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Functional Analysis of *Methylomonas* sp. DH-1 Genome as a Promising Biocatalyst for Bioconversion of Methane to Valuable Chemicals

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Abstract: *Methylomonas* sp. DH-1, newly isolated from the activated sludge of a brewery plant, has been used as a promising biocatalytic platform for the conversion of methane to value-added chemicals. *Methylomonas* sp. DH-1 can efficiently convert methane and propane into methanol and acetone with a specific productivity of 4.31 and 0.14 mmol/g cell/h, the highest values ever reported, respectively. Here, we present the complete genome sequence of *Methylomonas* sp. DH-1 which consists of a 4.86 Mb chromosome and a 278 kb plasmid. The existence of a set of genes related to one-carbon metabolism and various secondary metabolite biosynthetic pathways including carotenoid pathways were identified. Interestingly, *Methylomonas* sp. DH-1 possesses not only the genes of the ribulose monophosphate cycle for type I methanotrophs but also the genes of the serine cycle for type II. *Methylomonas* sp. DH-1 accumulated 80 mM succinate from methane under aerobic conditions, because DH-1 has 2-oxoglutarate dehydrogenase activity and the ability to operate the full TCA cycle. Availability of the complete genome sequence of *Methylomonas* sp. DH-1 enables further investigations on the metabolic engineering of this strain for the production of value-added chemicals from methane.

Keywords: complete genome sequence; methane; *Methylomonas* sp. DH-1; secondary metabolites

1. Introduction

Methane is the principal component of natural/shale gas and biogas, and recently, has attracted much attention as a chemical feedstock [1]. The chemical conversions of methane to other chemicals generally require the input of high amounts of energy because of the high stability of the carbon–hydrogen bond (C–H bond), while the biological conversion of methane to chemicals using methanotrophs can be conducted in ambient conditions [2]. In addition, the bioconversion of methane showed higher conversion of up to 75% [1,3].

In an effort to develop a biocatalytic platform strain, we have isolated a type I methanotroph, *Methylomonas* sp. DH-1, from the activated sludge of a brewery plant [4]. *Methylomonas* sp. DH-1 was reported as a highly efficient biocatalyst for the bioconversion of methane to methanol, which can be directly used as alternative fuels, antifreeze and as a precursor to other compounds, with a specific productivity of 4.31 mmol/g cell/h [4]. Furthermore, *Methylomonas* sp. DH-1 has high potential for

methanol production due to its high tolerance to methanol of up to 7% (*v*/*v*) [4]. *Methylomonas* sp. DH-1 has also been evaluated for its catalytic capability to convertpropane to acetone, which is used as an industrial solvent for polymers, in acetylene storage and in the pharmaceutical industry [5]. Moreover, the accumulation of acetone in the absence of chemical inhibitors is advantageous for biocatalytic gas-to-liquid conversion technology. Additionally, *Methylomonas* sp. DH-1 can produce yellow-to-red pigments which are expected to be numerous carotenoids (unpublished report). Thus, *Methylomonas* sp. DH-1 can be an important biocatalyst for methane bioconversion to chemicals/fuels. In this study, we sequenced, assembled and annotated the whole genome sequence of *Methylomonas* sp. DH-1 as the first step for the development of a methanotrophic platform strain. *Methylomonas* sp. DH-1 was also used for the production of succinate as a model compound from methane.

2. Results

2.1. Genome Statistics and General Features

The complete genome of *Methylomonas* sp. DH-1 consists of a circular chromosome of 4,849,532 bp (56.5% G + C) and a plasmid of 277,875 bp (51.7% G + C) (Figure 1).

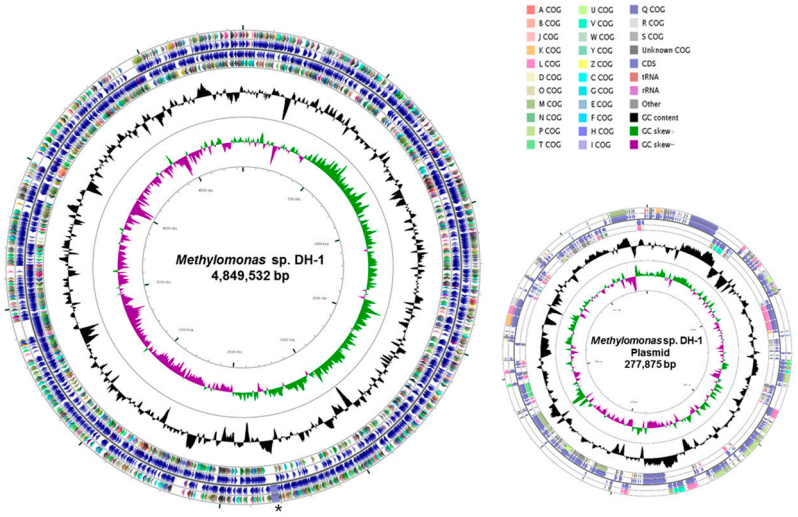

Figure 1. A circular representation of the *Methylomonas* sp. DH-1 chromosome and plasmid. The rings represent (from inner to outer) the nucleotide position ruler, GC skew, %GC, coding sequences (CDSs) transcribed in the counterclockwise direction, and the clockwise direction, respectively. The CDSs are colored according to the assigned clusters of orthologous genes (COG) classes. The image was rendered using the CGView server (http://stothard.afns.ualberta.ca/cgview_server/).

Methylomonas sp. DH-1 was shown to be phylogenetically closely related to *Methylomonas koyamae* Fw12E-Y^T based on 16S sequence similarity [4]. Electronic DNA–DNA hybridization (DDH) estimate between DH-1 and Fw12E-Y^T (=JCM 16701^T), calculated by the Genome-to-Genome Distance Calculator (http://ggdc.dsmz.de/distcalc2.php), was 73.9%, which suggests that DH-1 belongs to the *Methylomonas koyamae* species, while average nucleotide identity (ANI) between these two strains was calculated to be 97.76% using JSpecies [6]. Moreover, MUMMER whole-genome alignment [7] between DH-1 and Fw12E-Y^T showed the close similarity of these two strains, aligning 298 out of 382 scaffolds of the Fw12E-Y^T genome assembly (96.68% of the total length) on the DH-1 reference genome sequence (Figure 2A).

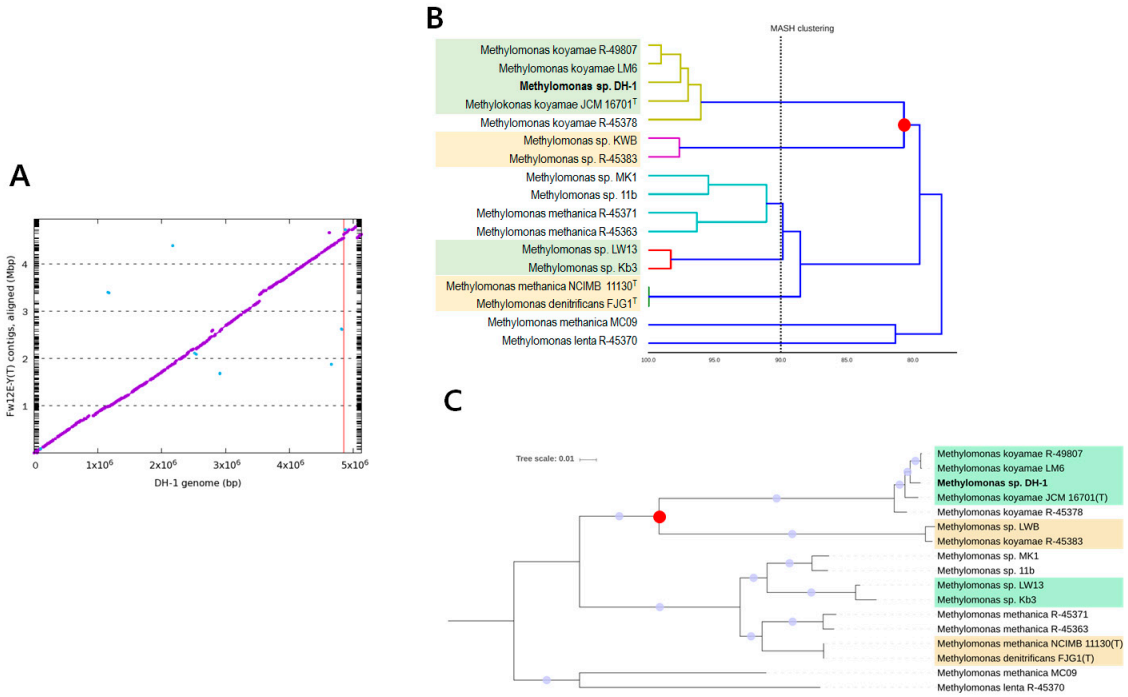

Figure 2. Comparative genomic and phylogenetic analyses of 17 *Methylomonas* strains. (**A**) Whole-genome alignment between DH-1 (*x*-axis) and Fw12E-Y^T (*y*-axis). The red vertical line at the 4.85 Mb position represents the concatenation junction between the chromosome and the plasmid sequences. Ticks on the *y*-axis denote the ends of aligned contigs. (**B**) The average nucleotide identity (ANI) dendrogram based on genome sequence similarities. Strains in one colored box belong to one species. (**C**) Phylogeny of *Methylomonas* strains based on the concatenated 37 marker gene sequences, visualized using the Interactive Tree Of Life server (http://itol.embl.de/). Colored circles on branches represent local support values based on the Shimodaira–Hasegawa test (>0.998). Red circles indicate clades whose subtree topologies are congruent in both trees, which accommodate all strains with the original label *M. koyamae*.

As of February 2018, there are 17 publicly available genome sequences in the genus *Methylomonas* (four at the 'Complete Genome' assembly level), including three strain types: *M. denitrificans* FJG1^T, *M. koyamae* Fw12E-Y^T, and, *M. methanica* NCIMB 11130^T. The three strains *Methylomonas* sp. LW13, *Methylomonas* sp. MK1, and *Methylomonas* sp. 11b were all isolated from a single geographical location [8]. The seven genome sequences of *M. methanica* (NCIMB 11130^T, R-45363, and R-45371), *M. koyamae* (R-45378, R-45383, and R-49807), and *M. lenta* (R-45370), isolated from different terrestrial ecosystems, were reported by a single research group [9]. The genome sizes range from 4.70 Mb (*M. lenta* R-45370, 171 contigs) to 5.48 Mb (*M. methanica* R-45371, 120 contigs).

The ANI-based genome analysis clustered 17 strains in eleven species, the *M. koyamae* group being the largest one, accommodating strains Fw12E-Y^T, DH-1, LM6, and R-49807 (Figure 2B,C). Multiple non-type strains originally labeled as *M. koyamae* or *M. methanica* were classified into separate groups, which implies that repositioning is required for these strains. It was found that two strain types, *M. methanica* and *M. denitrificans*, appear to form a conspecific group (100.0% ANI; 92.3% DDH estimate), while 16S rRNA sequence similarity is at 98.9%. The complete genome sequences of DH-1 and LM6 could be aligned with each other collinearly without any indication of gross rearrangement of insertion/deletion both in chromosome and in plasmid (data not shown).

The genome annotation predicted 4669 protein-coding genes, 47 tRNA and 9 rRNA (Table 1). Furthermore, there were 3638 genes assigned to different function categories based on the clusters of orthologous genes (COG) designation (Table 2) [10]. The most abundant COG category was "General function prediction only" (381 CDSs), followed by "Signal transduction mechanisms" (349 CDSs).

A single gigantic gene (AYM39_10365, 32.6 kb) was found to encode a hypothetical transmembrane protein with repetitive domains which have Ca^{2+} and carbohydrate binding property. Eight contigs from Fw12E-Y^T were aligned consecutively with this sequence and complete gene sequences were found from strains LM6, R-49807, and R-45378, while no homologous sequence could be found from other genomes, which suggests that this is a common characteristic of the *Methylomonas koyamae* species that could increase bacterial fitness under specific environmental niches [11].

Table 1. The general genome features of *Methylomonas* sp. DH-1.

Feature	Chromosome	Plasmid [1]
Size (bp)	4,849,532	277,875
G + C content (%)	56.47	51.66
Protein coding genes [2]	4441	228
Pseudogenes	85	13
tRNAs	47	0
rRNAs	3, 3, 3 (16S, 23S, 5S)	0
ncRNAs	4	0
CRISPR arrays	4	0
GenBank accession	CP014360	CP014361

[1] For the prediction of plasmidic genes, only Prokaryotic Genome Annotation Pipeline (PGAP) annotation was accepted without integration of multiple predictions. [2] Including pseudogenes.

Table 2. The COG function classification of the *Methylomonas* sp. DH-1 genome.

Category	Functional Classification	Chromosome	Plasmid
A	RNA processing and modification	1	1
B	Chromatin structure and dynamics	2	0
C	Energy production and conversion	200	1
D	Cell cycle control, cell division, chromosome partitioning	53	4
E	Amino acid transport and metabolism	191	3
F	Nucleotide transport and metabolism	58	0
G	Carbohydrate transport and metabolism	111	0
H	Coenzyme transport and metabolism	157	1
I	Lipid transport and metabolism	73	0
J	Translation, ribosomal structure and biogenesis	172	0
K	Transcription	183	12
L	Replication, recombination and repair	242	32
M	Cell wall/membrane/envelope biogenesis	248	13
N	Cell motility	123	0
O	Posttranslational modification, protein turnover, chaperones	162	5
P	Inorganic ion transport and metabolism	223	9
Q	Secondary metabolites biosynthesis, transport and catabolism	63	2
	General function prediction only	366	15
S	Function unknown	329	10
T	Signal transduction mechanisms	340	9
U	Intracellular trafficking, secretion, and vesicular transport	127	9
V	Defense mechanisms	83	5

All genes required for a type I methanotrophic lifestyle were identified. One functional operon encoding particulate methane monooxygenase (pMMO, *pmoCAB*), and the pxm operon (*pxmABC*), encoding the copper membrane monooxygenase [12] was determined in the DH-1 genome. All genes for carbon fixation via the ribulose monophosphate pathway were predicted. Genes encoding PQQ-dependent methanol dehydrogenases (*mxaFJGIRSACKLDEK*) along with the PQQ biosynthesis gene cluster (*pqqBCDE*) for methanol oxidation were detected. The tetrahydrofolate (H4F)- and tetrahydromethanopterin (H4MPT)-mediated formaldehyde oxidation pathways and formate dehydrogenase were encoded. Notably, the genome of DH-1 possesses two types of the

gene cluster encoding 3-hexulose-6-phosphate synthase (*hps*) and 6-phospho-3-hexuloisomerase (*phi*) including a *hps-phi* operon and another *hpsi* gene encoding an hps-phi fused protein [13].

2.2. Functional Analysis of the Complete Genome Sequence of Methylomonas sp. DH-1 and the Production of Succinate from Methane

The existence of the complete Embden–Meyerhof–Parnas (EMP) pathway, the pentose-phosphate pathways (PPPs), and the Entner–Doudoroff pathway (EDD) along with a complete TCA cycle were confirmed. Interestingly, a complete set of genes for the serine cycle was identified together with the gene encoding phosphoenolpyruvate carboxylase (*ppc*) which plays a key role in the serine cycle by converting phosphoenolpyruvate (PEP) to oxaloacetate with the addition of CO_2. Some type I methanotrophs have been predicted to have a partial serine cycle due to the absence of *ppc* [8,14]. The existence of PEP carboxylase together with pyruvate carboxylase and acetyl-CoA carboxylase indicates that DH-1 possesses more potential for CO_2 fixation compared to other type I methanotrophs. The ability to convert PEP to oxaloacetate, a key intermediate in the TCA cycle, is also advantageous in the production of TCA-derived products such as succinic acid. Additionally, most type I methanotrophs have no remarkable accumulation of succinate in aerobic conditions because 2-oxoglutarate cannot be converted to succinyl-CoA due to poor activity of 2-oxoglutarate dehydrogenase. It forms an incomplete "horseshoe" shaped TCA cycle [15], and consequently succinate could not be accumulated in aerobic conditions. Unusually, *Methylomicoribum buryatense*, Type I methanotroph, accumulated a detectable amount of succinate in aerobic conditions, because it has three different genes for succinate synthesis [16]. Even though DH-1 has only a TCA cycle as a succinate generation pathway, a large amount of succinate of up to 80 mM was successfully accumulated under aerobic growth conditions, indicating that DH-1 has 2-oxoglutarate dehydrogenase activity and the ability to operate a full TCA cycle (Figure 3).

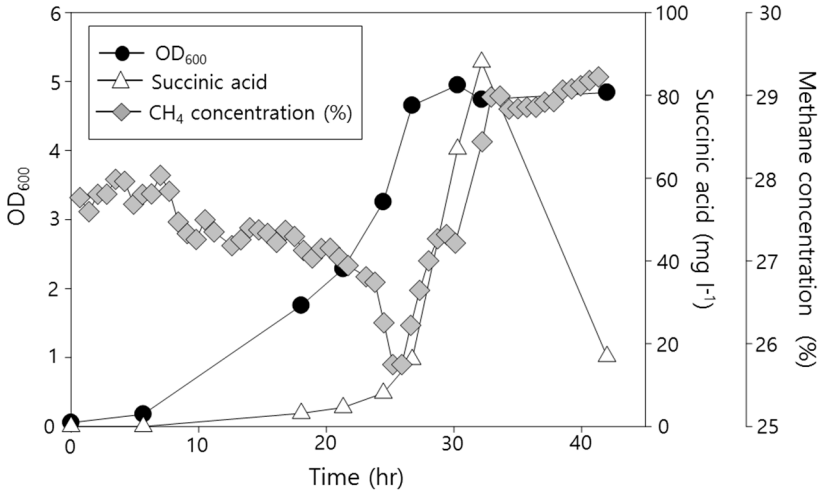

Figure 3. The growth curve and succinic acid production of *Methylomonas* sp. DH-1. The cells were grown in nitrate mineral salts (NMS) media with a bioreactor where a gas mixture of 30% CH_4, 55% N_2, 15% O_2, was continuously fed at the speed of 40 mL min^{-1}. The methane concentration in the reactor off-gas was indicated in the off-set *y*-axis.

The existence of a set of genes related to various secondary metabolite biosynthesis pathways via the methylerythritol 4-phosphate (MEP) pathway, including isoprenoid and carotenoid pathways, was confirmed (Figure 4). Notably, the DH-1 genome contains two genes encoding 1-Deoxy-D-xylulose 5-phosphate synthase catalyzing the first step of the MEP pathway. However, the carotenoids biosynthesis pathway in DH-1 has not been fully discovered. From the genome mining analysis, squalene/phytoene synthase (*sqs*) which is committed in the carotenoid synthesis pathway and the

gene cluster related to 4,4′-diapolycopene biosynthesis including diapolycopene oxygenase (*crtP*), phytoene desaturase (*crtI*) and aldehyde dehydrogenase (*ald*) were identified in DH-1. Other potential secondary metabolites that can be synthesized by *Methylomonas* sp. DH-1 were identified using antiSMASH [17]. The results indicated that eight possible gene clusters encoding secondary metabolites were identified in *Methylomonas* sp. DH-1 including aryl polyene, bacteriocins, terpene, hserlactone, and T1pks-Nrps. Among them, aryl polyene can protect the bacterium from reactive oxygen species, similar to the functionality of carotenoids [18].

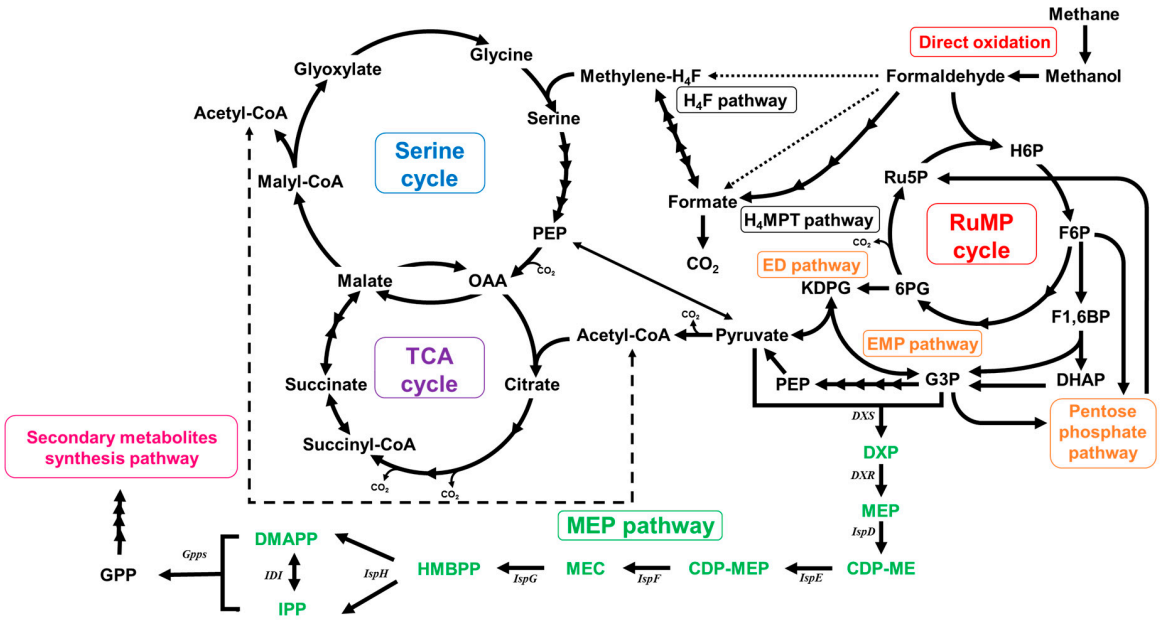

Figure 4. The proposed metabolic pathway of C1 assimilation and the secondary metabolites biosynthesis pathway of *Methylomonas* sp. DH-1. DXP: 1-deoxy-D-xylulose-5-phosphate; DXS: DXP synthase; MEP: 2-C-methyl-D-erythritol-4-phosphate; CDP-ME: 4-diphosphocytidyl-2-C-methyl-D-erythritol; CDP-MEP: 4-diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate, MEC: 2-C-methyl-D-erythritol-2,4-cyclopyrophosphate; HMBPP: (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; DXR: DXP reductoisomerase; IspD: CDP-ME synthase; IspE: CDP-ME kinase; IspF: MEC synthase; IspG: HMBPP synthase; IPP: isopentenyl pyrophosphate; DMAPP: dimethylallyl pyrophosphate; IspH: HMBPP reductase; IDI: isopentenyl diphosphate isomerase; GPP: geranyl pyrophosphate; Gpps: GPP synthase.

2.3. Nucleotide Sequence Accession Number

The completed genome sequence of *Methylomonas* sp. DH-1 was deposited at GenBank under accession number CP014360 and CP014361. In addition, the strain was deposited at the Korean Collection for Type Culture under the KCTC number 13004BP.

3. Conclusions

We sequenced and analyzed the whole genome of a newly isolated type I methanotroph, *Methylomonas* sp. DH-1 consisting of a 4.86 Mb chromosome and a 278 kb plasmid. *Methylomonas* sp. DH-1 accumulated a large amount of succinate (up to 80 mM) under aerobic conditions most probably due to 2-oxoglutarate dehydrogenase activity, showing its biocatalytic potential for methane bioconversion. The existence of PEP carboxylase, pyruvate carboxylase and acetyl-CoA carboxylase can enable the DH-1 strain to fix CO_2 more efficiently compared to other type I methanotrophs. A set of genes related to various secondary metabolite biosynthesis pathways via the MEP pathway was also identified. The availability of a complete genome sequence of *Methylomonas* sp. DH-1 contributes

to a system-level understanding of methanotrophic metabolism which provides valuable resources for metabolic engineering of this strain for overproduction of value-added chemicals from methane.

4. Materials and Methods

4.1. Bacterial Growth, DNA Isolation, Genome Assembly and Annotation

Methylomonas sp. DH-1 was isolated from the activated sludge of a brewery plant based in a nitrate mineral salts (NMS) medium using enrichment culture with methane as a sole carbon source as described by Hur et al. [4]. Liquid pre-cultures were grown in a 180 mL baffled-flask with a 10 mL NMS medium containing 10 μM $CuSO_4$ with a supplement of 30% methane (v/v) as a sole carbon source at 30 °C and 230 rpm, sealed with a screw cap. The pre-cultures were then inoculated into 50 mL of fresh medium in a 500 mL baffled-flask for large-scale cultivation.

The genomic DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). The library construction and sequencing were carried out at NICEM, Seoul, Republic of Korea. The general genome properties were first obtained using Illumina HiSeq 2500 platform-based draft genome sequencing (2 × 151 nt; 1.6 Gb) and then, the PacBio RS II platform was used to obtain the complete genome sequence, which was polished and verified using the previously generated Illumina reads. The 807.6 Mb filtered polymerase reads, produced from the PacBio RS II sequencing using P6-C4 chemistry with 119-fold average coverage was assembled into two contigs using the hierarchical genome assembly process RS_HGAP.3 [19]. The identification of sequence overlap at both ends and the alignments with the Illumina assemblies revealed their circular structures. The genome annotation was performed by integrating results from Prokaryotic Genome Annotation Pipeline (PGAP) (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/), Integrated Microbial Genomes (IMGs) (http://jgi.doe.gov/data-and-tools/img/), Rapid Annotation using Subsystem Technology (RAST) (http://rast.nmpdr.org/) and PROKKA [20] on the basis of stop codons. The priority for choosing functional annotation was in the order of PGAP, IMG, PROKKA, and RAST (Tables S1 and S2). The genome sequence information for comparative and phylogenomic analyses was downloaded from the RefSeq database. The ANI-based genome comparison and clustering were done using DREP [21]. The universal prokaryotic marker gene sequences, identified using the PHYLOSIFT [22], were concatenated into one and an approximately maximum-likelihood tree was constructed using the FASTTREE 2 [23].

4.2. Analytical Methods

The supernatant of cultures was separated by centrifugation. The succinate was quantified using a HPLC equipped with an Aminex HPX-87 column (Bio-Rad, Hercules, CA, USA) and a refractive index detector. Sulfuric acid to the amount of 0.005 M was used as the mobile phase with a flow rate of 0.7 mL/min at 60 °C. The bioreactor off-gas was connected to the GC (Agilent 7890A, Santa Clara, CA, USA) and the methane composition was analyzed by a GC equipped with a TCD detector.

Supplementary Materials: Supplementary materials can be accessed at: http://www.mdpi.com/2073-4344/8/3/117/s1, Table S1: Annotated genes (legend in the following worksheet), Table S2: Secondary metabolite related genes.

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Author Contributions: Anh Duc Nguyen annotated whole the genome sequence and prepared a draft of the manuscript. Dong Hoon Hur, In Yeub Hwang, Young Chan Jeon and Ok Kyung Lee isolated *Methylomonas* sp. DH-1 and conducted the genome analysis-related experiments. Susila Hadiyati and Min-Sik Kim cultivated *Methylomonas* sp. DH-1 in the bioreactor and analyzed the succinate production. Sung Ho Yoon and Haeyoung Jeong contribute to the genome assembly, annotation, and bioinformatics analysis. Eun Yeol Lee coordinated the study and finalized the manuscript. All authors have read and approved the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

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