Alternative fate of glyoxylate during acetate and hexadecane metabolism in *Acinetobacter oleivorans* DR1

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The glyoxylate shunt (GS), involving isocitrate lyase (encoded by *aceA*) and malate synthase G (encoded by *glcB*), is known to play important roles under several conditions including oxidative stress, antibiotic defense, or certain carbon source metabolism (acetate and fatty acids). Comparative growth analyses of wild type (WT), *aceA*, and *glcB* null-strains revealed that *aceA*, but not *glcB*, is essential for cells to grow on either acetate (1%) or hexadecane (1%) in *Acinetobacter oleivorans* DR1. Interestingly, the *aceA* knockout strain was able to grow slower in 0.1% acetate than the parent strain. Northern Blot analysis showed that the expression of *aceA* was dependent on the concentration of acetate or H$_2$O$_2$, while *glcB* was constitutively expressed. Up-regulation of stress response-related genes and down-regulation of main carbon metabolism-participating genes in a Δ*aceA* mutant, compared to that in the parent strain, suggested that a Δ*aceA* mutant is susceptible to acetate toxicity, but grows slowly in 0.1% acetate. However, a Δ*glcB* mutant showed no growth defect in acetate or hexadecane and no susceptibility to H$_2$O$_2$, suggesting the presence of an alternative pathway to eliminate glyoxylate toxicity. A lactate dehydrogenase (LDH, encoded by *ldh*) could possibly mediate the conversion from glyoxylate to oxalate based on our RNA-seq profiles. Oxalate production during hexadecane degradation and impaired growth of a Δ*ldh*Δ*gbc* double mutant in both acetate and hexadecane-supplemented media suggested that LDH is a potential detoxifying enzyme for glyoxylate. Our constructed LDH-overexpressing *Escherichia coli* strain also showed an important role of LDH under lactate, acetate, and glyoxylate metabolisms. The LDH-overexpressing *E. coli* strain, but not wild type strain, produced oxalate under glyoxylate condition. In conclusion, the GS is a main player, but alternative glyoxylate pathways exist during acetate and hexadecane metabolism in *A. oleivorans* DR1.
pathway is essential for defense against antibiotic action in *M. tuberculosis*, and antibiotic-induced oxidative stress could be protected against using an antioxidant in GS-deficient cells. A recent study also showed that the MS is critical for *M. tuberculosis* during fatty acid assimilation; furthermore, the elimination of MS in *M. tuberculosis* could prevent acute and chronic infections in mice. In another posecomial pathogen, *Pseudomonas aeruginosa*, both aceA and glcB were induced under oxidative stress, antibiotics treatment, and iron-limiting conditions. When glyoxylate is supplied, *P. aeruginosa* is more resistant against tobramycin than in fumarate-supplemented media. Further analysis showed that both reduced respiration rate and proton motive force confer decreased tobramycin uptake and tobramycin resistance.

Although *Acinetobacter baumannii* is a major infectious bacteria and global concerns of its spread have increased due to its multidrug resistance, the GS systems of *Acinetobacter* species remain poorly understood. Reduced persister cell formation appears to be linked to inhibition of ICL, which suggests the importance of ICL in antibiotic resistance (AR) of pathogenic bacteria. However, in a previous study, when colistin or colistin with curcumin was added to *A. baumannii*, ICL expression was highly upregulated, and there was limited persister cell formation. There is no general agreement about the role of ICL on persister formation. Living in natural environments also confers many stresses to microorganisms, such as chemical and oxidative stresses, and nutrient deficiencies, which often induces activation of the GS under those harsh conditions. Thus, to gain a better understanding, further research of the GS is required for not only pathogens, but also environmental microorganisms.

Previously, our lab has shown that GS-participating genes in a soil-borne bacterium, *A. oleivorans* DR1, are upregulated upon exposure to ampicillin, paraquat (PQ), phenazine methosulfate (PMS), hexadecane (Hex), and triacontane (TRI)-treated condition. Examination of an aceA-deficient strain in triaconatane (C30 alkane)-containing minimal salt basal (MSB) media showed retarded growth with a long lag phase in contrast to that observed for the parent strain, and the susceptibility of the ICL-lacking mutant to H$_2$O$_2$, was considerably increased. Thus, alkane or ROS-generating substances can induce carbon-metabolic shift to GS in *A. oleivorans* DR1. However, detailed mechanisms are still in its early stage. In this study, we focused on analyzing unpredicted growth of GS-deficient DR1 cells under 0.1% acetate and Hex. Our data suggested that low toxicity of 0.1% acetate and low solubility of Hex enable ICL-lacking DR1 cells to grow using an unknown alternative pathway and MS-lacking cells produce oxalate under the same conditions for detoxifying accumulated glyoxylate.

**Results and Discussion**

**Comparative growth of WT and GS null-strains in the presence of acetate and Hex.** Previously, it was shown that GS-related genes in DR1 are highly upregulated when cells degrade Hex and TRI. The ΔaceA mutant grew at a slower rate than the WT in Hex-, hexadecanoic acid-, and TRI-supplemented media, and no growth was observed in 1% NaAc-amended media. However, the susceptibility of the aceA mutant under H$_2$O$_2$ treatment and the induction of aceA expression in the WT as a result of increased H$_2$O$_2$ concentration indicates that aceA is also critical for survival of the DR1 strain under conditions of oxidative stress. On the other hand, deletion of the glcB gene did not increase the sensitivity to H$_2$O$_2$ (Fig. S1). This result led to the assumption that an alternative GS pathway is present.

Growth assays were conducted under 1% sodium acetate (NaAc) and Hex. Surprisingly, the WT strain and glcB null-mutant strains of *A. oleivorans* DR1 grew normally with similar growth rates under both conditions (Fig. 1.A,C). On the other hand, growth of aceA-lacking mutant was not observed under the same conditions (Fig. 1.A,C). Due to extreme toxicity of glyoxylate generated during NaAc and Hex metabolism, and no growth was observed in 1% NaAc-supplemented media (Fig. 1.B,E,F). From the above, it is apparent that ICL, but not MS, is essential, and an alternative pathway of glyoxylate in MS-lacking cells exists under 1% NaAc and Hex metabolism.

*Escherichia coli*, aceA and aceB, encoding ICL and MS, are clustered as an operon, so that they are expressed and regulated simultaneously. However, many aceA and glcB (or aceB) genes had dispersed from one another in the genomes of many bacteria, indicating that divergently evolved ICL and MS might participate in the same process accidentally. In this context, it is possible that other enzymes could play a role in glyoxylate detoxification generated by ICL.

**Evaluation of acetate toxicity in GS-deficient mutants.** Similar growth patterns of WT and ΔglcB mutant strains were observed; the highest growth rate and the longest lag phase were observed in 1% NaAc among the surveyed conditions of 0.01–1% NaAc-supplemented media (Fig. 2.A,C). Surprisingly, growth of the aceA knockout strain has been observed under 0.1% NaAc and a final OD$_{600}$ could reach 0.25 (Fig. 2.B). We hypothesized that the essentiality of the aceA gene under acetate metabolism in many bacteria requires reconfirmation because all experimental reports used high concentrations of acetate and fatty acids. The sensitivity of the aceA mutant in 1% NaAc would be due to the toxicity of acetate because the aceA-null strain also showed high susceptibility to acetate even when another carbon source, sodium succinate (NaSc), which activates the TCA cycle, is present (Fig. 2D). The WT and ΔglcB mutant strains grew in NaAc and NaSc-mixed media (0.1% NaAc and 1% NaSc), however, retarded growth of both strains was shown in the presence of 1% NaAc plus 1% NaSc (Fig. 2DF). Furthermore, the aceA mutant could not grow in 1% NaAc and NaSc supplemented media, and growth was slightly delayed in 0.1% NaAc and 1% NaSc media compared to that in only 1% NaSc-supplemented media (Fig. 2E). Retarded growth of the WT and ΔglcB strains and inhibited growth of the aceA mutant were observed in the presence of 1% NaAc with or without NaSc (Fig. 2A, B, E, F), indicating that 1% NaAc is sufficiently toxic to the DR1 strain.

Acetate toxicity to cells has mainly two outcomes: 1. release of protons in the cytoplasm, and 2. intercalation of undissociated acetate into the lipid bilayer at low external pH. A previous study has shown that 1% NaAc...
did not lower external pH\textsuperscript{17}, thus it is expected that the growth deterioration of DR1 cells is due to intracellular acidification in high concentration of acetate (>1% NaAc). Interestingly, the ΔaceA mutant could not grow in the presence of 1% NaAc but showed slight growth in 0.1% NaAc-supplemented media (Fig. 2C,D), implying that a low concentration of NaAc (0.1%) could be metabolized without ICL, but not as efficient as in the GS pathway. Unlike growth in the presence of NaAc, the growth rate and the expression of aceA and glcB were not significantly different between 1% and 0.1% Hex (Fig. 3). In fact, several obstacles are present when DR1 metabolizes Hex; the Hex solubility in media, the diffusion rate of Hex through the cell membrane, and the activation rate of inert Hex. Thus, the observation of no differences in growth rate or gene expression on 1% and 0.1% Hex are probably due to the above limited factors.

**Expression of GS-participating genes and growth under high and low concentrations of NaAc and Hex.** To confirm the importance of the GS during acetate and Hex metabolism, the expression of aceA and glcB was quantified using a Northern blot assay. Previously, many studies have reported that oxidative stress induces activation of the GS pathway in *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, as well as *A. oleivorans* DR1\textsuperscript{13,17}. It is generally agreed that the GS pathway helps cells avoid unnecessary NADH production, which prevents excess ROS generation from unbalanced oxygen consumption during stress conditions\textsuperscript{20}. The aceA-deficient strain is highly sensitive to H₂O₂ compared to the WT or glcB-knockout mutant (Fig. S1A–C). The expression of aceA and glcB was examined under oxidative stress conditions (0-, 0.1-, 0.2-, 0.5-, and 1 mM H₂O₂).
The expression of aceA was considerably increased only when a high concentration of H$_2$O$_2$ (1 mM) was added (Fig. S1D). Low concentrations of H$_2$O$_2$ might be quickly eliminated by various catalases present in the genome of DR1 cells$^{23}$. On the other hand, glcB showed constitutive low-level expression (Fig. S1E). The expression of aceA was considerably increased only when a high concentration of H$_2$O$_2$ (1 mM) was added (Fig. S1D). Low concentrations of H$_2$O$_2$ might be quickly eliminated by various catalases present in the genome of DR1 cells$^{23}$. On the other hand, glcB showed constitutive low-level expression (Fig. S1E). The expression of aceA was considerably increased only when a high concentration of H$_2$O$_2$ (1 mM) was added (Fig. S1D). Low concentrations of H$_2$O$_2$ might be quickly eliminated by various catalases present in the genome of DR1 cells$^{23}$. On the other hand, glcB showed constitutive low-level expression (Fig. S1E).
aceA was also significantly increased in the presence of NaAc and Hex compared to that observed in the presence of NaSc (Fig. 3A). Interestingly, aceA was expressed in a NaAc concentration-dependent manner, but the expression level under Hex was not increased even at a high concentration because of the low solubility of Hex. Constitutive expression of glcB gene was observed on 0.1 and 1% NaAc, however, expression was decreased in 1% Hex compared to that in 0.1% Hex (Fig. 3A). A relatively longer lag time, but higher optical density values in 1% NaAc (OD$_{600}$~1.0) compared to that in 0.1% NaAc (OD$_{600}$~0.3) were observed at the stationary phase, which also offers an insight into acetate toxicity at high concentration. However, similar growth rates and optical density values were observed in 0.1 and 1% Hex-supplemented media (OD$_{600}$~1.1) (Fig. 3B). Our expression and deletion analyses suggested that the aceA product plays significant protective roles under conditions of oxidative stress by operating the GS and an unrecognized pathway might be present for replacing the role of the glcB product.

**Transcriptome analysis of aceA mutant during acetate metabolism.** To analyze the mechanism for detoxifying acetate in GS-deficient strains, transcriptomic analysis was performed. Previous RNA-seq data of DR1 under succinate-supplemented media were retrieved and compared with transcriptome data of WT and aceA mutant strains when they were grown in 0.1% NaAc-supplemented media in this study. The information of analyzed RNA-seq data is supplied in Table S1. Compared to that of the WT strain, 272 and 1,096 genes were up- and down-regulated, respectively, in the aceA mutant. In addition, 63.8% of all genes showed no change at the transcriptional level, implying that no significant metabolic alterations would occur (Fig. S3A). Six of the ten most upregulated genes encoded hypothetical proteins, and the remaining genes were annotated as a bacterial RNaseP, ferredoxin, a murain hydrolase, and an aldehyde dehydrogenase (Fig. S3B). The highest upregulated gene, encoding a hypothetical protein, showed only a 2.3-fold increase in the aceA mutant compared to that in the control (Fig. S3B).
In WT cells, downstream genes (succinate to oxaloacetate) of the TCA cycle (succCD, sdhABCD, fumBC, mpo, and mdh) were down-regulated during acetate metabolism, compared to that under succinate-supplemented conditions (Fig. S4). Upstream genes (citrate to succinyl CoA) of the TCA cycle (gltA, acnB, tcd, and succAB) including GS-participating genes (aceA, and glcB) in WT strain were upregulated in the NaAc-supplemented media compared to that in NaSc-supplemented media, confirming that acetate metabolism occurs actively through the GS and upstream of TCA cycles (Fig. S4). Furthermore, aacC, encoding a succinyl-CoA:acetate CoA transferase, was highly upregulated in the presence of NaAc (WT RPKMNaAc 409.2; WT RPKMNaSc 11.1; ΔaceA RPKMNaAc 1701.8, Fig. S5A). These results indicate that acetate is also possibly metabolized by succinyl-CoA:acetate CoA transferase generating succinate and succinyl CoA in A. oleivorans DR1 (Fig. S5).

Notably, fadB and fadA, encoding an enoyl-CoA hydratase/3-hydroxybutyryl-CoA epimerase/a 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase, respectively, showed slightly higher expression levels in the aceA-null mutant compared to that of the WT. In addition, poly-3-hydroxybutyrate (PHB) synthesis participating genes, phaA (Acetyl-CoA acetyltransferase) and phaB (Acetoacetyl-CoA reductase), were also slightly upregulated in the aceA mutant strain (Fig. S5B). However, expression of phaC (PHA synthase) and phaZ (PHB depolymerase) was not significantly increased in the aceA-lacking strain (phaC 1.0-fold; phaZ 0.9-fold). Thus, acetyl CoA might be forced to 3-hydroxy butyryl-CoA via aceA-CoA due to a retarded consumption rate for acetyl-CoA.

Cells lacking the GS need to maintain the levels of NADH and ATP by operating other cellular metabolism pathways. Iron is an essential element for enzymes participating in the TCA cycle and electron transport chain (ETC)16. It has been shown that iron limitation induces the GS cycle and acetate-triggered GS operating cells also have lower intracellular iron content17. In the ΔaceA mutant, genes related to iron transporters (fex, and fecA), iron regulatory protein (fis), iron-binding protein (ftr), and glutaredoxin (grx) were upregulated (Table S2). Furthermore, the expression levels of genes involved in the ETC (cybB, cytochrome O ubiquinol oxidase; nuoI, NADH-quinone oxidoreductase subunit I; and etfD, probable electron transfer flavoprotein-ubiquinone oxidoreductase) and ATP synthase subunits (atpE, atpB, atpC, and atpA1) were slightly increased with high RPKM (>1,000) in the aceA strain (Table S2). Taken together, increased expression level of genes related to iron uptake, respiration, and ATP synthesis occur in the aceA mutant because of compensation for GS malfunction.

RNA-seq data implied that the aceA-null mutant is under severe stresses compared to the WT strain. Three cold shock protein-coding genes (cpsA, cpsE, and cpsG) were upregulated when the aceA was deleted. Notably, cpsA, and cpsE are considerably upregulated in the knockout strain (1.5-, and 1.4-fold, respectively) with high RPKM (>1,000). In addition, DNA repair proteins (encoded by recO, and uvrC), DNA/RNA helicases (encoded by hreA, and rveX), and oxidative stress response proteins (encoded by aphF, and katG) were expressed to a greater extent in the aceA knockout mutant, indicating that the lack of the GS confers acetate susceptibility to the mutant. RNA-seq profiling showed that activated Pta-AckA (reversible pathway from acetate to acetyl CoA) and the GS are main pathways during acetate metabolism (Fig. S4). Furthermore, the high expression of aacC in NaAc implies an alternative contributing factor for acetyl-CoA utilization in the presence of acetate (Fig. S5A). It was reported that aacC confers acetate resistance and assimilation to Acetobacter aceti, a GS-lacking bacterium18. A. baumannii also possesses an aacC gene and the function of Succinyl-CoA:acetate CoA transferase was examined20. Therefore, aacC could be a candidate for acetate metabolism when glutamate is inactivated in A. oleivorans. The expression level of aacC in the ΔaceA mutant strain is slightly lower than that of the WT in the presence of NaAc, resulting in the retarded growth of the aceA-null mutant compared to that of the WT strain (Figs 2 and S5A). Furthermore, the ΔaceA mutant may synthesize acetoacetyl CoA and 3-hydroxybutyryl CoA to avoid acetyl CoA accumulation (Fig. S5B). Uproregulated genes for alkane degradation are ambiguous, the genes maybe induced due to the acetate accumulation in the aceA mutant17. Previous studies have revealed that respiration- and proton motive force-related genes were downregulated in P. aeruginosa when glutamate is a sole carbon source, because it uses the GS cycle rather than the TCA cycle10. Thus, blocking of the GS by elimination of the aceA gene may cause cells to sustain respiration and proton motive force. Higher expression of cytochrome oxidase, Fe-S cluster proteins, and iron transporter was observed in the aceA mutant compared to that of the wild type. Marine heterotrophic bacteria has evolved to survive in iron-limited conditions by activating the GS and reducing iron consumption rate (reducing the expression of Fe-S cluster participating in the TCA cycle and ETC)24.

Expression of genes for motility, such as cheY (Chemotaxis protein), pilY (Twitching motility protein), pilW (Pilus assembly protein), pilF (Pilus assembly protein), and pilS (Sensor protein), was increased in the aceA-null strain compared to that in the WT strain, suggesting that lack of GS might be responsible for active motility. Although the relationship between motility and GS is unclear, a recent study demonstrated that cold shock protein C (CspC) positively regulates the expression of GS (aceA) and motility (fjNLK, pilA, and cheW) related genes21. Thus, it could be hypothesized that abundant CspS within the aceA mutant cell confers increased expression of motility-related genes directly or indirectly.

**Alternative glyoxylate detoxification by lactate dehydrogenase (LDH).** The phenotypic differences between WT and glcB knockout strains were not noticeable, although the GS is not operating in the glcB mutant under NaAc- or Hex-supplemented media or under conditions of oxidative stress (Figs 1 and S1). Thus, we assumed that DR1 possesses an alternative pathway for detoxifying accumulated glyoxylate during NaAc- or Hex metabolism. Using BlastP, possible candidate enzymes for glyoxylate detoxification were searched, and seven genes which are homologous to hydroxypyruvate/glyoxylate reductase, glyoxylate carboligase, alanine glyoxylate transaminase, lactate dehydrogenase (LDH), and tartronic semialdehyde dehydrogenase were found (Table S3, Fig. 4A). Furthermore, previous RNA-seq data were retrieved, and the gene expression of candidates under 0.1 and 1 mM H2O2, 0.2 mM PMS, 1 mM PQ, 0.1% NaAc, 0.1% TRI, and 1% Hex treatment was investigated15-17. Uprogulation of L-ldh and D-ldh genes were observed in most of these conditions (1 mM H2O2, PMS, PQ, NaAc,
and HEX = 5.9-, 1.1-, 2.4-, 2.2-, 0.9-, and 1.1-fold changes for L-ldh; 2.9-, 1.2-, 1.4-, 2.0-, 1.0-, and 0.9-fold changes for D-ldh) (Fig. 4A). Many previous studies have suggested that glyoxylate is a substrate for a LDH in human erythrocytes, human plasma, rabbit muscle, rat liver, spinach, and pig heart, resulting in the production of oxalate

In addition, the expression of genes encoding a lactate dehydrogenase (L-ldh, and D-ldh) is relatively higher among potential enzymes for glyoxylate as a substrate during NaAc-, and alkane metabolism (Fig. 4A). Thus, we hypothesized that a LDH is one of the promising enzymes for glyoxylate detoxification in A. oleivorans DR1 although no studies have elucidated the activity of LDH for glyoxylate as a substrate in bacteria. Interestingly, only oxalate (0.15 mM) was detected in the supernatant of Hex-supplemented media, implying that oxalate can be produced during Hex metabolism, which is one of the GS-activating conditions in the DR1 strain (Fig. 4B, C).
ldh knockout strains (ΔL-ldh and ΔD-ldh) could grow (Fig. 5A,C), but the growth of ldh-, glcB double knockout strains (ΔΔL-ldhΔglcB and ΔΔD-ldhΔglcB) was completely impaired in both NaAc and Hex-supplemented media, implying that the ldh gene product compensates the lack of the glcB gene during acetate and Hex metabolism by detoxifying glyoxylate (Fig. 5B,D). To conduct further analyses of LDH, we cloned the L-ldh gene of A. oleivorans DR1 into pRK415 vector and generated Escherichia coli Top10 expressing L-ldh gene (hereinafter referred to as Top 10 (pRK415::L-ldh)). Because LDH is known to mediate the reaction from lactate to pyruvate, vice versa. Growth of Top 10 (pRK415::L-ldh) under lactate, acetate or glyoxylate was monitored. Control strain having only the vector could not grow well under all tested conditions, but LDH from A. oleivorans DR1 cells boosted the growth of Top 10 (pRK415::L-ldh) under the same conditions (Fig. 6A). Poor growth of control strain under acetate or glyoxylate might be due to their toxicities to cells, which could not be observed in the presence of LDH (Fig. 6A). Reverse transcription-polymerase chain reaction (RT-PCR) confirmed that L-ldh gene in Top10 (pRK415::L-ldh) strain was expressed during lactate and acetate metabolisms (Fig. 6B). Surprisingly, Top10 (pRK415::L-ldh) strain, but not Top10 (pRK415), produce oxalate (0.79 mM) during acetate metabolisms. These data supports the possible role of LDH in the alternative fate of glyoxylate during acetate metabolism.

In summary, we demonstrated that the aceA mutant strain could grow at a low concentration of acetate and suggested that succinyl-CoA:acetate CoA acetyl transferase is a candidate for acetate metabolism. Furthermore, it was concluded that glyoxylate detoxification is conducted by a lactate dehydrogenase in the glcB mutant. Although the GS pathway is a main carbon flux in DR1, an alternative pathway via lactate dehydrogenase also exists to enable survival under several dynamic environments.
Methods

Bacterial strains, chemical reagents, and culture conditions. All growth tests for *A. oleivorans* DR1 were conducted at 30 °C in MSB media with medium-intensity agitation for 100 sec every 1 h. Seed culture of DR1 strain was grown at 30 °C in nutrient broth (NB) medium. Growth was monitored by measuring the optical density at 600 nm (OD₆₀₀). All data show the average of three replicates, and the error bar indicates the standard deviation. (B) The expression profile of 16S rRNA (193 bp) and *L-ldh* (191 bp) in Top 10 (pRK415) and Top 10 (pRK415::L-ldh). The expression of Top 10 (pRK415::L-ldh) is highlighted by asterisk. After PCR from 1/10-diluted samples, 5 μL of samples were loaded to ethidium bromide-stained gel. (C) Intracellular oxalate concentration (mM) of Top 10 (pRK415) and Top 10 (pRK415::L-ldh) during 1% NaAc metabolism. ND indicates 'not detected'.

Construction of mutants. *aceA* knockout and complemented strains were produced as described previously. To construct the cloning vector for knockout, the internal region of the *glicB*, *L-ldh*, *D-ldh* genes was amplified using PCR from genomic DNA (Table S4). A *glicB* PCR product and pVIK 112 plasmid were digested using KpnI and SmaI restriction enzymes, fragments of *L-ldh* and *D-ldh*, and pEX18Gm were treated with KpnI.
and EcoRI restriction enzymes. Each fragment was subsequently cloned into the pVIK 112 plasmid for glcB genes and the pEX18Gm plasmid for L-ldh and D-ldh genes via ligation. The constructed plasmids were then transformed into E. coli S17-1Δ pir strain, extracted from E. coli, and transformed into A. oleivorans DR1. Knockout mutants were screened on nutrient agar (NA) containing antibiotics (50 μg/ml kanamycin, or 15 μg/ml gentamycin). To produce ldh and glcB double KO mutants, vectors were constructed (pEX18Gm::ldh) as above, introduced into glcB single KO strain, and then the double KO mutant was selected on NB agar containing 50 μg/ml kanamycin and 15 μg/ml gentamycin.

**RNA extraction, library construction, and sequencing.** Both WT and aceA knockout strains were grown to exponential phase (WT OD600~0.5, ΔaceA OD600~0.15) in NaAc-supplemented MSB media. Total RNA was isolated from 10 ml of cells using the RNeasy Mini Kit (Qiagen, USA) according to the manufacturer’s instructions. All procedures for RNA sequencing were conducted by Chunlab (South Korea). The RNA was subjected to a subtractive Hyb-based rRNA removal process using the MICROBExpress Bacterial mRNA Enrichment Kit (Ambion, USA). A library was constructed as described previously. RNA sequencing was performed with two runs of the Illumina Genome Analyzer Ix to generate single-ended 100-bp reads. Quality-filtered reads were aligned to the reference genome sequence using CLC Genomics Workbench 6.5.1 (CLC bio, USA). Mapping was based on a minimal length of 100 bp with an allowance of up to two mismatches. Relative transcript abundance was measured in RPKM. RNA-seq data has been deposited in NCBI under Gene Expression Omnibus (GEO) accession number GSE124640.

**Gene expression analysis by northern blotting.** After total RNA was extracted using an RNeasy Kit according to the manufacturer’s instructions, a northern blot analysis was performed as described previously. Briefly, the quantified total RNA samples (2.5 μg) were loaded onto denaturing agarose gels including 0.25 M formaldehyde, separated by gel electrophoresis, and then stained with ethidium bromide to visualize 23S and 16S rRNA. The RNA bands were transferred to nylon membranes (Schleicher and Schuell, Germany) using a TurboBlotter (Schleicher and Schuell, Germany). The membrane was hybridized with a specific 32P-labeled probe (Takara, Japan) based on PCR amplification with each primer pair. Autoradiography was performed using an IP plate (Fujifilm, Japan) and a Multiplex Bio-Imaging System (Fujifilm, Japan).

**High-performance liquid chromatography (HPLC) analysis for organic acid detection.** DR1 was incubated in 50 ml MSB media containing 1% Hex for 24 h and then centrifuged (1 min, 10,000 x g) to acquire a clear supernatant for the organic acid analysis using UV/visible HPLC (1525, 2707, 2489, 2414, CHM, Waters Co., USA). As previously described, the type of column used was Aminex HPX-87H (Bio-Rad, USA), and the temperature was set to 30 °C. Sulfuric acid (H2SO4) was used as the solvent, and the flow rate was 0.6 ml/min. For the quantification of intracellular concentration of pyruvate and oxalate, exponentially-grown cells in 100 ml of NaAc-added MSB media were collected, and supernatant was discarded. The cell pellet was resuspended in 1 ml of boiling distilled water (DW). After boiling for 5 min, the sample was immediately cooled in ice for 5 min. Cell debris was removed after centrifugation, supernatant was filtered with 0.22 μm filter. The sample was stored at −80 °C before injecting 20 μL into UV/visible HPLC system (Waters Co. USA) equipped with C18-column (2.1 x 100 mm, 2.1 μm). The condition of temperature, solvent, and the flow rate was the same as above.

**Construction of L-ldh expression strain.** The whole region of L-ldh gene was amplified using the L-ldh forward and reverse expression primers based on DR1 strain (Table S3). The PCR product was digested using EcoRI and BamHI restriction enzymes. Fragments were inserted into and ligated with pRK415 vector. The recombined plasmids were transformed into E. coli Top10 strain, and L-ldh expression strains were screened on Luria-Bertani broth agar media containing 20 μg/mL tetracycline. Confirmation was conducted using polymerase chain reaction (PCR) and DNA sequencing.

**Reverse transcription polymerase chain reaction (RT-PCR).** Total RNA was extracted from 10 ml of cell cultures using an RNeasy minikit according to the manufacturer’s instructions. Synthesis of cDNA was conducted from 1 μg of RNA with primers for 16S rRNA (b341, b534 primers) and L-ldh gene (Table S3). cDNA was diluted by 10-fold, and amplified following PCR condition; 95 °C for 5 min, followed by 35 cycles of 15 s at 95 °C, 15 s at 60 °C, and 1 min at 72 °C. Each 5 μl of PCR samples was loaded onto 0.8% agarose gel.

**Susceptibility tests.** Wild type and mutant strains were grown overnight in nutrient broth and subsequently diluted 100-fold. The cells reached mid-exponential phase, washed with phosphate buffer saline (PBS, pH 7.4) twice, and approximately 10⁶ cells per ml was resuspended in 1 ml PBS. Serially diluted cells were spotted on nutrient agar containing 0.1–0.4 mM H₂O₂.

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Author Contributions
C.P. and W.P. designed and coordinated the study. C.P. and B.S. performed the experiments and collected the data. C.P. wrote the first complete draft of the manuscript. W.P. provided substantial modifications, and all authors contributed to and approved the final version of the manuscript.

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