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ORIGINAL PAPER

Sucrose metabolism in halotolerant methanotroph *Methylomicrobium alcaliphilum* 20Z

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Abstract Sucrose accumulation has been observed in some methylotrophic bacteria utilizing methane, methanol, or methylated amines as a carbon and energy source. In this work, we have investigated the biochemical pathways for sucrose metabolism in the model halotolerant methanotroph *Methylomicrobium alcaliphilum* 20Z. The genes encoding sucrose-phosphate synthase (Sps), sucrose-phosphate phosphatase (Spp), fructokinase (FruK), and amylosucrase (Ams) were co-transcribed and displayed similar expression levels. Functional Spp and Ams were purified after heterologous expression in *Escherichia coli*. Recombinant Spp exhibited high affinity for sucrose-6-phosphate and stayed active at very high levels of sucrose ($K_i = 1.0 \pm 0.6$ M). The recombinant amylosucrase obeyed the classical Michaelis–Menten kinetics in the reactions of sucrose hydrolysis and transglycosylation. As a result, the complete metabolic network for sucrose biosynthesis and re-utilization in the non-phototrophic organism was reconstructed for the first time. Comparative genomic studies revealed analogous gene clusters in various Proteobacteria, thus indicating that the ability to produce and metabolize sucrose is widespread among prokaryotes.

Keywords Methylotrophic bacteria · *Methylomicrobium alcaliphilum* 20Z · Sucrose metabolism · Sucrose-phosphate phosphatase · Amylosucrase

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Introduction

Sucrose is one of the most abundant products of photosynthesis. Until recently, sucrose was thought to be accumulated mainly in phototrophic organisms, including plants, unicellular algae, and cyanobacteria (Salerno and Curatti 2003; Klähn and Hagemann 2011). Sucrose synthesis upon the salt stress has been also demonstrated for the marine planctomycete *Rhodopirellula baltica* (d'Avó et al. 2013). The biochemical pathways for sucrose metabolism have been investigated mostly in higher plants and cyanobacteria where the disaccharide biosynthesis involves the sucrose-phosphate synthase (Sps, UDP glucose: D-fructose-6-phosphate 2-α-D-glucosyltransferase, EC 2.4.1.14) and the sucrose-phosphate phosphatase (Spp, sucrose-6^F-phosphohydrolase, E.C. 3.1.3.24) (Bruneau et al. 1991; Page-Sharp et al. 1999; Lunn et al. 2000). The new pathway for sucrose breakdown was described in the cyanobacterium *Synechococcus* sp. PCC 7002 (Perez-Cenci and Salerno 2014). However, little is known about the enzymes catalyzing the

biosynthesis and further metabolism of sucrose in non-phototrophic prokaryotes (Chua et al. 2008).

Our earlier studies have shown that a number of methylotrophic bacteria utilizing methane, methanol, or methylated amines can accumulate sucrose as a primary or secondary solute (Khmelenina et al. 1999; Doronina et al. 2003a, b; But et al. 2013b). *Methylomicrobium alcaliphilum* strain 20Z, a halotolerant gammaproteobacterial methanotroph, is one of the prominent methanotrophic species detected in saline and alkaline ecosystems (Khmelenina et al. 1997, 2010). The methanotroph synthesizes sucrose in response to the increased salinity of growth media (Khmelenina et al. 1999). A cluster of four genes encoding the putative Sps, Spp, fructokinase (FruK), and amylosucrase (Ams) has been identified in the genome of the strain (But et al. 2012, 2013b). The colocation of the four genes implied an unusual organization of sucrose metabolism in *M. alcaliphilum* 20Z. It has been shown that the mutation of *sps* abolished the ability of the methanotroph to accumulate sucrose, thus confirming the key role of Sps in sucrose biosynthesis (But et al. 2013b). The *fruK* gene product has been characterized as an ATP-dependent fructokinase (But et al. 2012). Here, we have further refined the sucrose metabolic pathway in *M. alcaliphilum* 20Z via biochemical and genetic studies. Additionally, we have surveyed the distribution of analogous functional modules among different bacterial phyla.

Materials and methods

Bacteria and growth conditions

Methylomicrobium alcaliphilum 20Z (VKM B-2133 = NCIMB 14124) was grown at 30 °C under methane–air atmosphere (1:1) in a mineral medium containing (g/L) KNO_3 (2), $MgSO_4$ (0.2), $CaCl_2$ (0.02), Na_2-EDTA (0.01), $FeSO_4 \cdot 7H_2O$ (0.004), $ZnSO_4 \cdot 7H_2O$ (0.0002), $MnCl_2 \cdot 4H_2O$ (0.00006), $CuCl_2 \cdot 5H_2O$ (0.0002), $CoCl_2 \cdot 6H_2O$ (0.0004), $NiCl_2 \cdot 6H_2O$ (0.00004), Na_2MoO_4 (0.00006), H_3BO_3 (0.0006) with the addition of 0.1 M $NaHCO_3$ and 3 % (w/v) NaCl (Khmelenina et al. 1999). *Escherichia coli* cells were routinely grown at 37 °C in the Luria–Bertani (LB) medium (Sambrook and Russell 2001). The following antibiotics were added if required such as kanamycin, 50–100 μg/ml; chloramphenicol, 25 μg/ml; and tetracycline, 10 μg/ml.

DNA manipulations

Plasmid isolation and cleavage, agarose gel electrophoresis, ligation, and transformation of *E. coli* cells were performed according to the standard protocols (Sambrook and Russell 2001). Restriction enzymes, T4 DNA ligase, *Taq* DNA polymerase, and dNTP mixture were purchased from Thermo scientific (Lithuania).

Expression and purification of Spp and Ams

Chromosomal DNA from *M. alcaliphilum* cells was prepared as described previously (Kalyuzhnaya et al. 1999). The putative *spp* (MALCv4_0615) and *ams* (MALCv4_0617) genes were amplified by PCR using primer pairs *Spp20zF-Spp20zR* and *Ams20zF-Ams20zR* (Suppl. Table 1). PCR products were purified by the Wizard kit (Promega, USA). The fragments were digested with endonucleases, the *Nde*I and *Hind*III sites for the *ams* gene, and the *Nco*I and *Hind*III sites for the *spp* gene to generate sticky-ends and ligated into the expression vectors pET30(a)+ or pET28b (Novagen, USA), respectively. The resulting constructs were used to transform *E. coli* Rosetta (DE3) (Novagen, USA). *E. coli* strains harboring the *ams* or *spp* expression systems were grown overnight at 37 °C in 20 mL of the LB medium and then transferred into a fresh LB medium containing 50 μg/ml kanamycin and 25 μg/ml chloramphenicol and cultivated until OD_{600} 0.6–0.7. The cells were transferred to 17 °C, and the protein expression was induced by isopropyl β-D-1-thiogalactopyranoside at a final concentration of 0.5 mM. After overnight incubation at 17 °C, the cells were harvested by centrifugation at 6,000*g* for 20 min (4 °C). The His_6-tagged proteins were purified by affinity chromatography on a Ni^{2+}-NTA agarose column as described earlier (But et al. 2012), and their purity was analyzed by 12 % SDS–PAGE (Laemmli 1970).

Sucrose-phosphate phosphatase activity assay

The activity of sucrose-phosphate phosphatase was measured by determining the concentration of orthophosphate released from sucrose-6P by the following two methods: (1) the reaction mixture containing 50 mM 2-(*N*-morpholino)ethanesulfonate (MES) buffer, pH 6.5, and 5 mM $MgCl_2$, and 400 μM sucrose-6-phosphate (total volume 200 ml) was incubated at 30 °C and stopped by adding 600 μl of the Bencini reagent (Bencini et al. 1983). This procedure was used to follow the effects of Mg^{2+}, pH, and temperature on the enzyme activity. The effect of pH was tested using the following buffers (50 mM): MES–KOH (pH 5.0–7.0), Tris–HCl (pH 7.5–9.0), and sodium carbonate (pH 9.0–10.0); (2) the kinetics of the enzyme was studied by measuring its activity at 35 °C in 1 ml of the reaction mixture containing 50 mM MES buffer (pH 6.5), 8–400 μM sucrose-6-phosphate, 1 mM fructose-1,6-bisphosphate, 5 mM $MgCl_2$, 0.5 mM $NADP^+$, 0.25 U of phosphoglucoisomerase (PGI), 2 U of glucose-6-phosphate dehydrogenase (GPDH), and 2 U pyrophosphate-dependent 6-phosphofructokinase obtained as a His_6-tagged protein

from *M. alcaliphilum* 20Z as described earlier (Rozova et al. 2010). The reduction of $NADP^+$ at 340 nm was monitored with a Shimadzu UV-1700 spectrophotometer (Japan).

Amylosucrase activity assay

The total activity of amylosucrase was measured at 30 °C by determining the velocity of fructose formation with the auxiliary enzymes PGI, GPDH, and recombinant fructokinase (FruK-His_6) obtained from *M. alcaliphilum* 20Z as indicated (But et al. 2012). 1 ml of the standard assay mixture contained 50 mM MES–KOH buffer (pH 7.0), 5 mM ATP, 5 mM $MgCl_2$, 200 mM sucrose, 0.5 mM $NADP^+$, 1 U of FruK-His_6, 0.25 U PGI, 2 U GPDH, and 0.1 mg glycogen (Fermentas) if required. The hydrolytic activity of amylosucrase was determined by measuring the rate of glucose formation using the coupling enzymes hexokinase (HK) and GPDH in 1 ml of the reaction mixture containing 50 mM MES–KOH buffer (pH 7.0), 5 mM ATP, 5 mM $MgCl_2$, 200 mM sucrose, 0.5 mM $NADP^+$, 0.1 mg glycogen if required, 1 U HK and 2 U GPDH. The $NADP^+$ reduction rate was registered at 340 nm. One unit of total or hydrolytic activity of amylosucrase corresponded to the amount of the enzyme that catalyzed the production of 1 μmol of fructose or glucose, respectively, per min under the assay conditions. Transglycosylation activity of amylosucrase was calculated as a difference between the total and hydrolytic activities. The effect of pH on the activity was tested by using the following buffers (50 mM): potassium phosphate (pH 5.5–6.5), MES–KOH (pH 6.0–7.0), *N*-Tris(hydroxymethyl)methyl-3-aminopropanesulfonate–KOH (pH 7.5–9.0), and sodium carbonate (pH 9.0–10.0). The kinetic parameters were determined by measuring the enzyme activities with different concentrations of sucrose (1–400 mM). The enzyme kinetics module of the SigmaPlot 11 software was used for the calculation of V_{max} and K_m.

The ability of amylosucrase to catalyze the formation of polyglucan from sucrose in vivo was verified using the iodine test. Briefly, the *ams* gene from *M. alcaliphilum* 20Z was cloned into the low copy number vector pHSG575 (Takeshita et al. 1987) under the *lac* promoter, and the cells of *E. coli* Top 10 (Invitrogen, USA) were transformed by the resulting plasmid pHSG575/ams. *E. coli* harboring the plasmid was grown on LB plates containing 150 mM sucrose and 1 mM IPTG, and the colonies were stained with iodine vapors as described (Buttcher et al. 1997).

Transcriptomic studies

In this study, the RNAseq data obtained from the previously described RNAseq experiments were used (NCBI's GEO accession number GSE51145). Transcriptional organization of sucrose-metabolizing genes was additionally confirmed by RT-PCR. Total RNA was isolated from exponentially grown *M. alcaliphilum* cells ($OD_{600} = 0.8$) as described earlier (Reshetnikov et al. 2006). For cDNA synthesis, a mixture of 2 μg total RNA and 0.1 μM reverse primer was heated for 5 min at 70 °C and immediately placed on ice. The mixture was supplemented with 4 μl of the reverse transcriptase buffer (5×), 1 mM of each dNTP, 10 units RNase inhibitor (Fermentas), and 10 units HMinus M-MuLV reverse transcriptase (Fermentas), and the sample was incubated at 44 °C for 1 h and then at 70 °C for 15 min. For cDNA amplification, 5 μl of this reaction mixture was added to 50 μl of the amplification buffer containing 0.2 mM of each dNTP, 0.25 μM of the forward primer, and 1 unit of *Taq* DNA polymerase (Fermentas). In each case, PCR performed without the RT step was used for controlling DNA contamination in the RNA preparations. After 1-min incubation at 94 °C, the samples were subjected to 35 amplification cycles (30 s at 94 °C, 20 s at 65 °C, and 2 min at 72 °C) followed by the final incubation at 72 °C for 2 min. The reaction products were resolved in 1 % agarose gel and quantified using the Gene Ruler™ 100-bp DNA Ladder Plus (Fermentas). The primers used in this study are specified in Suppl. Table 1.

Phylogenetic analysis

The concatenated full-length amino acid sequences of Sps, FruK, and Ams, as well as the full-length sequences of Spp from the protein databases in the National Center for Biotechnology Information (NCBI), were used for phylogenetic analyses. The sequences were aligned using Clustal X software (version 1.8) (Thompson et al. 1997). The phylogenetic tree was generated with MEGA 4 using maximum parsimony, neighbor-joining, and UPGMA methods. The topologies of the trees constructed using different approaches were similar.

Results

Characterization of the recombinant sucrose-phosphate phosphatase

The putative Spp (ORF MALCv4_0615) was purified after heterologous expression in *E. coli* Rosettta (DE3). The molecular mass of the Spp-His_6-tagged protein determined by SDS–PAGE was in a good agreement with the value calculated from the predicted amino acid sequence (32.7 kDa). The recombinant Spp catalyzed the hydrolysis of sucrose-6-phosphate to sucrose and inorganic phosphate with apparent $K_m = 36 \pm 4$ μM and $V_{max} = 18.9 \pm 0.6$ U/mg. The enzyme activity depended on Mg^{2+}; the highest activity was obtained at 5 mM Mg^{2+}. The enzyme did not hydrolyze

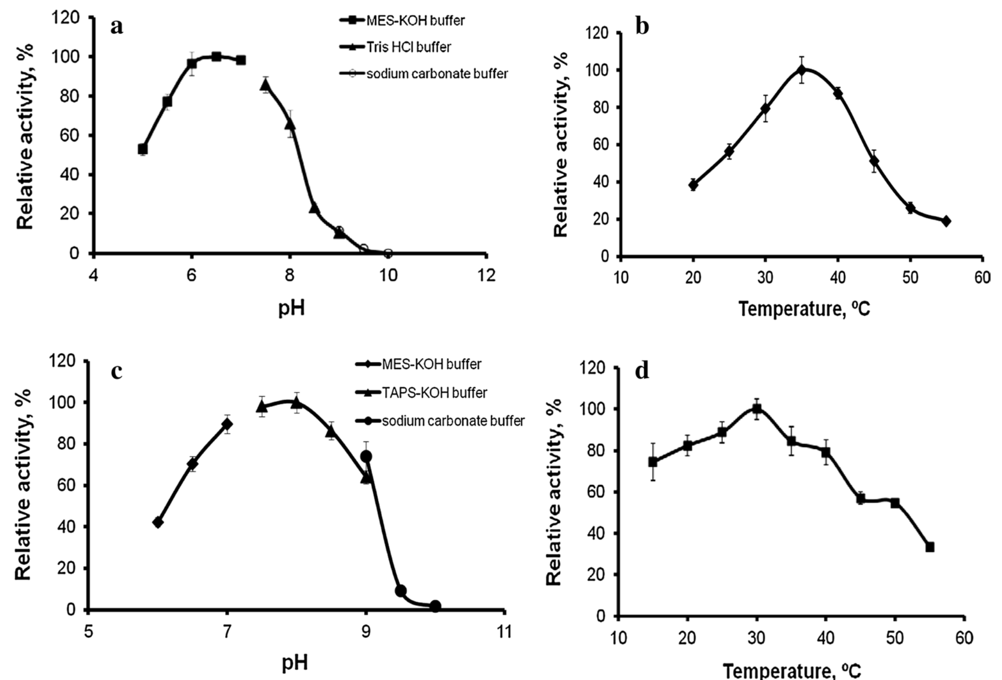

Fig. 1 Dependence of the recombinant sucrose-phosphate phosphatase (**a**, **b**) and the recombinant amylosucrase activity (**c**, **d**) on pH (**a**, **c**) and temperature (**b**, **d**). All the measurements were taken in triplicate. The *bars* represent the standard error

Table 1 Properties of characterized sucrose-phosphate phosphatases

Parameter	*M. alcaliphilum* 20Z	*Synechocystis* sp.6803[a]	*Anabaena* sp. PCC 7120[b]	*Oryza sativa*[c]	*Pisum sativum*[d]
Subunit molecular mass, kDa	31.4	27	28	50	55
Subunits structure	Monomer, dimer, tetramer, hexamer	Monomer	Monomer	Dimer	Dimer
pH optimum	6.5	6.8	6.5	6.5	6.8
Temperature optimum (°C)	35	nr	nr	nr	nr
K_m (mM)	0.036	0.0075	0.35	0.065	0.25
V_{max} (U/mg)	18.9	46	15.7	1,250	nr
K_i (sucrose) (mM)	1,000	161	80	nr	nr

[a] Data from Lunn (2002)

[b] Data from Cumino et al. (2001)

[c] Data from Lunn et al. (2000)

[d] Data from Whitaker (1984)

nr not reported

fructose-1-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, glucose-1-phosphate, glucose-6-phosphate, ribose-5-phosphate, ribulose-5-phosphate, or erythrose-4-phosphate as a substrate. The maximal Spp activity was found at 35 °C and pH 6.5 (Fig. 1a, b). Unlike Spp from other bacteria, the *M. alcaliphilum* Spp showed a very high tolerance toward sucrose, the end product of the reaction ($K_i = 1 \pm 0.6$ M) (Table 1).

Characterization of the recombinant amylosucrase

The genome of *M. alcaliphilum* 20Z has only one copy of the gene homologous to *ams* (MALCv4_0617). It consists of 1956 nucleotides encoding a 651 amino acid protein with a theoretical molecular mass of 76.6 kDa. The gene was expressed in *E. coli* Rosettta (DE3) as described in "Materials and methods." The molecular mass of the purified His_6-tagged protein calculated from electrophoresis under both native and denaturing conditions was ~75 kDa.

The recombinant Ams catalyzed the following two reactions: (i) sucrose cleavage into fructose and glucose, and (ii) transglycosylation with the transfer of the glycopyranosyl residue of sucrose to the glycan polymer accompanied by the formation of free fructose. Both Ams activities were maximal at pH 8.0 and 30 °C (Fig. 1c, d). In contrast, the described amylosucrases have the higher temperature optima

Table 2 Properties of characterized bacterial amylosucrases

Parameters	*M. alcaliphilum* 20Z		*Neisseria polysaccharea*[a]	*Deinococcus radiodurans*[b]	*Deinococcus geothermalis*[c]	*Alteromonas macleodii*[d]
Subunits structure	monomer (1 × 76 kDa)		monomer (1 × 70 kDa)	nr	nr	nr
pH optimum	8.0		6.0	nr	7.0	7.0 (hydrolysis) 8.0 (transglycosylation)
Temperature optimum (°C)	30		42	36	47	45
K_m (mM)	Without glycogen	In the presence of 0.1 mg/ml glycogen				
Total activity	8.1	11.3	4.0 ([sucrose] < 20 mM) 71 ([sucrose] > 20 mM)	10 ([sucrose] < 41 mM) 84 ([sucrose] > 41 mM)	nr	nr
Transglycosylation	6	11.2	8.1 ([sucrose] < 20 mM) 112 ([sucrose] > 20 mM	nr	nr	nr
Hydrolysis	11	11	2.5 ([sucrose] < 20 mM) 29 ([sucrose] > 20 mM)	nr	nr	nr
k_{cat} (min^{-1})	Without glycogen	In the presence of 0.1 mg/ml glycogen				
Total activity	8.7	11.2	44 ([sucrose] < 20 mM) 84 ([sucrose] > 20 mM)	49.2 ([sucrose] < 41 mM) 121 ([sucrose] > 41 mM)	nr	nr
Transglycosylation	4.6	7.5	26 ([sucrose] < 20 mM) 54 ([sucrose] > 20 mM)	nr	nr	nr
Hydrolysis	4.1	4.0	21 ([sucrose] < 20 mM) 31 ([sucrose] < 20 mM)	nr	nr	nr

[a] Data from Jung et al. (2009) and van der Veen et al. (2006)

[b] Data from Pizzut-Serin et al. (2005)

[c] Data from Emond et al. (2008) and Jung et al. (2009)

[d] Data from Ha et al. (2009)

nr not reported

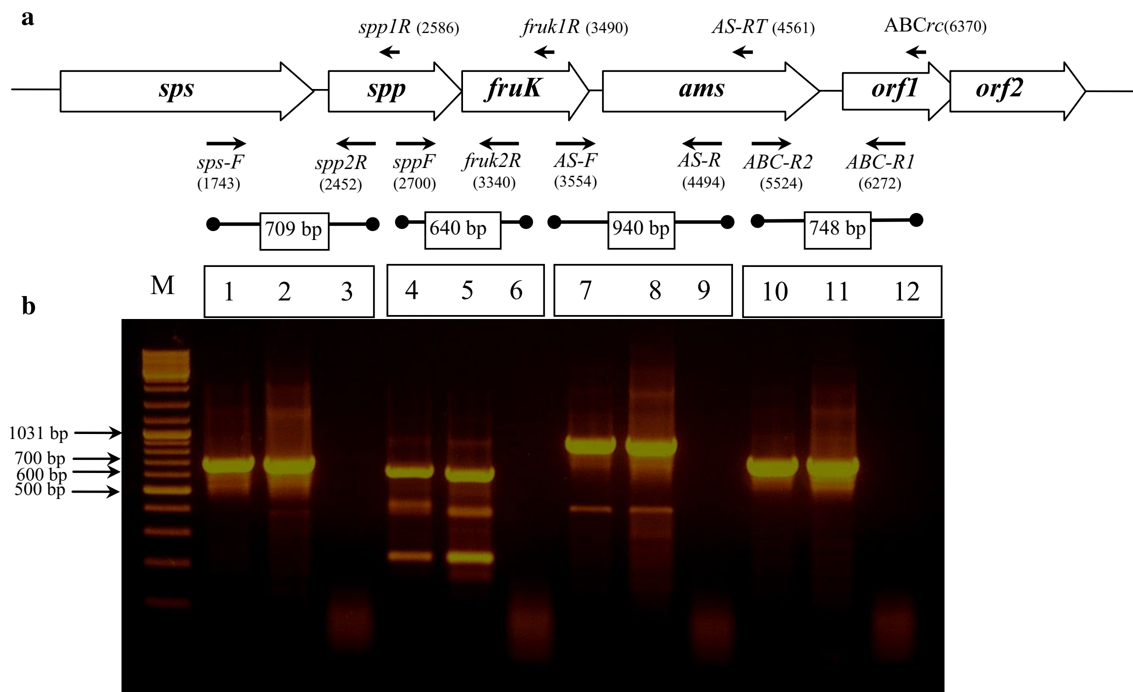

Fig. 2 Organization of genes for sucrose metabolism in *Methylomicrobium alcaliphilum* 20Z. **a** Localization of the genes in cluster and positions of the oligonucleotide primers used for cDNA synthesis and amplification in RT-PCR assays. **b** Gel electrophoresis of the RT-PCR products obtained with the primers sps-F/spp2R (*lanes 1*, *2*, *3*); sppF/fruk2R (*lanes 4*, *5*, *6*); AS-F/As-R (lanes 7, 8, 9); ABC-R2/ABC-R1 (*lanes 10*, *11*, *12*). Primers spp1R, fruk1R, AS-RT, and ABCrc were used for cDNA amplification. The negative controls for PCR containing the total RNA, primers, and *Taq*-polymerase, but not reverse transcriptase, are shown on *lanes 3*, *6*, *9*, *12*. The positive controls for PCR containing the genomic DNA and the same primers are shown on *lanes 1*, *4*, *7*, *10*. The *numbers in brackets* indicate DNA nucleotides downstream of the first *sps* nucleotide. The expected sizes of cDNA are indicated in *squares*. The sequences of oligonucleotide primers used for cDNA synthesis and amplification are listed in Suppl. Table 1

(Table 2). The dependence of Ams activity on sucrose concentrations obeyed the classical Michaelis–Menten kinetics (Suppl. Fig. 1). The *M. alcaliphilum* amylosucrase showed the twice lower $K_m^{sucrose}$ value (6 mM) for transglycosylation reaction compared to the value for hydrolysis ($K_m^{sucrose}$ = 11 mM). In the absence of glycogen, the transglycosylation and hydrolytic activities were comparable (60 and 53 mU/mg, respectively). The addition of 0.1 mg/ml glycogen primer increased transglycosylation activity to 98 mU/mg, while the hydrolysis rate remained constant. Earlier, we showed that inactivation of the *ams* gene using insertion mutagenesis resulted in a 30 % increase in sucrose level in *M. alcaliphilum* cells, thus indicating the in vivo involvement of amylosucrase in sucrose cleavage (But et al. 2013b).

Additional test was performed in order to validate the transglycosylation activity of the enzyme in vivo. The *E. coli* Top10 cells were transformed by the pHSG575/ams plasmid and grown in the presence of sucrose. Only the cells harboring the pHSG575/ams plasmid were stained blue with iodine vapors, while the cells carrying the empty pHSG575 vector remained non-colored (Suppl. Fig. 2). This fact confirmed the participation of Ams from *M. alcaliphilum* 20Z in polyglycan formation from sucrose.

Analysis of transcriptional organization of the sucrose-metabolizing genes

In a single DNA locus of *M. alcaliphilum* 20Z, an 18-bp intergenic region separates the *sps* and *spp* genes. The *spp* and *fruK* genes overlap by 4 bp, and *fruK* is separated from *ams* by 82 bp. Two additional Orfs, predicted to encode a putative ABC transporter (orf1, 102 bp downstream of *ams*) and a conserved membrane protein (orf2, overlapped with orf1by 13 bp), were identified (Suppl. Fig. 3). The previously performed RNAseq experiments (GEO #GSM 1239656-1239658) showed good mapping coverage for the whole cluster *sps–spp–fruK–ams–orf1–orf2* (Suppl. Fig. 3). The transcriptional start site predicted from the RNA transcript mapping data is located 25 bp upstream of the putative translation start site. The promoter elements −10 (TACAAT) and −35 (TTGAGA) were similar to the sigma-70 factor. RT-PCR analysis was used to verify an operon organization. The RT-PCR products of the expected size with correct sequences were obtained across the *sps–spp, spp–fruK, fruK–ams,* and *fruK–orf1* gene pairs (Fig. 2). The controls for DNA contamination of the RNA preparations using direct PCR

without the RT step were negative. These data confirmed that all six genes are co-transcribed.

Distribution and organization of the sucrose-metabolizing genes in bacteria

The predicted amino acid sequences for the functionally validated sucrose-phosphate synthase from two cyanobacteria *Synechocystis* sp. PCC 6803 (Lunn et al. 2003) and *Synechococcus* sp. 7002 (Cumino et al. 2010), two methylotrophs *Methylobacillus flagellatus* KT and *M. alcaliphilum* 20Z (But et al. 2013a, b), and the Gram-positive heterotrophic bacterium *Halothermothrix orenii* (Huynh et al. 2005) were used to search for similar proteins in the sequenced microbial genomes using the NCBI database. The BLAST search revealed Sps-like ORFs in 101 bacterial genomes (Suppl. Table 2). Most of them belonged to Proteobacteria (64 genomes) and Cyanobacteria (22). The rest were Planctomycetes (6 genomes), Firmicutes (6), Deferribacteres (1), Nitrospinae (1), and Chrysiogenetes (1). However, only 37 of them had the same set of the four genes (*sps, spp, fruK,* and *ams)*; in 27 bacteria, they clustered analogously to *M. alcaliphium* 20Z.

The gene order varied throughout the Proteobacteria (see Suppl. Fig. 6 for full details of the gene organization in 50 bacteria). The Sps homologs were identified in 11 out of 19 sequenced genomes of gammaproteobacterial methanotrophs. Eight of them possess the four-gene cluster *sps–spp–fruK–ams* (Suppl. Fig. 6). The concatenated full-length protein sequences of Sps, FruK, and Ams of methanotrophs comprise a separate branch on the phylogenetic tree (Suppl. Fig. 4) being closely related to the enzyme from *Synechococcus* sp. 7002 (55 % AA sequence identity). The sequences from the genus *Methylophaga* (methanol-utilizing gammaproteobacterial methylotrophs) form a separate branch together with the proteins from non-methylotrophic members of beta- and deltaproteobacteria (Suppl. Fig. 4).

Surprisingly, the *spp* gene was not found in genomes of 44 *sps*-possessing bacteria (Suppl. Table 2). We showed earlier that the bifunctional Sps of the methylotrophic bacterium *M. flagellatus* KT catalyzed both phosphorylation of sucrose and dephosphorylation of sucrose-6-phosphate to free sucrose (But et al. 2013a). Notably, the Sps–FruK–Ams polypeptides of the bacteria lacking Spp are separated on the phylogenetic tree from those of the species possessing the four enzymes (Suppl. Fig. 4). The methanotrophic Spp sequences are divided into two main groups. The first group combines the Spp proteins from halotolerant methanotrophs, such as *M. alcaliphilum* 20Z, *Methylobacter marinus* A45, and *Methylomonas methanica* MC09. The second group includes the sequences of salt-independent methanotrophs (*Methylomicrobium album* BG8, *Methylobacter tundripaludum* SV96, and *Methylosarcina fibrata* AML-C10). The only exception is *Methylobacter luteus* IMV-B-3098^T isolated from soil; its Spp is related to the proteins of halotolerant methanotrophs (Suppl. Fig. 5). All methanotrophic Spp proteins are well conserved, and they share 63–93 % AA sequence identities but display very low similarity to the enzyme from *Zea mays* and *Nicotiana tabacum* (15.9 and 16.7 % identity, respectively) (Lunn et al. 2000; Chen et al. 2005).

The Ams-like sequences were revealed in 53 bacterial species: 21 Proteobacteria, 18 Cyanobacteria, and all Firmicutes lacked the *ams* genes. The function of Ams has been proved mainly in the sucrose-utilizing heterotrophic bacteria *Neisseria polysaccharea*, *Deinococcus radiodurans,* and *Deinococcus geothermalis* (Jung et al. 2009; van der Veen et al. 2006; Pizzut-Serin et al. 2005; Emond et al. 2008; Ha et al. 2009), and in the sucrose-synthesizing cyanobacterium *Synechococcus* sp. 7002 (Perez-Cenci and Salerno 2014). Many alpha-, beta-, gamma-, and deltaproteobacteria, including the halophilic methanotroph *Methylohalobius crimeensis,* possess a three-gene cluster, which includes *fruK, sps,* and *sus*, the latter coding for the reversible sucrose synthase (Sus) functionally replacing Ams. Ams from *M. alcaliphilum* 20Z shared about 37 % AA identity with the characterized enzymes of heterotrophs and 62 % identity with the enzyme from *Synechococcus* sp. 7002. The amino acid sequence alignment confirmed that the methanotrophic Ams possesses the consensus motifs characteristic of the glycoside hydrolase family 13 of the enzymes (Svensson 1994) and conserved residues (Asp394, Arg415, Phe436) in the active site (in the B'-domain), which are characteristic for amylosucrases from various microorganisms (Pizzut-Serin et al. 2005).

The absolute majority of bacteria possessing the *ams* or *sus* genes also contain *fruK* with the exceptions of *Desulfocapsa sulfexigens* and *Planctomyces brasiliensis* (Suppl. Table 2). The *M. alcaliphilum* FruK has the highest similarity to the putative FruK of other methanotrophs, i.e., *M. methanica* MC09, *M. album* BG8, and *M. tundripaludum* SV96 (54.4–61.4 % AA identities). Other close relatives of *M. alcaliphilum* FruK are the putative proteins from methylotrophic bacteria unable to grow under methane: *Methylophaga thiooxydans* DMS010, *Methylophaga lonarensis* MPL, *Methylophaga aminisulfidivorans* MP, *Methylophaga* sp. JAM1, *Methylobacillus flagellatus* KT, and *Methylovorus glucosetrophus* SIP3-4 (40.3–47.6 %), as well as from the autotrophic *Thiomicrospira halophila* (43.8 %) and *Cyanobium gracile* (46.6 % identity). FruK has been functionally characterized in *Synechococcus* sp. 7002 (Perez-Cenci and Salerno 2014).

Two additional Orfs (an ABC transporter and a conserved membrane protein of unknown function found in *M. alcaliphilum* 20Z) were revealed only in the halotolerant methanotroph *M. buryatense* 5G.

Discussion

The ability of halotolerant methylotrophs to accumulate sucrose is well documented (Khmelenina et al. 1999; Doronina et al. 2003a, b; But et al. 2013a, b). However, the pathways for sucrose production and further metabolism in non-phototrophic microbes remain elusive. Recently, we have demonstrated that the Sps and FruK enzymes are involved in sucrose conversion in the halotolerant methane-utilizing bacterium *M. alcaliphilum* 20Z (But et al. 2012, 2013b). In this work, we finalize the reconstruction of the sucrose metabolic pathway in this strain via additional biochemical, genomic, and transcriptomic studies. We have shown that the kinetic characteristics of the two enzymes, Spp and Ams, predicted to be involved in sucrose conversions in *M. alcaliphilum* 20Z, differ from those investigated previously. The activity of Spp is inhibited by very high sucrose concentrations, while the described cyanobacterial enzymes are more sensitive to sucrose. For example, much lower K_i values for sucrose were reported for Spp from *Anabaena* sp. PCC 7120 and *Synechocystis* sp. 6803 (80 and 161 mM, respectively) (Cumino et al. 2001; Lunn 2002). The enhanced resistance of the enzyme to sucrose probably allows the halophilic methanotroph to accumulate high intracellular concentrations of the disaccharide under osmotic stress. Contrary to the sucrose-utilizing *D. radiodurans* and *N. polysacharea*, with amylosucrases displaying the higher K_m for transglycosylation activity compared to sucrose hydrolysis (Pizzut-Serin et al. 2005; van der Veen et al. 2006), the kinetic properties of the *M. alcaliphilum* Ams indicate that the enzyme mostly contributes to the polyglycan biosynthesis in vivo.

Herewith, we have shown that sucrose production and cleavage in *M. alcaliphilum* 20Z are determined by a single operon. The metabolic network includes two stages. Similarly to higher plants and cyanobacteria, the methanotroph converts fructose-6-phosphate and UDP glucose into sucrose with the involvement of Sps and Spp. At the next stage, a glycogen-like polymer is synthesized, and free fructose is formed with the participation of Ams. The fructose can be converted back into fructose-6-phosphate via the ATP-dependent FruK (Fig. 3). Thus, the metabolic network probably represents a 'sucrose cycle.'

Glycogen synthesis via sucrose as an intermediate requires two NTP molecules for attaching the glucosyl residue to the glycogen primer. This pathway is less energy efficient compared to the glucose pyrophosphorylase (GlgC)/glycogen synthase (GlgA) route, which requires only one NTP molecule for glycogen elongation. The genome of *M. alcaliphilum* 20Z possesses and expresses the necessary genes being inventory for the latter route (Suppl. Table 3). Intracellular accumulation of sucrose in *M. alcaliphilum* 20Z is one of the responses to the decrease in water activity (Khmelenina et al. 1999). It would be reasonable to suggest

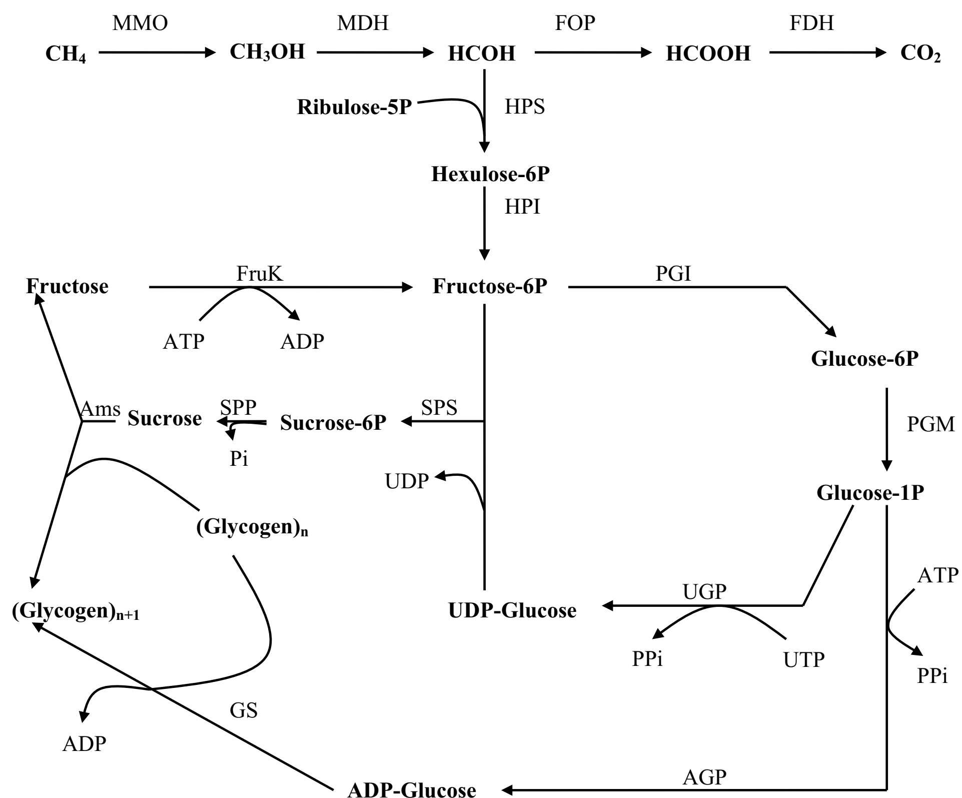

Fig. 3 Overview of sucrose metabolism in *M. alcaliphilum* 20Z. *MMO* methane monooxygenase, *MDH* methanol dehydrogenase, *FOP* formaldehyde oxidation pathways, *FDH* formate dehydrogenase, *HPS* hexulose-phosphate synthase, *HPI* hexulose-phosphate isomerase, *PGI* phosphoglucoisomerase, *PGM* phosphoglucomutase, *UGP* UDP glucose pyrophosphorylase, *AGP* ADP glucose pyrophosphorylase, *GS* glycogen synthase

that the main function of the pathway is *de novo* sucrose biosynthesis. This suggestion is supported by the atypical kinetic behavior of Spp with respect to the inhibition of the enzyme activity by an extremely high sucrose level. Nevertheless, the genes involved in sucrose biosynthesis (Sps/Spp) and sucrose breakdown/reutilization (Ams/FruK) display similar relative expression levels (Suppl. Fig. 3). It is possible that the sucrose cycle could be envisioned as a dynamic mechanism that balances the internal concentration of sucrose via posttranscriptional activation or repression of the Sps/Spp or Ams/FruK branches of the cycle.

All of the sequenced genomes of halotolerant methanotrophs and three of the four genomes of methanotrophs isolated from soils possess the sucrose biosynthesis genes. On the other hand, in the nine genomes of methanotrophs isolated from freshwater sediments or sewage systems, the sucrose biosynthetic genes were identified only in *Methyloglobulus morosus* DSM 22980. However, organization of the genes for sucrose metabolism in this species is different, since the corresponding gene cluster lacks *fruK* (Suppl. Fig. 6). It might be speculated that the distribution of sucrose metabolism in methanotrophic bacteria is driven by the conditions of natural habitat such as salinity and/or seasonal exposure to a low-water activity under dry conditions or high temperature.

No *sps*-homologous genes were found in the alpha- or betaproteobacterial methylotrophs assimilating the reduced C_1 compounds via the serine pathway and the methanotrophs belonging to *Verrucomicrobia* and candidatus "Methylomirabilis oxyfera" utilizing methane carbon at the level of CO_2 via the CBB cycle (Khadem et al. 2011; Rasigraf et al. 2014). Thus far, the ability to produce sucrose is restricted to methylotrophic gamma- and betaproteobacteria employing the energy-efficient RuMP pathway for C_1 assimilation.

Interestingly, the majority of the sequenced genomes of *Methylophilaceae*, mostly of freshwater species, possess the sucrose-metabolizing genes (Suppl. Fig. 6). The salt- and temperature-dependent sucrose biosynthesis has been observed in *M. flagellatus* KT (But et al. 2013a). It should be noted that sucrose accumulation provides only moderate salt and temperature tolerance of this methylotroph. On the other hand, all species of *Methylophilaceae* capable of sucrose biosynthesis display highly active growth on methanol, while the sucrose biosynthesis genes are not present in those species (*Methylotenera mobilis* JLW8, *Methylotenera versatilis* 301, *Methylotenera* sp 1P/1, and marine bacterium *Methylophilaceae* spp. HTCC 2181) showing no growth or very poor growth on methanol. It is tempting to speculate that sucrose metabolism provides bacteria with some advantages for efficient and active assimilation of reduced C_1 compounds, since the sucrose synthesis in all members of the *Methylophilaceae* is linked to the initial steps of formaldehyde assimilation via the RuMP pathway (Fig. 3). The conversion of fructose-6-phosphate into sucrose can stimulate the incorporation of formaldehyde into glycogen, the main carbon storage compound. It is also possible that glycogen synthesis via the sucrose cycle may function like a "futile cycle" for dissipation of energy excess.

Among non-methylotrophic bacteria, the homologs of the *sps* genes were identified in the genomes of photo- and chemolithotrophs capable of fixing CO_2 via the CBB cycle (such as *Nitrosomonas europaea*), the Wood–Ljungdahl pathway (*Desulfobacterium autotrophicum*), and the reverse Krebs cycle (*Magnetococcus marinus*). The genes for sucrose-synthesizing enzymes can also be identified in the genomes of Bacteroidetes, Chloroflexi, Planctomycetes, and Firmicutes. The exact role of sucrose biosynthesis/utilization genes in these poorly characterized microbial clades still has to be elucidated. Bearing in mind that these bacterial phyla were isolated from a variety of marine, freshwater, and terrestrial ecosystems, a more intricate picture of sucrose as an intermediate of microbial metabolic networks might be evolved in the future.

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