공학박사 학위논문

Metabolic Engineering of *Methylomonas* sp. DH-1 for the production of D-lactate from methane

Methylomonas sp. DH-1의 대사공학을 통한 메탄으로부터 D형 젖산 생산

2023 년 8 월

서울대학교 대학원

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이종관의 공학박사 학위논문을 인준함

2023 년 8 월

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Abstract

Metabolic Engineering of *Metyhlomoans* sp. DH-1 for the production of D-lactate from methane

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Biological conversion of methane to valuable chemicals such as a lactic acid is considered as a promising strategy regarding the abundance of methane. Here, a newly isolated Type I methanotroph, *Methylomonas* sp. DH-1 was used as a host strain for D-LA production from methane. By using evolutionary and metabolic engineering, highly efficient D-LA producing methane biocatalyst was developed.

Firstly, to improve lactate tolerance of *Methlyomonas* sp. DH-1, adaptive laboratory evolution was performed by gradually increasing the lactate concentrations in the culture medium. The LA evolved strain (JHM80) survived in the presence of 8 g/L of lactate while the wild-type strain barely grows in the presence of 0.5 g/L. By introducing stereospecific D-lactate dehydrogenase gene (*Lm.LDH*) into the chromosome of JHM80 while deleting the *glgA* gene encoding glycogen synthase, 750 mg/L of D-LA was

produced with the periodic methane supply, which was 7.5-fold higher than the wild-type strain. LA production was further improved by medium neutralization and optimization, resulting in a titer of 1.19 g/L and a yield of 0.245 g/g CH_4.

Secondly, to demonstrate the LA tolerance mechanisms in the LA evolved strains, whole genome sequencing was carried out. Genome analysis revealed up-regulation of AYM39_21120 (*watR*) gene encoding a LysR-type transcription factor, by 2bp (TT) deletion in the promoter region is partly responsible for LA tolerance of JHM80. Overexpression of *watR* gene improved LA tolerance of wild-type strain while the deletion of watR gene abolished LA tolerance of JHM80. Transcriptomic analysis further identified the overexpression of RND-type efflux pump was a major LA tolerance contributor mediated by WatR transcription factor.

Lastly, inducible promoters were used for enhancing LDH expression while minimizing the growth defects by lactate accumulation in the early growth phase. Under the control of IPTG inducible tac promoter, LDH expression was increased by 3.1 folds than the native *glgA* promoter. The *glgC* gene encoding enzyme related to ADP-glucose synthesis was further deleted to prevent the growth inhibition by ADP-glucose accumulation, resulting the final LA producing strain, JHM806. In the continuous gas fermentation, JHM806 produced 6.16 g/L of D-LA with the productivity of 0.057 g/L/h, which are highest ever reported in methanotrophs.

Keywords: Methane, D-LA, *Methylomonas* sp. DH-1, Adaptive evolution, Metabolic engineering

Student Number: 2017-21205

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List of Abbreviations

AOM : anaerobic oxidation of methane

CBB : calvin benson bassham

DHAP : dihydroxyacetone phosphate

ED : entner doudoroff

EMC : ethylmalonyl coA

EMP : embden Meyerhof parnas

FADH : flavin adenine dinucleotide

F 1,6BP : fructose-1,6-bisphosphate

G3P : glyceraldehyde-3-phosphate

HPLC : high performance liquid chromatography

H6P : hexulose-6-phosphate

LA : lactic acid

LAB : lactic acid bacteria

LB : luria-bertani

LDH : lactate dehydrogenase

KDPG : 2-keto-3-deoxy-6-phosphate

MMO : methane mono oxygenase\

MDH: methanol dehydrogenase

NADH : nicotineamide adenine dinucleotide

NMS : nitrate mineral salt

OD : optical density

PHA : poly hydroxyl alkanoate

PHB : poly hydroxyl butyrates

PLA : poly lactic acid

pMMO: particualte methane monooxygenase

PP : pentose phosphate

qRT-PCR : quantitative reverse transcription PCR

RuMP : ribulose monophosphate

Chapter 1.

Research background and objective

Lactic acid (LA) has wide range of applications due to their various properties. In recent decades, LA has gained much attention as a monomer of poly lactic acid (PLA), one of the major biodegradable plastics in the current bioplastic market. Since the thermal and mechanical properties of PLA are determined by the ratio of L-LA and D-LA, microbial fermentation is favored for optically pure LA production [1, 2]. Lactic acid bacteria (LAB) was majorly used for LA fermentation, but they require complex media compositions and culture conditions. As a new host for LA production, genetically engineered bacteria and yeasts were used based on sugar fermentation [3-5]. However, there are issues about the high cost of sugar and using human feedstock as a carbon sources.

Recently, methane, a main component of natural gas and biogas, has gained much attention as a next generation feedstock [6-8]. Methane is also a greenhouse gas with a high global warming potential. Methatnorophs are considered as a promising biocatalyst due to their ability using methane as a sole carbon and energy sources. In methaotrophs, methane is oxidized to methanol by methane monooxygenase (MMO) and methanol further converted to formaldehyde by methanol dehydrogenase (MDH). Formaldehyde can be assimilated by 3 different pathways; the Ribulose monophosphate (RuMP) pathway in Group I (Type I and Type X) methnoatoprhs, the serine cycle in Group II (Type II) methanotrophs, and Calvin-Benson-Bassham (CBB) cycle in Group III (Type IV) methanotrophs [9]. Most studies have focused on Group I methanotrophs since they relatively grow fast and possess glycolytic pathway which is beneficial for applying traditional pathway engineering strategies. Several chemicals including astaxanthin, butyrate, succinic acid, cadaverine, sesquiterpenoids but with very low titers [10-14].

Some efforts have been made to produce LA in Group I methanotrophs by expressing heterologous lactate dehydrogenase (LDH). In *Metyhlomicrobium buryatense* 5GB1S, by episomal expression LDH from *Lactobacillus helveticus*, 0.8 g/L of L-LA was produced in continuous gas fermentation [15]. In the same strain, various promoters and ribosome binding sites were used for LDH expression, producing 0.6 g/L L-LA in small-scale bottles with periodic methane supply [16]. The maximum lactate tolerance of *M. buryatense* was 0.8 g/L which coincided with the maximum titer of LA, and the toxicity of lactate was major limiting factor for LA production. Another Group I methanotroph, *Metyhlomicrobium alcaliphilum* $20Z^R$, was engineered by deleting pyruvate dehydrogenase as a competing pathway forming Acetyl-CoA from pyruvate [17] . However, titer was below 0.8 g/L, confirming increase of lactate tolerance is important for LA production in methanotrophs. Furthermore, little is known about the toxicity mechanism of lactate and the LA adaption response in methanotrophs.

In this study, *Methyloonas* sp. DH-1, a newly isolated Type I methanotroph, was used as a host strain for LA production. It has several advantages including fast growth and availability of genome sequences. In addition, *Methylomonas* sp. DH-1 does not have its own LDH gene, which means optically pure D-LA production can be achieved by introducing D-specific LDH genes.

The ultimate aim of this study is to develop the efficient D-LA producing strain of *Methylomona*s sp. DH-1 by improving its lactate tolerance. The first objective is the increase of lactate tolerance by adaptive evolution and production of D-LA in the evolved strains using genome and transcriptomic analysis. The last objective is improvement of LA production by fine-tuned expression of LDH and reducing toxic effects of ADP-glucose accumulation.

Chapter 2.

Literature review

2.1. Importance of biological conversion of methane

2.1.1. Methane

Methane (molecular formula of CH4) is important fuel providing energy and heat via combustion. Methane is primarily used for generating electricity in gas turbines or steam boilers but is also pied into homes for heating and cooking. However, emission of methane has negative effects to atmosphere since it has 20 times higher global warming potential than carbon dioxide [18, 19]. Methane concentration has been increased to 1857 bbp in 2018, which is 2.6 times higher than in preindustrial times [20]. Methane is emitted both from natural and anthropogenic sources. Anthropogenic activity accounts for 63% of global methane emission and natural sources account for remainder [21]. Fosiil fuels, livestock faming, landfilling, and biomass burning are the major sources of anthropogenic methane emission. Natural methane emissions are originated from abiogenic, thermogenic, biogenic microbial sources, and wild animals [22-24] Methanogenic archaea liberate methane as end-product from degrading organic matter and they are present in diverse environments including wetlands, peatlands, agriculture soil, landfills, and ocean [21, 25]. Most recent estimations indicate total average net methane production 737 Tg CH_4/year and total terrestrial and aquatic sinks 625 737 Tg CH_4/year, resulting emission of 112 Tg CH_4/year [20]

In the other aspects, methane is most reduced form of carbon which means it can generate more available electrons for target product formation [26]. Furthermore, methane is the most abundant carbon sources composing 80~95% of natural gas and shale gas. In this context, many efforts have been made for chemical or biological conversion of methane to methanol due to ease of storage [27]. Chemically, methane is transformed

into methanol by two-step syngas process. Briefly, methane is converted to syngas consisting H2 and CO2 by hydro-reforming process, and syngas is further converted into methanol by metal catalysts. In terms of cost, the equipment accounts almost 60% of the total capital costs [28, 29]. Furthermore, the overall conversion and selectivity are below 25% and 70%, respectively [30-32]. Therefore, biological conversion of methane has gained much attention since it can be conducted in the mild conditions with simple process.

2.1.2. Biological conversion of methane

In biological system, methane is oxidized by methanotrophic cells (methanotrophs). Methanotrophs are bacteria or archaea which can utilize methane as sole carbon and energy sources. They can oxidize methane under both aerobic and anaerobic conditions using wide range of electron acceptors. After the initial report of aerobic methanotrophs in 1906, aerobic oxidation of methane was considered as a sole biological pathway and all methanotrophs belongs to protebacteria. However, in last two decades, archaea which anaerobically oxidize methane using sulfate, nitrate, iron and manganese as alternative electron acceptors have been identified [33, 34] . In ocean sediments, anaerobic oxidation of methane (AOM) is coupled to sulfate-reduction (AOM-SR), and some microbes were characterized [35-37] .The methanogen-like anaerobic methanotrophic archaea (ANME) have also been reported to perform AOM with sulfate-reducing bacteria. Reverse reaction of final step in methanogenesis (producing methane) was identified as a mechanism [38-40]. Denitrification can also be coupled to AOM (AOM-D) (Fig. 2.1). AOM-D is thermodynamically more favorable than AOM-SR and has been demonstrated in lake sediments with high NO_3^- [41, 42]

In aerobic methanotrophs, methane is oxidized by methane mono-oxygenase (MMO) using oxygen as electron acceptor. There are two kinds of MMO; particulate form (pMMO) within intracellular membrane and a soluble form (sMMO) within the cytoplasm. The expression levels of pMMO and sMMO are regulated by copper ions. Under the low concentrations of copper, sMMO is expressed while pMMO is expressed when copper-to-biomass ratio is high [43-46]. The sMMO contains di-iron center in the actie sites and can be produced by various α- and γ- proteobacteria with broad substrate specificity including propane, cyclohexane, toluene, naphthalene, and CO [47, 48]. It is believed that copper center in the active site of pMMO is important for methane oxidation and pMMO is produced in most methanotrophs with exceptions such as *Methylocella* spp. expressing sMMO exclusively[49, 50] .

2.1.3. sMMO and pMMO

The sMMO catalyzes methane oxidation using reduced form of nicotine amide dinucleotide (NADH) as an electron donor. The sMMO consists of three components; a reductase (MMOR), a regulatory protein (MMOB), and a hydroxylase (MMOH). MMOR contains [2Fe-2S]-ferredoxin cofactor, and a bound Flavin adenine dinucleotide (FAD) which transfer electrons to reduce the Fe ions in MMOH (Fig 2.1.) [51-53]. Di-iron center in MMOH is cyclically changed including intermediates P ad intermediate Q with various iron states [54].

The membrane-associated pMMO consists of three subunits pmoA (β), pmoC (γ), and pmoB (α) with trimeric $(\alpha\beta\gamma)_3$ polypeptide arrangements [55, 56]. The subunits of pmoA and pmoC with helix structure exist in membrane. The pmoB subunit has two

transmembrane helix and soluble domain which plays important role in catalysis [57] . In *Methylococcus capsulatus*, pMMO structure of dinuclear copper site (coordinated by His 133, His 137, His 139), mononuclear copper site (coordinated by His 48 and His 72), and zinc site was identified in the active site to activate O2 with various oxidation states [56]. Three possible mechanisms have been proposed for methane oxidation; 1) redox arm: ubiquinol (UQH_2) from electron transport chain drives methane oxidation 2) Uphill electron transfer: electrons from methanol oxidation transferred to pMMO by respiratory chain complex III 3) direct coupling: electrons from methanol oxidation partially drives methane oxidation by feeding back to ubiquinol (Fig 2.2) [58, 59]

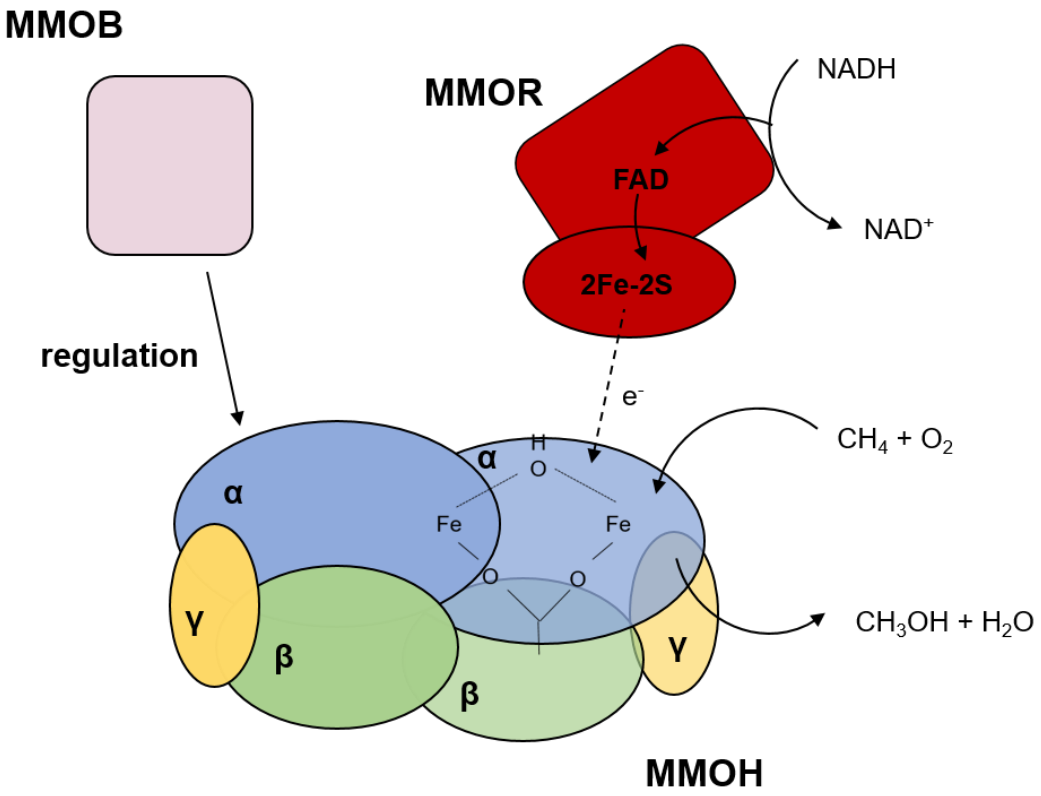


Figure 2.1 The structure of Soluble methane monooxygenase (sMMO)

The sMMO structure consists of three subunits, MMOB: regulatory protein, MMOR: reductase, MMOH: hydroxylase. The MMOR contains [2Fe-2S] ferredoxin cofactor and bound FADH, which transfers electron from NADH to the di-iron center of MMOH subunits.

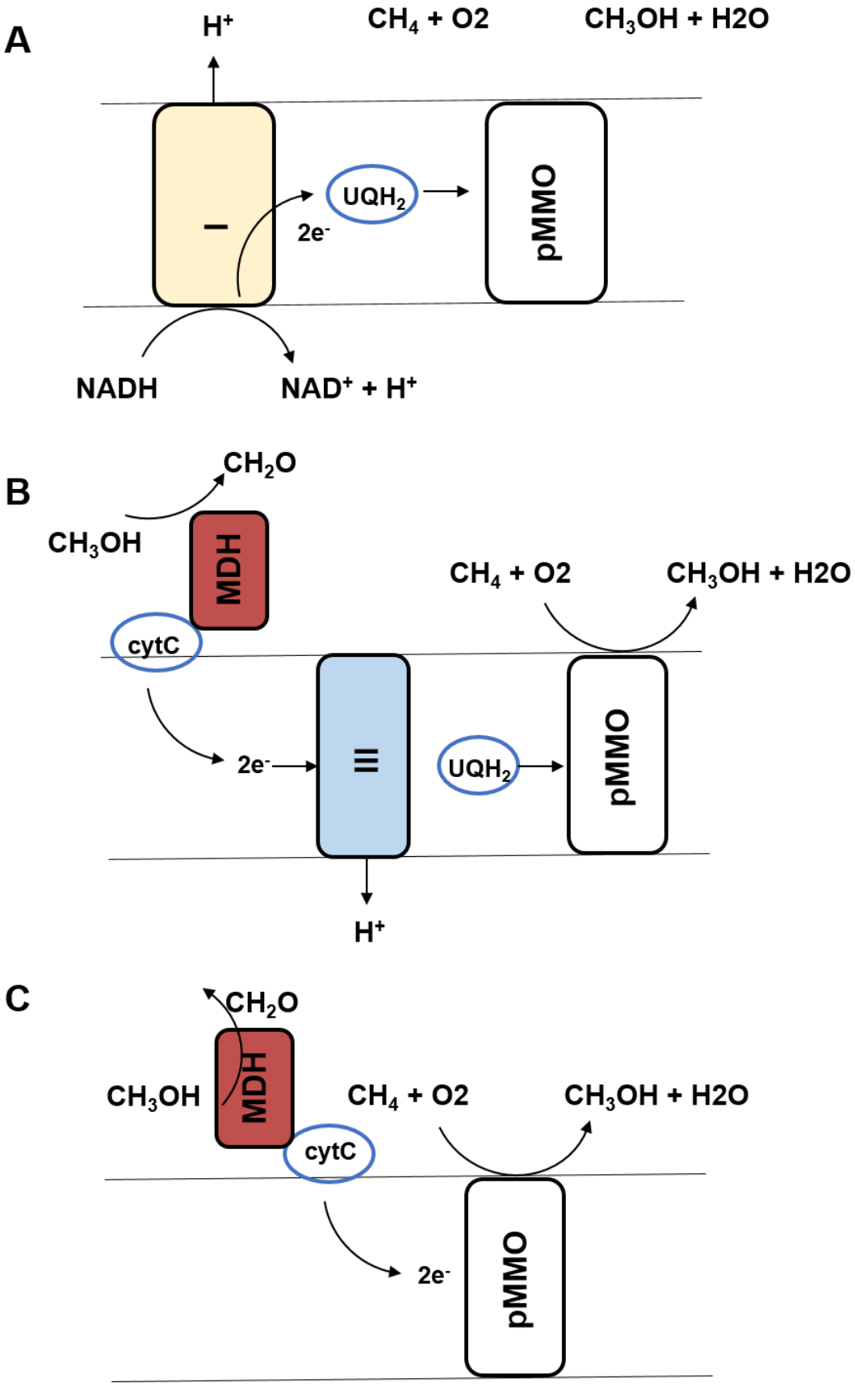

A
H+
CH4 + O2
CH3OH + H2O
UQH2
2e-
pMMO
NADH
NAD+ + H+
B
CH2O
CH3OH
MDH
CH4 + O2
CH3OH + H2O
cytC
2e-
UQH2
pMMO
H+
C
CH2O
CH3OH
MDH
CH4 + O2
CH3OH + H2O
cytC
2e-
pMMO

Figure 2.2 Three possible mechanisms of methane monooxygenase in pMMO

Schematic illustration of the three possible methane oxidation mechanisms

(a): redox arm, (b): uphill electron transfer, (c): direct coupling

acids [2]. *ARO9* gene encodes an aromatic aminotransferase II involved in the first catabolic step of tryptophan, phenylalanine and tyrosine [39]. *ARO10* gene encodes a decarboxylase [7]. The transcription of *ARO9* and *ARO10* is induced in the presence of aromatic acids and in the presence of a poor nitrogen source such as urea and proline, which is repressed in the presence of a good nitrogen source such as glutamine or ammonia [2]. Some branched chain amino acids induce the expression of *ARO9* and *ARO10* via Ehrlich Pathway in *S. cerevisiae* [9].

Ehrlich pathway is a well-known pathway for amino acid catabolism in *S. cerevisiae* (Fig 2.2). In Ehrlich pathway, following transamination of an amino acid into the corresponding 2-keto acid, the 2-keto acid decarboxylates to an aldehyde [5,40]. And then the aldehydes can be either oxidized by aldehyde dehydrogenases to organic acids or reduced by alcohol dehydrogenases to alcohols, which are called fusel alcohols.

Especially, *S. cerevisiae* can utilize aromatic amino acids as a sole nitrogen source via Ehrlich pathway, with the main products of their catabolism being tryptophol, 2-phenethyl alcohol or tyrosol, respectively, which are collectively known as fusel alcohols [5]. Aromatic amino acids directly activates transcriptional factor Aro80, where Aro80 is required for expression of *ARO8* and *ARO9* (encodes aromatic transaminases) and *ARO10* (encodes aromatic decarboxylase). Yeast cells cannot use the tryptophan as a sole nitrogen source by deletion of *ARO80* gene [2]. It means that aromatic amino acid utilization by Ehrlich pathway requires the expression of *ARO9* and *ARO10* via transcription factor Aro80.

2.2. Recent developments in methanotrophs

2.2.1. Assimilation pathways of aerobic methanotrophs

In aerobic methanotrophs, methane is oxidized by methane mono-oxygenase (MMO) using oxygen as electron acceptor. Methanol is further oxidized to formaldehyde by methanol dehydrogenase. [60, 61]. By the metabolic pathway of formaldehyde assimilation, aerobic methanotrophs are classified as three groups (Group I – Type I, Type X, Group II- Type II, and Group III- Type IV) [62]. In Type I methanotrophs (γ-proteobacteria), formaldehyde is condensed to Ribulose 5-phosphate (Ru5P) by RuMP pathway, and Ru5P is further converted to either Fructose 1,6-bisphosphate (F 1,6BP) by EMP pathway or 2-keto-3-deoxy-6-phosphate (KDPG) by Entner-Doudoroff (ED) pathway. F 1,6 BP is cleaved to dihydroxyacetone phosphate (DHAP) and converted to glyceraldehyde 3-phosphate (G3P) and KDPG is cleaved to G3P and pyruvate by KDPG aldolase [63]. In Type II methanotrophs (α-proteobacteria), formaldehyde is assimilated by serine cycle coupled with TCA cycle and ethylmalonyl-CoA (EMC pathway) (Figure 2.3).

Briefly, formaldehyde is converted to Methylene-H_4F by H_4F pathway and condensed with glycine to form serine. Seine is further converted to oxaloacetate (OAA) by serine cycle. OAA can be used for TCA cycle or is further converted to malyl-CoA for re-generation of glycine through EMC pathway [64-66]. Group III (verrucomicrobia) methanotroophs convert formaldehyde to CO2 by formaldehyde dehydrogenase (*fdh*), which is assimilated by Cavin-Bassham-Benson (CBB) cycle.

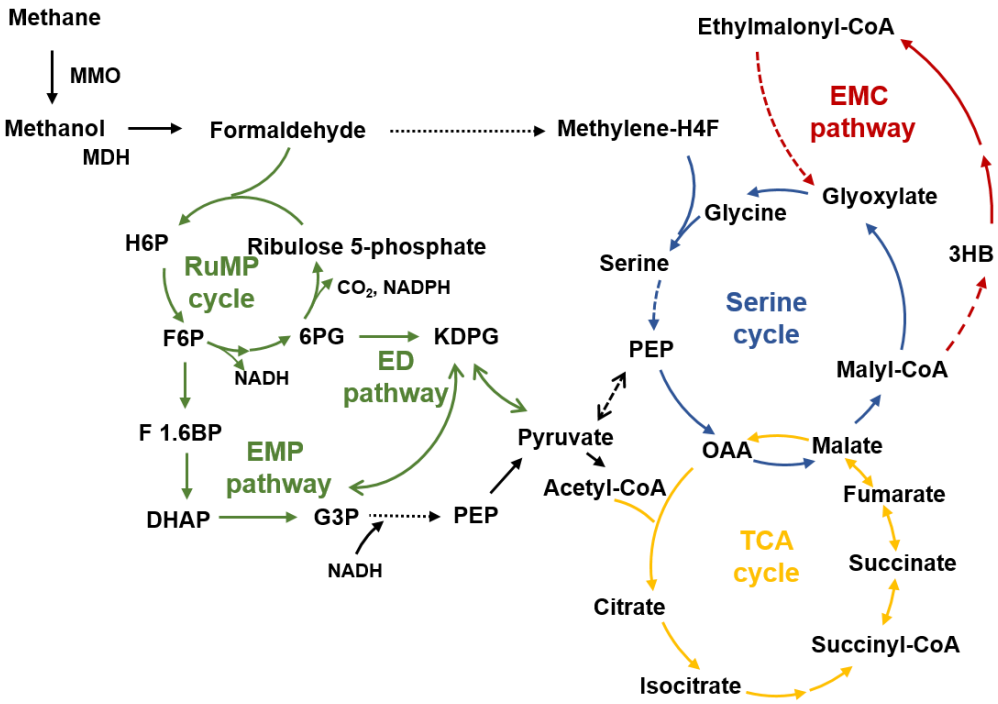


Figure 2.3 Metabolic pathway of Type I methanotroph (RuMP pathway, green) and Type II methantoroph (Serine cycle, blue and EMC pathway, red)

2.2.2. Genetic manipulation tools in methanotrophs

For production of non-native chemicals and higher production of native metabolites, genetic tools are necessary to apply synthetic biology and metabolic engineering strategies. There are some replicable plasmids, mostly incompatibility group P (IncP) group with RP2/RP4 origins including pAWP78 and pVK100. Integration and deletion vectors using homologous recombination were also developed. Conjugation method was firstly used for introducing foreign DNA in methatnotrophs. An electroporation based gene manipulation system was also developed in Type I methanotroph (*M. buryatense* 5GB1) and Type II (*M. silvesris* BL2) [67-69].

For multiple gene manipulation, *sacB* or mutant *pheS* based counter-selection system was also developed. By plating cells in medium containing sucrose or 4-chlorophenylalanine, only cells that has lost antibiotic markers with negative selection markers (*sacB*/mutant *pheS*) was isolated. [70, 71] Along with this, the strength of various promoters was investigated using reporter genes. It was determined that native *mxaF* promoter and tac promoter (P*tac*) are most effective [69]. Inducible promoter such as anhydrotetracycline-inducible promoter (P*tet*) has been used for LDH expression in *M. buryatense* 5GB1S [15]. Recently, gene editing system using CRISPR/Cas9 has been reported in *M. capsulatus* Bath [72].

2.2.3. Chemicals produced in methanotrophs

With the growing interests on the potential of methanotrophs, many efforts have been made for bioconversion of methane to value-added chemicals. During the second half of the last decade, biopolymers (mainly polyhydroxyalkanoates, PHA), and methanol was main focus [73]. PHAs are thermoplastic polyesters produced in wide range of bacteria as a carbon storage in nutrient-deficient conditions [74]. In methanotrophs, majorly polyhydroxybutyrates (PHB), belonging to PHA, is produced from acetyl CoA by sequential reactions by phaA, phaB, phaC [75-77]. Most studies focused on mixed culture of methanotrophs for PHB production. PHB was efficiently accumulated when mixed cultures were dominated by Type II methanotrophs with producing 22.5 % PHB/DCW [78]. To improve the quality of PHA, volatile fatty acids were used as co-substrates, producing co-PHA with increased flexibility [79]. The poly (3-hydroxybutyrate-co-3-hydroxyvalerate, PHBV) was produced by addition of 3-hydroxyvalerate as a co-substrate [80]. Another intensively studied chemical is methanol which produced as the first reaction of methane oxidation. In methanotrophs, methanol is converted to formaldehyde by methanol dehydrogenase (MDH). Since MDH is activated by Ca2+, inhibitors such as phosphate, MgCl2, cyclopropanol, and EDTA to lower calcium concentrations [81, 82]. However, disruption of MDH activity leads to growth inhibition and electron depletion for methane uptake. To overcome these problems, formate is added to provide reducing agents with producing 485 mg/L of methanol [83].

Recent developments of genetic manipulation tools enabled the bioconversion of methane to other valuable chemicals such as biodiesel (mainly fatty acids), and organic acids, and important precursors is positioned as new trends. In methanotrophs, fatty acids

and glycerolipids is produced from Acetyl-CoA during the growth and are incorporated into membranes. Due to robust growth and high lipid content, *Methylomicrobium buryatense* is considered as promising host for fatty acids production. In continuous stirred bioreactor with limitation of CH_4 and O_2, wild-type of *M. buryatense* showed fatty acid content of 10.7% of DCW. To improve production of fatty acids, phosphokeotolase was overexpressed to increase acetyl-CoA pool and glycogen synthesis as competitive pathway was deleted in *M. buryatense*. However, total fatty acid contents were similar to that of wild-type. By modifying fatty acid biosynthesis regulation, 11% increase of fatty acid production has been reported, but restriction of metabolic flux upstream of fatty acid and low carbon conversion efficiency remains metabolic issues. Chemicals produced from methanotrophs are listed in table. 2.1

Table 2.1 Chemicals produced in methanotrophs

Strains	Acuumulation (%), /Titer (g/L)	Reference
PHB		
Heterotrophic-methanotrophic consortium (dominated by *Methylocystis* sp.)	8.4% (84.17 mg/g DCW)	[84]
Methylocystis parvus OBBP	49.4%	[85]
M. trichosporium OB3b	52.5%	[86]
Methlyocystis sp. WRRC1	78% (58 mol% of 3HV fraction)	[87]
Other PHAs	,	
M. parvus OBBP	- 50% P(3HB-co-4HB) 9.5 mol% of 4HB fraction - 48% P(3HB-co-5 HV-co-3 HV), 1.4 mol% of 3 HV and 3.6% mol% 5 HV fraction	[88]
*M. trichosporium OB3b/*SYOI mutant strain	7.01% P(3HB-co-4HB) 3.08% of 4HB fraction	[89]
Methanol		
Methylosinus trichosporium OB3b	12.28 mM (0.393 g/L)	[82]
Strain 14B isolated from SS-AD reactor (similar to *Methylocaldum* sp.)	13.44 mM (0.43 g/L)	[90]
Methylomonas sp. DH-1	41.86 mM (1.34 g/L)	[91]
Strain AS1 isolated from active anaerobic sludge	50 mM (1.6 g/L)	[92]
Fatty acids and lipids		
Methylomicrobium buryatense 5GB1 (FAME)	10.7%	[93]
M. buryatense 5GB1(Lipids)	9.5%	[94]
M. buryatense 5G(B1) mutant Strain (Fatty acids)	11% (111 ± 2 mg/g DCW)	[95]

Table 2.1 Chemicals produced in methanotrophs (continued)

Products	Strain	Accumulation, Titer (g/L)	Reference
Organic acids			
L-Lactic acid	*Methylomicrobium buryatense* 5GB1S	0.8	[15]
L-Lactic acid	*Methylomicrobium alcaliphilum 20Z mutant strain*	0.027 g/g DCW/h	[17]
Crotonic acid Butyric acid	*M. buryatense* 5GB1C	0.06 0.08	[11]
Muconic acid	*M. buryatense* 5GB1	0.012	[96]
Succinic acid	*Methylomonas* sp. DH-1	0.195	[12]
3-HP acid	*Methylosinus trichosporium* OB3b	0.061	[97]
Other products			
2,3-butanediol	*Methylomicrobium alcaliphilum* 20ZM3	0.086	[98]
Putrescine	*M. alcaliphilum* 20ZE4	0.098	[99]
Cadaverine	*Methylosinus trichosporium* OB3b	0.284	[13]
α-humulene	*Methylotuvimicrobium alcaliphilum* 20Z	0.56 mg/g DCW	[14]
α-bisabolene	*Methylotuvimicrobium alcaliphilum* 20Z	12.24 ± 0.43 mg/g DCW (24.55 ± 0.86 mg/L)	[14]

2.2.4. *Methylomonas* sp. DH-1

Methylomonas sp. DH-1, a newly isolated Type I methanotrophs from activated sludge of a brewery plant. It possesses pMMO and four alcohol dehydrogenase including PQQ-dependent methanol dehydrogenase (PQQ-MDH, mxaFI), NAD^+-dependent MDH (NAD+-MDH)), one propanol-preferring NAD^+-dependent ADH (ADH1), and another alcohol dehydrogenase (ADH2) but not sMMO. *Methylomonas* sp. DH-1 efficiently converts alkanes (methane and propane) into the corresponding alcohols (methanol and propanol) [100, 101]. Along with the efficient conversion of methane, fast growth and availability of genome sequences makes this strain as a promising biocatalysts.

This promising strain has been used for production of non-native chemicals such as succinate and cadaverine. Deletion of *sdh* (succinate dehydrogenase) genes which convert desired products succinate to fumarate improved succinate production by 10-folds compared to wild-type strain. By integrating isocitrate lyase and malate synthase, two key enzymes of glyoxylate pathway, succinate production was further enhanced and 195 mg/L of succinate was produced in fed-batch gas fermentation [12] . Another group developed tunable library consisting of 33 promoters with different strengths and by optimizing expression of *cadA* (lysine decarboxylase, converting lysine to cadaverine) and *cadB* (lysine/cadverine antiporter, exporting cadaverine) genes using various combinations of promoter, 18.12 mg/L of cadaverine was produced [102]. In addition, the shift of central metabolism in which formaldehyde oxidation and serine cycles was enhanced to produce acetyl-coA and NADH when carbon sources are changed from methane to methanol was revealed by transcriptomic analysis [103].

2.3. Microbial production of lactic acid

2.3.1. Lactic acid

Lactic acid (LA, 2-Hydroxypropanoic acid) is a three carbon organic acid with the molecular formula C3H6O3. The molecular structure of LA contains one chiral carbon at the center and two terminal carbons which are the part of carboxylic acid and hydroxyl group, respectively (Fig. 2.4). LA is approved by U.S Food and Drug Administration as GRAS (Generally recognized as safe) and it is utilized in various industries including food, cosmetic, chemical, and pharmaceutical fields. Recently, LA has attracted much attention as a monomer of poly lactic acid (PLA), a biodegradable polyester which is a major component of bioplastics [1, 104-106]. Global market of LA is predicted to grow from 1,220 kilotons to 1,960 kilotons in 2025 with increasing demand of PLA [107]. LA can be produced either by chemical synthesis or microbial fermentation. There are many routes for chemical synthesis of LA including sugar degradation, oxidation of propylene glycol, and nitric acid oxidation of propylene [108]. Due to the economical unfeasibility of above mentioned methods, LA is industrially produced by nitronitrile hydrolysis. In brief, lactonitrile is first produced by addition of hydrogen cyanide to the acetaldehyde. Methyl lactate is then produced by hydrolysis of lactontrile with H2SO4 and esterification with methanol. Finally, methanol is separated from LA by distillation, and racemic D/L-LA is purified [1, 109, 110]. However, the racemic D/L-LA has limitations for applications in food and pharmaceutical industries because D-LA cannot be metabolized by human. In addition, optically pure LA is preferable for production of PLA. L-LA is main precursor of PLA and physical properties and biodegradability of PLA can be changed by the ratio of D-LA [111-113]. Optically pure LA can be produced by microbial

production with expressing optically specific LDH genes. With the increasing demand of pharmaceutical use of LA and PLA, several companies such as Corbion Purac (Netherlands), Nautre Wokrs LLC (USA), Pyramid Bioplastics Guben GMBH (Germany), Galctic S. A. (Belgium), Archer Daniels Midland Company (USA), and chinese companies produce almost 90% of LA through fermentation process [1, 114].

2.3.2. LA production by lactic acid bacteria

Lactate dehydrogenase (LDH) reduces pyruvate to lactate using NADH as a cofactor and there are wide range of microorganisms which possess LDH including bacteria, fungi, and algae. Lactic acid bacteria (LAB) are gram-positive bacteria which is the main producers of LA. LAB are divided into two groups, homo-fermentative and hetero-fermentative strains depending on metabolic pathway. In homo-fermentative LAB, one molecule of glyceraldehyde 3-phosphate (G3P) is produced by degradation of one molecule of glucose through Embden-Meyerhoff-Parnas (EMP) pathway. One molecule of G3P is further converted to 2 molecules of LA without forming by-product, resulting theoretical yield of 1.0 g/g (2.0 mol/mol). On the other hand, hetero-fermentative LAB consume one molecule of hexose by phosphogluconate pathway and produce one molecule of LA with one molecule of carbon dioxide (CO_2) and one molecule of ethanol or acetate as by-products, resulting theoretical yield of 0.5 g/g (1.0 mol/mol) (Fig. 2.5). Due to the high yields and high optical purity, homo-fermentative LAB is preferred for commercial production of LA [115-117]. There are many factors affecting LA production using LAB. Though glucose was most preferred substrates for LA fermentation, high concentrations of glucose exhibit growth inhibitory effect with high

cost [118, 119]. In this context, many researchers investigated the raw materials for LA production.

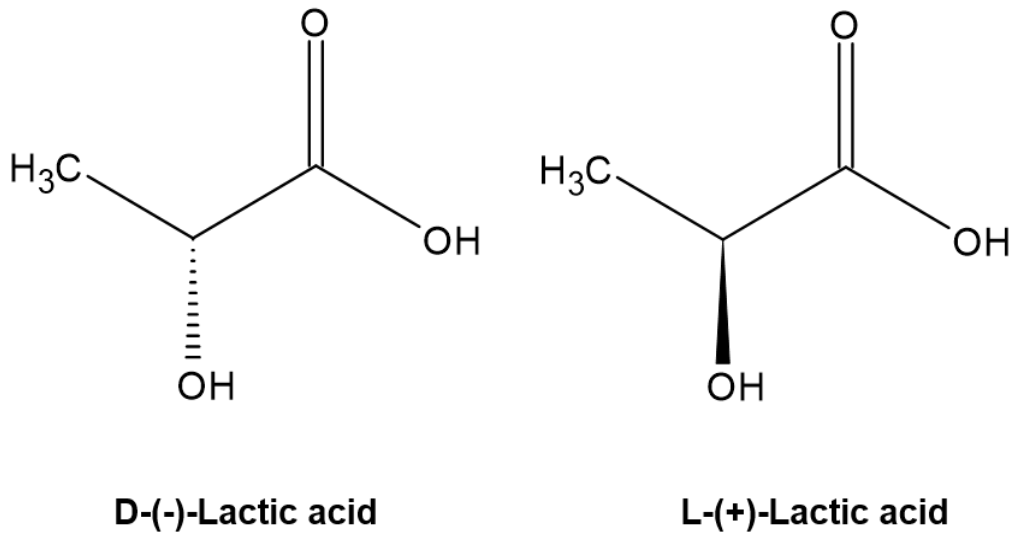


Figure 2.4 Structure of stereo-isomers of lactic acid

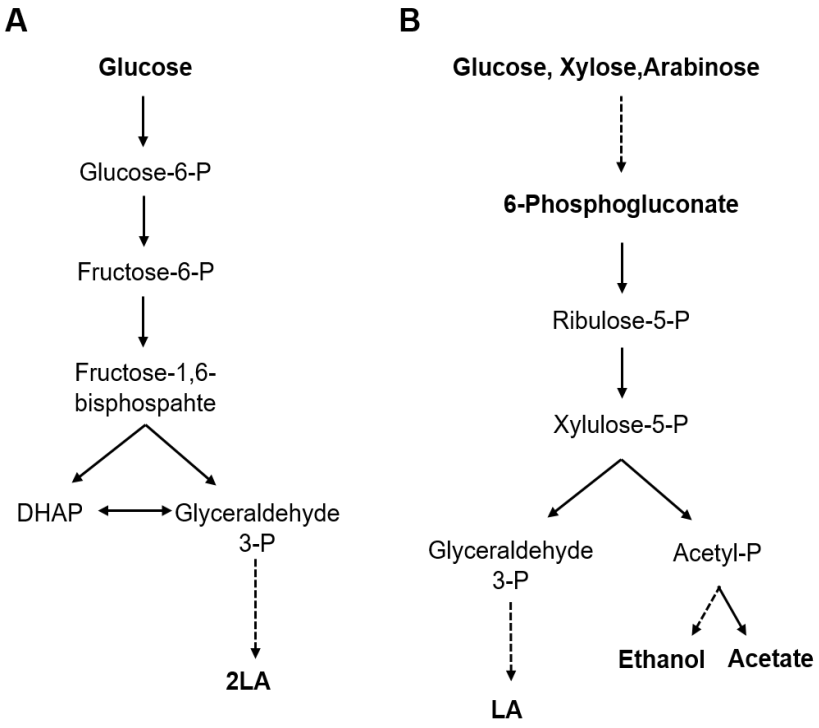


Figure 2.5 Metabolic pathways of LAB for LA production

A. LA fermentation in homo-fermentative LAB

B. LA fermentation in hetero-fermentative LAB

Starchy materials such as wheat, corn, potato, rice are potential raw materials. Approximately, 90% of the commercial LA is produced from corn fermentation [120]. Lignocellulose, the most abundant biomass, is also attractive carbon sources. However, pretreatment process, inhibitory compounds generated during hydrolysis process, and production of mixed pentose exist as challenge [121, 122]. Nitrogen is another important factor for LA fermentation. LAB requires nutritionally rich media because of their limited ability to synthesize vitamins and amino acids [106, 123]. Complex nitrogen sources such as peptone, meat extracts, and yeast extract were used to fulfill nutritional requirements, however, this addition significantly increases the cost. In this context, several studies have investigated the alternative nitrogen sources. In *Lactobacillus* sp. MTKLC878, addition of corn steep liquor as a nitrogen source showed increase of LA production [124]. The acidity of medium is another important factor. Since, general optimum pH of LAB is around 5~7, acidification by LA production inhibits fermentation. Therefore, neutralizing agent such as calcium carbonate and trimethylamine was used for pH maintenance [125, 126]. However, industrial LA production prefers fermentation below the pH 3.8 because undissociated LA is more easy to purify. To overcome this problem, some researchers developed acid-tolerant strains by genome shuffling or error-prone PCR of whole genome [127, 128].

Table 2.2 Chemicals produced in methanotrophs

Microorganism	Substrate	Titer (g/L)	Reference
Bacillus coagulans C106	Municipal solid wastes	60.7	[146]
B. coagulans WCP 10-4	Glucose	210	[147]
Corynebacterrium glutamicum	Glucose	120	[148]
Escherichia coli W3110 SZ40	Glucose	51	[149]
E. coli AC-521	Sucrose	85	[150]
E. coli strain CICIM B0013-070	Glycerol	111.5	[151]
E. coli ATCC11303 TG114	Glucose	118	[152]
Rhizopus oryzae NRRL 395	Glucose	77.5	[153]
R. oryzae NRRL 395	Glucose	140	[145]
R. oryzae NRRL 395	Glycerol	48	[154]
R. oryzae NRRL 3584	Glucose	231	

Table 2.3 Chemicals produced in methanotrophs

Microorganism	Strategies	Titer (g/L)	Reference
Methylomicrobium buryatense *5GB1S*	Episomal expression of L-Ldh/ continuous gas fermentation	0.8	[15]
Methylomicrobium buryatense *5GB1S*	Promoter and RBS engineering/ continuous gas fermentation	0.6	[16]
Corynebacterrium glutamicum	Deletion of competitive pathway(*Δpdh*)/ Continuous gas fermentation	< 0.75	[17]

Chapter 3.

Materials and methods

3.1. Strains and culture conditions

All strains used in this study are listed in Table. 3.1. *E. coli* strain DH5α was used for genetic cloning. DH5α was cultured in Luira-Bertani LB) medium (10 g/L of tryptone, 5 g/L of yeast extract, and 10 g/L NaCl) with 30 μg/mL of kanamycin as a selection marker. *Methylomnas* sp. DH-1 (KCTC13004BP) was used as a parental strain for LA production. *Methylomonas* sp DH-1 was cultured in nitrate mineral salts (NMS) medium (1.0 g/L MgSO4·7H2O, 1.0 g/L KNO_3, 0.2 g/L CaCl2·H2O, 3.8% (w/v) Fe-EDTA, 0.1% (w/v) NaMo·4H2O, trace element complex, phosphate solution, and vitamin solution) supplemented with 20% (v/v) methane at 30℃ with shaking at 170rpm. Methatnorophic strains were grown in 3 mL NMS medium in 30 mL serum bottle capped with a butyl rubber stopper or 12.5 mL NMS medium in a 125 mL baffled flask or 50 mL NMS medium in a 500 mL baffled flask sealed with rubber type screw cap. For LA production. 10 μg/mL of kanamycin was added to a medium.

Table 3.1 Strains used in this study.

Strain	Genotype	Reference
E. coli		
DH5α		
***Methylomonas* sp. DH-1**	Wild-type strain	[100]
JHM11	DH-1 AYM39_05845::P_{watR} (*ΔTT*)-*watR*-T_{rrnB} (OE1)::AYM39_05850	This study
JHM12	DH-1 AYM39_05845::P_{watR} (*ΔTT*)-*sdmM, rstM*-T_{rrnB} (OE2)::AYM39_05850	This study
JHM13	DH-1 AYM39_05845:: P_{watR} (*ΔTT*)-*watR, sdmM, rstM*-T_{rrnB} (OE3)::AYM39_05850	This study
JHM14	DH-1 *ΔglgA::Lm.LDH-Kan*^{R}	This study
JHM30	Evolved strain from DH-1	This study
JHM31	JHM30 *ΔfliE::Kan*^{R}	
JHM80	Evolved strain from DH-1	This study
JHM81	JHM80 *Δ*(*sdmM-rstM*)*::Kan*^{R}	This study
JHM82	JHM80 *Δ(watR-smtM-rstM)::Kan*^{R}	This study
JHM83	JHM80 *ΔglgA::Lj1.LDH-Kan*^{R}	This study
JHM84	JHM80 *ΔglgA::Lj3.LDH-Kan*^{R}	This study
JHM85	JHM80 *ΔglgA::Ld.LDH-Kan*^{R}	This study
JHM86	JHM80 *ΔglgA::Lm.LDH-Kan*^{R}	This study
JHM87	JHM80 *Δ(AYM39_17380,17385,17390,17395,17400)-KanR*	This study
JHM801	JHM80 *ΔglgA::Kan*^{R}	This study
JHM802	JHM80 *Δ(glgC-glgB-glgA)::Kan*^{R}	This study
JHM803	JHM80 *ΔglgA::Ptet-Lm.LDH-Kan*^{R}	This study
JHM804	JHM80 *ΔglgA::Ptac-Lm.LDH-Kan*^{R}	This study
JHM805	JHM80 *ΔglgA::focA-Ptet-Lm.LDH-Kan*^{R}	This study
JHM806	JHM80 *ΔglgA::Ptac-Lm.LDH-Kan*^{R} *ΔglgA::Amp*^{R}	This study

3.2. **Plasmids**

Plasmid used in this study are listed in Table. 3.2. The 1-kb upstream DNA fragment of the *glgA* (AYM39_03770) gene (U_{glgA}), LDH gene from *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC8293 (*Lm.LDH*), and *rrnB* terminator (T_{rrnB}) from *Escherichia coli* were prepared by PCR amplification from *Methylomonas* sp. DH-1 genomic DNA, p425-ADH-*Lm.ldhA* [155], and *E. coli* DH-5α genomic DNA, respectively. These PCR products were cloned between the *Nhe*I and *Eco*RI sites of pCM184 by using AccuRapid™ Cloning Kit (Bioneer, Korea), generating pU_{glgA}-Lm.LDH. The 1-kb downstream DNA fragment of the *glgA* gene (D_{glgA}) was amplified by PCR and cloned between the *Apa*I and *Sac*I sites of pCM184, generating pD_{glgA}. The DNA fragments encoding pBR322 replication origin with or without ampicillin resistance gene (Amp^R), U_{glgA}-*Lm.LDH*-T_{rrnB}, and kanamycin resistance gene (Kan^R) with D_{glgA} were prepared by PCR amplification from pCM184, pU_{glgA}-Lm.LDH, and pD_{glgA}, respectively, and ligated using AccuRapid™ Cloning Kit, generating pDel-glgA-Lm.LDH (with Amp^R) and pDel2-glgA-Lm.LDH (without Amp^R). For the integration of other heterologous LDH genes, LDH genes from *Lactobacillus jensenii* (*Lj1.LDH* and *Lj3.LDH*) and *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842 (*Ld.LDH*) were prepared by PCR amplification from p425ADH-*Lj.ldh1*, p425ADH-*Lj.ldh3*, and p425ADH-*Ld.ldhA* [155], and cloned between the *Nde*I and *Mlu*I sites of pDel-glgA-Lm.LDH, resulting in pDel-glgA-Lj1.LDH, pDel-glgA-Lj3.LDH, and pDel-glgA-Ld.LDH, respectively.

To construct plasmids for inducible expression of LDH, [U_{glgA}] flanked by MauB1 was amplified by PCR from DH-1 genome and replaced [UglgA] in pDel2-glgA-Lm.LDH, resulting in pDel3-glgA-Lm.LDH. The tet promoter and tac promoter were cloned between MauB1 and BamH1 sites, resulting in pDel3-glgA-P_{tet}-Lm.LDH and pDel3-

glgA-P_{tact}-Lm.LDH, respectively.

To construct plasmid for DNA integration into a noncoding region of *Methylomonas* sp. DH-1 chromosome, two consecutive DNA fragments between AYM39_05845 and AYM39_05850 (U_{Ins} and D_{Ins}) were amplified by PCR and sequentially cloned into the *Not*I/*Spe*I and *Apa*I/*Sac*I sites of pDel2-glgA-Lm.LDH, generating pIns. The *watR*, *sdmM-rstM*, and *watR-sdmM-rstM* operon genes were prepared with the 500-bp upstream sequence including a deletion of 2 bp (TT) [P_{watR} (*ΔTT*)] by PCR amplification or overlap extension PCR using JHM80 genomic DNA as a template, and then cloned between the *Spe*I and *Kpn*I sites of pIns plasmid, resulting in pIns-mW, pIns-mSR, and pIns-mWSR, respectively.

To construct plasmid for gene deletion, U_{Ins} and D_{Ins} sequences in pIns plasmid were replaced with 1-kb upstream and downstream sequences of the target genes by using *Not*I/*Spe*I and *Apa*I/*Sac*I sites, respectively.

Table 3.2 Strains used in this study.

Plasmid	Description	Reference
pCM184	*Amp*^{R}, *Kan*^{R}, *Tet*^{R}; broad-host range allelic exchange vector	
pDel-glgA-Lm.LDH	pCM184-U*glgA*-[*Lm.LDH*-T*rrnB*-*Kan*^{R}]-D*glgA*	This study
pDel-glgA-Lj1.LDH	pCM184-U*glgA*-[*Lj1.LDH*-T*rrnB*-*Kan*^{R}]-D*glgA*	This sutdy
pDel-glgA-Lj3.LDH	pCM184-U*glgA*-[*Lj3.LDH*-T*rrnB*-*Kan*^{R}]-D*glgA*	This study
pDel-glgA-Ld.LDH	pCM184-U*glgA*-[*Ld.LDH*-T*rrnB*-*Kan*^{R}]-D*glgA*	This study
pDel2-glgA-Lm.LDH	pDel-glgA-Lm.LDH without *Amp*^{R}	This study
pDel3-glgA-Lm.LDH	pDel2-glgA-Lm.LDH containing additional MauB1sequeces in [UglgA]	This study
pDel3-glgA-Ptet-Lm.LDH	PDel3-U*glgA*-[Ptet-*Lm.LDH*-T*rrnB*-*Kan*^{R}]-D*glgA*	This study
pDel3-glgA-Ptac-Lm.LDH	PDel3-U*glgA*-[Ptet-*Lm.LDH*-T*rrnB*-*Kan*^{R}]-D*glgA*	
pDel3-glgA-focA Ptac-Lm.LDH	PDel3-U*glgA*-[*focA*-Ptet-*Lm.LDH*-T*rrnB*-*Kan*^{R}]-D*glgA*	This study
pIns	Plasmid containing [UIns-T*rrnB*-*Kan*^{R}-DIns] cassette for integration into noncoding region between AYM39_05845 and AYM39_05850	This study
pIns-mW	pIns-[P*watR* (*ΔTT*)-*watR*-T*rrnB*]	This study
pIns-mSR	pIns-[P*watR* (*ΔTT*)- *sdmM, rstM*-T*rrnB*]	This study
pIns-mWSR	pIns-[P*watR* (*ΔTT*)- *wat, sdmM, rstM*-T*rrnB*]	This study
pDel2-fliE	pDel2-U*fliE*-[T*rrnB*-*Kan*^{R}]-D*fliE*	This study
pDel2-SR	pDel2-U*sdmM*-[T*rrnB*-*Kan*^{R}]-D*rstM*	This study
pDel2-WSR	pDel2-U*watR*-[T*rrnB*-*Kan*^{R}]-D*rstM*	This study
pDel2-MT	pDel2-U*mdtD*-[T*rrnB*-*Kan*^{R}]-D*tolC*	This study
pDel2-glgA	pDel2-U*glgA*-[T*rrnB*-*Kan*^{R}]-D*glgA*	This study
pDel2-glgCAB	pDel2-U*glgC*-[T*rrnB*-*Kan*^{R}]-D*glgA*	This study
pDel2-glgC	pDel2-U*glgC*-[T*rrnB*-*Amp*^{R}]-D*glgC*	This study

3.3. **Gene manipulation in *Methylomonas* sp. DH-1**

Gene deletion or integration of DNA in *Methylomonas* sp. DH-1 was achieved by homologous recombination between the chromosome and plasmid vector containing 1 kb each of upstream and downstream regions of the target integration site. Proper integration of the target DNA or gene deletion was confirmed by PCR analysis using confirmation primers. Plasmid DNA was introduced by electroporation as previously reported with some modifications. OD_{600} of 0.2 cells were spread onto a NMS plate and cultured for 3 days while supplying 30% methane. The biomass was harvested from the plate with distilled water and then transferred to 1.5 mL microcentrifuge tubes. After centrifugation at 14,000 rpm for 2 min, cells were washed twice with distilled water. 50 μL of cell suspension was mixed gently with 3 μL DNA and the mixture was transferred to an ice-cold 2-mm-gap cuvette (Bio-Rad, USA). Electroporation was performed using a Gene Pulser II system (Bio-Rad, USA) at preprogrammed Ec2 setting. Immediately after electric shock, cells were resuspended with 1 mL of NMS medium and then transferred to 30 mL bottle supplied with additional 2 mL of medium and 20% (v/v) methane. After overnight incubation in a shaking incubator, cells were harvested by centrifugation at 14,000 rpm for 2 min, and then spread onto a selective NMS plate containing 10 μg/mL of kanamycin.

3.4. **Adaptive laboratory evolution**

To develop LA-tolerant mutants of *Methylomonas* sp. DH-1, cells were adapted to LA by growing in NMS medium with gradually increasing concentrations of LA from 0.5 g/L to

8 g/L during 35 subcultures. The pH of the NMS medium containing LA was adjusted to 6.8 with NaOH.

3.5. **Whole genome sequencing analysis**

Genomic DNA of *Methylomonas* sp. DH-1 and evolved strains JHM30 and JHM80 was isolated using a bacteria genomic DNA extraction kit (iNtRON Biotechnology, Korea). DNA libraries were generated using a Truseq Nano DNA LT kit (Illumina, USA) and sequenced using PE 2x300-Miseq (Illumina, USA). The raw reads were processed with Trimmomatic to remove adapters and poor quality reads (quality score < 20). Reads shorter than 36 bp after processing were discarded. The quality of the processed data was evaluated using FastQC. Reads were mapped to the reference genome (wild-type strain of *Methylomonas* sp. DH-1) using Burrows-Wheeler Aligner (BWA) software. Duplicates were removed with the MarkDuplicates program of the Picard package. Indels were located and realigned with Realigner Target Creator/Indel Realginer of Genome Analysis Toolkit (GATK). Single nucleotide variants (SNVs) were detected using MuTect. Indels were detected using VaScan software.

3.6. **Quantitative transcription PCR (qRTP-PCR) and RNA sequencing analysis**

Methanotrophic cells were cultured in 12.5 mL NMS medium supplied with 20% (v/v) methane in a 125 mL flask for 16 h. Total RNA was extracted using RNeasy Mini kit (Qiagen, USA) according to the manufacturer's instructions. The relative amount of

mRNA was determined by quantitative reverse transcription PCR (qRT-PCR) as previously described [156] with minor modifications. 1 μg of total RNA was used for reverse transcription in a 25 μL reaction volume containing 200 unit of M-MLV reverse transcriptase (Thermo Fishers Scientific, USA), 0.1 μg of random hexamer, and 2 μL each of 10 mM dNTPs. After incubation at 25°C for 10 min and 42°C for 60 min, reverse transcription was terminated by heating at 70°C for 10 min. For qRT-PCR analysis, 1 μL of cDNA (diluted 1:20) was amplified by SYBR Green I maser mix (Roche-Applied Science, USA) using 0.75 pmol each of gene-specific primers with 45 cycles of 95°C for 20 s, 55°C for 20 s, and 72°C for 20 s on a Lightcylcer 480 II System (Roche Applied Science, USA). Primer sequences used for qRT-PCR are listed in supplementary Table. 3.3.

For RNA sequencing, 1 μg/mL of total RNA was processed to rRNA depetion using NEBnext rRND depletion kit (Bacteria) (#750, NEB). Resulted mRNA was used for sequencing library construction by TruSeq standard mRNA library prep kit (#20020594, Illumina). The prepared sequencing library was sequenced using NovaSeq 6000 (Illumina). The sequencing adapter removal and quality-based trimming on raw data was performed by Trimmomatic with default parameter. Cleaned reads were mapped to reference genome (Methylomonas sp. DH-1, GCF_001655685.1) using hisat2 v.2.2.1 with '—no-spliced-alignment' option. For counting reads which mapped to each CDS, featureCounts in Subread package was used. Finally, normalization of retrieved counts and fold change calculation between groups were performed by DESeq2 package.

3.7. **Fermenter culture condition**

Bioreactor fermentation was performed in a 5L Bioreactor (BioCNS, Daejeon, Republic of Korea) containing 3L NMS medium at 30 °C with agitation speed of 800 rpm. The gas mixture of 20% methane and 80% air controlled by a mass flow controller (Brooks Instrument, Hatfiel,PA) was supplied using microgas sparger at the rate of 320 mL/min.

Table 3.3 Primers used for qRT-PCR

Primers	Sequence (5'-3')
RT_15615 (mxaF) F	CCGCTTTCAACATCAAGGAC
RT_15615 (mxaF) R	GCTGTCGTAAGCGTACCAGC
RT_21110 F	TCCGCATTTATTGGTGGTGC
RT_21110 F	TGCTGGAAACTTCGCCTTCC
RT_21115 F	AGCAGCGCAAACAACAGTCG
RT_21115 R	CTAGTTCCTGGTGCGCCAAC
RT_21120 (watR) F	TATCTGGAACGCTGCCAGCA
RT_21120 (watR) R	TGACCGCCTTTCAGCACCAT
RT_21125 (smtM) F	GGCTAAGCCTGAGCGTCAAC
RT_21125 (smtM) R	GGGCCGTGTTGGTCAAGCTT
RT_21130 (rstM) F	AATCCCAACGCCGTGCTGAT
RT_21130 (rstM) F	ACAGAACGTTGTCCGCTGCG
RT 03770 (glgA) F	TGGAAGGCAAACAGGCCAAT
RT 03770 (glgA) F	GTACTCTATGCTCTTGTCGC
RT 17380 (mdtO) F	TGGCGCAATTTCTCAGCGCC
RT 17380 (mdtO) R	GCGTAAACCGAACGCCAACG
RT 17385 (hlyD) F	TCGATCCGCGGCCTTACCAG
RT 17385 (hlyD) R	CTGCAAACGGTGGTGCAGAT
RT 17390 F	GATGTTGAGCCTGCAAGGCT
RT 17390 R	TGCTGAAGGTGCACAAGGCC
RT 17395 F	GCCTTGGCCTCCCGGTACTC
RT 17395 R	CGCTATTTTTACGCAACGCG
RT 17400 (tolC) F	GCGCTTGGTACCCGATGCTG
RT 17400 (tolC) R	CTGCAAACGGTGGTGCAGAT

Chapter 4.

Production of D-lactate using lactate tolerant strain of *Methylomonas* sp. DH-1

4.1. Introduction

With the global concerns of plastic pollution, biodegradable plastics such as poly lactic acid (PLA) have attracted much attention. PLA can be synthesized by direct condensation polymerization of LA, dehydration condensation, and lactide (dimer form of LA) ring-opening polymerization [158]. LA, a monomer of PLA, has been produced by carbohydrates fermentation using metabolically engineered bacteria and yeasts [3-5]. Recently, methane, a major component of natural gas and biogas, is considered as a next-generation feedstock. In addition, methane is greenhouse gas with high global warming potential. Therefore, bioconversion of methane to LA is considered as promising strategy.

Methanotrophic bacteria can utilize methane as sole carbon and energy sources. In methanotorphs, methane is oxidized to methanol by methane monooxygenase and methanol is further converted to formaldehyde by methanol dehydrogenase. By the form aldehyde assimilation pathway, methanotrophs are classified as three groups (Group I, Group II, Group IIII). In Group I methanotrophs, formaldehyde is converted to pyruvate via RuMP cycle and EMP pathway [9]. In addition, they exhibit relatively fast growth rate compared to other methanotrophs. In this context, there were several efforts to produce LA from methane using Type I methanotrophs. In *Methylomicrobium buyatense* 5GB1S, heterologous lactate dehydrogenase (LDH) was expressed as a episomal plasmid and 0.8 g/L of L-LA was produced under continuous culture [15]. In another study, promoter and RBS were engineered for LDH expression in the same strain with the titer of 0.6 g/L [16]. These studies suggested that lactate toxicity was major limitation of LA production.

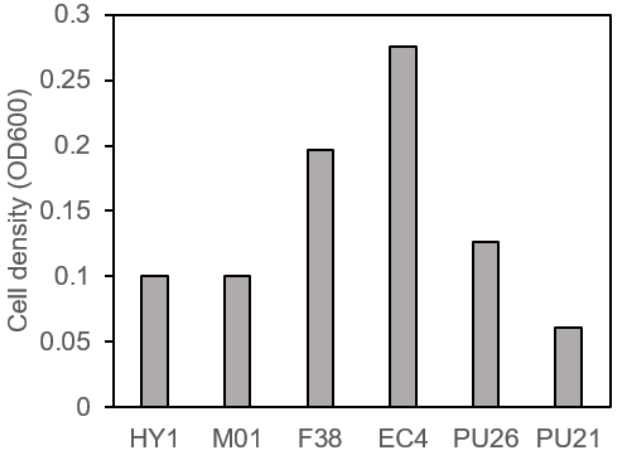
In this chapter, another Type I mehthanotroph, *Methylomonas* sp. DH-1 was used as a host strain for D-LA production from methane. DH-1 strain has several advantages including relatively fast growth, and availability of genome sequence. In addition, it does not possess natural LDH gene which is beneficial for production of optically pure LA. First, the lactate tolerance of *Methylomonas* sp. DH-1 (JHM80) was dramatically improved by adaptive laboratory evolution. Next, antibiotic resistance genes were investigated for use of selection markers. Lastly, D-LA was produced by integrating D-specific LDH gene from *L. mesenteroides* subsp. *mesenteroides* ATCC 8293 into *glgA* site of JHM80. By optimizing culture condition, 1.19 g/L of D-LA was produced with the productivity of 0.008 g/(L·h).

4.2. **Development of LA tolerant strain of Methylomonas sp. DH-1**

Although many methanotrophs grow optimally at neutral pH, industrial LA production prefers fermentation below the pH 3.8 sine high proportion of undissociated form of LA is easy to purify. Therefore, six acidophilic methanotrophic bacteria; *Methylovirgula ligni* HY1, *M. ligni* M01, *M. lingi* F38, *Methylobacter tundripaludum* SV96 EC4, *M. tundripaludum* SV96 PU26, *M. tundripaludum* SV96 PU21 were cultured to investigate availability for LA production. As shown in Fig. 4.1 all the stains showed very poor growth on methane. On the contrary, *Methylomonas* sp. DH-1, a newly isolated Type I methanotroph showed relatively fast growth rate with high cell densities. DH-1 strain was selected as a host strain for LA production.

The growth inhibition by lactate, dissociated form of LA, is major limitation for LA production in methanotorphs. To examine lactate tolerance of *Methylomoans* sp. DH-1, wild-type DH-1 strain was cultured in the NMS medium containing various concentrations of lactate. The growth of DH-1 was severely inhibited in the presence of 0.5 g/L of lactate, though pH ws neutralized to 6.8, indicating poor LA tolerance. Therefore, adaptive laboratory evolution was used to increase LA tolerance of wild-type DH-1 strain by gradual increase of LA from 0.5 g/L to 8.0 g/L. During the evolution, the strains JHM30 and JHM80 which can survive up to 3.0 g/L and 8.0 g/L of LA, respectively. In the absence of LA, wild type, JHM30, and JHM80 showed comparable growths. On the other hand, in the presence of 3 g/L of LA, only evolved strains survived. In the medium containing 8 g/L of LA, JHM80 showed higher tolerance than JHM30.

A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0.15
0.1
0.05
0
HY1
M01
F38
EC4
PU26
PU21

B

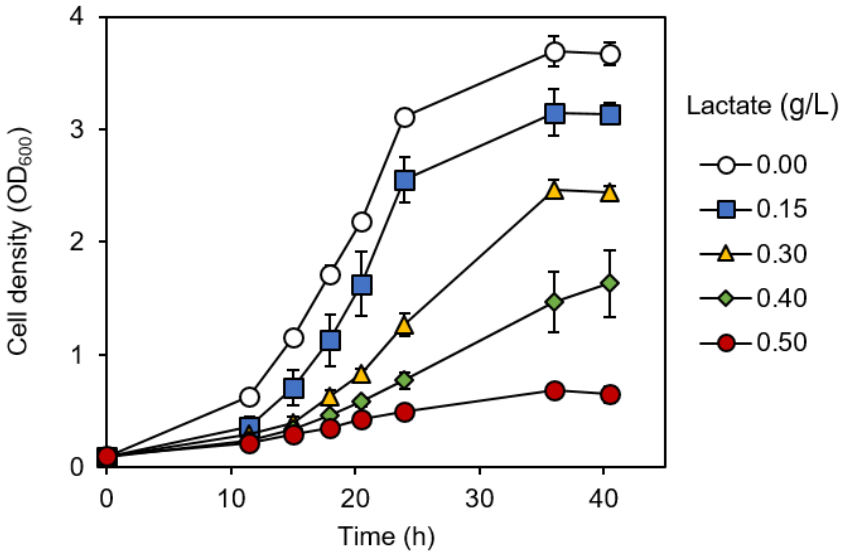
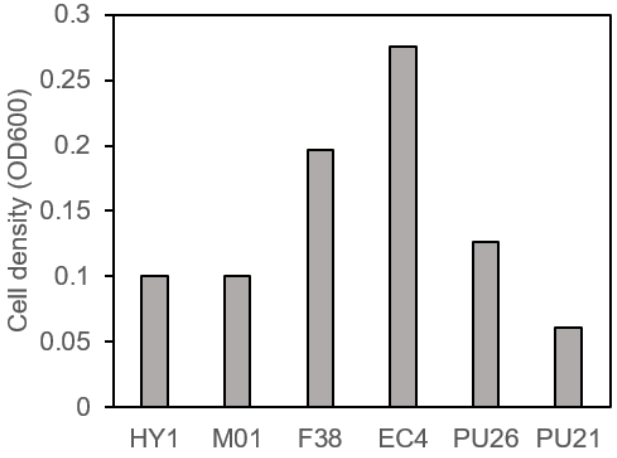

Cell density (OD600)
4
3
2
1
0
0
10
20
30
40
Time (h)
Lactate (g/L)
0.00
0.15
0.30
0.40
0.50

Figure 4.1 Growth profiles of methanotrophic cells.

A. *Methylovirgula ligni* HY1, *M. ligni* M01, *M. lingi* F38, *Methylobacter tundripaludum* SV96 EC4, *M. tundripaludum* SV96 PU26, *M. tundripaludum* SV96 PU21 were grown in the NMS medium containing 20% (v/v) methane for 7 days. The medium pH was adjusted to 4.0

B. LA tolerance of *Methylomonas* sp. DH-1. Cells were grown in NMS medium containing 20% (v/v) methane and indicated concentrations of LA. The medium pH was adjusted to 6.8.

A


Cell density (OD600)
0.3
0.25
0.2
0.15
0.1
0.05
0
HY1
M01
F38
EC4
PU26
PU21

B

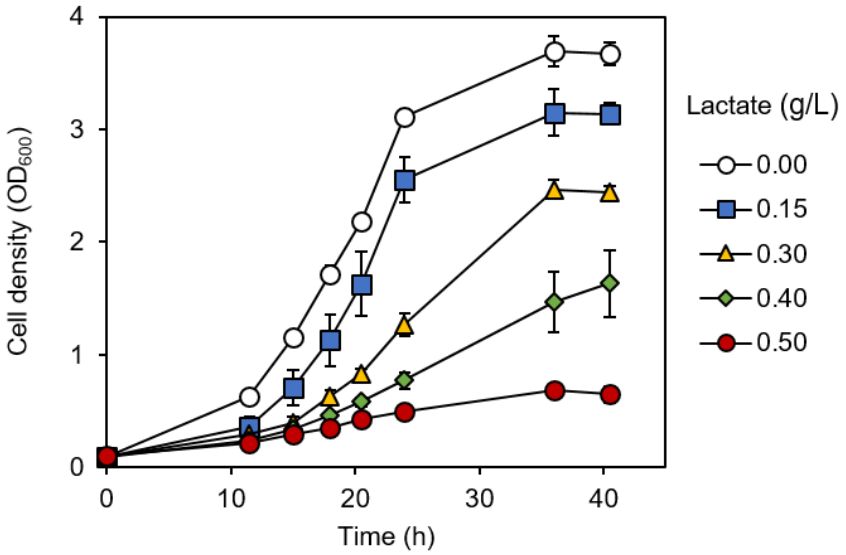

Cell density (OD600)
4
3
2
1
0
0
10
20
30
40
Time (h)
Lactate (g/L)
0.00
0.15
0.30
0.40
0.50

Figure 4.2 Improved LA tolerance in the evolved strains

Wild type *Methylomonas* sp. DH-1, JHM30, and JHM80 cells were grown in the NMS medium containing 0 g/L (A) or 3 g/L lactate (B) or 8 g/L lactate (C). 20% (v/v) methane was supplied with initial inoculum and the medium pH was adjusted to 6.8. Error bars indicate the standard deviations of two independent experiments.

4.3. **Selecting antibiotic resistance genes as selection marker**

Development of genetic manipulation tools are important for pathway engineering in methanotrophs. By several researchers including this study, electroporation based gene deletion or insertion methods have been developed in *Methylomonas* sp. DH-1. In addition, some efforts have been made to develop replicable plasmids in this strain including removal of its native plasmid, however, episomal DNA expression has not been reported in this strain. It means that marker-free genome editing by CRISPR/Cas9 or Cre recombinase which are transiently expressed by plasmid is difficult in this strain.

For multiple genomic manipulation, I investigated the availability of negative selection system consisting two-step selection (using kanamycin resistance gene) and counter-selection (using *sacB* and mutant *pheS* gene). The *sacB* (levansucrae) gene converts sucrose to levan which is toxic in gram negative cells, and the mutant *pheS* (phenylalanine tRNA ligase) gene incorporating 4-chlorophenylalnine (4-CP) into tRNA instead of phenylalanine. As shown in Fig. 4.3, kanamycin resistance gene with *sacB* or mutant *pheS* (*pheS**) genes was integrated into noncoding region (insert site on the figure) of wild-type strain. Compared to wild-type strain, transformed cells showed severe growth defects in plate containing 4-CP (Fig. 4.3) or sucrose (data not shown). After selection, linear DNA fragments consisting insert DNA fragment flanked by 1kb-up/down-stream DNA sequences for homologous recombination was introduced into mutant strains to remove kanamycin marker gene. After 2nd selection, cells were streaked on NMS plate containing kanamycin. Unexpectedly, all colonies survived in the kanamycin plate, implying that counter-seletion system is also unavailable in this strain. Further studies are necessary for effective genetic manipulation in *Methylomonas* sp. DH-1.I investigated the availability of negative selection system consisting two-step

selection (using kanamycin resistance gene) and counter-selection (using *sacB* and mutant *pheS* gene). The *sacB* (levansucrae) gene converts sucrose to levan which is toxic in gram negative cells, and the mutant *pheS* (phenylalanine tRNA ligase) gene incorporating 4-chlorophenylalnine (4-CP) into tRNA instead of phenylalanine. As shown in Fig. 4.3, kanamycin resistance gene with *sacB* or mutant *pheS* (*pheS**) genes was integrated into noncoding region (insert site on the figure) of wild-type strain. Compared to wild-type strain, transformed cells showed severe growth defects in plate containing 4-CP (Fig. 4.3) or sucrose (data not shown). After selection, linear DNA fragments consisting insert DNA fragment flanked by 1kb-up/down-stream DNA sequences for homologous recombination was introduced into mutant strains to remove kanamycin marker gene. After 2^{nd} selection, cells were streaked on NMS plate containing kanamycin. Unexpectedly, all colonies survived in the kanamycin plate, implying that counter-seletion system is also unavailable in this strain. Further studies are necessary for effective genetic manipulation in *Methylomonas* sp. DH-1. On the other hand, wild-type strain showed sensitivity to ampicillin and gentamicin which means corresponding resistance genes (Amp^R, Gen^R) can be used as additional selection markers (Fig. 4.4).

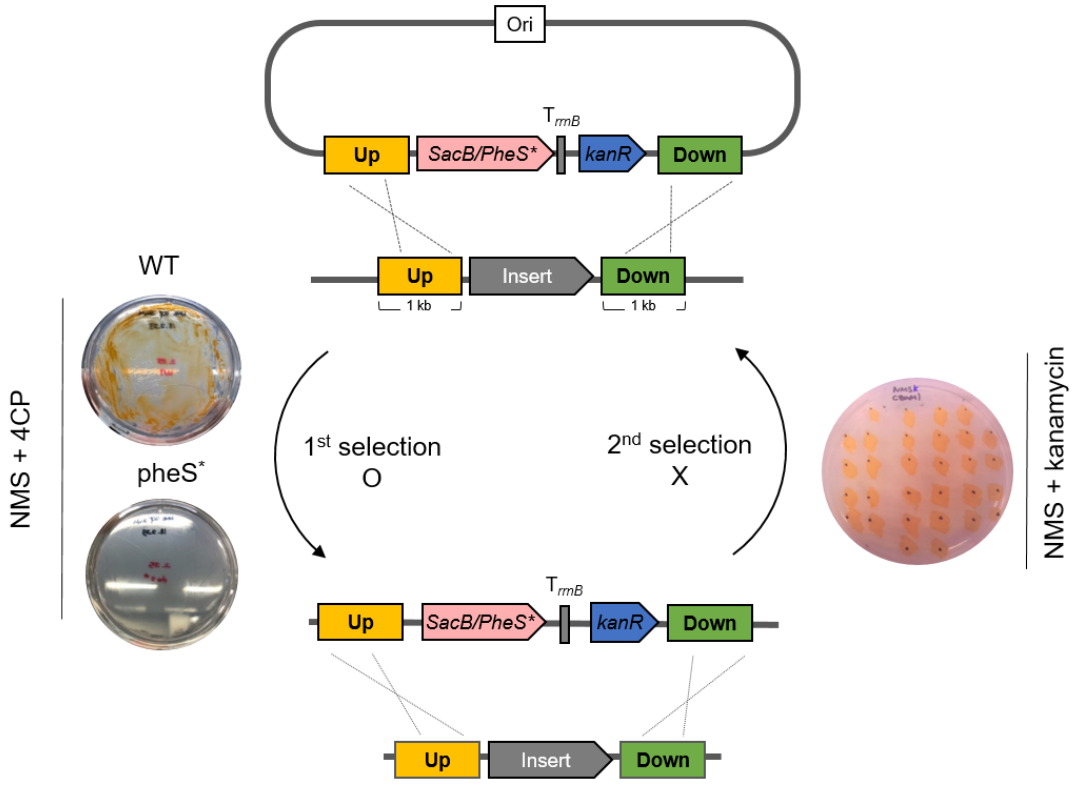


Figure 4.3 Strategy for negative selection in *Methylomonas* sp. DH-1

Plasmid containing kanamycin resistance gene (selection marker) with *sacB* or mutant *pheS* gene (counter selection marker genes) were transformed into noncoding region (Insert) of wild-type strain. After selection on kanamycin plate, linear DNA fragments were transformed to mutant cells for removal of selection marker.

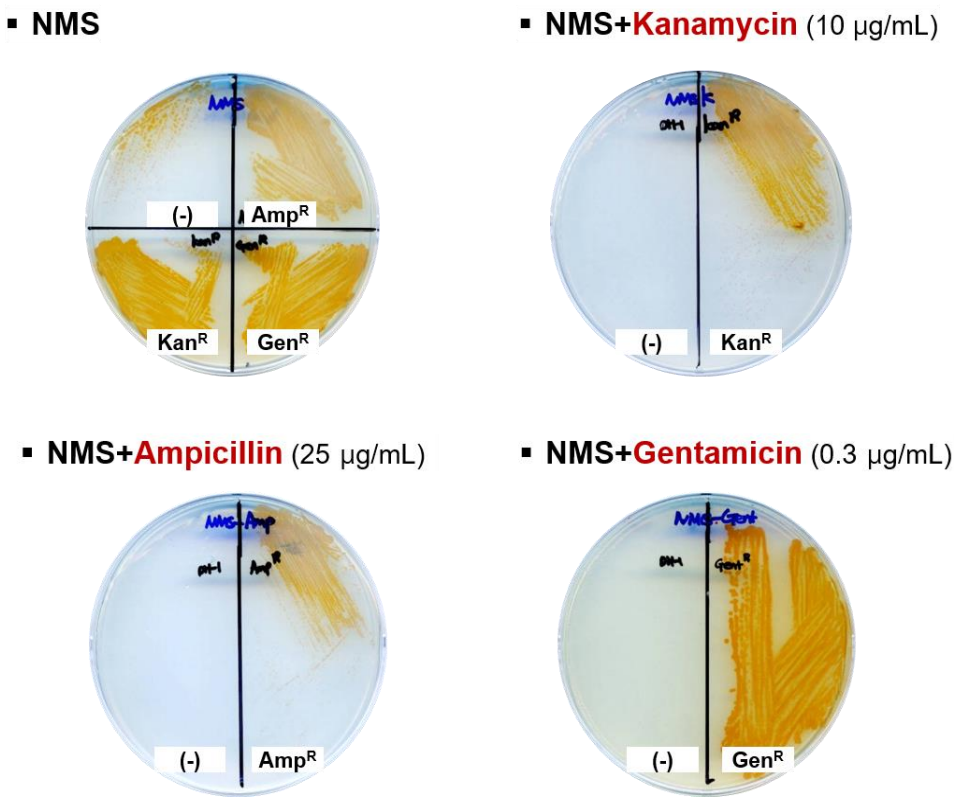


Figure 4.4 Antibiotic sensitivity of *Methylomonas* sp. DH-1

Wild-type strain (-) and antibiotic resistance gene integrated cells (Amp^R, Kan^R, Gen^R) were streaked on NMS plate with or without corresponding antibiotics (Ampicillin, Kanamycin, Gentamicin).

4.4. **Expression of heterologous D-lactate dehydrogenase in JHM80**

Methylomonas sp. DH-1 naturally produces pyruvate from methane through RuMP and EMP pathways. However, it lacks lactate dehydrogenase (LDH) enzyme which converts pyruvate to LA. To produce D-LA, four heterologous D-form specific LDH genes from *L. jensenii* (*Lj1.LDH* and *Lj3.LDH*), *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 (*Ld.LDH*), *L. mesesnteroides* subsp. *mesenteroides* ATCC 8293 (*Lm.LDH*) were integrated into the genome of JHM80 while deleting *glgA* genes encoding glycogen synthase (Fig. 4.5). The *glgA* gene consists an operon with *glgB* and *glgC* genes. The LDH gene were expressed under the control of native *glgA* promoter. JHM80 strain expressing *Lm. LDH* showed highest LA production (192 mg/L) followed by *Ld. LDH*, and *Lj1. LDH* after 48h (Fig. 4.6).

To investigate the effect of increased lactate tolerance of JHM80 on LA production, LA production of JHM80 expressing *Lm. LDH* (JHM84) was compared to wild-type expressing *Lm. LDH* (JHM14). In the 30 mL serum bottle, methane was fed every 24h by exchanging the head space with 20% (v/v) of methane. As shown in Fig. 4.6, JHM14 produced only 100 mg/L of D-LA with severe growth inhibition. On the contrary, JHM86 produced 750 mg/L of D-LA after 118h, which was 7.5-fold increase than wild type. These results clearly suggest that the increase of LA tolerance is a key factor for LA production in methanotrophs (Fig. 4.6).

In a 125 mL baffled flask with supplying 20% (v/v) methane every 24h, JHM86 produced 860 mg/L of D-LA after 144h. Since the medium pH decreased as the accumulation of D-LA during culture, NaOH was added to the medium as a neutralizing agent. In this pH controlled condition, 1.04 g/L of D-LA was produced with 15% increase.

Despite the continuous supply of methane and pH neutralization, the growth of JHM86 stopped at 96h which might result from the depletion of other nutrients such as nitrogen sources. Therefore, modified NMS medium containing 2x KNO_3 was used for LA production. Under this optimized condition, JHM86 produced 1.19 g/L of D-LA at 144h with productivity of 0.008 g/(L·h). The consumption of methane was also analyzed by GC chromatography and 60.8 mg of methane was consumed by JHM86 which was 51% of the supplied methane, achieving yield of 0.245 g/g CH_4 (Fig. 4.7).

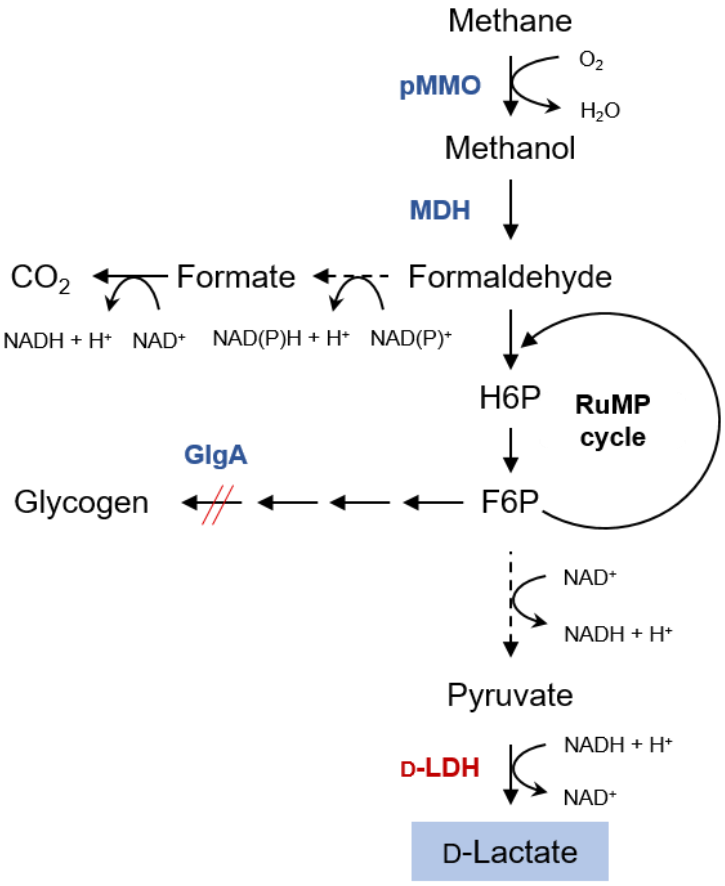


Figure 4.5 Metabolic pathway for the D-LA production from methane in *Metyhlomonas* sp. DH-1

Wild-type strain (-) and antibiotic resistance gene integrated cells (Amp^R, Kan^R, Gen^R) were streaked on NMS plate with or without corresponding antibiotics (Ampicillin, Kanamycin, Gentamicin).

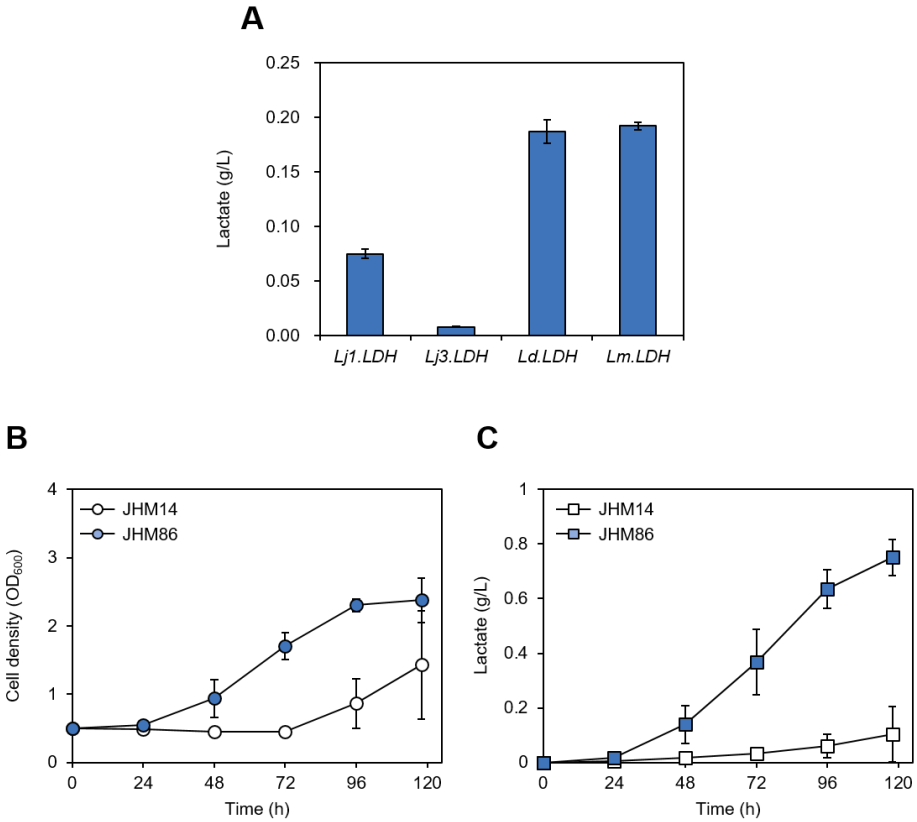


Figure 4.6 D-LA production by expressing lactate dehydrogenase in JHM80

A. The indicated heterologous D-LDH genes were integrated into the chromosome of JHM80, and LA production was measured after growing cells in NMS medium containing 20% (v/v) methane and 10 μg/mL of kanamycin for 48h.

B. Wild-type strain integrated with *Lm. LDH* (JHM14) and JHM80 integrated with *Lm. LDH* (JHM86) were grown in a 30 mL serum bottle containing 3 mL NMS medium while feeding 20% (v/v) metahen every 2h. Cell growth (B) and LA production (C) were monitored during growth. Error bars indicate standard deviations of two independent experiments.

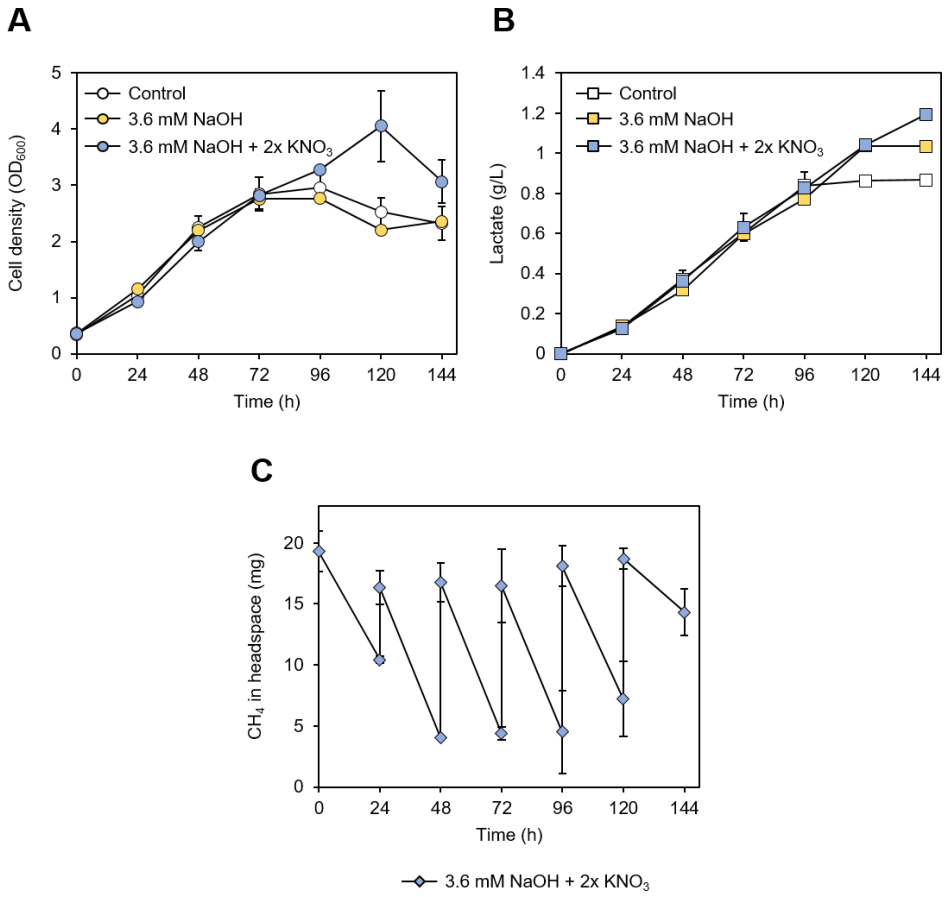


Figure 4.7 Improvement of LA production by neutralization and medium optimization

JHM86 was grown in a 125 mL flask containing 12.5 mL NMS medium (Control), NMS medium neutralized with 3.6 mM NaOH with or without additional nitrogen source. 20% (v/v) methane was fed every 24h. Cell growth (A) and LA production (B) were monitored during culture. (C) Methane consumption in the NMS medium with 3.6 mM NaoH and 2 KNO_3 was determined by GC chromatography. Methane levels were measured before and after feeding 20% (v/v) methane. Error bars indicate standard deviations of two independent experiments.

4.5. Conclusion

Due to the unique ability of utilizing methane as a sole feedstock, methanotrophs are considered as a promising biocatalyst for the conversion of methane to value-added chemicals. In this chapter, the lactate tolerant strain of *Metyhlomonas* sp. DH-1 (JHM80) was developed by adaptive laboratory evolution. For D-LA production, four heterologous LDH genes from *Lactobacillus* and *Leuconostoc* species were screened and *Lm. LDH* gene showed highest activity in JHM80. Compared to wild-type strain, JHM80 showed 7.5-fold higher LA production when *Lm. LDH* gene was integrated into the genome, suggesting that improving the LA tolerance is the key strategy for LA production in methanotrophs

Chapter 5.

Analysis of LA tolerance mechanism in evolved strains

5.1. **Introduction**

With the recent development of genetic tools, some none-native chemicals have been produced in methanotrophs by expressing heterologous genes and engineering metaolic pathways [12, 13, 88, 95] . Particularly, several approaches have been suggested for production of LA including modulating LDH expression and deletion of competitive pathway [15, 16] . However, the high-level production of LA is limited by growth inhibitory effects of LA in this novel bacteria. Moreover, little is known about the physiology of methanotrophs including toxic mechanisms of organic acids and its cellular response.

Typically, organic acids penetrate into the cytoplasm by diffusion and are dissociated to the proton and acid anion inside of cells where pH is maintained above the pKa value of organic acids. Both the proton and anion of organic acids have negative effects in cells [159]. In *E. coli*, several toxic mechanisms and its responses are demonstrated. The intracellular acidity caused by accumulation of proton can affect the integrity of purine bases and denature the necessary enzyme [160-162]. The accumulation of anion resulted to import of potassium ions which is related to increased turgor pressure [163, 164]. Besides the general effects of anion, anion-specific effects also have been demonstrated.

In this chapter, to investigate the genes related to the LA tolerance of evolved strains, genome sequences of evolved strains (JHM30 and JHM80) and wild-type strain were analyzed. Whole genome sequencing revealed the overexpression of AYM39_21120 encoding LysR-type transcription factor (named as *watR*) by 2bp deletion in the promoter region was one of the major LA contributor in JHM80.

In addition, overexpression of *watR* alone increased the LA tolerance of WT, implying the successful reverse engineering in *Methlyomonas* sp. DH-1. To further elucidate the WatR target genes for LA tolerance, RNA sequencing was performed in wild-type, JHM80, and *watR* overexpressing strain. The transcriptomic analysis identified the overexpression of RND-type efflux pump by *watR* gene. Moreover, disruption of this efflux pump abolished the LA tolerance of JHM80, implying that WatR mediated overexpression of efflux pump was responsible for LA tolerance in JHM80. To further demonstrate the function of this transporter, phosphate buffer containing 4g/L of LA was treated to JHM80 and mutant strain lacking efflux pump. In the mutant strain, LA accumulation was observed at 2h, while the extracellular LA concentrations was not changed in JHM80, implying that RND-type efflux pump contributed LA tolerance by pumping out lactate to outside of cells.

5.2. **Whole genome sequencing**

To identify mutated genes involved in increased LA tolerance, whole genome sequencing of JHM30 and JHM80 was performed with wild-type strain. In both JHM30 and JHM80, 2bp (TT) was deleted in the intergenic region between the AYM39_21115 and AYM39_21120 genes. In JHM80, the nucleotide position 145 of *fliE* gene was changed from C to T, resulting nonsense mutation (Gln49*) (Table. 5.1). Since the TT deletion was found in the promoter region, I supposed that it might affect the expression of downstream genes, AYM39_21115 and AYM39_21120, which are parts of operon structures transcribed in the opposite directions. As shown in Fig 5.1, compared to wild type, JHM80 with TT deletion showed dramatic increase in expression levels of AYM39_21120 and its downstream genes in the same operon, AYM39_21125 and AYM39-21130. On the other hand, expression levels of AYM39_21115 and AYM39_21110 was similar in WT and JHM80, indicating the TT deletion only overexpressed the operon including AYM39_21120 and the downstream genes. The AYM39_2120 gene (named as *watR*: weak acid tolerance regulator) genes encodes a LysR-type transcription factor, while the AYM39_21125 (named as *smtM*) and AYM39_21130 (named as *rstM*) encodes protein with homology of SAM (S-adenosyl-L-methionine)-dependent methyl transferase and rhodanese related sulfur transferase, respectively.

Table 5.1 Results of whole genome sequencing analysis of the evolved strains

	WT	Ev 3.0	Ev 8.0
Intergenic region (ΔTT) deletion	-	+	+
FliE nonsense mutation (AYM39_13220) Gln49*	-	-	+

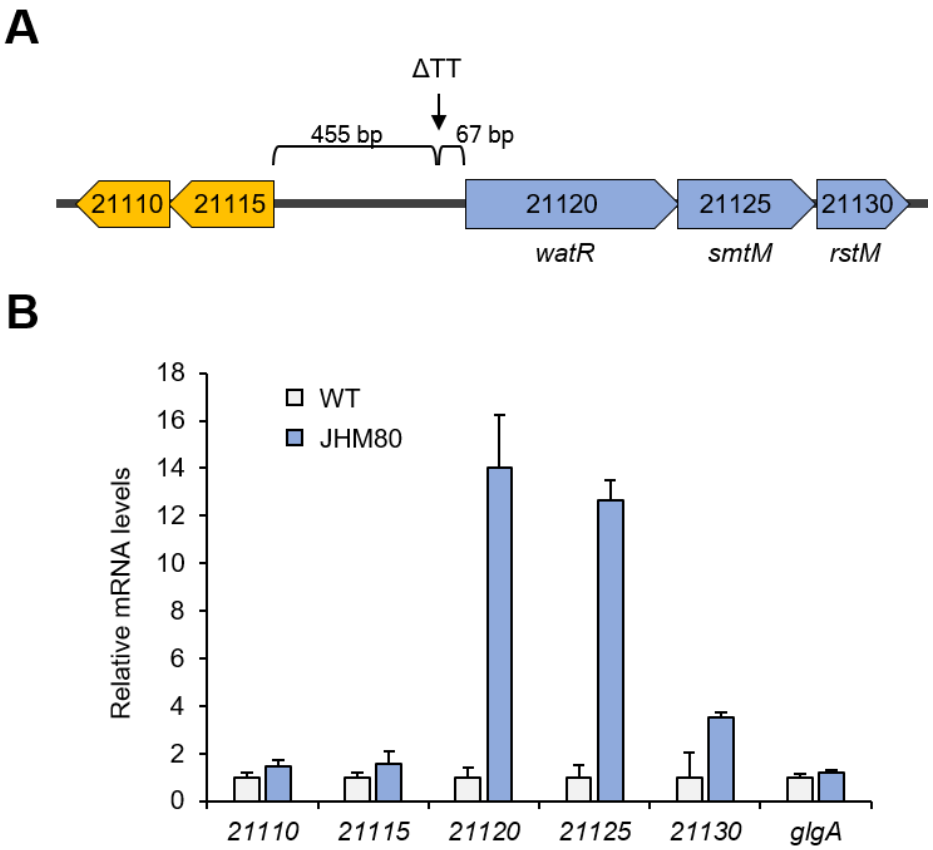


Figure 5.1 Effect of TT deletion on transcription of the downstream operons

A. Schematic illustration of the operon structures with TT deletion

B. The mRNA levels of the indicated genes in wild-type and JHM80 were analyzed by qRT-PCR and normalized to the mRNA level of *mxaF*. The mRNA level of *glgA* were used as a control.

5.3. **Functional analysis of mutated genes**

To elucidate the role of these up-regulated genes in the LA tolerance of JHM80, I first deleted all three genes (*watR*, *smtM* and *rstM*) or the last two genes (*smtM* and *rstM*) in JHM80 and evaluated LA tolerance. In the NMS medium containing 8 g/L of LA, JHM80 with *smtM* and *rstM* deletion (JHM81) showed only slightly reduced growth compared to JHM80. On the other hand, deletion of all three genes abolished the LA tolerance of JHM80, suggesting that the elevated expression of *watR* is major contributor for the LA tolerance (Fig. 5.2). The role of *watR* gene was further verified by overexpression of *watR*, *smtM*, and *rstM* genes with different combinations in wild-type strain. Using the 500-bp upstream region of the *watR* gene containing TT deletion as a promoter, overexpression cassettes for watR (OE1), smtM and rstM (OE2), and all three genes (OE3) was generated, then integrated into the noncoding region of wild-type chromosome. The overexpression of the target genes was confirmed by qRT-PCR. Integration of OE1 and OE3 increased LA tolerance compared to wild-type. On the other hand, cells integrated with OE2 failed to recover LA sensitivity of the wild-type strain (Fig 5.3). These results clearly imply that overexpression of the *watR* gene, but not the *smtM* and *rstM* genes, is mainly responsible for LA tolerance.

The effect of *fliE* nonsense mutation identified in JHM80 was also examined. The *flIE* gene is related to the formation of flagella. Since the *fliE* mutation was only found in JHM80, but not JHM30, I hypothesized that the additional *fliE* mutation might be responsible for the higher LA tolerance of JHM80. Therefore, *fliE* gene was deleted in JHM30 to mimic the nonsense mutation, but *fliE* deletion failed to improve the LA tolerance of JHM30 (Fig. 5.4). Further studies are necessary for identifying the casual mutations responsible for higher LA tolerance of JHM80 than JHM30.

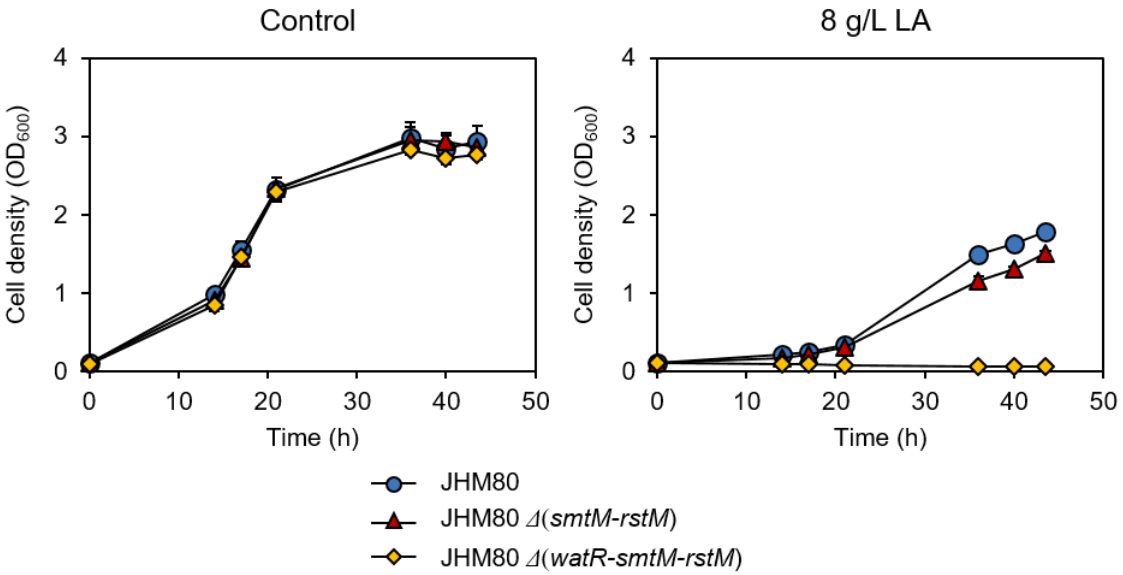


Figure 5.2 Effect of *watR* gene deletion on LA tolerance of JHM80

JHM80 and JHM80 with the indicated gene deletions were grown in the absence or the presence of 8.0 g/L LA. Error bars indicate standard deviations of three independent experiments.

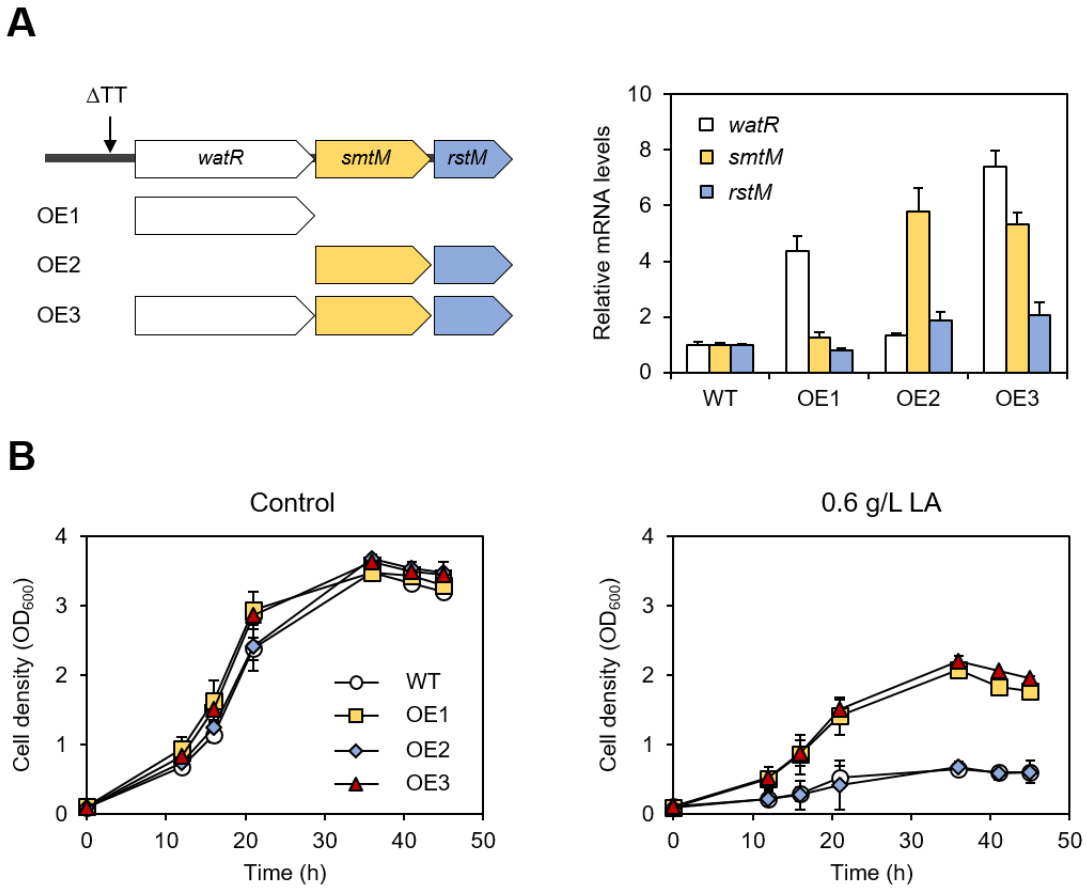


Figure 5.3 Effect of *watR* gene overexpression on LA tolerance

A. The indicated overexpression cassette OE1, OE2, or OE3 was integrated into the gnome of wild-type strain and mRNA levels of the overexpressed genes were detected by qRT-PCR.

B. LA tolerance was determined by growing cells in the absence (Control) or presence of 0.6 g/L LA. Error bars indicate standard deviations of three independent experiments.

5.4. **RNA sequencing of watR overexpressed strain**

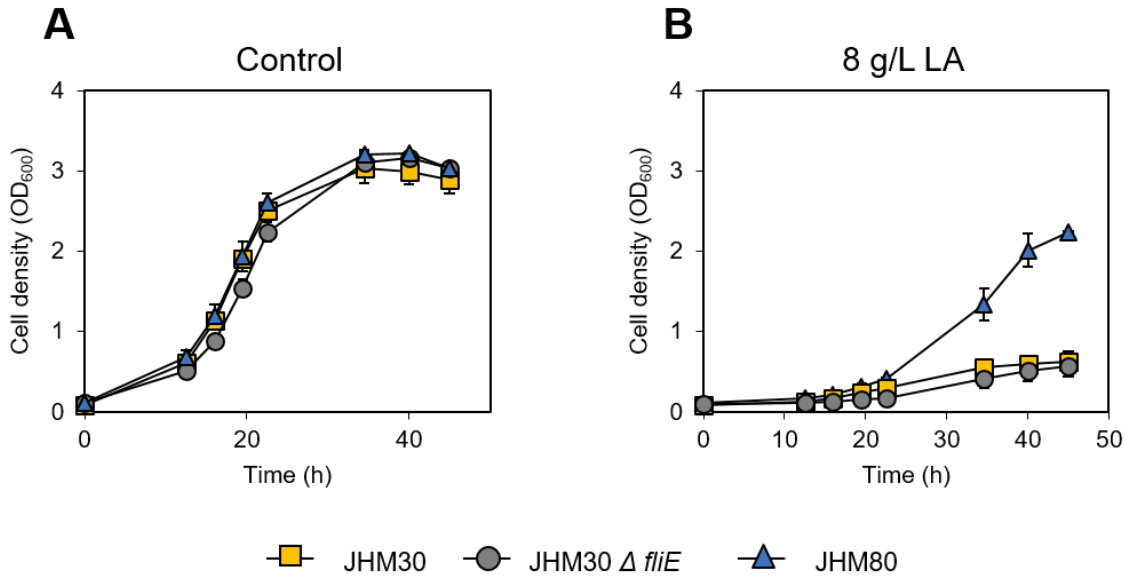


Figure 5.4 Effect of *fliE* gene deletion on LA tolerance of JHM30

JHM31(JHM30 *ΔfliE::Kan*^{R}), JHM80 were grown in the absence or the presence of 8.0 g/L. Error bars indicate standard deviations of three independent experiments.

To further identify genes of *watR* target genes related to LA tolerance, gene expression levels of JHM80, *watR* overexpressed strain (JHM11) were compared to wild-type strain by performing RNA-sequencing. RNA sequencing data was filter by fold change of 2.0 and 1.2 in JHM80 and JHM11, respectively. In JHM80, 165 genes were up-regulated and 13 genes were down-regulated compared to WT. In JHM11, 256 genes were up-regulated and 224 genes were down-regulated (Fig 5.5). Since there exist other mutations besides the intergenic TT deletion which overexpressed *watR* in JHM80, I supposed that not only *watR* but other factors might affect the gene expression in JHM80. On the ther hand, because the overexpression of *watR* was not sufficient in JHM11, target genes which was identified in JHM80 are more likely related to LA tolerance. Therefore, genes which were overexpressed or down-regulated both in JHM80 and JHM11 were selected as a *watR* target and a contributor to LA tolerance. Consequently, overexpression of an operon encoding resistance-nodulation-division (RND)-type efflux pump was identified as a candidate. The operon structure consisted of AYM39_17380 (encoding multidrug resistance protein *mdtO*), AYM39_ 17385 (encoding secretion protein *hlyD*), AYM39_17400 (outermembrane protein, *tolC*), and AYM39_17390, 17395 with unknown function (Fig 5.6).

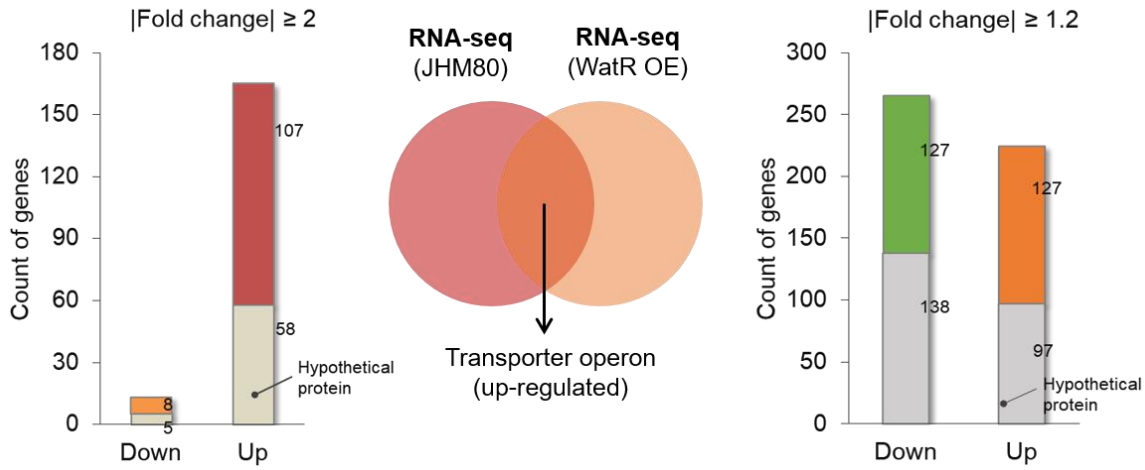
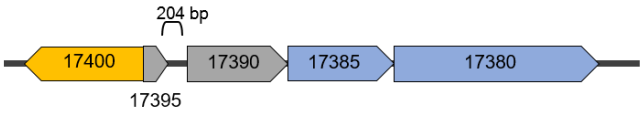


Figure 5.5 RNA sequencing of JHM80 and JHM11

The total gene expression levels of JHM80 and JHM11 was analyzed by RNA-sequencing. The indicated count of genes was filtered by fold change of 2.0 and 1.2 in JHM80 and JHM11, respectively.

A



Gene	Definition
AYM39_17380	Fusaric acid resistance protein
AYM39_17385	Secretion protein (HylD)
AYM39_17390	Hypothetical protein
AYM39_17395	Hypothetical protein
AYM39_17400	Transporter

B

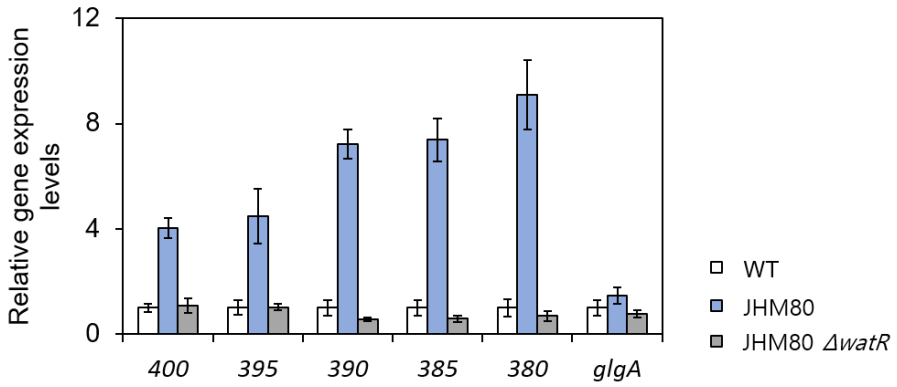


Figure 5.6 RNA sequencing of JHM80 and JHM11

A. Schematic illustration of operon structure of RND-type efflux pump

B. The mRNA levels of the indicated genes in wild-type, JHM80, JHM80 *ΔwatR* were analyzed by qRT-PCR and normalized to the mRNA level of *mxaF*. The mRNA level of *glgA* was used as a control

5.5. **RND-type efflux pump contributes to organic acid tolerance in *Methylomonas* sp. DH-1**

To verify the role of *watR* on expression of efflux pump, the *watR* gene was deleted in JHM80 and the mRNA expression level was compared to wild-type strain and JHM80. As shown in Fig. 5.6, whole genes consisting the operon of efflux pump were highly overexpressed in JHM80. On the other hand, deletion of *watR* reduced the expression levels of these genes to the level of wild-type, suggesting that the activation of the efflux pump operon is dependent on *watR*.

Next, I investigated the role of the efflux pump (named as Organic Acids Pump in Methanotroph, OapM) on LA tolerance in JHM80. Whole 5 genes consisting the operon were deleted in JHM80 and the LA tolerance was compared to JHM80. In the absence of lactate, both the strains showed comparable growth rate. However, JHM80 which can survive in the presence of 8 g/L of LA, totally abolished the LA tolerance by the deletion of OapM, implying that the efflux pump is a major contributor for LA tolerance, possibly by pumping out the lactate outside the cells (Fig 5.7). To further demonstrate the function of OapM, the phosphate buffer containing 4 g/L of buffer was treated to the JHM80 and JHM80 with efflux pump deletion and the extracellular LA concentrations were measured every 30min. As shown in Fig.5.8, extracellular LA concentrations was maintained at 4 g/L in JHM80. On the contrary, intracellular LA concentrations increased in efflux pump deficient strains, suggesting the lactate efflux function of OapM. Since RND-type efflux pump is known to pump out wide spectrum of chemicals including antibiotics and organic compounds, I supposed OapM might be related to not only lactate but other weak organic acids tolerance in JHM80.

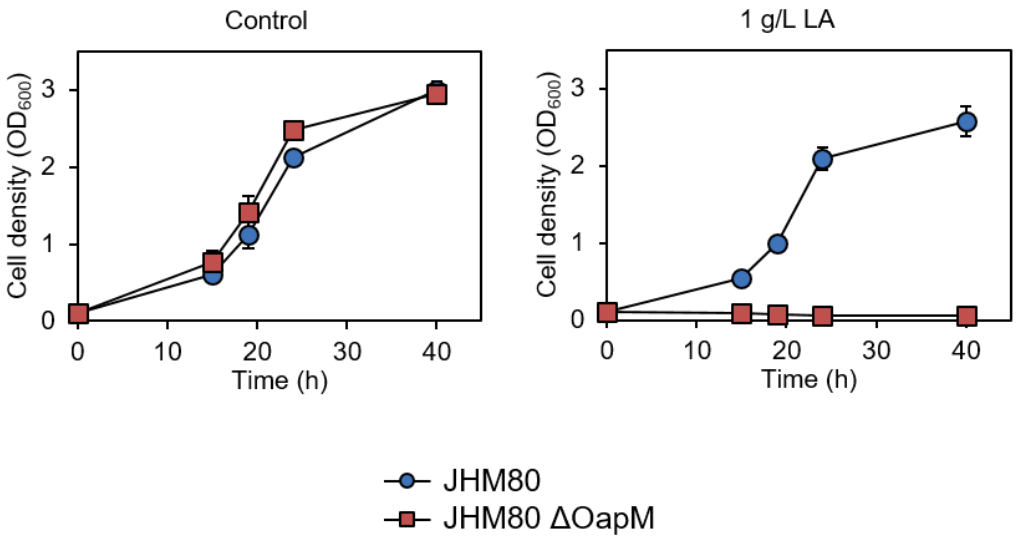


Figure 5.7 RNA sequencing of JHM80 and JHM11

JHM80 and JHM80 lacing OapM efflux pump were grown in the presence or absence of 1.0 g/L of LA. Error bars indicated standard deviations of two independent experiments.

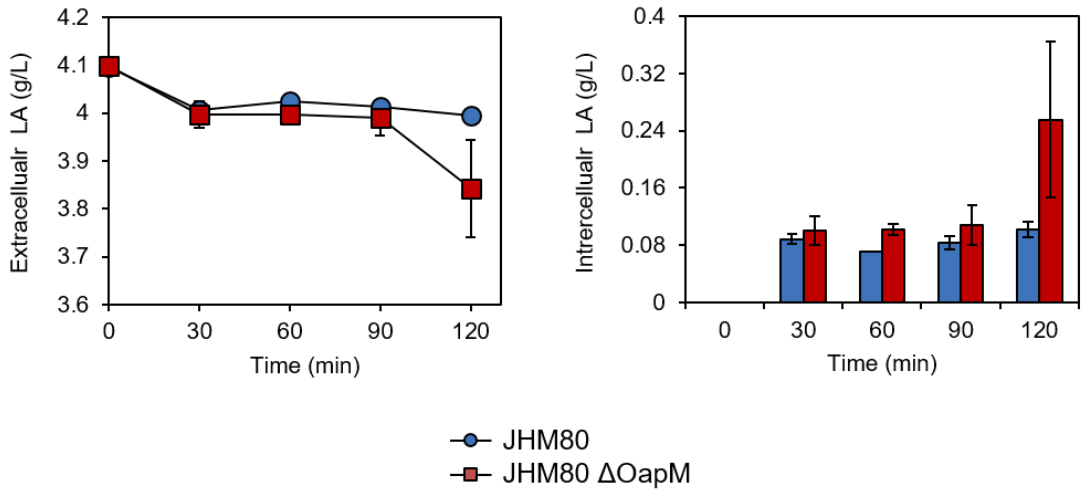


Figure 5.8 Lactate efflux function of RND-type efflux pump

JHM80 and JHM80 lacing OapM efflux pump were grown in the phosphate buffer containing 4 g/L of LA. Extracellular LA concentrations was measured every 30 min. Error bars indicate standard deviations of three independent experiments.

As shown in Fig. 5.9, disruption of OapM reduced formate, acetate, and propionate tolerance in JHM80. In addition, deletion of *watR* also decreased weak organic acids tolerance in JHM80, confirming that *watR*-mediated overexpression of OapM was a major contributor of organic acids tolerance in JHM80. However, wild-type strain showed higher acetate tolerance than JHM80 and deletion of *watR* dramatically increased acetate tolerance of JHM80, assuming that there are other genes regulated by WatR which is responsible for acetate tolerance. Further studies should focus on unveiling the WatR dependent acetate tolerance mechanisms.

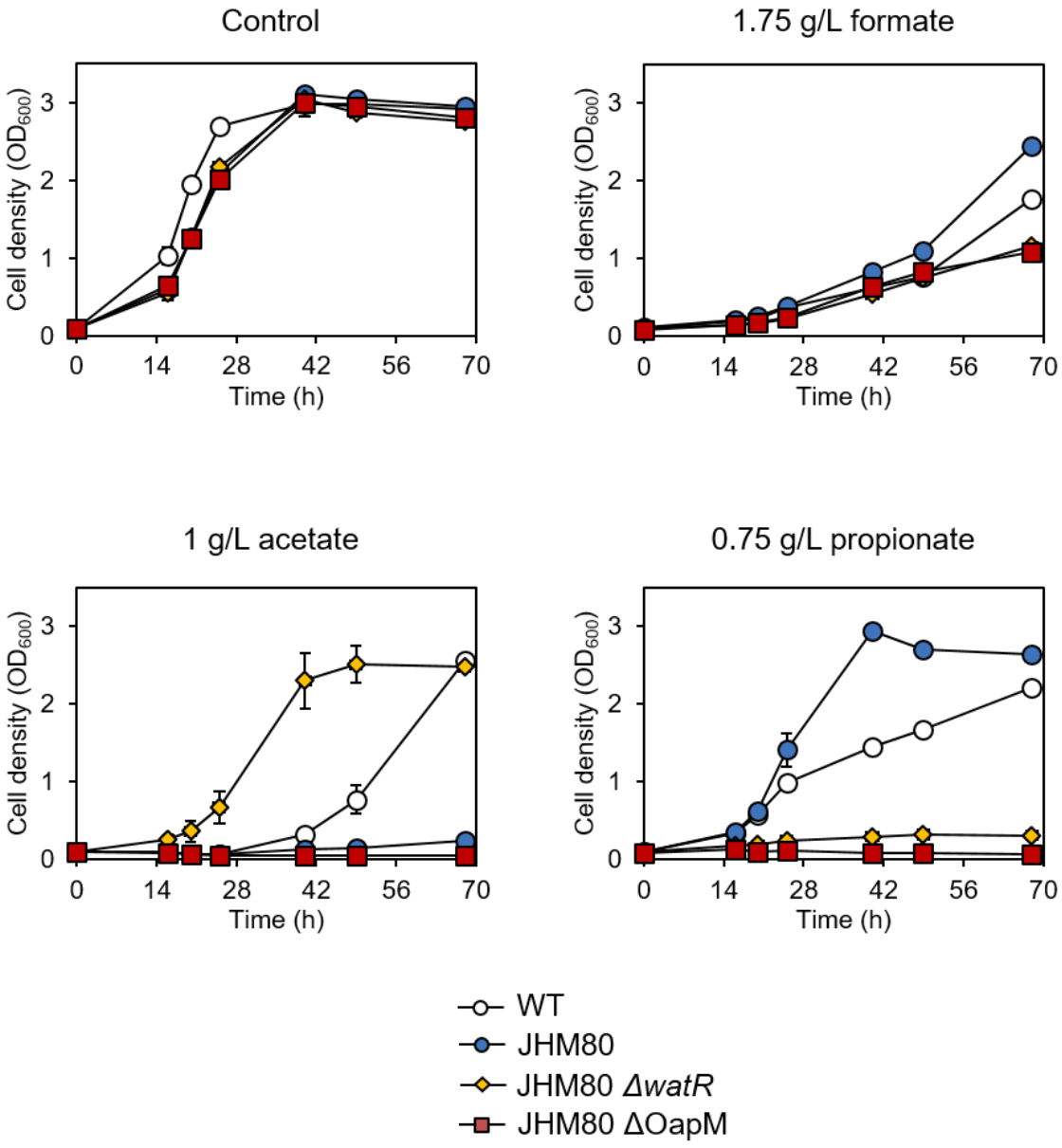

Control
1.75 g/L formate
1 g/L acetate
0.75 g/L propionate
Cell density (OD600)
Time (h)
0
14
28
42
56
70
1
2
3
WT
JHM80
JHM80 ΔwatR
JHM80 ΔOapM

Figure 5.9 Effects of watR gene and efflux pump deletion on organic acid tolerance

Wild type strain, JHM80, and JHM80 with indicated gene deletion were grown in the NMS medium with or without organic acids. Error bars indicated standard deviations of three independent experimetns.

5.6. **Conclusion**

In this chapter, genomic and transcriptomic analysis were performed to demonstrate the LA tolerance mechanisms JHM80. Whole genome sequencing identified overexpression of AYM39_21120 gene encoding LysR-type regulation transcription factor (*watR*) by 2-bp (TT) deletion in promoter region was responsible for LA tolerance in JHM80. RNA-sequencing further demonstrated WatR-mediated overexpression of RND-type efflux pump was major LA contributor in JHM80. Moreover, WatR transcription regulator and efflux pump were also related to other weak organic acids tolerance including formate and propionate. On the other hand, efflux pump was not related to acetate tolerance. In fact, deletion of *watR* dramatically increased acetate tolerance of JHM80, suggesting that other genes regulated by *watR* are responsible for acetate tolerance.

Chapter 6.

Improvement of LA production by fine-tuned expression of LDH and reducing toxic effects

6.1. **Introduction**

LA is one of the most industrially successful chemicals produced by microbial fermentation. Pyruvate, a precursor of LA, biochemically occupies important position in central carbon metabolism. It is end-product of glycolysis and is oxidized to acetyl-CoA which enters TCA-cycle for energy generation or providing precursors for fatty acid and steroids biosynthesis. Pyruvate also can be converted carbohydrate through gluconeogenesis or alanine for amino acid synthesis [165]. Therefore, overexpression of lactate dehydrogenase (LDH) which converts pyruvate to lactate is important to increase pyruvate flux to LA.

Promoters are DNA fragments which enable expression of downstream genes by binding of RNA polymerase and transcription factors. They can be classified into three types 1) constitutive, 2) spatio-temporal: tissue-specific activation of genes, and 3) inducible promoters: activates gene in response to inducer chemicals based on their activity [166]. Inducible promoters have advantages for microbial-based chemical production since toxic gene products can be expressed after reaching high cell densities, which is suitable for producing toxic chemicals such as LA production in methanotrophs

Poly-3-hydroxyburyrate (PHB) or glycogen is accumulated as major carbon storage in certain conditions. *Methylomoans* sp. DH-1 synthesizes glycogen rather than PHB. Therefore, deletion of glycogen biosynthetic gene (*glgA*) for competitive use of carbon sources can be important strategy for LA production in *Methlyomonas* sp. DH-1. Instead, ADP-glucose, a precursor of glycogen, can be accumulated and it has been reported that phosphate intermediated can be toxic to cells. For example, galactose-1-phsopahte was involved in depletion of ADP in *E. coli*. In cyanobacteira, ADP-glucose accumulation reduced photosynthetic capacity and adenylate energy charge, severely inhibiting the growth.

In this chapter, to enhance the LA production, anhydrotetracylcine (aTc) inducible tet promoter and isopropyl-β-D-thiogalctoside (IPTG) inducible tac promoter were evaluated for LA production, and tac promoter was selected due to its ability of strong expression. However, growth inhibition was observed by ADP-glucose during the scale-up processes. To overcome this problem, *glgC* gene encoding enzyme which synthesizes ADP-glucose was deleted. In the LA-evolved strain with LDH expression under tac promoter and additional deletion of *glgC*, 6.16 g/L of D-lactate was produced under continuous culture, which is highest ever reported in methanotrophs.

6.2. **Expression of Lm. LDH using different promoters**

To improve LA production, 5 constitutive promoters with different strengths including 1) 50s ribosomal protein L31 (AYM39_19855, relative strength of 0.07 compared to *glgA*), 2) *glgA* promoter for control, 3) elongation factor Tu (AYM39_17865, relative strength of 1.5, 4) transaldolase (AYM39_02490, relative strength of 5.1, and 5) methanol dehydrogenase (AYM39_15615, relative strength of 54.9). The above mentioned promoters with LDH was integrated to *glgA* site in JHM80 and LA production was compared. However, expressing LDH using strongest promoter, *mxaF*, was failed, possibly due to the lactate toxicity. Strains expressing LDH with different promoters were cultured in the NMS medium containing 20% (v/v) of methane. As shown in Fig. 6.1, highest amount of LA was produced in *glgA* promoter, followed by promoter of AYM39_17865, AYM39_02490, and AYM39_15615. Since the strength of AYM39_15615 promoter was weakest among 4 promoters, it is acceptable of smallest production of LA. On the contrary, the LA production-levels of other 3 promoters was opposite with the strength of promoters. The loss of LDH which might result from genetic instability and lactate toxicity might be responsible for it (Data not shown). If this is the case, pathway engineering strategies aimed to increase pyruvate flux to LA might not be successful for improvement of LA production,

Inducible promoters have advantages for expression of interested gene to desired level at desired time. Therefore, tet promoter (P_{tet}) and tac promoter (P_{tac}), which are induced by anhydrotetracycline (aTc) and IPTG, respectively, were utilzed for LA production in JHM80.

A

Gene	Definition	Relative strength
AYM39_19855	50S ribosomal protein L31	0.07
glgA	Glycogen synthase	1
AYM39_17865	Elongation factor Tu	1.5
AYM39_02490	Transaldolase	5.1
AYM39_15615	Methanol dehydrogenase	54.9

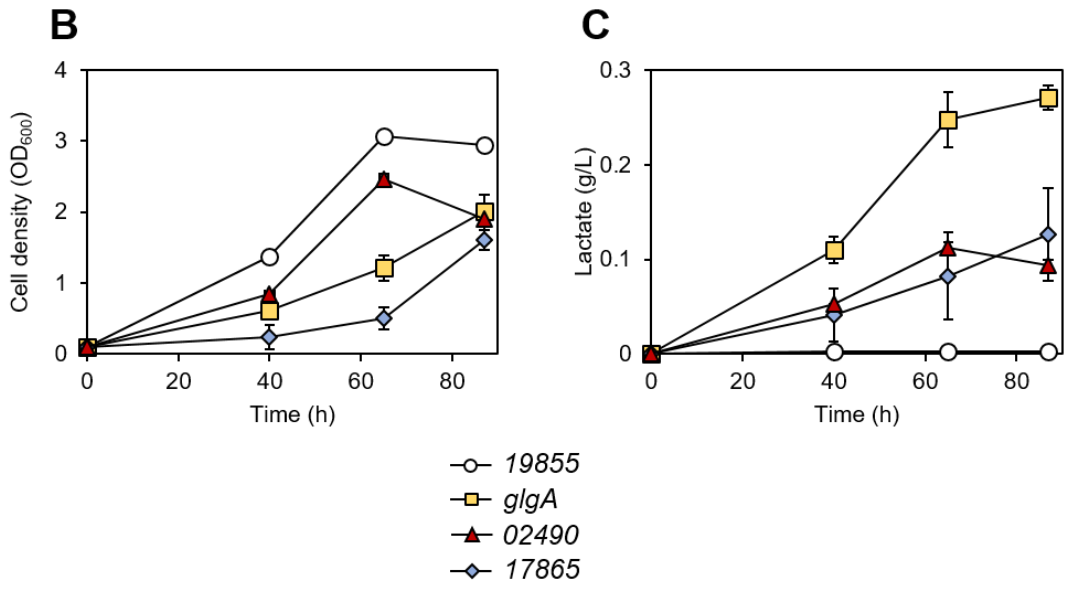


Figure 6.1 D-LA production by expressing Lm. LDH by constitutive promoters with different strengths.

A. Five different constitutive promoters with indicated strengths

B. Indicated promoters with Lm. LDH were integrated to chromosome of JHM80. Cells were grown in NMS medium containing 20% (v/v) and 10 μg/mL of kanamycin. Cell growth (B) and LA production (C) were measured during the growth. Error bars indicated standard deviations of two independent experimetns.

By integrating DNA fragments of LDH under the control P_{tet} and P_{tac} into JHM80, the strains JHM803 (JHM80 *ΔglgA::P_{tet}-LDH*) and JHM804 (JHM80 *ΔglgA::P_{tac}-LDH*) were generated. The aTc, a derivative of tetracycline with reduced antimicrobial activity, exhibited cellular toxicity at higher concentrations in *M. buryatense* 5GB1S. In *Methylomonas* sp. DH-1, there were no antimicrobial activity in the presence of ~1 μg/mL of aTc, whereas 2 μg/mL of aTc severely inhibited the growth of wild-type strain (Fig. 6.2). To evaluate the P_{tet} for LA production, JHM803 was grown in the NMS medium containing various concentrations of aTc. As shown in Fig.6.3, in the absence of aTc, JHM803 produced only 2 mg/L of D-LA. When induced by 0.5 mg/L of aTc, JHM803 produced 29 mg/L of D-LA at 48h, implying the tight regulation of tet promoter. On the other hand, leaky expression of LDH was found in tac promoter, producing 92 mg/L of LA in the absence of IPTG. In the presence of 10 μM of IPTG, JHM804 produced 187 mg/L of LA even with the significant growth inhibition, suggesting that tac promoter provides the highly strong expression of LDH (Fig. 6.3). Therefore, tac promoter was used for further experiments.

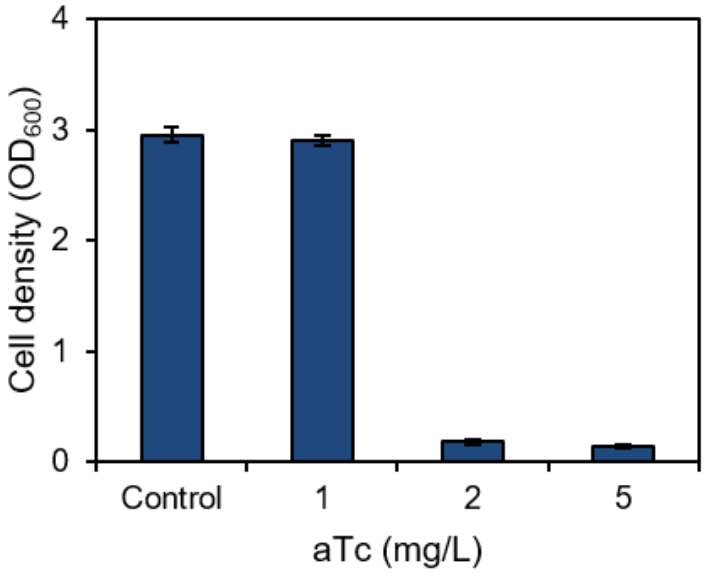


Figure 6.2 Growth inhibitory effect of anhydrotetracyclien in JHM80

JHM80 was grown in the NMS medium containing indicated concentrations of anhydrotetracycline (aTc) and growth was measured after 40h. Error bars indicate standard deviations of two independent experiments.

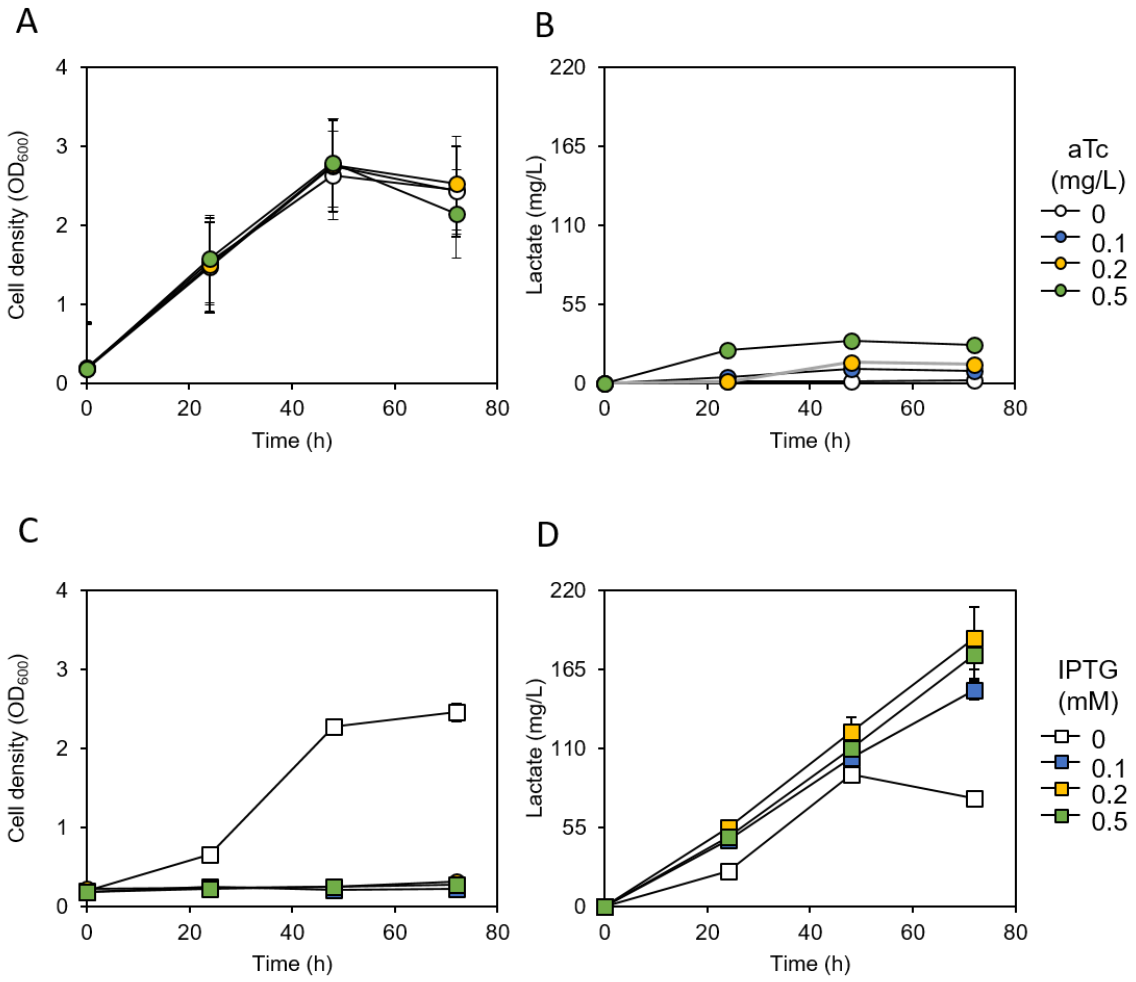


Figure 6.3 D-LA production by expressing Lm. LDH using inducible promoters

JHM80 with expressing Lm. LDH under Tet promoter or Tac promoter were grown in NMS medium containing indicated concentrations of anhydrotetracycline (A,B) or IPTG (C,D). The growth (A,C) and LA production (B,D) were measured during the growth. Error bars indicate standard deviations of two independent experiments.

6.3. **Evaluation of tac promoter for LA production**

To further enhance the LA production, JHM804 was cultured in the NMS medium containing various concentrations of IPTG with supplying 20% (v//v) methane every 24h. As shown in Fig.6.4, the highest amount of D-lactate was produced in the presence of 5 μM of IPTG. In the presence of higher amount of IPTG, JHM804 showed growth inhibition and produced less amount of LA. On the contrary, relative LA production to cell concentrations (LA production/OD_{600} of cells) increased in proportion to IPTG concentrations. At 96h, JHM804 showed 6.3 times higher relative LA production level in the presence of 25 μM IPTG compared to un-induced condition. Moreover, it was 3.1 times higher than the native *glgA* promoter (JHM86). These results suggest that tac promoter enables strong expression of LDH in JHM80 but failed to increase LA production due to growth inhibition by lactate accumulation, acidification of medium, and possibly by depletion of nitrogen sources (Fig. 6.4).

To improve LA production, additional nitrogen sources were added to the medium with pH neutralization. In the modified NMS medium containing 3x KNO_3, JHM803 produced 1.59 g/L of LA at 80h (Fig. 6.5). In *E. coli*, formate chanel encoded by *focA* is known to export not only formate but other organic anion such as acetate and lacate. Therefore, *focA* was expressed in JHM804 to reduce lactate accumulation in cells, generating the strain JHM805. The expression of *focA* improved both growth and LA production, resulting 1.86 g/L of LA which was 56% increase than D-LA production in JHM86 (JHM80 expressing LDH using native *glgA* promoter) (Fig. 6.6).

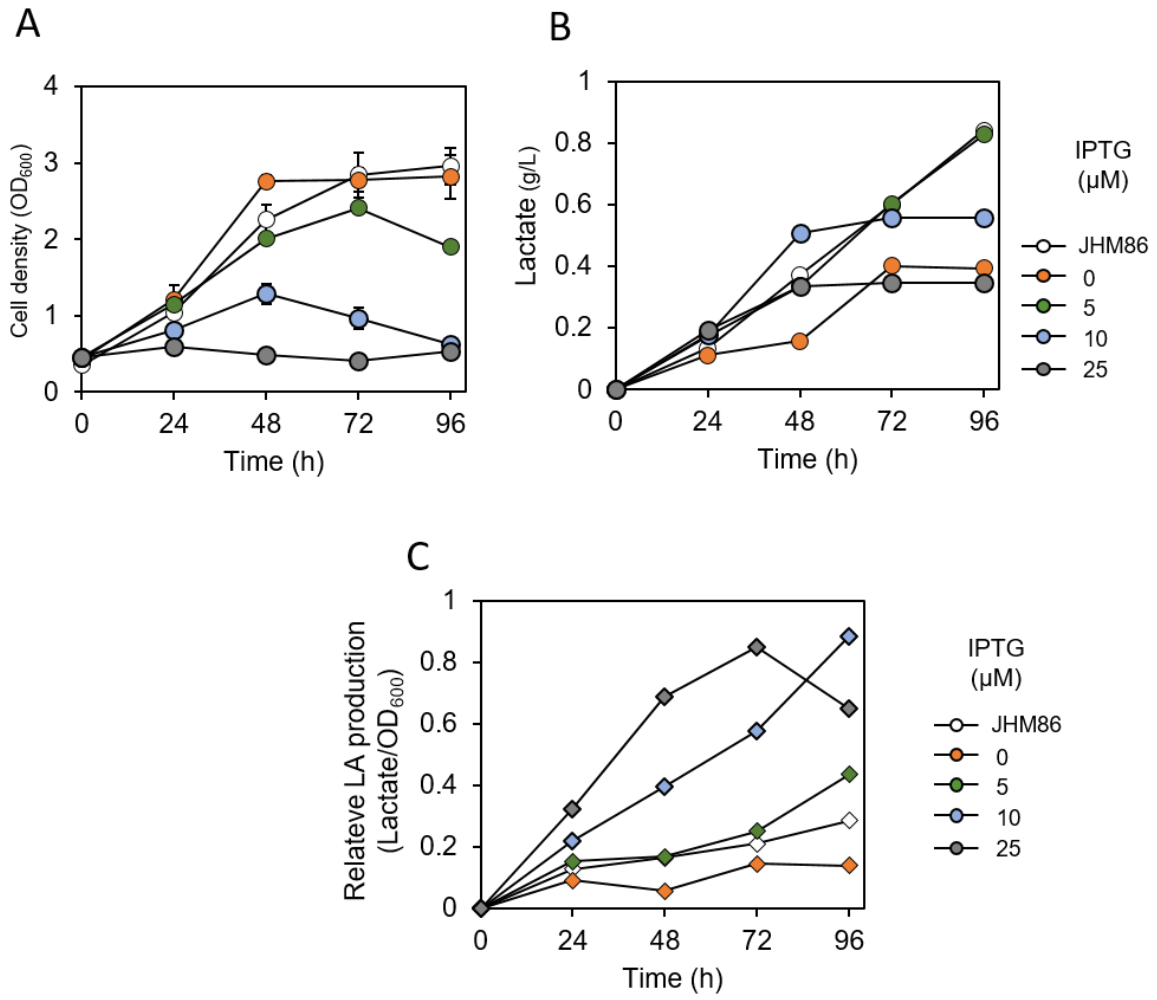


Figure 6.4 Evaluation of Tac promoter for LA production

JHM86 (JHM80 expressing LDH with native *glgA* promoter) and JHM804 (JHM80 expressing LDH with tac promoter) were grown in NMS medium containing indicated concentrations of IPTG. The growth (A) and LA production (B) were measured during the growth. Relative LA production (C) was measured by dividing LA by cell concentrations. Error bars indicate standard deviations of two independent experiments.

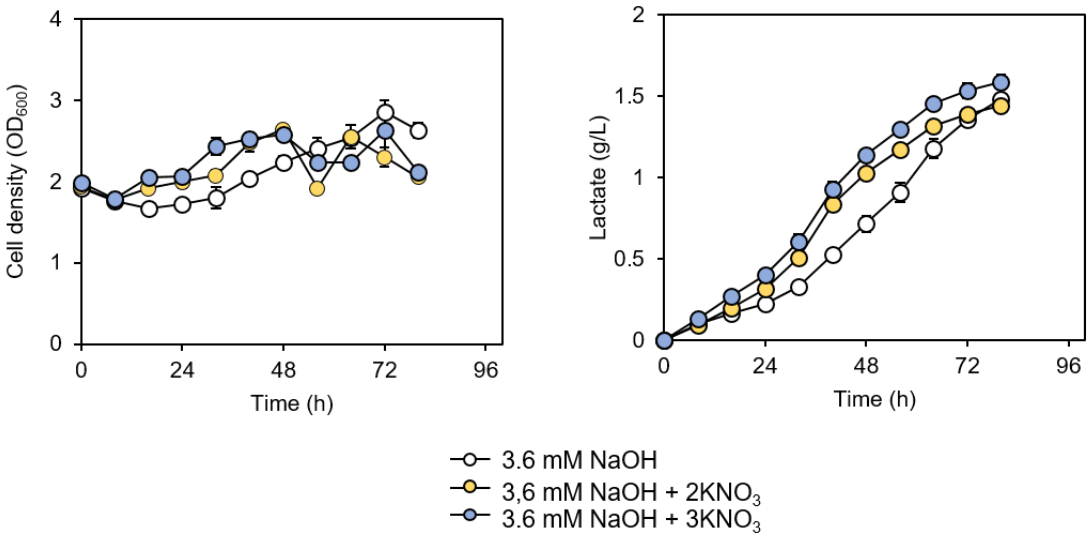


Figure 6.5 Effect of culture optimization on LA production in JHM803

JHM804 was grown in NMS medium or NMS medium containing additional nitrogen sources (2x or 3x KNO_3) neutralized with 3.6 mM NaOH 20% (v/v) methane was fed every 24h. Cell growth (A) and LA production (B) were measured during the growth. Error bars indicate standard deviations of two independent experiments.

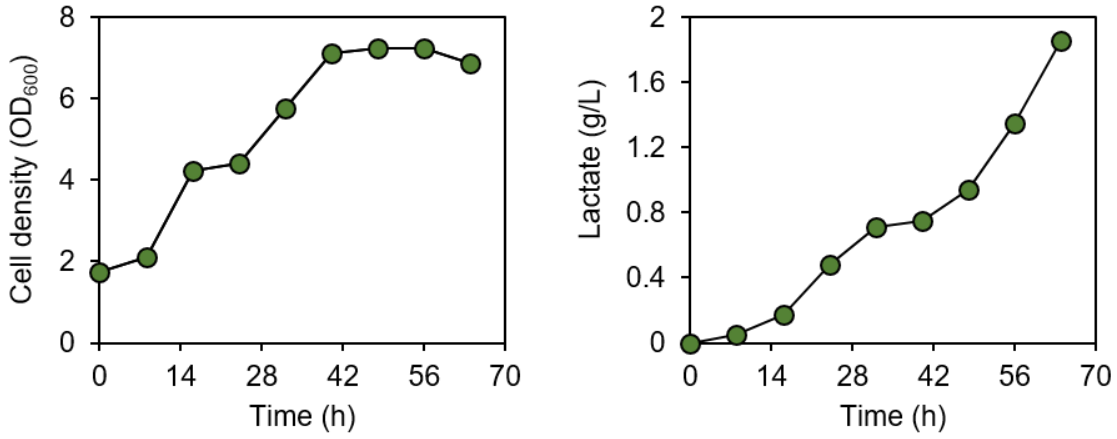


Figure 6.6 Effect of *focA* expression on LA production in JHM804

JHM804 expressing formate channel (*focA*) was grown in NMS medium containing additional nitrogen sources (3x KNO_3). 20% (v/v) methane was fed every 24h. Cell growth (A) and LA production (B) were measured during the growth. Error bars indicate standard deviations of two independent experiments.

6.4. **Improvement of D-LA production by disruption of glucose 1-phosphate adenylyltransferase**

For continuous supply of methane and pH neutralization, JHM805 was used for fermenter culture. However, the growth of JHM805 was severely inhibited in 500 mL flask even in the absence of the inducer, whereas JHM80 showed comparable growth both in 125 mL and 500 mL flask (data not shown). I supposed that deletion of *glgA* which is the only different genetic background between JHM80 and JHM805 is related to growth inhibition. In *Methylomonas* sp. DH-1, methane is converted to glucose-1-phosphate (G1P) by RuMP and glycolytic pathway. Glycogen is synthesized from G1P by serial reaction of *glgCAB* operon. The *glgC* gene encodes glucose-1-phosphate adenylyltransferase which converts G1P to ADP-glucose. ADP-glucose is further converted to glycogen by glycogen synthase encoded by *glgA* (Fig. 6.7).

The deletion of *glgA* can result to accumulation of ADP-glucose in *Methylomonas* sp. DH-1. In addition, some phosphate intermediates showed growth inhibition in bacteria. ADP depletion by galactose-1-phospahte was observed in *E. coli*. In cyanobacteria, accumulation of ADP-glucose was lethal to cell and the growth was recovered by disruption of ADP-glucose synthesis pathway. To investigate the effect of ADP-glucose accumulation on the methanotrophic cells, *glgA* gene or *glgCAB* operon were deleted in JHM80 and the resulting strains were grown in the 125 mL or 500 mL flask. In the 125 mL flask, all strains showed comparable growth rate. However, *glgA* deleted strain showed severe growth inhibition compared to JHM80 in 500 mL flask, while the *glgCAB* deleted strain showed similar growth levels to JHM80.

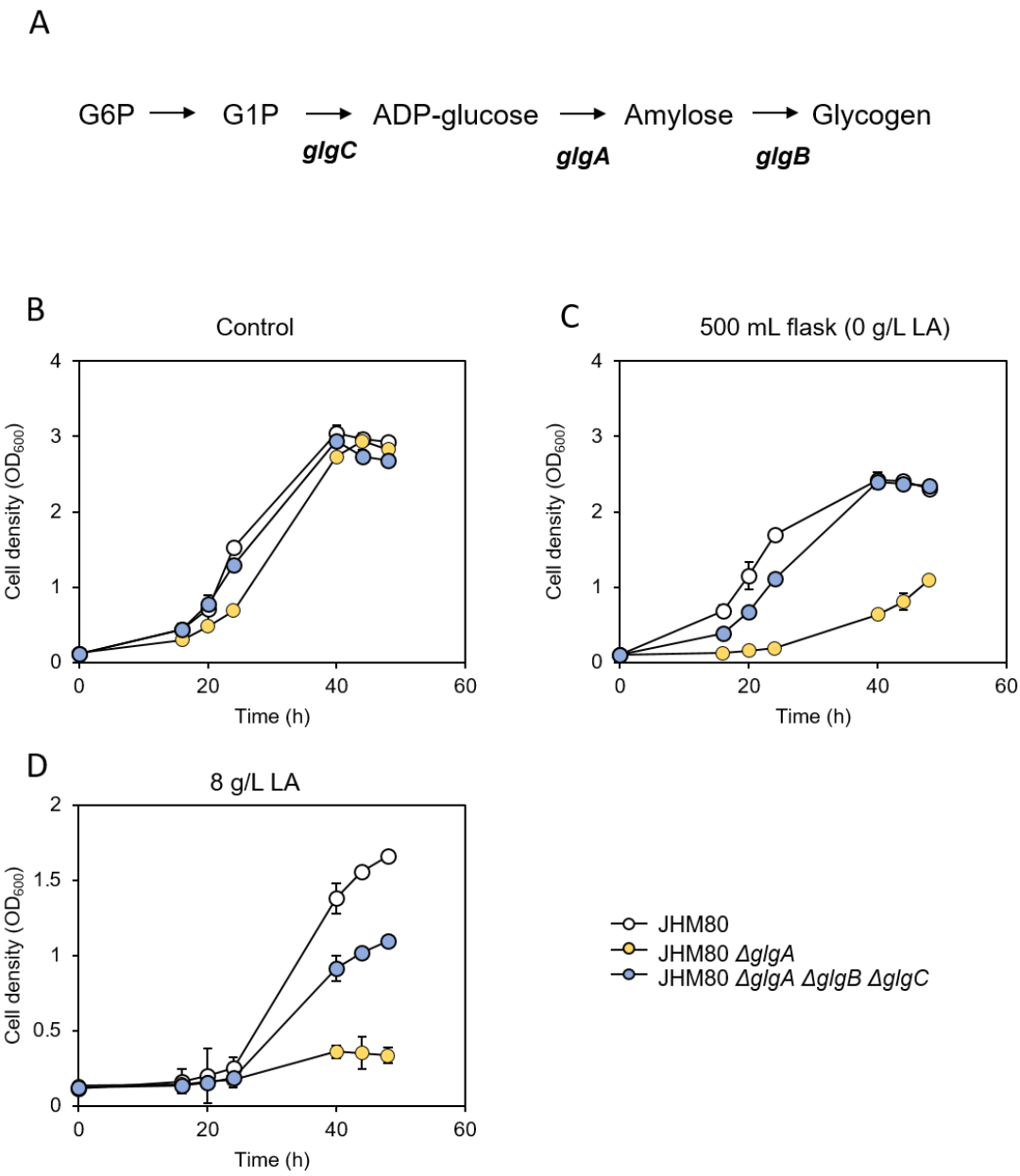

A
G6P
G1P
glgC
ADP-glucose
glgA
Amylose
glgB
Glycogen
B
Control
C
500 mL flask (0 g/L LA)
D
8 g/L LA
Cell density (OD600)
Time (h)
JHM80
JHM80 ΔglgA
JHM80 ΔglgA ΔglgB ΔglgC

Figure 6.7 D-LA production by expressing Lm. LDH by constitutive promoters with different strengths.

A. Glycogen synthesis pathway in *Methylomonas* sp. DH-1. F6P, fructose-6-phosphate; G1P, Glucose-1-phosphate

B. JHM80 and JHM80 with indicated gene deletions were grown in 125 mL flask containing 12.5 mL NMS medium in the absence (B) or presence of 8g/L of LA (C) or 500 mL flask containing 50 mL NMS medium (D).

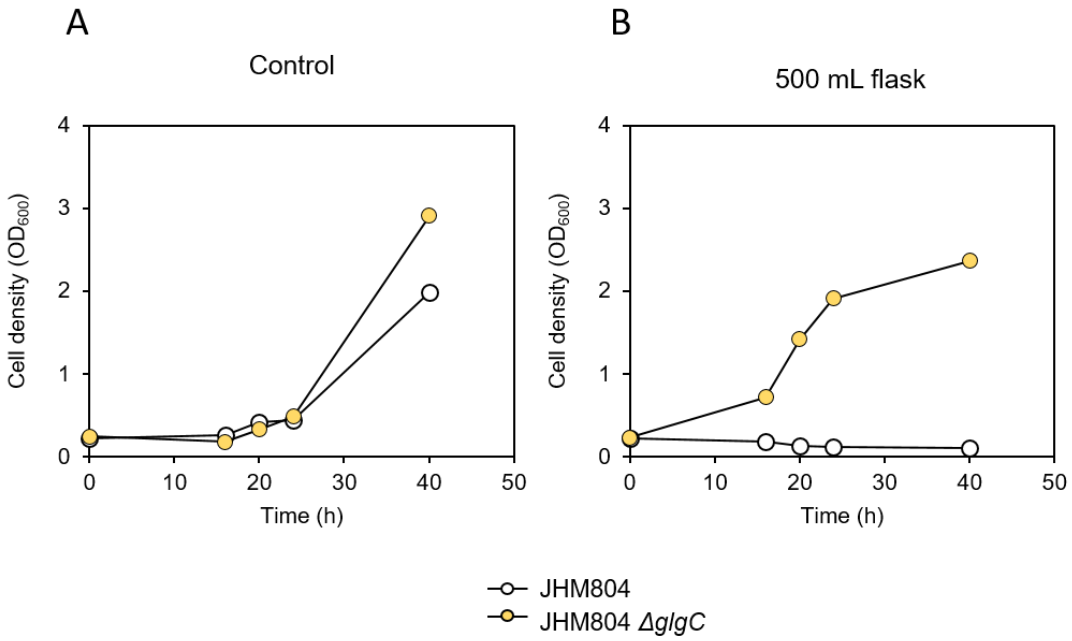


Figure 6.8 Effect of *glgC* gene deletion on the growth of JHM804

JHM804 and JHM804 with *glgC* deletion were grown in the 125 mL flask containing 12.5 mL NMS medium (A) or 500 mL flask containing 50 mL NMS medium (B) with supplying 20% (v/v) methane. Cell growth was measured during the growth. Error bars indicate standard deviations of two independent experiments.

These results imply that ADP-glucose accumulation is toxic to *Methylomona*s sp. DH-1, and the toxicity can be alleviated by disruption of glucose-1-phosphate adenylyltransferase (*glgC*). The LA tolerance of three strains was also measured. Interestingly, accumulation of ADP-glucose also had negative effects on LA tolerance, partly responsible for the growth inhibition of JHM805 during the scale-up culture.

As a final LA producing strain, deletion of *glgC* was tried in JHM805, however strain construction failed. Instead, *glgC* gene was deleted in JHM804, resulting the strain JHM806. Growth inhibition was not observed during the culture of JHM806 in the 500 mL flask (Fig. 6.8). In the 5L-scale continuous stirred bioreactor with continuous supply of 20% (v/v), JHM806 produced 2.06 g/L of LA at 108h (Fig. 6.9). Since JHM806 stopped growth when the nitrate was depleted (48h), modified NMS medium containing 8x of KNO*3* was used for LA production. In the modified NMS medium, JHM806 produced 6.16 g/L of D-LA at 108h with the productivity of 0.057 g/L/h (Fig. 6.10)

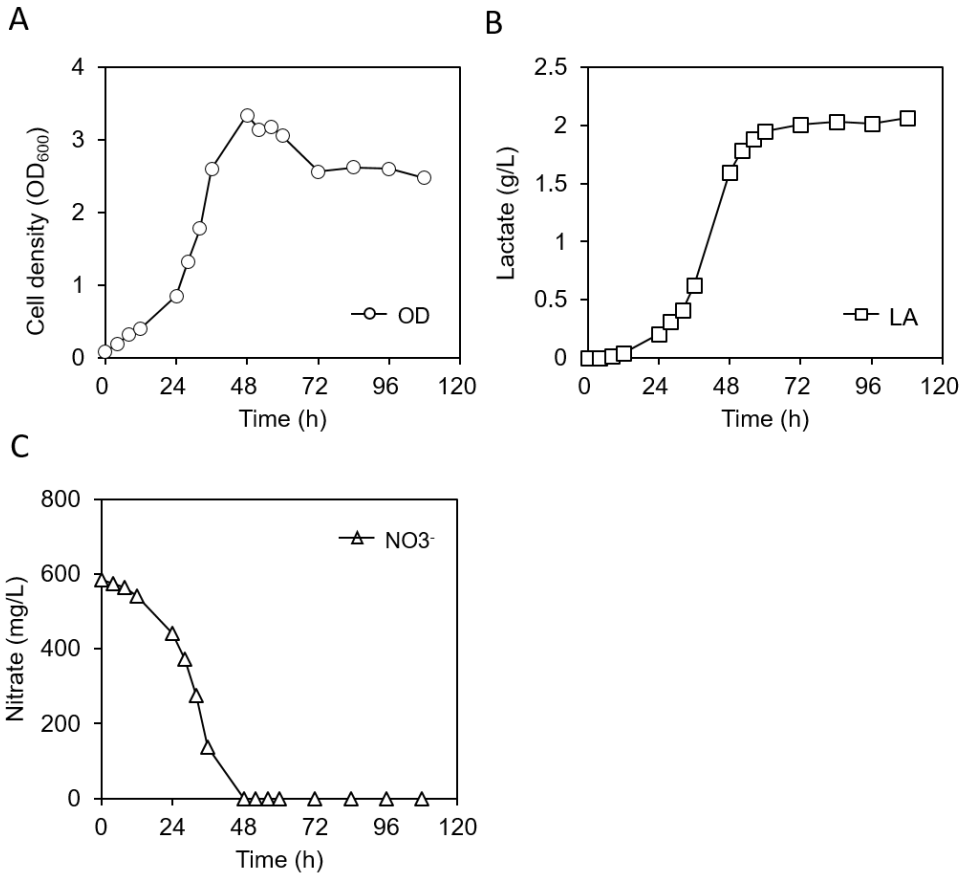


Figure 6.9 Fermenter culture of JHM806

JHM806 was grown in 5L scale fermenter containing 3L NMS medium with 10 μg/mL of kanamycin and 50 μM of IPTG. The pH of medium was maintained at the range of 6.9~7.1 with 2N Hydrochloric acid and 5N Sodium hydroxide. 20% (v/v) methane was continuously supplied to the medium. Cell growth (A), LA production (B), and nitrate concentrations (C) were measured during the growth

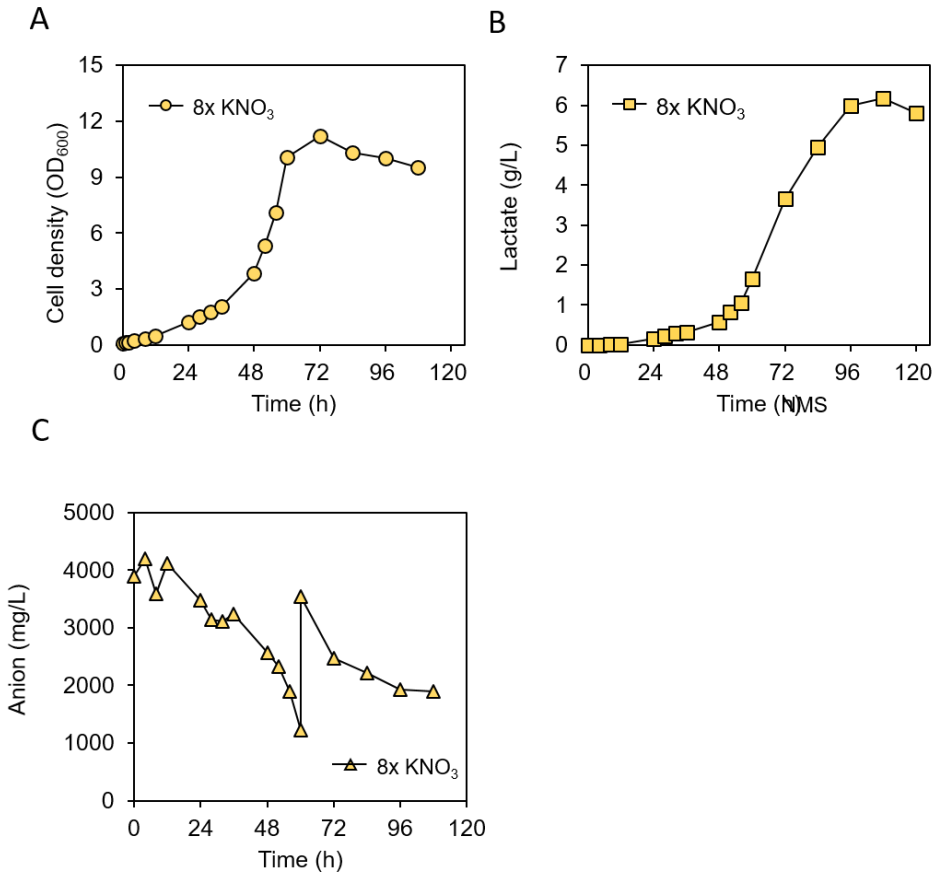


Figure 6.10 Improvement of D-LA production by optimizing culture condition

JHM806 was grown in 5L scale fermenter containing 3L modified NMS medium (8x KNO_3) with 10 μg/mL of kanamycin and 50 μM of IPTG. The pH of medium was maintained at the range of 6.9~7.1 with 2N Hydrochloric acid and 5N Sodium hydroxide. 20% (v/v) methane was continuously supplied to the medium and feeding solution (100 g/L of potassium nitrate) was supplied before nitrate was depleted. Cell growth (A), LA production (B), and nitrate concentrations (C) were measured during the growth.

6.5. **Conclusion**

In this chapter, various promoters were evaluated to improve LA production. It was revealed that IPTG inducible tac promoter effectively overexpressed LDH with minimizing growth inhibition during the early growth phase. The expression of formate channel (*focA*) also increased LA production with cell growth. In this study, it has been shown that accumulation of ADP-glucose by deletion of *glgA* severely inhibited growth during the scale-up process and also reduced LA tolerance. The deletion of *glgC* gene encoding glucose-1-phosphate adenylyltransferase prevented growth inhibitory effects of ADP-glucose accumulation. Lastly, JHM806 developed in this study produced 6.16 g/L of D-LA with productivity of 0.057 g/(L·h), which are highest ever reported in methanotroph

Chapter 7.

Discussion

In this study, a highly efficient D-LA producing methane biocatalyst was developed using *Methylomonas* sp. DH-1 as a parental strain. Adaptive laboratory evolution was performed to improve lactate tolerance of *Methylomoans* sp. DH-1 and metabolic engineering strategies including fine-tuned expression of LDH, reduction of byproducts and toxic intermediates was used for efficient production of D-LA.

In the first part, LA-tolerant evolved strains were developed by adaptive laboratory evolution. The resulting strains JHM30 and JHM80 tolerated 3.0 g/L and 8.0 g/L LA, respectively, while wild-type *Methylomonas* sp. DH-1 barely survived in the presence of 0.5 g/L of lactate. D-LA was produced by integrating LDH from *L. mesenteroides* subsp. *msesnteroides* ATCC 8293 into the JHM80. Compared to wild-type strain integrated with the same LDH gene, evolved strain showed 7.5-fold increase in D-LA production, demonstrating the importance of LA tolerance in LA production. In fed-batch culture with pH neutralization and medium optimization, 1.19 g/L of D-LA with a yield of 0.245 g/g CH_4, which are 48% increase than previous world record.

In the second part, LA tolerance of JHM80 was investigated. Genome and transcriptomic analysis revealed overexpression of AYM39_21120 (LysR-type transcription factor, named as *watR*) was partly responsible for LA tolerance. LA tolerance of the wild-type strain was improved by overexpressing the watR gene alone, demonstrating successful reverse engineering. The AYM39_21125 (*smtM*) and AYM39 (*rstM*) genes, constituting an operon structure with the *watR* gene, were also up-regulated in JHM80, but deletion or overexpression of these genes did not affect LA tolerance. RNA sequencing was performed to further elucidate the target of *watR* gene and identified the overexpression of efflux pump encoded by an operon consisting AYM39_17380, AYM39_17385, AYM39_17390, AYM39_17395, and

AYM39_17400 genes as a WatR-mediated LA tolerance contributor in JHM80. The deletion of efflux pump totally abolished the LA tolerance of JHM80 and the its function of exporting the lactate anion was demonstrated. This efflux pump also contributed to other organic acids tolerance including formate and propionate. The results of this study provide the understanding of organic acids tolerance mechanism in methanotrophs. In addition, overexpression of this efflux pump may be useful for organic acid production in methane utilizing bacteriaW.

In the third part, IPTG inducible tac promoter was employed to improve LA production. Under the control of tac promoter, LDH was effectively overexpressed with minimizing growth inhibition. However, the resulting strain (JHM804) showed severe growth inhibition during the scale-up culture. Deletion of glycogen synthase (*glgA*) significantly decreased the growth and LA tolerance of JHM80. On the other hand, additional deletion of *glgC* which is related to ADP-glucose synthesis fully recovered the growth defects, suggesting that accumulation of ADP-glucose was responsible for growth inhibition. By deleting *glgC* gene in JHM804, the final LA producing strain, JHM806 was generated. In the continuous gas fermentation, 6.16 g/L of D-LA was produced with the productivity of 0.057 g/(L·h), which is the highest ever reported in methanotrophs. Considering that glycogen is one of the major carbon storage in methanotrophs, disruption of glycogen synthesis can be important strategies as a competing pathway. The results of this study suggest that deleting the *glgCAB* operon instead *glgA* gene can improve chemical production without the growth inhibition.

By using adaptive laboratory evolution with metabolic engineering, an efficient LA-producing *Methylomonas* sp. DH-1 was developed. The resulting strain showed the highest D-LA titer, yield, and productivity in methanotrophs with increased lactate tolerance. Further

engineering including deletion of pyruvate dehydrogenase to increase pyruvate flux can improve LA production. Also, further understanding of gene expression system and development of inducible promoter using cheap chemicals or ligand might enhance the availability of commercial LA production from methane.

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Abstract in Korean

국문 초록

자연에서 가장 풍부한 탄소원 중 하나인 메탄을 생물학적으로 전환하여 젖산과 같은 가치있는 화학물질을 생산하는 것은 유망한 전략이다. 이 연구에서, 메탄으로부터 D 형 젖산을 생산하기 위해 새롭게 선별된 메탄자화균 (*Methlyomonas* sp. DH-1)을 모균주로 이용하여 적응 진화 전략과 대사공학 전략을 통해 효율적으로 D 형 젖산을 생산하는 바이오 촉매를 개발하였다. 수준에서 규명하였다.

첫 번째로 적응진화 전략을 통해 신규 메탄자화균주의 젖산 내성을 향상하였다. 야생형 균주는 0.5 g/L 농도의 젖산이 존재 하에 거의 성장하지 못하는 반면, 적응진화 균주 (JHM80)는 8 g/L 농도의 젖산이 첨가된 배지에서 생존하였다. D 형 젖산 생산을 위하여, 입체특이성이 매우 높은 *Leuconostoc mesenteroides* subsp. *mesenteroide*s ATCC 8293 율의 D 형 젖산 탈수효소를 도입하고 글리코겐 합성경로를 결손하였다. 야생형 균주와 비교하여, 이 전략을 통해 적응 진화 균주는 750 mg/L 농도의 D 형 젖산을 생산하였다. 배지 중화와 최적화를 통해 젖산 생산량을 1.19 g/L 농도로 향상하였다.

다음으로, 적응진화 균주의 젖산 내성 기작을 규명하기 위하여 전장 유전체 분석 (whole genome sequencing)을 수행하였다. 그 결과, 프로모터 (promoter) 영역의 2개의 염기 (TT) 결손에 의한 AYM39_21120 유전자 (*watR*)의 과발현이 젖산 내성에 부분적으로 기여하였다. *watR* 유전자의 과발현은 야생형 균주의 젖산 내성을 향상한 반면, *watR* 유전자의 결손은 적응진화 균주의 젖산 내성을 감소시켰다. RNA 분석 (RNA sequencing)을

통해 전사인자인 *watR* 유전자의 target 유전자로 RND 유형의 efflux pump가 젖산 내성에 기여함을 규명하였다.

마지막으로, 유도성 프로모터를 이용하여 젖산 생산에 의한 성장 저해를 최소화 하면서 젖산 탈수소 효소의 발현을 효율적으로 강화하였다. 추가로, ADP-glucose 축적에 의한 세포 독성을 감소하기 위하여 ADP-glucose 합성 효소를 결손하였다. 다음과 같은 전략을 통해 개발된 JHM806 균주는 발효기 배양 조건에서 6.16 g/L 농도의 D 형 젖산을 생산하였으며, 생산성은 0.057 g/(L · h) 이었다.

주요어: 메탄, D 형 젖산, *Methylomonas* sp. DH-1, 적응진화, 대사공학

학번: 2017-21482