Enzymes of an Alternative Pathway of Glucose Metabolism in Obligate Methanotrophs

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Research Article

Keywords: methanotropic bacteria, Entner–Doudoroff pathway, glucose-1-dehydrogenase, gluconate kinase, gluconate shunt

Posted Date: January 27th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-146733/v1>

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Version of Record: A version of this preprint was published at Scientific Reports on April 22nd, 2021. See the published version at <https://doi.org/10.1038/s41598-021-88202-x>.

Abstract

Aerobic methanotrophic bacteria utilize methane as a growth substrate but are unable to grow on any sugars. In this study we have shown that two obligate methanotrophs, *Methylothermobacter alcaliphilum* 20Z and *Methylobacter luteus* IMV-B-3098, possess functional glucose dehydrogenase (GDH) and gluconate kinase (GntK). The recombinant GDHs from both methanotrophs were homotetrameric and strongly specific for glucose preferring NAD^+ over NADP^+ . GDH from *Mtm. alcaliphilum* was most active at pH 10 ($V_{\text{max}} = 95 \text{ U/mg protein}$) and demonstrated very high K_m for glucose ($91.8 \pm 3.8 \text{ mM}$). GDH from *Mb. luteus* was most active at pH 8.5 ($V_{\text{max}} = 43 \text{ U/mg protein}$) and had lower K_m for glucose ($16 \pm 0.6 \text{ mM}$). The cells of two *Mtm. alcaliphilum* double mutants with deletions either of the genes encoding GDH and glucokinase (gdh^-/glk^-) or of the genes encoding gluconate kinase and glucokinase ($gntk^-/glk^-$) had the lower glycogen level and the higher contents of intracellular glucose and trehalose compared to the wild type strain. The $gntk^-/glk^-$ knockout mutant additionally accumulated gluconic acid. These data, along with bioinformatics analysis, demonstrate that glycogen derived free glucose can enter the Entner–Doudoroff pathway or the pentose phosphate cycle in methanotrophs, bypassing glycolysis via the gluconate shunt.

Introduction

Aerobic methanotrophs are a highly specialized group of bacteria that utilize methane as an energy and carbon source. Methanotrophs belong to the Alpha- or Gamma-classes of *Proteobacteria*, as well as to the phylum *Verrucomicrobia*. Only few representatives of alphaproteobacterial methanotrophs can utilize a limited range of polycarbon compounds and none of the methanotrophs can use sugars as growth substrates^{1–3}. Nevertheless, bioinformatics and biochemical studies have shown that at least three pathways for the cleavage of hexose phosphates are operative in gammaproteobacterial (Type I) methanotrophs: the modified pyrophosphate-dependent glycolysis, the Entner–Doudoroff pathway, and the oxidative pentose phosphate cycle; the phosphoketolase glycolytic pathway is also encoded by the methanotrophs⁴. These routes start from hexose phosphates, which are the first metabolites of the ribulose monophosphate (RuMP) pathway of C1 assimilation, and lead to the formation of C2, C3 and C4 compounds, as well as the redox cofactors NAD(P)H, and to ATP generation^{5,6}. Moreover, the enzymes for glucose and fructose phosphorylation (ATP-glucokinase and ATP-fructokinase and, additionally, polyphosphate-dependent glucokinase in several species) have been characterized in Type I methanotrophs^{7–9}. In spite of such a high universality of carbohydrate metabolism, the biochemical basis of the obligate dependence of methanotrophs on C1 compounds remains unclear. Moreover, the genomic analysis also indicates that some Type I methanotrophs encode the putative glucose-1-dehydrogenase (GDH) and gluconate kinase (GntK), whose functionality still has to be elucidated.

Glucose-1 dehydrogenases are the cofactor-dependent enzymes catalyzing oxidation of the first hydroxyl group of D-glucose to form D-glucono-1,5-lactone. The bacterial GDH (EC1.1.1.47) that catalyzes β -D-glucose oxidation, simultaneously converting NAD(P)^+ to NAD(P)H , belongs to the extended superfamily

of short-chain dehydrogenases/reductases (SDR)¹⁰. The enzymes of this family show the typical GXGXXG/A fingerprint motif found in most NADP⁺-binding proteins^{11,12}. Then D-glucono-1,5-lactone undergoes spontaneous hydrolysis in water (or by gluconolactonase) to gluconic acid, and the latter can be further phosphorylated to 6-phosphogluconate by gluconokinase (GntK) (EC 2.7.1.12). NADP⁺-GDHs have been described in gram-positive heterotrophic bacteria and archaea¹³. In archaea, however, GDH reaction product is further converted to 2-keto-3-deoxygluconate by gluconate dehydratase¹⁴. The Entner–Doudoroff pathway, which includes glucose dehydrogenase and gluconokinase, in addition to glycolysis and the oxidative pentose phosphate pathway, has been found in plants and cyanobacteria, and simultaneously anabolic (Calvin-Benson-Basham cycle) and catabolic processes (glycolytic pathways) have been demonstrated^{15,16}.

This paper reports for the first time on the characteristics of NAD(P)⁺-dependent glucose dehydrogenase and gluconokinase in gram-negative bacteria unable to use carbohydrates as growth substrates. Characterization of the mutants with knockout of the enzymes leading to 6-phosphogluconate from free glucose, as well as bioinformatics analysis, have shown the *in vivo* functioning of the gluconate branch linking glycogen metabolism and the Entner–Doudoroff pathway in obligate methanotrophs.

Results

GDH-His₆ expression, purification and structure.

The sequences encoding the proteins with ~ 39% identity to that of GDH characterized in *Bacillus subtilis* (BSU02830) were found in the *Mtm. alcaliphilum* and *Mb. luteus* genomes. The full sequences of these ORFs (CCE23386) and (WP_027157932) were expressed in *E. coli* Rosetta (DE3). The recombinant proteins, MtmGDH and MbGDH with 6 histidines at the N-termini, were purified by one-step affinity metal-chelating chromatography. SDS-PAGE of each enzyme revealed one band corresponding to a molecular mass of ~ 30 kDa, which is in accordance with the theoretically calculated masses of 28.6 kDa and 29.1 kDa for GDH from *Mtm. alcaliphilum* or *Mb. luteus*, respectively. Gel filtration of the purified enzymes indicated a tetrameric structure of both methanotrophic GDHs (with Mr 112 kDa for MtmGDH and 100 kDa for MbGDH) (Fig. S1).

Catalytic properties of GDHs.

Both recombinant GDHs catalyzed the D-glucose-dependent reduction of NAD⁺ or NADP⁺, and they were inactive with other sugars tested (L-glucose, fructose, mannose, galactose, sorbose and N-acetyl-glucosamine). The enzyme from *Mtm. alcaliphilum* was most active at pH 10.0 (Fig. 1A) and 20–30 °C (Fig. 1C). It lost 52% or 87% of activity after 1-h exposure at 40°C and 50°C, respectively. 50 mM NH₄Cl stimulated the enzyme activity 1.5-fold (Supplementary Table S1). In the presence of 0.2 M NaCl or 2 M KCl, the enzyme activity decreased by 50% and 88%, respectively, but chlorides of bivalent metals had no significant effect. The dependence of activity on the concentrations of substrates obeyed the Michaelis–Menten kinetics. The maximum reaction rate with NAD⁺ was higher than with NADP⁺ (95 versus 39 U/mg

protein) (Table 1). The apparent K_m values measured at 30°C and pH 10 were as follows: 92 ± 4 mM for glucose, 0.36 ± 0.02 mM for NAD^+ , and 0.15 ± 0.01 mM for NADP^+ .

Table 1
The kinetic properties of GDHs from methanotrophs

Parameter	<i>Mtm. alcaliphilum</i>		<i>Mb. luteus</i>
	His ₆ -tag at the N-terminus	Without His ₆ -tag	
M.m. (number of subunits), kDa	112 (29×4)		100 (35×4)
$V_{\max}$, U/mg protein (NAD^+)	95 ± 3		31 ± 2
$V_{\max}$, U/mg protein (NADP^+)	39 ± 2		43 ± 6
K_m , mM:			
Glucose	92 ± 4	86 ± 1	16 ± 1
NAD^+	0.36 ± 0.02	0.39 ± 0.06	0.12 ± 0.02
NADP^+	0.14 ± 0.01	0.34 ± 0.02	1.6 ± 0.3
k_{cat} NAD , 1/min	10.6		3
k_{cat} NADP , 1/min	0.35		4
$k_{\text{cat}}/K_m^{\text{glucose}}$, 1/(mM×min)	0.1		0.2
$k_{\text{cat}}/K_m^{\text{NAD}}$, 1/(mM×min)	30		26
$k_{\text{cat}}/K_m^{\text{NADP}}$, 1/(mM×min)	2.5		2.7
Specificity $(k_{\text{cat}}/K_m)^{\text{NAD}}/$ $(k_{\text{cat}}/K_m)^{\text{NADP}}$	12		10

Table 2
The kinetic properties of GntKs from methanotrophs

Parameter	<i>Mtm. alcaliphilum</i>	<i>Mb. luteus</i>
M.m. (number of subunits), kDa	20 (1)	20 (1)
$V_{\max}$, U/mg protein	25.8 ± 0.4	56.1 ± 0.9
K_m , mM:		
Gluconate	0.6 ± 0.04	0.24 ± 0.01
ATP	1.3 ± 0.2*(n = 1.6)	0.042 ± 0.002 (n = 2.3)
$k_{\text{cat}}^{\text{Gluconate}}$, 1/min	0.26	0.77
$k_{\text{cat}}^{\text{ATP}}$, 1/min	0.52	1.1
$k_{\text{cat}}/K_m^{\text{gluconate}}$, 1/(mM×min)	0.4	3.2
$k_{\text{cat}}/K_m^{\text{ATP}}$, 1/(mM×min)	0.4	26

GDH from *Mb. luteus* was most active at pH 8.5 (Fig. 1A) and 30 °C (Fig. 1C). The enzyme lost 37% and 61% of the activity after heating for 1 h at 50 °C and 70 °C, respectively. The activity slightly increased in the presence of 50 mM KCl, NaCl or NH₄Cl. However, in the presence of 0.25 M NaCl or 2 M KCl, the residual activity was 84 and 39%, respectively. The chlorides of Ni²⁺, Cd²⁺, Zn²⁺ and Co²⁺ completely inhibited *Mb. luteus* GDH (Supplementary Table S1). The maximum of activity with NADP⁺ was higher than with NAD⁺ (43 vs 31 U/mg protein) (Table 1). MbGDH had a higher affinity for glucose compared to the *Mtm. alcaliphilum* enzyme (K_m = 16 mM vs. 92 mM) but a lower affinity for NADP⁺ (K_m = 1.6 mM vs. 0.01 mM) (Table 1). The calculated catalytic constants k_{cat} and k_{cat}/K_m showed that both enzymes had low specificity for glucose and exhibited an order of magnitude higher specificity for NAD⁺ than for NADP⁺ (Table 1). The activities of both GDHs did not significantly vary in the presence of various organic metabolites (Supplementary Table S2). The activity of MtmGDH slightly increased (by 40%) in the presence of isocitrate (1 mM), and the activity of *Mb. luteus* GDH decreased by 40% in the presence of 10 mM glucose-6-phosphate.

As shown, recombinant form of *Bacillus thuringiensis* GDH with His₆ at the C-terminus displayed a 12-fold higher K_m for glucose compared to the protein possessing His₆ at the N-terminus¹⁷. We tested the kinetic parameters of MtmGDH after cutting off His₆-tags and found that the apparent K_m values for the substrates did not change compared to the His₆-tagged enzyme (Table 1).

Expression, purification and structure of GntK-His₆ from methanotrophs.

The gluconate kinase encoding the genes from *Mtm. alcaliphilum* (CE23379) and *Mb. luteus* (WP_051299846) were heterologically expressed in *E. coli* Rosetta (DE3). The recombinant proteins MtmGntK and MbGntK with 6 histidines at the C-terminus were purified by affinity Ni-NTA chromatography. SDS-PAGE of each enzyme showed one band corresponding to a molecular mass of ~ 20 kDa, which is consistent with the theoretically calculated masses. Gel filtration of the purified enzymes indicated a monomeric structure of both GntKs (Fig. S1).

Catalytic properties of GntKs from methanotrophs.

The recombinant MtmGntK demonstrated an activity of 26 U/mg of protein. The enzyme was active in the pH range from pH 7.5 to pH 9.5 with the optimum at pH 9.0 (Fig. 1B) and in the temperature range from 10 to 30 °C (Fig. 1C). It was quite stable, withstanding 2-h heating at 30–50°C without a decrease in activity. Nonetheless, its activity was fully lost after 5-min exposure at 70°C. No allosteric regulators were found for MtmGntK; however, various metals significantly reduced the activity (Supplementary Tables S1, S3). Thus, CuCl₂, CdCl₂, NiCl₂, ZnCl₂ and BaCl₂ at a concentration of 1 mM almost fully inhibited the activity, while CaCl₂ and CoCl₂ decreased the activity by 60 and 70%, respectively. 50 mM NH₄Cl and KCl reduced the activity by 30%. 50 mM NaCl had no influence on the enzyme activity whereas, in the presence of 0.5 M NaCl, decreased the activity of MtmGntK by 80%. The dependence of the MtmGntK activity on gluconate concentration obeyed the Michaelis–Menten kinetics: the apparent K_m value was 0.60 ± 0.03 mM. In response to the changes in ATP concentration, the enzyme behavior did not obey the Michaelis–Menten kinetics: the $S_{0.5}$ value, 1.3 ± 0.2 mM; the Hill coefficient, $n = 1.6$.

The maximum activity of MbGntK under optimal conditions (pH 9.0, 40 °C) was 56 U/mg of protein (Fig. 1). Unlike MtmGntK, the enzyme from *Mb. luteus* fully lost the activity after 5-min exposure at 50°C, while the activity did not change after 2-h exposure at 30°C. No allosteric regulator of the enzyme was found. CuCl₂ almost completely suppressed the MbGntK activity, while CdCl₂ and ZnCl₂ reduced its activity by 80 and 70%, respectively (Supplementary Table S1). The activity slightly increased in the presence of monovalent cations Na⁺ and NH₄⁺ (50 mM); however, in the presence of 0.5 or 1 M NaCl, the activity decreased by 70 or 10%, respectively. The apparent K_m value for gluconate was 0.23 ± 0.01 mM. Like for the enzyme from *Mtm. alcaliphilum*, the dependence of MbGntK activity on ATP concentration did not obey the Michaelis–Menten kinetics; $S_{0.5}$ was 0.042 ± 0.002 mM, the Hill coefficient $n = 2.3$.

The activities of GDH and Glk in cell-free extracts of methanotrophs.

The activity of GDH in cell-free extracts of *Mtm. alcaliphilum* was found to be ~ 20 mU/mg protein in the presence of 200 mM glucose in the reaction mixture, but the reaction quickly stopped if 20 mM glucose concentration was used to measure the activity. It corresponded with the extraordinary high apparent K_m value (92 mM) found for the purified enzyme. This implies the *in vivo* expression of glucose dehydrogenase in the cells of *Mtm. alcaliphilum*. The specific activity of GDH in the cell-free extracts of

Mb. luteus in the presence of 200 mM glucose was ~ 28 mU/mg protein; at 20 mM glucose, it was ~ 14 mU/mg protein.

The specific activity of glucokinase (Glc) in cell-free extracts of *Mtm. alcaliphilum* was ~ 30 mU/mg protein. In response to the increase in glucose concentration in the reaction mixture from 5 mM up to 200 mM, the enzyme activity increased to 60 ± 5 mU/mg protein. Due to low apparent K_m value for Glc from the methanotroph (0.32 mM)⁸, this increase can be explained by simultaneous functioning of Glc and GDH.

The analysis of activities of the putative promoter regions of the *gntk* and *amy* genes.

We were unsuccessful to find activity of gluconate kinase in the cell-free extracts of both methanotrophs. Therefore, using *cat* as a reporter gene (encoding chloramphenicol acetyltransferase), we analyzed the activities of the putative promoter region of the *gntk* gene in *Mtm. alcaliphilum*. In cell-free extracts of *Mtm. alcaliphilum* pMHA:P*gntk*-*cat* grown under methane or in the presence of methanol, the activities of chloramphenicol acetyltransferase were 2.6 ± 0.8 and 5.1 ± 0.3 mU/mg, respectively.

Since the *gntk* gene is located 10 bp upstream the *amy* gene encoding alpha amylase, we also analyzed the activity of the *amy* promoter. The activities of chloramphenicol acetyltransferase were 9.7 ± 0.5 or 6.0 ± 0.3 mU/mg, if *Mtm. alcaliphilum* pMHA:P*amy*-*cat* was grown on methanol or under methane, respectively. Consequently, both promoters work and one can expect that the *gntk* and *amy* genes are transcribed and subsequently translated by their mRNA. The *Mtm. alcaliphilum* pMHA:*cat* and WT strains lacked the activity of chloramphenicol acetyltransferase.

Phenotypic characterization of insertional mutants.

To prove the *in vivo* glucose metabolism throughout the GDH/GntK reactions in *Mtm. alcaliphilum*, the mutant with insertion in the *gdh* gene was obtained but its phenotype could not be identified. This can be due to the presence of glucokinase highly specific to glucose (Mustakhimov et al., 2017). Therefore, two double mutated strains with the *glk* gene disruption, *gdh*⁻/*glk*⁻ and *gntk*⁻/*glk*⁻, were designed. In the cell-free *gntk*⁻/*glk*⁻ strain, the GDH activity was 22 ± 5 mU/mg of protein but Glc activity was not detected. The *gdh*⁻/*glk*⁻ strain lacked both GDH and Glc activities.

In the presence of methane or methanol, these strains grew 1.5 times slower compared to the wild type (WT) strain 20Z (Fig. 2S). The same effect was observed in a single *glk*⁻ mutant⁸. In contrast to the *glk*⁻ strain, where the content of intracellular glucose did not actually differ from the WT strain (Mustakhimov et al., 2017), the pools of intracellular glucose in the *gdh*⁻/*glk*⁻ strain grown on methane or methanol increased 50- and 32-fold, respectively (Table 3). At the same time, the glucose content in the cells of the *gntk*⁻/*glk*⁻ strain increased only 3–4 times regardless of the growth substrate. Only a slight increase in glucose in the *gntk*⁻/*glk*⁻ mutant indicated the functioning of GDH, which converts excess glucose into gluconate. Another evidence of the functionality of GDH and GntK enzymes is the accumulation of

gluconic acid in the *gntk⁻/glk⁻* strain (Table 3). In the cells of all mutants grown in either methane or methanol, glycogen accumulation dramatically decreased (Table 3). It should be noted that the glycogen concentration in the cells of the *gdh⁻/glk⁻* strain grown under methane dropped 690 times compared to the WT strain, whereas a minor glycogen decrease was observed in the methanol-grown *gntk⁻/glk⁻* strain (~ 6 times). The *gntk⁻/glk⁻* strain growing on methanol accumulated the highest level of intracellular trehalose, but least of all when growing under methane (Table 3). The cells of the *gntk⁻/glk⁻* and *gdh⁻/glk⁻* strains growing on methanol were shown to contain a mixture of malto-derivatives of carbohydrates, but it was very difficult to identify these compounds by the H¹-NMR method.

Table 3

Accumulation of carbohydrates (mg per g of DCW) in the wild type (WT) cells of *Mtm. alcaliphilum* and mutant strains deficient in glucose dehydrogenase/glucokinase (*gdh⁻/glk⁻*) and gluconate kinase/glucokinase (*gntk⁻/glk⁻*)

Metabolite Growth substrate	Sucrose	Glycogen	Glucose	Gluconate	Trehalose	Glucose- 1P	Glucose- 6P
WT							
Methane	17.3 ± 0.9	138 ± 8	0.14 ± 0.04	ND	0.04 ± 0.01	0.12 ± 0.02	0.09 ± 0.01
Methanol	12.2 ± 0.3	322 ± 9	1.27 ± 0.03	ND	0.06 ± 0.01	0.10 ± 0.06	0.18 ± 0.03
<i>gdh⁻/glk⁻</i>							
Methane	4.9 ± 1.2	0.20 ± 0.04	7.3 ± 1.1	ND	0.56 ± 0.16	0.12 ± 0.01	0.13 ± 0.08
Methanol	0.82 ± 0.08	4.9 ± 0.5	32.2 ± 1.4	ND	0.83 ± 0.11	0.26 ± 0.11	0.41 ± 0.03
<i>gntk⁻/glk⁻</i>							
Methane	8.7 ± 2.0	1.24 ± 0.06	0.41 ± 0.04	0.83 ± 0.14	0.16 ± 0.1	0.14 ± 0.04	0.15 ± 0.01
Methanol	7.6 ± 1.2	56 ± 5	5.3 ± 1.1	1.30 ± 0.23	1.84 ± 0.18	0.24 ± 0.11	0.2 ± 0.02

The sequence and distribution of GDH in methanotrophs.

As can be judged from protein sequence, GDHs from *Mtm. alcaliphilum* and *Mb. luteus* belong to the superfamily of short-chain dehydrogenases/reductases (SDR). These enzymes show the typical GXGXXG fingerprint motif found in NADP⁺-binding proteins^{11,12} (Fig. 3S). There are some differences in the composition of amino acids forming the active center in GDH from *Bacillus megaterium* and those from

Mtm. alcaliphilum and *Mb. luteus* (marked * in Fig. 1S). The amino acids at the C-terminus of *B. megaterium* GDH (Q257, A258, R260 and G261) directly interact with glucose and thus determine the kinetic properties of the enzyme. The C-termini at GDHs from *Mtm. alcaliphilum* and *Mb. luteus* are significantly different from the C-terminus of the *B. megaterium* enzyme, which may be reflected in the kinetic properties of methanotrophic enzymes (Fig. 3S). The only difference in amino acids forming hydrogen bonds with the hydroxyl groups of glucose between the *Mtm. alcaliphilum* and *Mb. luteus* GDHs is the replacement of aspartate (D219) by glutamate (E219). The GDHs from other methanotrophs have more differences in amino acids presumably forming the active center and interacting with glucose (Fig. 3S). GDH homologs are present in some other Type I methanotrophs: in representatives of the genera *Methylocaldum* (~ 62% identity of translated amino acids to MtmGDH), *Methylomonas* (from 29 to 61%), as well as in *Methylosarcina fibrata* (Fig. 2). Interestingly, the genomes of *Methylomonas* and *Methylosarcina* have no gene for gluconate kinase.

Discussion

In this study we have characterized for the first time glucose dehydrogenase and gluconate kinase, the starting enzymes for glucose metabolism, in aerobic methanotrophic bacteria, which are unable to use sugars as growth substrates. The GDHs from *Mtm. alcaliphilum* and *Mb. luteus* are strongly specific for D-glucose and use NAD⁺ as the preferred cofactor. To date, microbial NAD(P)⁺-GDHs have been characterized in gram-positive bacteria, cyanobacteria, archaea, fungi and yeasts, as well as in plants, whereas GDHs investigated in gram-negative bacteria were PQQ-dependent²². GDHs from *Bacilli* can oxidize a broad range of substrates²³. The NAD(P)⁺-GDH from *B. thuringiensis* oxidizes glucose (K_m 14 mM) and 2-deoxy-D-glucose (K_m 12.2 mM)¹⁷, and the enzyme from *Lysinibacillus sphaericus* also oxidizes disaccharides such as maltose, lactose, and sucrose²¹. GDHs from the archaea *Thermoproteus tenax* and *Sulfolobus solfataricus* have K_m 0.3–0.4 mM for glucose, whereas K_m values for the enzymes from heterotrophic bacteria were in a wider range (0.5–42 mM)^{22,24}. Although the enzyme from *Mb. luteus* has a dehydrogenase with a K_m value for glucose similar to that of heterotrophs, the *Mtm. alcaliphilum* enzyme remains the absolute record holder for the highest K_m value for glucose (92 mM). Similarly to the GDHs from *Bacillus* sp. ZJ and *L. sphaericus*^{18,21}, methanotrophic GDHs are tetramers.

Previously, we have characterized a single *glk*[−] mutant exhibiting a drastic decrease in glycogen level (50 times compared to WT), while intracellular glucose remained at the same level⁸. In this work, a large pool of intracellular glucose was found in the *gdh*[−]/*glk*[−] mutant lacking the genes responsible for the activation of free glucose (Table 3). It is evidence that, despite the high K_m , *Mtm. alcaliphilum* GDH is involved in maintaining the intracellular pool of glucose. In addition, only a slight increase in the intracellular pool of glucose in the *gntk*[−]/*glk*[−] strain also confirms the participation of glucose dehydrogenase in utilization of free glucose. The occurrence of gluconate, a product of the glucose dehydrogenase reaction, in the *gntk*[−]/*glk*[−] strain, where the gene for gluconate kinase is turned off and GDH works, proves the *in vivo* participation of both routes of free glucose removal.

The increased trehalose levels during the growth of the *glk*, *gdh⁻/glk⁻* and *gntk⁻/glk⁻* strains on either methane or methanol compared to the WT cells could be evidence of glycogen degradation in the mutant. This follows from the fact that the *Mtm. alcaliphilum* genome has no *otsA-otsB* genes encoding trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, which are responsible for the synthesis of trehalose in many bacteria, fungi, insects and plants, but it harbors the *treY* and *treZ* genes encoding the putative maltooligosyltrehalose synthase (CCE23905) and trehalohydrolase (CCE23904), converting the terminal unit of the linear maltodextrin (glycogen or starch) into trehalose via maltooligosyltrehalose (Fig. 3). It also has the gene encoding the putative maltose alpha-D-glucosyltransferase (trehalose synthase, TreSCCE22655), which catalyzes the reversible conversion of maltose into trehalose described for the first time in mycobacteria²⁵. We propose that only glycogen can be a source of trehalose (for TreS) or maltodextrins (for the TreY-TreZ route). As a consequence, glycogen can be the source of free glucose for GDH (and Glk reactions), since the methanotroph has 4-alpha-glucanotransferase (MalQ, CCE25166). MalQ recognizes maltose and longer maltodextrins and preferentially removes glucose from the reducing ends of maltose and from small maltodextrins and transfers the remaining enzyme-bound dextrinyl residue to the non-reducing ends of other maltodextrins, thus forming longer maltodextrin chains²⁶. Two Glg enzymes are essential for endogenous maltotriose formation in *Mtm. alcaliphilum*: glycogen phosphorylase GlgP (CCE22814; CCE22130; CCE25654), which shortens the branch chains of glycogen to the lengths of maltotetraosyl and maltotriosyl units, and GlgX (CCE25166), which releases the branch chains. Some GlgPs can act as maltodextrin phosphorylases MalP²⁷ (Fig. 3). Since small traces of glucose and trehalose were found in wild type cells (Table 3), the process of glycogen degradation in *Mtm. alcaliphilum* most likely goes along with glycogen synthesis. It has been shown for *Corynebacterium glutamicum* and *E. coli* that glycogen synthesis and degradation have to be coordinated efficiently throughout cultivation since the glycogen level is set by the concerted action of glycogen-synthesizing and -degrading enzymes²⁸. Recent studies have proved that in plants and cyanobacteria the Entner–Doudoroff pathway shunt including glucose dehydrogenase and gluconate kinase, in addition to glycolysis and the oxidative pentose phosphate pathway, are simultaneously occurring^{15,16,29}.

In *Mtm. alcaliphilum* 20Z, glucose derived from glycogen degradation is utilized mainly by glucokinase due to its higher specificity to this sugar. The formed glucose-6-phosphate is further converted by phosphoglucoisomerase into fructose-6-phosphate, which enters glycolysis or the Entner–Doudoroff pathway by glucose-6-phosphate dehydrogenase. Since glucokinase activity is inhibited by ADP⁸ (K_i 2.34 mM), the accumulation of ADP allows the involvement of GDH in metabolic process. Recent studies have shown that in *Mtm. alcaliphilum*, in contrast to the closely related strain *Mtm. buryatense*, the Entner–Doudoroff pathway is a secondary pathway of catabolism of C6 compounds and the PPI-dependent glycolysis is always advantageous regardless of the growth substrate (methane or methanol). Among methanotrophs, the GDH homologues are present in other (but not all) Type I methanotrophs: in the genera *Methylocaldum*, *Methylomonas* and *Methylosarcina* (Fig. 2), while the Glk homologues are present in all representatives of Type I methanotrophs. The ability to obtain the key central metabolites from glycogen (glucose-6-phosphate/fructose-6-phosphate) probably allows these bacteria to survive the

lack of growth substrate in the environment at the expense of their own internal resources. It should be noted that 6-phosphogluconate is a substrate for 6-phosphogluconate dehydrogenase producing ribulose-5-phosphate, the latter being an acceptor of formaldehyde in the key reaction of carbon assimilation catalyzed by hexulosephosphate synthase (Fig. 3). Therefore, metabolism of glucose via the gluconate shunt can be an anaplerotic mechanism, which replenishes the RuMP cycle by tapping cellular carbohydrate reservoirs under fluctuations in methane (methanol) concentration in the environment. The redundancy of the pathways of sugar metabolism in obligate methanotrophs determines their ability to survive under the conditions of CH₄ deficiency, but the question about the causes of their inability to use glucose as a carbon and energy source for growth is still open.

Materials And Methods

Bacteria and growth conditions.

Mb. luteus (IMV-B-3098) and *Mtm. alcaliphilum* 20Z (VKM B-2133^T = NCIMB 14124^T) were grown at 30°C on a nitrate salt medium. For the growth of *Mtm. alcaliphilum* 20Z, the medium additionally contained 0.1 M NaHCO₃ and 0.3 M NaCl³⁰. Methane (50% in the gas phase) or methanol (0.5%, v/v) was used as a carbon source. *Escherichia coli* Rosetta (DE3) obtained from Stratagene (La Jolla) was grown at 37°C on a Luria–Bertani (LB) agar or in broth. Kanamycin (50 µg/mL) and/or chloramphenicol (25 µg/mL) was added to the growth medium as necessary.

Cloning, preparation and purification of the recombinant enzymes.

To amplify the gene encoding ubiquitin with 6 histidines at the N-terminus from the pHUE plasmid, the Ub-F/Ub-R primers (Supplementary Table S4) and vector pET28 for cloning were used. The *gdh* gene was amplified by PCR from the DNAs of *Mtm. alcaliphilum* or *Mb. luteus* using the primers presented in the Supplementary Table S4. The purified PCR product corresponding to the *Mtmgdh* gene was ligated into the pET28 or pET28Ub-shHis vectors at the NdeI/HindIII or SacII/HindIII restriction sites, respectively. The PCR product carrying the *Mbgdh* gene was ligated into pET28Ub-shHis at the SacII/HindIII restriction sites. The resulting vectors pET28:gdhMtm, pET28Ub-shHis:gdhMtm, or pET28Ub-shHis:gdhMb were transferred into *E. coli* Rosetta (DE3), enzyme synthesis was induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside, and cells were incubated for 15 h at 18°C. Recombinant enzymes with His₆ at the N-termini were purified as described³¹. To remove His-tags, the enzymes were incubated for 18 h with Usp2-His₆ protease (50 µg of protease per 1 mg of protein) at + 4 °C. The efficiency and completeness of hydrolysis were checked by SDS-PAAG electrophoresis. The final purification of GDH preparations from the cut-off His-tag and Usp2-His₆ were not carried out, since GDH activity was lost during dialysis.

The vector pET30 (Novagen) was used to amplify the *gntk* genes from methanotrophic DNAs by the respective primers (Supplementary Table S1). The resulting vector pET30:gntkMtm or pET30:gntkMb was transferred into *E. coli* Rosetta (DE3). The purification of the recombinant GntKs was carried out as described above. Purified recombinant enzymes were stored in 40% glycerol at -20°C.

Determination of molecular weight of recombinant enzymes.

Gel filtration was performed on the XK 16/100 Superdex 200 column (GE Healthcare) balanced with 0.02 M Tris-HCl (pH 7.0) containing 0.5 M NaCl. The following protein markers (Sigma-Aldrich) were used to build the calibration curve: carbonic anhydrase (29 kDa), albumin (66 kDa), alcohol dehydrogenase (150 kDa), β -amylase (200 kDa), apoferritin (443 kDa). GDHs with the cut-off His-tag were used to determine molecular weights of the enzymes.

Assay Of Enzyme Activities

GDH activity assay.

The activity of GDH from *Mtm. alcaliphilum* was measured by recording the rate of NAD^+ or NADP^+ reduction at 30°C. 1 mL of the reaction mixture routinely contained 50 mM Glycine-NaOH buffer, pH 10.0; 0.3 mM NAD(P)^+ and $\sim 1 \mu\text{g}$ of GDH. The reaction was started with 200 mM D-glucose. The activity of *Mb. luteus* GDH was measured using 20 mM D-glucose in 50 mM Tris-HCl, pH 8.5. Ribose, trehalose, deoxyglucose, galactose, fructose, arabinose, xylose, acetyl-glucosamine, maltose, sorbose, mannitol, mannose, sucrose, lactose and L-glucose at a final concentration of 10 mM were tested as potential substrates for glucose dehydrogenases. The effect of pH on the activity was studied using the following buffers (50 mM): Glycine-NaOH (pH 9.0–10.5), CHES-NaOH (pH 8.5–10.0), Tris-HCl (pH 7.5–9.0), and MES-NaOH (pH 5.0–7.0). KCl, NH_4Cl or NaCl at a final concentration of 50 mM were added to test the effect of monovalent ions on the enzyme activity. The effects of divalent metals were assessed using MgCl_2 , MnCl_2 , CoCl_2 , CuCl_2 , CdCl_2 , NiCl_2 or ZnCl_2 at a final concentration of 1 mM. Glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate (5 mM), pyruvate, PEP, oxaloacetate, lactate, malate, isocitrate, citrate, succinate, serine, α -ketoglutarate, ATP, ADP, AMP and PPI (1 mM) were examined as potential inhibitors or activators. To determine the optimal temperature for enzyme activity, the reaction was carried out in a temperature range of 10–70°C. To determine the apparent K_m values, substrate concentrations in the ranges of 0.391–400 mM (glucose), 0.0078–1 mM (NADP^+), 0.0078–0.75 mM (NAD^+) were used to measure the activity.

GntK activity assay.

The activity of GntK was measured in the reaction mixture containing 50 mM Tris-HCl buffer, pH 9.0; 8 mM MgCl_2 ; 6 mM gluconate; 5 mM ATP; 0.3 mM NADP^+ ; 5U 6-phosphogluconate dehydrogenase (6PGDH, Sigma-Aldrich) as a coupling enzyme and $\sim 15 \mu\text{g}$ GntK. The activity of *Mb. luteus* GntK was assayed in 50 mM Tris-HCl buffer (pH 8.5). The reaction was started with 6 mM sodium gluconate. To study the effect of pH, the following buffers were used (50 mM): Carbonate (pH 9.0–10.5), TAPS-NaOH (pH 8.0–9.0), Tris-HCl (pH 7.5–9.0), K-phosphate (pH 7.0–8.0), MES-NaOH (pH 5.0–6.0) or citrate (pH 4.0–5.0). Glucose-6-phosphate, glucose-1-phosphate, 6-phosphogluconate, PEP, ADP (5 mM at the final concentration), AMP (4 mM), pyruvate (3 mM), PPI (2 mM), oxaloacetate and α -ketoglutarate (1 mM)

were tested as potential inhibitors or activators. To determine the apparent K_m , the activity was measured in the concentration ranges of 0.031–8 mM gluconate and 0.015–4 mM ATP. The $\text{NAD}^+/\text{NADP}^+$ reduction rates were measured at 340 nm with a UV-1700 spectrophotometer (Shimadzu). The apparent K_m and V_{max} values were calculated using Sigma Plot (version 10). The exponentially grown cells were used to measure enzymatic activity in cell-free extracts.

Mutant generation.

The strains of *Mtm. alcaliphilum* with the double *gdh/glk* and *gntk/glk* gene mutations were generated by insertion mutagenesis. Intergenic regions flanking the *gdh* gene were amplified from DNA using primers dGDH2_f1/dGDH2_r1 and dGDH2_f2/dGDHr (Supplementary Table S4) and cloned in the pCM184 vector at the BglII/Acc65I and SacII/SacI sites. The resulting vector pCM184:gdhKm was introduced into *Mtm. alcaliphilum* cells by electroporation. The *gdh*[−] clones were selected by PCR using primers dGDH2_f1 and dGDHr.

The *gntk-gnl* DNA locus was amplified by PCR using primers dGnKinF and dGnKinR (Supplementary Table S4) containing the recognition sites for restriction endonucleases EcoRI and MluI and inserted into pCM184 with removal of the kanamycin resistance gene. The middle of this locus was removed at the sites of endonuclease PvuI and replaced by the kanamycin resistance gene. The kanamycin resistance gene was amplified by PCR from pCM184. The resulting plasmid pCM184:gntk-Km was introduced into *Mtm. alcaliphilum* cells by electroporation.

To obtain double *gdh*[−]/*glk*[−] or *gntk*[−]/*glk*[−] mutant strains, the previously generated plasmid pCM184:glkGm⁸ was introduced into either *gdh*[−] or *gntk*[−] strain by electroporation. The *glk*[−] clones were selected by PCR using primers dGlk (Acc65I) and dGlk (SacII) (Supplementary Table S4). The *gdh*[−]/*glk*[−] and *gntk*[−]/*glk*[−] strains were selected on methanol plates with Km (100 µg/mL) and Gm (10 µg/mL).

Isolation and analysis of carbohydrates.

The carbohydrates were extracted from 30–50 mg of freeze-dried cells by 80% methanol as described⁸. The dried purified methanol extract was dissolved in 540 µL H₂O, followed by the addition of 60 µL 4 M solution of 3-trimethylsilyl [2,2,3,3-2H₄] propionate (TSP) in D₂O, mixing in a shaker for 30 sec, and centrifugation at 15,000g for 5min. The spectra were recorded with an AVANCE III 600 NMR spectrometer (Bruker) at an operating frequency of 600 MHz, at 298 K, with a spectral width of 24 ppm and 90-degree pulse of 12.5 µs. The number of acquisitions was 128 scans; the interval between the scans was 10 s; free induction decay (FID) was recorded for 96 k points over 3.42 s. For correct signal assignment, we used both spectra acquired in the 1D and 2D COSY NMR experiments. The data were confirmed using the AMIX spectral software database (Bruker), as well as the Biological Magnetic Resonance Data Bank internet-based collection.

The concentration of metabolites such as glucose 1-phosphate (δ 5.46, m, 1H), glucose 6-phosphate (δ 5.37, m, 1H), trehalose (δ 5.20, d, J = 3.8 Hz, 1H, 1'H), maltose derivative/maltotriose (δ 5.41, br 1,1'CH), glucose (δ 5.24, d, J = 3.8 Hz, 1H), sucrose (δ 5.42, d, J = 3.8 Hz, 1H) were measured by using ^1H NMR. Additionally, sucrose and glucose concentration was determined using anthron reagent⁸ and ABTS assay³².

The concentration of gluconate was determined using GntK from *Mb. luteus* (the present work). In brief, the reaction mixture (1 mL) contained 50 mM Tris-HCl buffer (pH 9.0), 10 mM MgCl_2 , 2 mM ATP, 0.3 mM NADP^+ , 5U 6PGDH, 10 U *Mb. luteus* GntK and 5–25 μL intracellular extract. After 3-min incubation, OD_{340} was measured. To build the calibration curve, gluconate concentrations in the range of 2.925–23.4 $\mu\text{g/mL}$ were used.

Glycogen was extracted from 20 mg of freeze-dried cells as described³³. The glycogen content was estimated using “Glycogen Assay kit” according to the manufacturer's recommendations.

Analysis of the promoter activity of the *gntk* and *amy* genes.

The PCR products of 563 and 551 bp containing the sequences of putative *amy* or *gntk* promoters, respectively, were generated using primer pairs PamyF/PamyR or PglNF/PglNR (Supplementary Table S4). The PCR product with the *cat* gene was generated with primers SDglNK + CmF/CmR and plasmid p34S-Cm as the source of the *cat* gene. Primers PamyF/PglNF and CmR are tailed with XbaI and SphI restriction ends, respectively. PamyR/PglNR is tailed with a 20 bp sequence homologous to the first 20 bp of SDglNK + CmF containing the SD sequence of the *gntK* gene. PCR products with the *amy* or *gntk* promoters and the *cat* gene were used as a template in the third PCR reaction to produce the final fusion PCR products *Pamy-cat* or *Pgntk-cat* with XbaI and SphI restriction ends. The plasmid pMHA200 was digested with these endonucleases to remove a 752-bp segment, which was replaced by the *Pamy-cat* or *Pgntk-cat* fragments of DNA. The correct assembly was verified by sequencing, and the new plasmids were designated as pMHA200:*Pamy-cat* and pMHA200:*Pgntk-cat*. The resulting plasmids were introduced into *Mtm. alcaliphilum* cells by electroporation.

The activity of chloramphenicol acetyltransferase catalyzing the reaction of the acyl group transfer from acetyl-CoA to chloramphenicol was assessed by recording the 5-thio-2-benzoic acid accumulation as a result of interaction between 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and CoASH sulfhydryl groups at 30°C. The reaction mixture (1 mL) contained 50 mM Tris-HCl buffer (pH 7.5), 0.1 mM DTNB, 1 mM acetyl CoA, and 10 – 50 μL cell-free extract of the *Mtm. alcaliphilum*. The reaction was triggered by adding 2 mM chloramphenicol. The optical density was measured at 412 nm.

Declarations

Acknowledgements

This work was supported by the Russian Foundation for Basic Research (project No. 18-04-00728A).

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by O.N.R, A.S.R. and I.I.M. The first draft of the manuscript was written by V.N.K.; all authors commented on the previous versions of the manuscript. All authors have read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

Figure 1

The effect of pH on the activity of GDHs (A) and GntKs (B) from *Mtm. alcaliphilum* (solid line) and *Mb. luteus* (dashed line). The following buffers were used for activity measurement: MES-NaOH (red line), Tris-HCl (green line), K-P buffer (orange line), CHES-NaOH (purple line), and Glycine-NaOH (blue line). 100% activity of GDH corresponds to 90 U/mg protein for *Mtm. alcaliphilum* GDH and 28 U/mg protein for *Mb. luteus* GDH; 100% activity of GntK corresponds to 20 and 56 U/mg protein for *Mtm. alcaliphilum* and *Mb. luteus* GntK, respectively. (C) The effect of temperature on the activity of GDHs (solid line) and GntK (dashed line) from *Mtm. alcaliphilum* (purple line) and *Mb. luteus* (red line).

Figure 2

The phylogenetic tree built of the amino acid sequences of various putative and characterized bacterial NAD(P)⁺-dependent glucose dehydrogenases. The characterized enzymes are in bold: *Bacillus* sp. ZJ18, *Bacillus thuringiensis*17, *Bacillus megaterium*19, *Bacillus subtilis*20 and *Lysinibacillus sphaericus*21. The amino acid accession numbers in the Gene Bank are in brackets. Methane utilizing bacteria are underlined. The scale bar corresponds to the number of substitutions per site.

Figure 3

Schematic pathways for glycogen synthesis and degradation in *Mtm. alcaliphilum* 20Z. MMO methane monooxygenase, MDH methanol dehydrogenase, FADH formaldehyde dehydrogenase, FDH formate dehydrogenase, HPS hexulose-phosphate synthase, HPI hexulosephosphate isomerase, PGI phosphoglucose isomerase, PGM phosphoglucose mutase, Glk glucokinase, GntK gluconate kinase, FruK fructokinase, UGP uridine glucopyrophosphorylase, AGP adenosine glucopyrophosphorylase, GlgA glycogen synthase, GlgP glycogen phosphorylase, GlgX debranching enzyme, TreY maltooligosyl trehalose synthase, TreZ trehalohydrolase, MalP maltodextrin phosphorylases, TreS trehalose synthase, MalQ alpha-glucanotransferase, Ams amylosucrase, Sps sucrose-phosphate synthase, Spp sucrose-phosphate phosphatase, GDH glucose dehydrogenase, Gnl lactonase

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