate, and 10-formyltetrahydrofolate (11). **Formate may further be oxidized to carbon dioxide and genes encoding formate dehydrogenase are present in the genome.** The *gmas* homologue (encoding GMA synthetase) found in these non-methylotrophs is annotated as a putative glutamine synthetase; however, multiple sequence alignment of glutamine synthetases and characterized GMA synthetases, as shown in **Suppl. Fig. 1 and 2**, indicated that they are likely to be GMA synthetases. They cluster together with characterized GMA synthetases from methylotrophs and they lack key residues for ammonium binding, but contain

conserved domains for ATP and glutamate binding as do glutamine synthetases. Furthermore, the tyrosine residue (Tyr^{397}), commonly found in type I glutamine synthetases which is subjected to adenylylation, is missing. It is interesting to note that in two cases (*R. leguminosarum* bv. viciae 3841 and *Burkholderia phymatum* STM815), this gene cluster is encoded on a plasmid, indicating potential for horizontal gene transfer of this metabolic pathway in bacteria. Since the genetics of MMA metabolism in non-methylotrophs is not established, we therefore hypothesized that the genes in this cluster are involved in the utilization of MMA as sole N source by non-methylotrophs such as *A. tumefaciens*.

MMA can be used as sole nitrogen source for *Agrobacterium tumefaciens*

We first tested if MMA could support the growth of these bacteria as a sole N source since this has not been documented. The growth media used were: N-free medium of Kanvinde and Sastry for *A. tumefaciens* C58 (10), a minimal medium of Brown and Dilworth for *R. leguminosarum* bv. viciae 3841 and *M. loti* MAFF303099 (4) and a modified ammonium-free marine mineral salts medium for *Ruegeria pomeroyi* DSS-3 (8). In all cases, glucose (5 g l^{-1}) was used as C source and ammonium or MMA were added to a final concentration of 2 mM as sole N sources. A control for each growth experiment was set up without added N compounds in case of contaminating N in chemicals used for making media. All growth experiments were carried out at 30 °C with shaking at 150 rpm. Optical density at 540 nm was recorded.

All four strains tested could use MMA as sole N source (**Figure 2a**, **Suppl. Fig. 3**). Control experiments set up with no added N compounds did not yield any growth for the bacteria tested. In addition, the possibility of MMA being sole C source is ruled out since MMA alone did not support the growth of any of these four bacteria (data not shown).

The intracellular pool of amino acids was then analyzed to investigate whether GMA/ NMG is involved in MMA metabolism in these non-methylotrophs. Analyses were carried out at Alta Biosciences (Birmingham, UK) using ion-exchange chromatography followed by ninhydrin staining. GMA was detected in *A. tumefaciens* and *M. loti* when **they were grown** on MMA, but not on ammonium, as sole N source (**Suppl. Fig. 4**); whereas NMG was detected in *Ruegeria pomeroyi* when it was grown on MMA, indicating the importance of GMA/ NMG in MMA utilization in these non-methylotrophs. The reason for the accumulation of GMA or NMG is not well understood, but is probably related to the growth state as shown previously in *Pseudomonas* sp. MA, where GMA accumulated under low oxygen condition, whereas under high oxygen condition MMA was primarily converted to NMG (9).

Mutation of *mgdC* and *mgsC,* but not *gmas* abolishes MMA metabolism by *Agrobacterium tumefaciens*

To determine whether this gene cluster is essential in MMA metabolism in *A. tumefaciens*, we constructed three mutants, i.e. *mgdC*::*gm*, *mgsC*::*gm* and *gmas*::*gm*, by marker-exchange mutagenesis using a suicide vector pK18*mobsacB* (13). In each case, the upstream and downstream regions (~ 500 bp) of the target were PCR amplified using

the primers listed in **Suppl. Table 1** and cloned into pK18*mobsacB*. A gentamycin gene cassette from p34S-Gm (7) was then inserted in between these regions. The resulting plasmids were then electroporated into *A. tumefaciens*. Single homologous recombination mutants were selected on LB (lysogeny broth) plates with **kanamycin** (50 µg ml^{-1}). Colonies from these plates were then grown for 24 hours in LB liquid medium and plated out at different dilutions (10^{-2} – 10^{-4}) onto LB plates containing 10% (w/v) sucrose. Resulting **kanamycin**-sensitive colonies were then screened for double homologous mutation by PCR using primers targeting outside of the upstream and downstream regions. Mutation of the genes were confirmed by diagnostic PCR (**Suppl. Table 1**) and subsequently DNA sequencing. Mutants were checked for their abilities to use MMA as sole N source using NFDM medium.

Mutants of *mgdC* and *mgsC* lost their capacity to use MMA as sole N source (**Figure 2b**), although they grew normally using ammonium as the sole N source. The *gmas* mutant, in contrast, could still grow on MMA as N source; however, the growth rate of this mutant on MMA (0.024 hr^{-1}) was significantly reduced compared to the wild type strain (0.043 hr^{-1}) (**Figure 2a**). A fourth mutant (*mgsC_gmas::gm*) was therefore constructed to mutate *gmas* and one of the 'NMG synthase'-encoding genes simultaneously. This mutant could no longer grow on MMA as sole N source (**Figure 2b**).

Our results indicated that non-methylotrophs such as *A. tumefaciens* can use MMA as sole N source via the GMA/ NMG-mediated pathway which has been demonstrated in some methylotrophs. The *mgdABCD* and *mgsABC* genes seem to be essential in *A. tumefaciens* and mutation of representatives of these gene clusters

completely abolished its capacity to use MMA. GMA synthetase in this bacterium is important, but not vital, since mutation of *gmas* caused much slower growth of this bacterium on MMA. **It is likely that "NMG synthase" in *A. tumefaciens* (encoded by *mgsABC*) can use both GMA and MMA as a substrate as shown in Figure 1b. In fact it has been shown that the purified "NMG synthase" is specific for glutamate but not for MMA, and a number of amines can substitute for MMA (12).**The GMA/ NMG-mediated pathway for MMA uptake as N source is likely to be widespread in nature due to the potential for horizontal gene transfer of plasmids encoding this pathway. This, together with the importance of this pathway in MMA metabolism in the environment, warrants further investigation.

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Figure legends

Figure 1 (A) Gene organization of NMG dehydrogenase (*mgdABCD*), GMA synthetase (*gmas*) and "NMG synthase" (*mgsABC*) in representative methylotrophs and non-methylotrophs. *purU*, formyltetrahydrofolate deformylase.

(B) Proposed pathway of GMA and NMG-mediated MMA metabolism in bacteria. The substrate specificity of 'NMG synthase' is not well established (shown in dashed lines) and it is proposed that both MMA and GMA can be used as a substrate for this enzyme. MMA, monomethylamine; GMA, γ-glutamylmethylamide; NMG, N-methylglutamate; Glu, glutamate.

Figure 2 (A) Representative growth curves of wild type *A. tumefaciens* (dotted lines) and *gmas::gm* mutant (solid lines) grown on ammonium (square) or MMA (diamond) as sole nitrogen source. Means and standard deviation of three replicates are shown.

(B) Representative growth curves of *mgdC::gm*, *mgsC::gm* and *mgsC_gmas::gm* mutants of *A. tumefaciens* grown on ammonium (filled, empty and shaded squares, respectively) or MMA (filled, empty and shaded diamonds, respectively) as sole nitrogen source. Growth of wild type *A. tumefaciens* (dotted lines) is also shown for comparison. Means and standard deviation of three replicates are shown.